Biophys. Struct. Mechanism 2, 181–218 (1976)

Biophysics of Structure and Mechanism

Biophysics with Nitroxyl Radicals

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Abstract. The present status of the spin labeling method as applied to Biophysics is examined. After an outline of the chemical and physical properties of NO radicals, the analysis of linear and non-linear ESR spectra of spin labels and the informations it yields is described.

The possibilities of the method are critically discussed in the light of recent experiments.

Key words: Spin-label method – Spectra analysis – Biophysics.

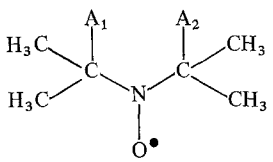
Introduction

Around 1960 a new class of stable free radicals was isolated independently by Russian and American researchers [1, 2]. These nitroxyl radicals (NO radicals, also called nitroxides or iminoxyls [3]) received considerable attention since it was discovered that chemical reactions without the participation of the unpaired electron are possible [4]. The development of the chemistry of the NO radical laid the basis for the spin label technique, originally suggested by H. M. McConnell [5, 6], which is now widely employed in physical chemistry and molecular biology [7–14]. This method consists in placing the stable NO group in the system of interest either by attaching it, by a chemical reaction leading to a covalent binding, onto a molecule of the system under study, or by introducing in this system a suitably tailored radical ("spin probe") which is the dissolved in the system without permanent bonding to any of its molecules (see § I). Since most media are diamagnetic, the electron spin resonance (ESR) signal of the NO group is the only signal in the ESR spectrum. The location of the spin label in the host system can generally be well defined and the spectrum can be used in a number of ways. The simplest information it contains is the concentration of spins, used to follow incorporation of labeled molecules or changes in time of their concentration. Analysis of line positions and line shapes, either in isotropic (liquid solutions) or anisotropic systems (liquid crystals, membranes, solids) can yield information about the rate of motion of the label, the structure, order, viscosity, polarity, of the host system (see § II and III). Examples of

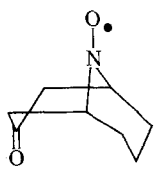
achievements with the spin labeling technique are the evidence of the bilayer structure of certain portions of biological membranes, the discovery of lateral diffusion and transverse diffusion ("flip-flop") in bilayers and membranes and the measurement of their diffusion constants, and the first measurement of the depth of an antibody active site. A further advantage in using spin labels is the high sensitivity of the ESR technique compared to conventional NMR which makes it possible to detect reasonable signals even in a 10^{-6} M spin label solution. In this review, the emphasis is on the physics and physical chemistry of the technique, its biological applications have been extensively discussed elsewhere [10, 12, 13]. We shall not deal with double resonance nor with polyradicals. Unless otherwise stated, the reported experiments have been performed at X band ($\nu_0 = 9.5$ GHz, $H_0 \simeq 3300$ G). Literature coverage goes to the end of 1975.

I. The Chemistry of NO Radicals

The chemical structure of a stable NO radical is given by the general formula:

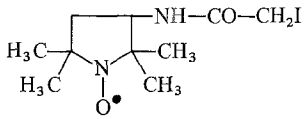


The unpaired electron is localized on the N—O bond and the radical may be attached to a larger molecule through A_1 and A_2. The detailed procedures for the synthesis of such nitroxyl radicals can be found in references 3, 4, 9 and 14. Radicals of this structure can be stored for months under ordinary conditions. This remarkable stability – characterized by a thermochemical bond energy for N–O of 100 kcal [3] – is due partly to the steric protection of the NO bond by the four adjacent methyl groups: also if one of the methyl groups is replaced by hydrogen, the radical decomposes by disproportionation into a hydroxylamine and a nitrone (which may be only an intermediate). An exception is the following bicyclic NO radical [36]:



Even though this molecule carries two hydrogens in α position to the N atom it is stable because the disproportionation would require a simultaneous ring cleavage. The sterically protected NO group is so unreactive that the other parts of the same molecule (i.e. the substituents A_1 and A_2) can easily be modified without involvement of the unpaired electron. Mainly due to the pioneering work of E. Rozantsev [3, 4] and coworkers in the USSR and A. Rassat [15, 16, 36] and coworkers in France, a

large number of NO radicals (mono- and polyradicals) is now available, even commercially. These radicals can be used as molecular probes in two ways. The first possibility is to attach a NO radical to the system of interest by a chemical reaction. A typical example is the radical



where the iodoacetamide group is a specific reagent for sulfhydryl groups [17]. This molecule can thus be bound covalently to the SH groups of proteins. This reaction may by called "*spin labeling*". A second method is simply to diffuse a nitroxyl radical into the system under investigation. In this case the molecule may be called a "*spin-probe*" since it is not bound covalently to its host system. Depending on the chemical structure of the NO carrier a spin probe can also be directed to a specific site in a biological system as has been shown for spin labeled analogues of haptens, enzyme substrates, coenzymes and lipids. Table 1 contains a selection of spin labels and spin probes currently used in physico-chemical and biological studies. A recent development is finally the biosynthetic incorporation of NO radicals into living systems [18–21].

The drawbacks of the chemistry of NO radicals are rather few in number. Nitroxides are very sensitive to acids and disproportionate at low pH values, they are often decomposed when heated above 80° C [3], or irradiated for several hours [3, 4]. They are also easily reduced, by ascorbic acid [22] for instance or certain intracellular media. This important property has been applied to the investigation of phospholipid transverse diffusion – "flip-flop" [22] – in bilayer vesicles or natural membranes.

Time constants of 6.5 h at 30° C in lecithin dispersions [22], 5 mn at 15° C in excitable membrane vesicles [127], 8 or 18 h depending on the reducing agent in inner mitochondrial membranes and red blood cells [128], are reported for this phenomenon.

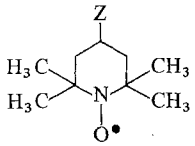
NO reduction has also been used to evidence tightly bound lipid in microsomal membranes [129].

II. Physical Properties of Nitroxyl Radicals and Spin Probes

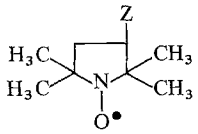
NO radicals are brightly coloured liquids or solids, the colour ranging from yellow to dark red depending on the ring size and the substituents. Their absorption spectrum has two bands at ~ 240 nm and ~ 460 nm [3]. They can be used as quenchers in fluorescence experiments [64], but since they are not fluorescent, the kinetics must be studied from the emission of the donor. The diffusion limited quenching which is observed seems to originate in an exchange process. In the IR spectrum, absorption at ~ 1350 cm^{-1} is produced by the vibration of the NO group [3]. The molecular geometry of a number of NO radicals has been investigated [130] by means of X-ray diffraction (in their cristalline state). The NO bond and adjacent N–C bond

Table 1. Spin labels and spin probes. The first reference generally contains the synthesis procedure

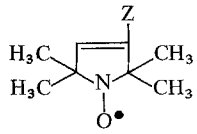
Abreviations:



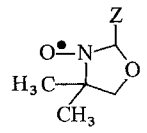
R_1



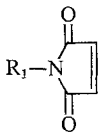
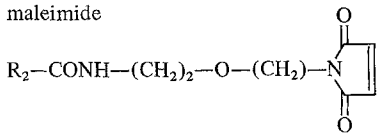
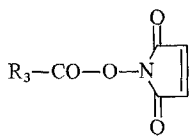
R_2

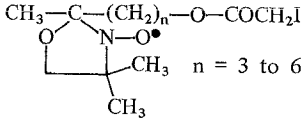
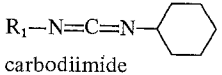
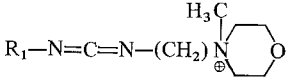
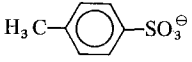


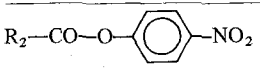
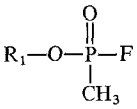
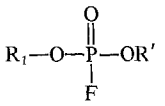
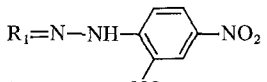
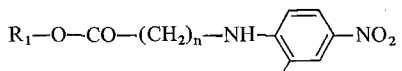
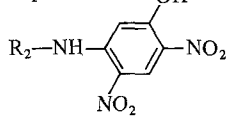
R_3

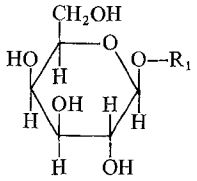
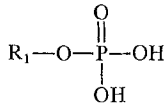


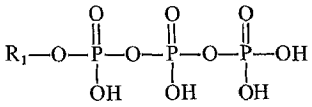
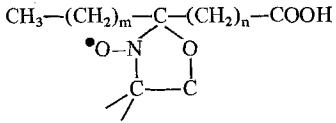
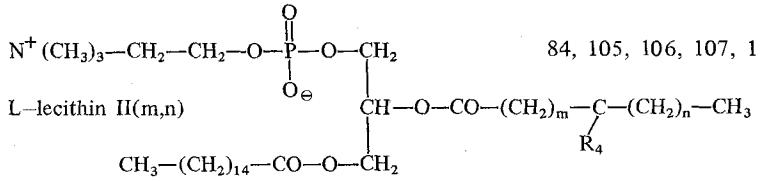
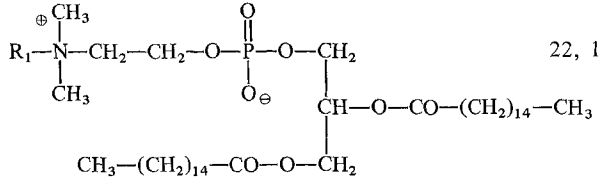
R_4
(oxazolidine ring)

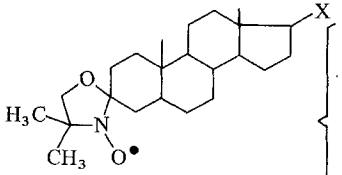
reactive substituent Z or spin label	reference for synthesis and use	target
	A. Covalent labeling	
 maleimide	23, 24 – 35	nucleic acids, proteins – SH groups – amino groups
	34	proteins – SH groups
 N–hydroxy succinimide	38, 39	α–amino group of AA ^{t}RNA

$R_2—NH—CO—CH_2—CHBr—COOH$	40, 26	nucleic acids
$R_1—$; $R_2—NH—CO—CH_2Br$	17, 26, 28–30, 35, 41–48	nucleic acids (specific for thiouridine in ^tRNA)
$R_1—$; $R_2—NH—CO—CH_2I$	42	proteins
haloacetamide	50, 51, 69	(–SH groups, histidine residues)
 n = 3 to 6	52, 53	– SH groups (GAPDH)
$R_1—N=C=S$ isothiocyanate	28, 29	– OH groups, – NH_2 groups
 carbodiimide	54	mitochondrial ATPase
  carbodiimide	55, 56, 57	nucleic acids (poly U)
$R_3—CO—O—CO—OC_2H_5$ mixed anhydride	58, 31, 59	amino groups

reactive substituent Z or spin label		reference for synthesis and use	target
		60, 61, 62	acylating agent
nitrophenol			
		63, 45	serine residues (specific)
fluorophosphate			
	$R' = C_2H_5$	65	serine residues, α–chymotrypsin
	$R' = CH_2(CH_3)_2$	66	esterases, acetylcholinesterase inhibitors
	$R' =$ 	66	
organophosphate			
		B. Non-covalent labeling	
R_1 $Z = -H_2$	TANANE or TEMPO	125, 137, 138, 140 – 145	physico–chemical studies
$Z = -HOH$	TANOL or TEMPOL	126	artificial and natural
$Z = O$	TANONE	1, 146	membranes
		67	antibodies
hapten 			
		68	antibodies
hapten  n = 1 to 5			
		69	oxidative phosphorylation system

$R_1—O—CO—(CH_2)_2—CO—O—NO_2$	70	chymotrypsin
succinic ester		
	71	galactosidase
$R_2—CH_2—$(NAG)		
$R_2—CH_2—(NAG)_2$	45	lysozyme
N–Acetylglucosamine		
$R_1—$(N)—adenosine monophosphate	72	DNA polymerase
$R_1—$(N)—adenosine triphosphate	72, 73, 74	DNA polymerase
$R_1—$ADP–Ribose	53, 75, 76	lactase dehydrogenase alcohol dehydrogenase
$R_2—CO—NH—(CH_2)_n—CO—NH—C_6H_4—SO_2H$	77	carbonic anhydrase
sulfonamide		
	78, 79	ribonuclease
phosphate		

reactive substituent Z or spin label	reference for synthesis and use	target
	80, 73, 74, 81	hemoglobin
triphosphate		
R_1—NH—CO—$(CHOH)_2$—COO^-	79	lipid vesicles (membrane potential)
tartric acid		
	18, 19, 35, 37, 44, 53, 65, 82–104, 129, 149, 164	artificial and natural membranes
fatty acid I(m,n) n = 2 to 14 m = 13 to 1		
	84, 105, 106, 107, 19, 32	artificial and natural membranes
L–lecithin II(m,n)		
	22, 128	artificial and natural membranes
L–lecithin III		



steroid–SL

X = H androstane	108, 109	artificial and natural membranes
X = OH androstanol	108, 109, 90, 36, 65, 99	thermotropic liquid crystals
X = $-CH(CH_3)(CH_2)_3CH(CH_3)_2$ cholestane	109, 86, 88, 110–115, 117, 118, 148	

$$\overset{+}{N}(CH_3)-(CH_2)_2-O-\underset{\underset{O}{\|}}{C}-(CH_2)_n-\underset{\underset{R_4}{|}}{C}-(CH_2)_m-CH_3$$ 119, 128

cholinergic receptor protein studies

(m,n) Acylcholine label

$$CoA-S-\underset{\underset{O}{\|}}{C}-(CH_2)_n-\underset{\underset{R_4}{|}}{C}-(CH_2)_m-CH_3$$ 120, 128

mitochondrial ADP carrier

(m.n) acyl CoA label

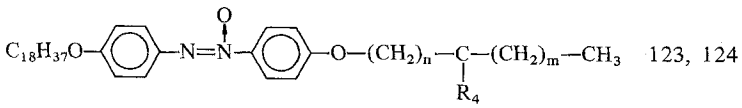
$$ATR-\underset{\underset{O}{\|}}{C}-(CH_2)_n-\underset{\underset{R_4}{|}}{C}-(CH_2)_m-CH_3$$ 128

mitochondrial ADP carrier

(m,n) acyl Atractyloside label

$$CH_3-(CH_2)_m-\underset{\underset{R_4}{|}}{C}-(CH_2)_n-CH_3$$

m = 3, n = 4 decane label	37	artificial and natural membranes
m = n = 6, m = 2 n = 8	122	liquid crystals



123, 124 liquid crystals

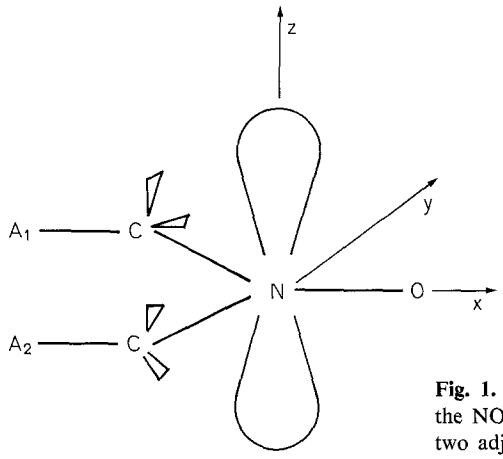


Fig. 1. The coordinate system (x, y, z) ascribed to the NO radical. In this drawing, The N, O and the two adjacent C atoms are assumed to be coplanar

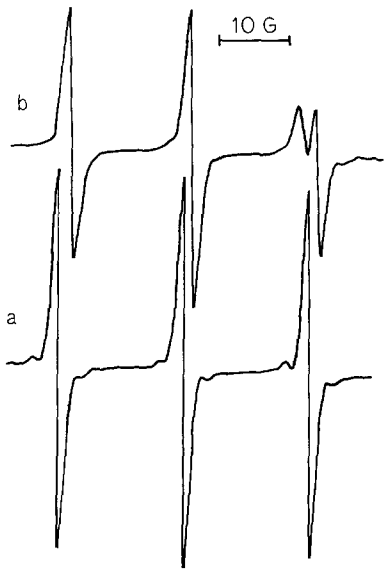


Fig. 2. Linear response spectrum of Tempo. (**a**) in aqueous solution, (**b**) the label is distributed between environments of different polarity

length are 1.25–1.30 Å and ~ 1.5 Å respectively. The nitrogen, the oxygen, and the two neighbour tertiary carbon atoms are often practically coplanar. Deviations from coplanarity have been observed [131, 132], but they are small and are not normally taken into account in the interpretation of ESR spectra. The nitrogen $2p\pi$ orbital carrying the unpaired electron is extended perpendicular to the plane of the four atoms (Fig. 1).

A cartesian coordinate system (x, y, z) is then usually ascribed to the NO radical in such a way that the x axis is extended along the NO bond, and the z axis along the $2p\pi$ orbital.

In the ESR experiment, the magnetic moment of the electron interacts with the external magnetic field $\vec{H}_0$ and also with the local magnetic fields $\vec{H}_l$ created at the

electron by surrounding nuclei, in fact almost entirely by the nitrogen nucleus (spin quantum number of ^{14}N: $I = 1$).

The basic spectrum of a rapidly tumbling NO radical in dilute solution therefore consists of just three lines of equal heights and equal separation (Fig. 2a). The separation between two adjacent resonance lines is called isotropic hyperfine splitting (*hfs*) constant a_N ($a_N \sim 13-16$ G for most spin labels), while the so-called *g*-factor determines the magnetic field value corresponding to the center of the spectrum. The success and the importance of the spin label method are then based on the fact that small changes in the environment of the NO radical (i.e. changes of the viscosity, polarity or structure of the surrounding medium) can cause dramatic effects in the shape of the ESR signal and that the spectral differences can be evaluated quantitatively with great precision.

1. Polarity of the N–O *Bond*

The solvent dependence of the ESR signal of the spin probe may serve as a first example to illustrate this. From measurements in organic solvents, it is known that the separation of the three lines in Figure 2a becomes larger with increasing polarity of the solvent [15, 133, 134], e.g. for fatty acid spin labels I(*m, n*) [135] a_N varies between 15.6 G in water and 13.8 G in a hydrocarbon solvent. This effect can be explained by a formal resonance between the non polar form *A* and the dipolar form *B*

$$\underset{A}{>N-\dot{O}} \leftrightarrow \underset{B}{>\dot{N}^{+}-O^{-}},$$

where the dipole character of the NO group manifests itself in a contribution of 3 Debye to the dipole moment of the molecule [4]. Since the *hfs* constant a_N is proportional to the unpaired spin density, increasing contributions from the dipolar form lead to increasing values of a_N. Polar solvents stabilize the dipole *B*, thus explaining qualitatively the observed variations of *a*. But motional effects [136] can also contribute to the *hfs* constant value, and this limits the possibility of accurately measuring the polarity of the immediate spin label environment with a_N.

This solvent dependence can be exploited in another way: a spin probe may be distributed between two environments of quite different polarity (e.g. a water-hexane sample). Then two superimposed ESR spectra with different *hfs* constants and slightly different *g*-factors are obtained. In a similar fashion, when a small spin probe (e.g. Tempo) is diffused into bilayer lipid vesicles [138] or natural membranes [137], it will partition between the aqueous buffer phase and the hydrophobic region according to its chemical and structural affinity (Fig. 2b). The equilibrium is strongly dependent on the dynamical state of the chain system, Tempo is excluded into the polar medium when the chains becomes immobilized. Yet if the hydrophobic decane label (see Table 1) is used, it will still be soluble in the hydrocarbon region when the relatively polar Tempo is already excluded, thus allowing the investigation of more rigid membranes [139].

The spectrum then obtained has well resolved high field components [137] and a partition coefficient for the label can be defined [139]. Resolution can be improved by working at Q band [140] ($\nu_0 = 35$ GHz, $H_0 \sim 12.5$ KG) where the peak separation is enhanced.

This method allows an estimate of the percent of "fluid lipid" [138], the detection of liquid crystalline phase transitions and lateral phase separation in artificial [141–143] and living membranes [144] and the demonstration of their relevance to perpendicular transport of small molecules (sugars...) [145, 139]*.

2. *Anisotropic Couplings of the Unpaired Electron*

Another important property of the NO radical is the anisotropy of the coupling of its unpaired electron to the permanent magnetic field $\vec{H}_0$ and to the magnetic field created by the magnetic moment of the nitrogen nucleus. These couplings are second order tensors, $\boldsymbol{g}$ and $\boldsymbol{A}$, and their principal axes – that is the reference frame in which they can be made diagonal – have been found to coincide with the x, y and z axes defined above. $\boldsymbol{g}$ determines the positional anisotropy, $\boldsymbol{A}$ the hyperfine anisotropy. These anisotropies are easily measured by studies of single crystals [84, 146–148] doped with a small amount of a NO radical, in which the orientation of the permanent magnetic field $\vec{H}_0$ is varied with respect to the molecular coordinate system. When $\vec{H}_0$ is oriented along the nitrogen $2p\pi$ orbital (z axis) a large hyperfine splitting of $A_{zz} = 32$ G is measured (at X band). Orienting $\vec{H}_0$ along the x or y axis yields separations A_{xx} and A_{yy} of approximately 6 G each. At the same time, the position of the center of the spectrum, characterized by g_{zz}, then g_{xx} or g_{yy} is also shifted. If the single crystal is dissolved in a fluid liquid, these anisotropies are averaged out by the very rapid isotropic tumbling of the molecules. The observed *hfs* a_N is then $a = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$ and the g factor $g = \frac{1}{3}(g_{xx} + g_{yy} + g_{zz})$. If however, the motion of the spin probe is anisotropic, the molecular anisotropies are not completely averaged out, instead they yield valuable information about the ordering of the probe in the host system.

These results can be described quantitatively in terms of the electron spin Hamiltonian $\mathscr{H}$

$$h\mathscr{H} = \beta_e \vec{H}_0 \boldsymbol{g} \vec{S} + \vec{I} \boldsymbol{A} \vec{S} \tag{1}$$

where β_e, $\vec{S}$, $\vec{I}$ are the electron Bohr magneton, the electron spin operator and the nuclear spin operator, respectively. A listing of these molecular parameters derived either from single crystal studies [84, 146–148] or from optimization of computer simulated spectra [149, 150] is given in Table 2.

From now, we shall suppose these parameters are known.

III. Physics with Spin Labels

We are now facing the following problems: First, how are produced and analyzed the label spectra, second, how is this going to help us in the understanding of the

* Jet its use can be perturbed by differential changes in the rate of motion and degree of order in the two environments [218]

Table 2. g and hyperfine coupling tensor elements for some currently used spin labes.
DTBN = ditertiobutyl nitroxide $(CH_3)_3$–C–N(•O)–C–$(CH_3)_3$; PADS = Fremy's salt $(K_2(SO_3)_2NO)$;
k = value not reported; l = Gauss at X band; m = in MHz

Molecular species	DTBN	DTBN	Tanone	Cholestane-SL
Origin of data	single cryst.	single cryst.	single cryst.	single cryst.
Reference	[146]	[147]	[146]	[84]
g_{xx}	2.0089	2.00872	k	2.0089
g_{yy}	2.0061 $\pm 3 \cdot 10^{-4}$	2.00616 $\pm 0.5 \cdot 10^{-4}$	k	2.0058 $\pm 10 \cdot 10^{-4}$
g_{zz}	2.0027	2.00270	k	2.0021
$g = \frac{1}{3}(g_{xx} + g_{yy} + g_{zz})$	$2.0060 \pm 2 \cdot 10^{-4}$	$2.00586 \pm 0.5 \cdot 10^{-4}$	$2.0062 \pm 2 \cdot 10^{-4}$	$2.0056 \pm 10 \cdot 10^{-4}$
$\Delta g = \frac{1}{6}(2g_{zz} - g_{xx} - g_{yy})$	$-16 \cdot 10^{-4}$	$-15.8 \cdot 10^{-4}$	k	$-17.5 \cdot 10^{-4}$
$\delta g = \frac{1}{2}(g_{xx} - g_{yy})$	$14 \cdot 10^{-4}$	$12.8 \cdot 10^{-4}$	k	$15.5 \cdot 10^{-4}$
Unit for A values	l	l	l	m
A_{xx}	7.1	7.59	5.2	16.2 ± 2
A_{yy}	5.6 ± 0.5	5.95 ± 0.05	5.2	16.2 ± 2
A_{zz}	32	31.78	31	86 ± 2
$a = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$	15.1 ± 0.5	15.11 ± 0.05	14.3 ± 0.5	39.47 ± 2
$\Delta a = \frac{1}{6}(2A_{zz} - A_{xx} - A_{yy})$	8.6	8.34	8.6	23.27
$\delta a = \frac{1}{2}(A_{xx} - A_{yy})$	0.75	0.82	0	0

Molecular species	Cholestane-SL	I(m, n)	PADS	PADS
Origin of data	single cryst.	simulation	simulation	simulation
Reference	[148]	[149]	[150]	[150]
g_{xx}	2.0090	2.00872	2.0081	2.00785 ± $2 \cdot 10^{-4}$
g_{yy}	2.0060 $\pm 1 \cdot 10^{-4}$	2.00616	2.0057 $\pm 2 \cdot 10^{-4}$	2.0059 ± $2 \cdot 10^{-4}$
g_{zz}	2.0024	2.00270	2.0025 ± $1 \cdot 10^{-4}$	2.00265 ± 10^{-4}
$g = \frac{1}{3}(g_{xx} + g_{yy} + g_{zz})$	$2.0058 \pm 1 \cdot 10^{-4}$	2.00586	$2.00543 \pm 2 \cdot 10^{-4}$	$2.00547 \pm 2 \cdot 10^{-4}$
$\Delta g = \frac{1}{6}(2g_{zz} - g_{xx} - g_{yy})$	$-17.0 \cdot 10^{-4}$	$-15.8 \cdot 10^{-4}$	$-14.6 \cdot 10^{-4}$	$-14.1 \cdot 10^{-4}$
$\delta g = \frac{1}{2}(g_{xx} - g_{yy})$	$15.0 \cdot 10^{-4}$	$12.8 \cdot 10^{-4}$	$12 \cdot 10^{-4}$	$9.75 \cdot 10^{-4}$
Unit for A values	m	l	l	l
A_{xx}	17.7 ± 0.3	6.95	5.5 ± 0.5	5.5
A_{yy}	16.4 ± 0.3	5.35	4.0 ± 0.5	5.0
A_{zz}	89.4 ± 0.06	33	29.8 ± 0.3	28.7
$a = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$	41.17 ± 0.22	15.1	13.1 ± 0.4	13.1
$\Delta a = \frac{1}{6}(2A_{zz} - A_{xx} - A_{yy})$	24.12	8.95	8.35	7.82
$\delta a = \frac{1}{2}(A_{xx} - A_{yy})$	0.65	0.8	0.75	0.25

molecular properties of the system under investigation, especially how reliable is the information we get (see part IV).

1. The ESR *Spectra of* NO *Radicals*

The principles of ESR spectroscopy have been extensively described elsewhere [151–153]. We shall only briefly recall here the main physical ideas and define the

parameters which will be necessary during our discussion – in a deliberately elementary fashion.

In the ESR experiment, the paramagnetic sample is in the resonant cavity tuned for the constant frequency ν_0, placed in a permanent magnetic field $\vec{H}_0$, the direction of which is chosen as Z axis in the laboratory reference frame, and ν_0 is defined by

$$E_0 = h\nu_0 = g\beta H_0 . \tag{2}$$

E_0 is the distance between the two energy levels of the electron in $\vec{H}_0$. The oscillating microwave field $2H_1 \cos 2\pi\nu_0 t$ perpendicular to $\vec{H}_0$ is created in the cavity by a klystron and a small low frequency modulation $H_m \cos \omega_m t$ is superimposed on $\vec{H}_0$ along Z. The total field H along Z felt by the electron is then

$$H = H_0 + H_m \cos \omega_m t + H_l^Z(t) + \Delta H_Z , \tag{3}$$

where $H_l^Z(t)$ represents the component of the local magnetic field sensed by the electron as a result of its anisotropic interaction with neighbouring nuclei (mainly N) and depends on molecular orientation. ΔH_Z is an instrumentally adjustable increment to H_0 necessary to slowly scan the spectrum (e.g. $\Delta H_Z = 100$ G in 4 mn).

In the absence of $\vec{H}_0$, the spins are randomly oriented and the magnetization $\vec{M}$ of the sample, defined as the vector sum of the electronic magnetic moments is zero. $\vec{H}_0$ polarizes the spins, creating a new equilibrium where there exists a population difference n_0 between the two energy levels of the electron and a magnetization $\vec{M}_0$ of the sample, aligned with $\vec{H}_0$. M_{Z0} is proportional to n_0, that is to the number difference of the magnetic moments parallel and antiparallel to $\vec{H}_0$, and the transverse magnetization is $M_{X0} = M_{Y0} = 0$. The system has then acquired the magnetic energy $-M_{Z0}H_0$ and we note that the changes in energy of the spin system are associated with longitudinal processes which change M_Z or n.

A perturbation can bring the system out of equilibrium, creating a small non-zero transverse magnetization (M_X, M_Y) and changing M_{Z0} to M_Z. The return to equilibrium of the system, still in $\vec{H}_0$ viewed from a frame rotating around H_0 at the angular velocity $2\pi\nu_0$ can be described as follows: M_Z relaxes to M_{Z0} with an intrinsic characteristic time constant T_{1e} called spin lattice or longitudinal relaxation time, with nitroxides it is of the order of 10^{-6} s, the transverse magnetization relaxes to zero with T_{2e} called transverse or spin-spin relaxation time, of the order of 10^{-8} s. This evolution of the magnetization can in many cases be described by the so-called Bloch equations. Likewise, n relaxes after

$$\frac{dn}{dt} = -\frac{n - n_0}{T_{1e}} \tag{4}$$

and T_{1e} also characterizes the time scale of magnetic energy changes in the system.

The values of 0.43 μs for Tanol in secondary butylbenzene and of 6.6 μs for maleimide labeled hemoglobin [154] are reported at room temperature (see Table 1).

When M_Z returns to its value M_{Z0}, the electronic spins on the higher energy level release magnetic energy to degrees of freedom of the molecular motion of the whole system, usually called "the lattice", via, here, the Zeeman interaction or the hyperfine interaction between the electron and the N nucleus. This happens efficiently if the molecular motion which modulates this interaction (between the electron and the lattice) has motional components with such a frequency that they can receive the energy $h\nu_0$ given away by the spin.

The relaxation of M_X and M_Y to their zero equilibrium value involves no exchange of energy with the lattice.This process can be viewed as a loss of coherence in the individual magnetic moments, and is related to the entropy changes of the spin system.

The oscillating microwave field $H_1 \cos 2\pi\nu_0 t$ induces transitions to the higher energy level for those electron spins for which the resonance condition

$$h\nu_0 = g\beta\, H^* \tag{5}$$

is fullfilled, where H^* is the resonance value of H. The *resonant response* to H_1 of the spin system in H_0 has a real and an imaginary part. Both modify the complex impedance of the cavity at resonance: there is some microwave *energy absorption* by the spins as well as a small frequency shift in the cavity tuning called *dispersion.* Both signals from absorption and dispersion carry information about the spins and can be detected with appropriate techniques [153].

When H_1 is gradually increased, n dimishes, then goes to zero – relaxation is not able to carry off the absorbed energy – the system is said to be partially then totally *saturated* (typically at $H_1 \sim 0.3$ G).

a. In the most common type of ESR *experiments,* resonance is observed using a weak H_1 (e.g. $H_1 \sim 0.01$ G), the spin system reaches an equilibrium steady state where n is very close to n_0 (no saturation) and its response, hence the signal height is proportional to H_1 ("*linear response*" experiment). There are little or no energy changes and spin-spin relaxation (T_2) processes are the dominant ones. The modulation field serves here only for detection, it is kept at a low level (for us, $H_m < 1$ G, $\omega_m = 2\pi\ 10^5$ Hz). With standard ESR spectrometers equipped with phase sensitive detection, one usually observes the absorption, the signal is recorded at the first harmonic of the modulation, in phase with it. The spectrum has the shape of the first derivative of the absorption curve.

It is convenient to define the correlation time τ_c of a random isotropic molecular motion as the inverse frequency of its main motional or reorientational component. If one nitroxyde spin label is oriented with its $2p\pi$ orbital parallel to $\vec{H}_0$ at a given time t_0, then perpendicular to $\vec{H}_0$ at $t_0 + \tau_c$ due to molecular reorientation, it has exchanged between two resonance lines of the spectrum distant of Δa (in frequency units), the anisotropy of the hyperfine coupling (see Table 2 and § II.2). Thus Δa defines the time scale of the linear response experiment: in an isotropic medium, for rapid tumbling, $\tau_c \ll \frac{1}{\Delta a}$, the anisotropies of the couplings are averaged out (three line spectrum) (Fig. 2a), for slower motion $\tau_c \lesssim \frac{1}{\Delta a}$, contributions from the different orientations appear in the spectrum, then as τ_c increases, the spectrum asymptotically approaches the rigid powder spectrum arising from a fixed random orientation-

al distribution. In an anisotropic fluid (e.g. liquid crystalline host system) the reorientation of the label is severely hindered in one or more directions and this anisotropic restricted motion results in more important contributions to the spectrum of certain orientations of the label.

In conclusion, the ESR linear response signal is largely insensitive to motions significantly slower than $\frac{1}{\Delta a}$, one can measure correlation times up to 10^{-7} s only. Yet, spectra from strongly immobilized labels have been obtained by several workers, in oriented viscous nematic liquid crystals [155], in smectic lipids as well as in biological systems [119], and for the elucidation of the molecular dynamics of such systems, a technique showing sensitivity on a slower time scale appeared desirable.

b. Non linear ESR *techniques* have been recently developed, due to the pioneering work of Hyde, Dalton, McConnell and coworkers [156–159]. In this type of experiment, a larger H_1 is used to create conditions of moderate or strong saturation. One looks now at the energy changes in the spin system where n is different from its equilibrium value n_0, and spin lattice (T_1) is the dominant relaxation mechanism.

The important physical fact here is that under these conditions of saturation, the system is sensitive to the *rate* at which H goes through its resonance value H^*. Let us consider Equation (3) for fixed values of H_0 and ΔH_Z:

(1) for a spin label of given orientation this rate depends on the amplitude and frequency of the modulation field $\left(\frac{dH}{dt} \alpha\ \omega_m H_m\right)$.

The modulation field has now a double function: the detection of the signal as before, and the creation of a proper rate of passage of H through resonance, which in many cases, effects in the so-called "adiabatic rapid passage" situation. With phase sensitive detection, one observes the dispersion of the first harmonic of the modulation, or the absorption at the second, the signal is found now *out of phase* with the modulation.

(2) for given modulation conditions, this rate also depends on the changes in H_i^Z, due to the reorientation of the spin label molecule: H_i^Z is modulated by molecular motion of correlation time τ_c which thus also sweeps H past its resonance value and influences the signal $\left(\frac{dH_i^Z}{dt} \alpha\ \frac{1}{\tau_c} \cdot H_i^Z\right)$.

Since the spin system loses all memory in a time T_{1e}, it is because this type of ESR experiments depends on the rate of passage of H that correlation times even longer than T_{1e} can be detected. Indeed, the spectra are sensitive to changes in τ_c up to 10^{-4}–10^{-3} s.

With nitroxydes, T_{1e} depends little on molecular motion. The signal height can be shown to vary approximatively as $\frac{\omega_m \tau_c}{1 + \omega_m^2 \tau_c^2}$ [157] (see Fig. 5).

Typically at X band one uses for example $H_1 = 0.1$ G, $H_m = 5$ G, $\omega_m = \pi \cdot 10^5$ Hz or less [156].

c. Other important factors influencing the spectra are:

– the label concentration. Molar concentrations of label above 0.5% usually result in label-label interaction which causes dramatic changes in the spectrum (see III § 3),

– the shape of the carrier molecule, the structural orientation of the NO $2p\pi$ orbital with respect to the long molecular axis [176],

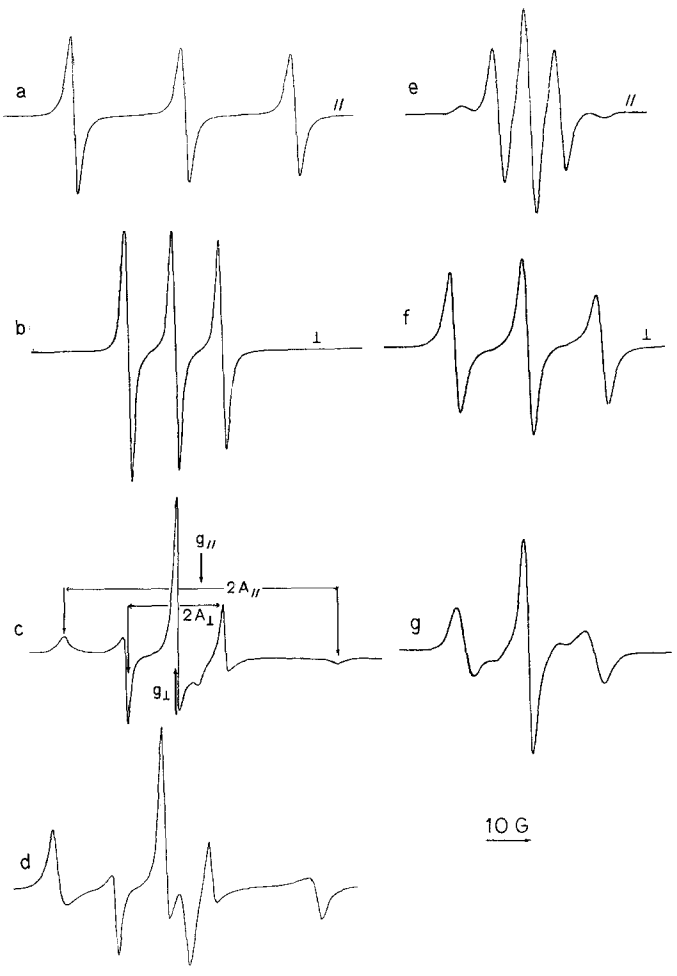


Fig. 3. Linear response spectra of *I*(13.2) {a) to d)} and the androstanol label {e) to g)} in a multilamellar soap system at room temperature. Planar oriented samples with the applied field $\vec{H}_0$ parallel (perpendicular) to the bilayer normal: a and e (b and f). Random distribution of bilayers: c and g, cylindrical distribution of bilayers: d (from ref. [149] with permission)

– the overall geometry of the sample. In studies of lipid smectic mesophases for example, planar oriented arrangements obtained by pressing the material between quartz plates [149], cylindrical geometries obtaines by sucking it into thin capillaries [160], and random isotropic distributions investigated with the same label will yield very different spectra. With a planar arrangement, a single orientation of the sample with respect to the magnetic field $\vec{H}_0$ contributes to the spectrum. The spectrum of an isotropic distribution can be understood as a weighted sum of spectra corresponding to many single orientations, and because of this averaging, it contains less information about the system.

The influence of these factors on linear response spectra is illustrated in Figure 3.

Theories describing the effects of all these parameters within certain regions of their variation are based either on the equation of evolution of the spin density matrix, or on the phenomenological Bloch equations modified to include diffusion and modulation effects. Then numerical calculations allow a simulation of the spectra.

We shall now proceed to describe the analysis of the spectra, distinguishing the fast tumbling $\tau_c < 10^{-9}$ s, intermediate 10^{-9} s $< \tau_c < 10^{-7}$ s and slow motion $\tau_c > 10^{-7}$ s regions.

2. *Analysis of the Spectra: Dilute Samples (Non Interacting Spins)*

In liquid crystalline systems the motion of the dissolved spin label is highly anisotropic, very often it retains cylindrical symmetry around one axis z'; as we shall see below, it is then customary to use an orienting potential [161] to describe some of the effects caused by the anisotropy of these fluids. In isotropic media, where no such orienting forces exist anymore, the label freedom can still be restricted but the main parameter influencing the spectra is the correlation time τ_c. The analysis developed for the case of anisotropic fluids remains valid by setting the orienting potential equal to zero. In the following, we denote θ_3 the angle between the z and z' axes, β the angle between z' and $\vec{H}_0$ (Z axis) and θ the angle between z' and $\vec{n}$, the director of the mesophase.

a. Fast Motion Region $\tau_c < 10^{-9}$ s

This is the range of τ_c values for which linear response ESR spectra show excellent sensitivity.

1. "hand made" Analysis and Orientational Properties. The hamiltonian of the electron spin [Eq. (1)] has the time dependance of the molecular motion; we can write [84, 148]:

$$h\mathscr{H}(t) = h\overline{\mathscr{H}(t)} + h(\mathscr{H}(t) - \overline{\mathscr{H}(t)}) = h\mathscr{H}_{\text{eff}} + h\mathscr{H}_1(t) \tag{6}$$

(in all the following bars indicate time averages).

The analysis rests on the conditions that $|X_1(t)|$ is very small,

$$|\mathscr{H}_1(t)|\tau_c \ll 1 \quad \text{or} \quad \tau_c \leq 3 \cdot 10^{-9} \text{ s} \tag{7}$$

so that time dependant perturbation theory can be applied with $X_1(t)$ and the position of the lines is determined by the eigenvalues of the time averaged or effective hamiltonian H_{eff} or $\mathscr{H}$.

In a planar oriented lamellar sample, for example, the observed hyperfine splitting is

$$A_{\text{obs}}(\beta) = (A_{\parallel}^2 \cos^2\beta + A_{\perp}^2 \sin^2\beta)^{1/2} \tag{8}$$

and if the spectrum is taken with $\vec{H}_0$ aligned with the axis of motional averaging z' ($\beta = 0$)

$$h\mathscr{H}_{\parallel} = \beta_e H_0 g_{\parallel} S_z + h A_{\parallel} S_z M \tag{9}$$

(where M is the nuclear spin quantum number $(+1, 0, -1)$, $A_{\parallel}$ can be measured from the spectrum (Fig. 3a). $A_{\perp}$ is obtained in the same way ($\beta = \frac{\pi}{2}$). Both $A_{\parallel}$ and $A_{\perp}$, can be obtained from samples having cylindrical or isotropic geometry if the motion is sufficiently anisotropic (Fig. 3c and d). A field standard (Hall probe or other free radical of accurately known g factor) is necessary to get $g_{\parallel}$ and $g_{\perp}$.

The order matrix $\|S_{ij}\|$ first defined by Saupe [162] is currently used to describe the mean orientation of the nitroxide molecular axes ($x\,y\,z$) with respect to z'.

$$\left.\begin{array}{l} S_{ii} = S_i = \frac{1}{2}\,\overline{(3\,\cos^2\theta_i - 1)} \quad i = 1, 2, 3 \\ \cos\theta_1 = \vec{x}\cdot\vec{z} \quad \text{etc.} \\ \sum\limits_{i\,=\,1,\,2,\,3} S_i = 0 \end{array}\right\} \tag{10}$$

The knowledge of $A_{\parallel}$ and $A_{\perp}$, thus allows a hand made evaluation of the order parameter S_3 [83, 84]

$$S_3 = \frac{A_{\parallel} - A_{\perp}}{3\Delta a} \cdot \frac{a}{a'} \tag{11}$$

with

$$a' = \tfrac{1}{3}\,(A_{\parallel} + 2\,A_{\perp}) \tag{12}$$

where the factor $\frac{a}{a'}$ is a correction for polarity effects and the approximation $\delta a = 0$ is used. S_1 and S_2 can be determined using the measured value of g and (10) from

$$g_{\parallel} = g + 2\,\Delta g\,S_3 + \tfrac{2}{3}\,\delta g\,(S_1 - S_2)\,. \tag{13}$$

Both $A_{\parallel}$ and S_3 have been used to characterize lipid bilayers, artificial and biological membranes vesicles. They are sensitive to phase transitions. In smectic soap water systems, S_3 as measured with label I(m, n) was found to depend exponentially on n [83]

$$S_3 = S_0\,S_{\alpha}^{n} \tag{14}$$

and this behaviour could approximately be verified [163] in many artificial lipid systems.

Defined by (10), S_3 varies from $+1$ to $-\frac{1}{2}$. In nematics and in most smectic lipids only positive order parameters have been obtained. However, negative order parameters have been found from oriented spectra having $A_{\parallel} < A_{\perp}$, in the central part of unsaturated soap water systems, and interpreted in terms of a bending of the chain below the position of the double bond [164].

Order parameters, for example S_3

$$S_3 = \tfrac{1}{2}\,\overline{(3\,\cos^2\,\theta_3(t) - 1)} \tag{15}$$

are time averaged quantities. In the course of calculations, the averaging on time is replaced by a statistical averaging on θ_3 using a probability function $P(\theta_3)$ the shape of which we have to assume.

The function

$$P(\theta_3)\;\alpha\;\sin\,\theta_3\;e^{-\frac{q_3\,\cos^2\,\theta_3}{RT}} \tag{16}$$

which is analogous to a Boltzmann distribution with an orienting potential U,

$$U = q_3\,\cos^2\,\theta_3 \tag{17}$$

has been used successfully by several workers to interpret data from magnetic resonance measurements in liquid crystals [161, 164–166]. Inserting (16) into (15), one has:

$$S_3 = -\,\tfrac{1}{2} - \tfrac{3}{2}\,\frac{\int_0^{\pi}\cos^2\,\theta_3\;e^{-\frac{q_3\,\cos^2\,\theta_3}{RT}}\,\sin\,\theta_3\;d\theta_3}{\int_0^{\pi} e^{-\frac{q_3\,\cos^2\,\theta_3}{RT}}\,\sin\,\theta_3\;d\theta_3} \tag{18}$$

and analogous equations for S_1 and S_2. The one to one correspondance between S_3 and q_3 can easily be tabulated and, knowing S_3 one has then an evaluation of the orienting potential acting on the label still without computer simulation. q_3 values of 3 Kcal/mole for saturated and 2.4 Kcal/mole for unsaturated soap water smectic systems measured with I(13, 2) are reported [149, 164]. These values are temperature independant within the range 20–70° C which gives confidence that (16) is adequate for the description of the temperature behaviour of the order parameter.

When in a mesophase z' and $\vec{n}$ are colinear, the maximum splitting $A_{\max}$ measured from a sample having isotropic geometry is identical to $A_{\parallel}$ measured on an oriented sample. If the inspection of the spectra shows $A_{\max}$ to be greater than $A_{\parallel}$ one has a strong indication of the presence of a tilt $\bar{\theta}$ [167, 168], that is a bending of z', with respect to $\vec{n}$ which is long lived compared to the time scale of the linear response ESR experiment. A tilt can also be detected when the angular dependance of A_{obs} on β deviates from (8) [169]. But a precise evaluation of $\bar{\theta}$ requires accurate computer simulation of the spectra using the distribution function

$$P(\theta)\;\alpha\;\sin\,\theta\;e^{-\frac{(\theta-\bar{\theta})^2}{2\,\theta_0^2}}. \tag{19}$$

The existence of tilted spin-labeled fatty acid chains in phospholipid bilayers has first been reported by McConnell and McFarland [167], with a value $\bar{\theta} \sim 25°$ close to the polar head at room temperature for natural lecithin. This data has been confirmed using a different computational method (moment analysis [170]). In soap water

mesophases, the spin-labeled chains become tilted when the temperature is decreased, the tilt angle is 18° at −8° C with I(13, 2) as calculated by computer simulation [149].

From this section we can conclude that mainly orientational and structural information can be extracted from spin label spectra of anisotropic fluids by a simple "hand made" analysis based on a static hamiltonian.

Some criticism has been exerted about the validity of the use of the time independant $\mathscr{H}_{\text{eff}}$ for the evaluation of S_3 and a'. It has been shown that, when the averaging of the hamiltonian is effected by a motion which is fast around an axis z', a slowing down of this motion (still in the range under discussion now) leading to a decreased averaging, can result in an increase in the "hand made" observed $A_{\parallel}$, a' and S_3 without any change in microscopic order, so that the apparent trace and order parameter obtained are overestimates [136]. As we already mentioned this phenomenon hampers the use of a' for polarity measurements.

2. Dynamical Features and Simulation of the Spectra. Still starting from Equation (6), we can comment it a little too simply by saying that $\mathscr{H}_{\text{eff}}$ alone gives rise to a spectrum of infinitely sharp lines, the width of which originates in $\mathscr{H}_1(t)$ modulated by the molecular motion of correlation time τ_c.

When $\tau_c \leqslant 3 \cdot 10^{-9}$ s (Redfield limit) the linewidth T_2^{-1} can be calculated accurately applying perturbation theory till the second order only to the equation of evolution of the spin density matrix, and using a Lorentzian lineshape [149, 151, 166].

In the course of this calculation it appears necessary to make some hypothesis about the type of molecular reorientation that occurs in the system under investigation, or, equivalently, about the shape of the correlation function of the random molecular motion. The models of reorientation considered are: isotropic or anisotropic Brownian diffusion characterized respectively by diffusion coefficients D or $D_{\parallel}$ and $D_{\perp}$ if cylindrical symmetry is retained, jump diffusion and free diffusion [150]. The correlation function depends on the model chosen and, in anisotropic fluids, on the orienting potential described above [171, 177]. In the fast tumbling region the results of spectral computation show little or no sensitivity to the choise of the model. One then satisfactorily describes the reorientation with Brownian diffusion for which the correlation function is

$$f(\tau) \alpha\ e^{-\frac{|\tau|}{\tau_{cm}}} \quad \text{with} \tag{20}$$

$$(\tau_{cm})^{-1} = 6\ D_{\perp} + (D_{\parallel} - D_{\perp})\, m^2 \quad m = 0.2\,, \tag{21}$$

where different correlation times τ_{cm} describe the reorientation of the m different tensor components of anisotropic motion. Finally the calculated linewidth T_2^{-1} is

$$T_2^{-1} = T_2^{-1}\ (S_3,\ S_1,\ \bar{\theta},\ \theta_0,\ \beta,\ a,\ M,\ \tau_{cm}) \tag{22}$$

It depends on the anisotropy and order, as well as on the rate of molecular motion. We note the dependance on β [172, 177] so that, for an isotropic geometry of the sample, the proper average must be taken on β.

In isotropic media where no orienting forces are present, the main parameter influencing the spectra is the correlation time which is probably the most important dynamical parameter. It is even more characteristic of the events happening at a molecular level than the T_1 and T_2 currently obtained from NMR experiments and simpler to use, because it allows in many cases a determination of the local viscosity η of the surrounding medium

$$\eta = \tau_c \frac{kT}{\frac{4}{3}\pi r^3}, \tag{23}$$

(the molecular dimensions e.g. here, r for a sphere, being known) and also of the translational diffusion coefficient D_{tr} [173].

Several authors have proposed calculations leading to computer simulation of the ESR linear response spectra. Their basic assumptions are generally the same, they differ by the description of molecular motion. Instead of Brownian diffusion, for example, a model with rapid motion within a cone [174], or a restricted random walk model [175] is used (this last one, unfortunately does not include the correlation time as a parameter). The fitting of computed with experimental spectra is then a method of measurement also of the order parameters S_3, S_1, tilt and spread angles $\bar{\theta}$ and θ_0 and the only really accurate way of obtaining τ_c, also $D_{\parallel}$ and $D_{\perp}$. The agreement is good in general, it can be impressive [149].

For example, τ_c values of $1 \cdot 10^{-10}$ s to $2.8 \cdot 10^{-9}$ s have been obtained by this method in soap water smectics investigated with I(13, 2) in the temperature range 45° C to −8° C [149], $D_{\parallel} = 8.3 \cdot 10^8$ s^{-1} and $D_{\perp} = 1.0 \cdot 10^8$ s^{-1} are found with the androstanol label in the same system at room temperature [176]. In mixed multibilayers of dipalmitoyl lecithin and 10% cholesterol, studied with the cholestane label $D_{\parallel} = 1.4 \cdot 10^8$ s^{-1}, $D_{\perp} = 3.4 \cdot 10^6$ s^{-1} at 39° C [178]. At 21° C, values of $\tau_c = 2.3 \cdot 10^{-10}$ s, $\eta = 4.8 \cdot 10^{-2}$ poise and $D_{tr} = 1.5 \cdot 10^{-6}$ cm^2 s^{-1} in saturated soap systems, $\tau_c = 2.5 \cdot 10^{-9}$ s, $\eta = 0.5$ poise, $D_{tr} = 3.6 \cdot 10^{-8}$ cm^2 s^{-1} for aqueous dispersions of dimyristoyl lecithin are reported both with I(13, 2). This last value is in good agreement with membrane viscosities and lateral diffusion constants as determined by entirely different methods. Also the activation energy for the reorientational process can be obtained from the temperature dependance of τ_c [149, 178].

Owing to the difficulties involved in the simulation work, approximations have been designed, which can give good estimates of τ_c. Equation (22) can be written [179]:

$$(T_2(M))^{-1} = A + BM + CM^2. \tag{24}$$

At this point one must be careful, for the dependance of A, B and C on the τ_{cm} is not simple in anisotropic fluids [150], so that an approximation based on Equation (24) would give only a rough estimate of an equivalent single correlation time (except if the diffusion could be considered locally isotropic and ordering effects negligible). But in isotropic media (24) yields [6]:

$$\frac{T_2(0)}{T_2(M)} = 1 + \tau_c\,(B'M + C'M^2) \quad \text{hence} \tag{25}$$

$$\tau_c = K\left(\frac{T_2(0)}{T_2(M)} - 1\right) = K\left(\sqrt{\frac{h(M)}{h(0)}} - 1\right), \tag{26}$$

where $h(M)$ is the peak height of the first derivative absorption spectrum and K a numerical factor. Equation (26) has been used extensively in physicochemical as well as biological studies [18, 92, 93]. Arrhenius plots of τ_c obtained by this method allow the detection of conformational transitions [38, 39].

The formula

$$\tau_c = \frac{K'}{T_2(-1)}\left(\sqrt{\frac{h(+1)}{h(-1)}} - 1\right) \tag{27}$$

has also been proposed [180]*.

b. Intermediate Motion Region 10^{-9} s $< \tau_c < 10^{-7}$ s

Slower molecular motion effects in an increase in τ_c and an increase in $\mathscr{H}_1(t)$ due to decreased motional averaging of the hamiltonian. When $|\mathscr{H}_1(t)\,\tau_c| \sim 1$, the simple relaxation theory outlined above cannot be applied anymore. We shall not attempt a detailed description of the theoretical analysis of the linear response ESR lineshapes in this region.

One is based on the powerful stochastic Liouville method applied to the spin density matrix equation of motion, it has been developed for both anisotropic and isotropic media and allows accurate simulation of the spectra with the set of parameters of Equation (22) [181–184, 150]. It includes as a particular case, in the limit of short τ_c the analysis of § a. Remarkably enough, the spectra are now sensitive to the model of reorientation chosen during the calculation [150, 185]. In fact, molecular reorientation depends not only on τ_c, but also on the relative size and shape of the solute and solvent. Brownian diffusion satisfactorily accounts for the spectra of a macromolecule in aqueous solution (even for $\tau_c > 10^{-7}$ s), while jump diffusion is favoured when solute and solvent are of comparable size. The other way of computing the spectra is to use Bloch equations modified by adding a diffusion term [186].

Several methods have been proposed to evaluate τ_c without a complete simulation of the spectra, which is now longer and more difficult than in the rapid motion region. But before we explain them, let us mention a very important result pertaining to the spectrum of a random distribution of labels undergoing very anisotropic motion in this intermediate motion region we are considering. This situation is found for spin labeled hemoglobin in a viscous solvent, or for certain spin labeled phospholipids.

It has been shown [9, 84] that the outer hyperfine extrema of the first derivative spectrum have the absorption lineshape of an array of labels with their $2p\pi$ orbital

* But here also, even a slight slowing down of molecular motion can lead to artefactual results [217]

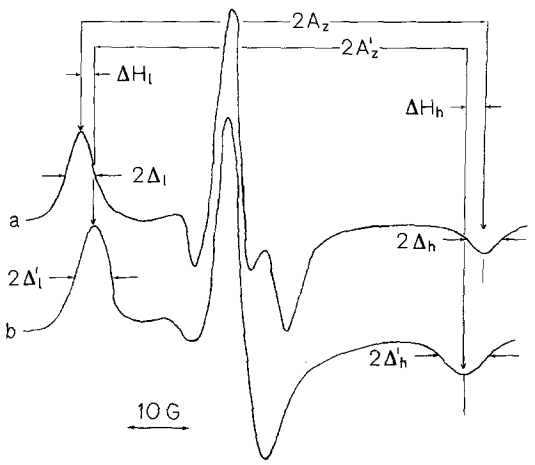


Fig. 4. Rigid limit (**a**) and slow tumbling (**b**) nitroxide linear response spectra demonstrating the measurements required for the parameters S, ΔH, W (see text)

parallel to $\vec{H}_0$, in other words, contribute to these outer peaks those labels oriented with the principal axis of their $\boldsymbol{g}$ and $\boldsymbol{A}$ tensors nearly parallel to $\vec{H}_0$. As a consequence, deviations from axial symmetry, which would affect mostly the center of the spectrum have no effect on these extrema which can be fitted separately in a calculation. For instance using an isotropic Brownian diffusion model, we can have from the simulation of these outer wings, information on the reorientation of the principal axis z even in an anisotropic situation, and measure the corresponding τ_c.

With the first calibration, a parameter S is defined [187]

$$S = \frac{A'_z}{A_z} \tag{28}$$

as the ratio of the distance between the outer hyperfine extrema of the spectrum in the conditions of the study A'_z and in a situation of complete immobilization of the label A_z. S is shown to be a sensitive monotonically increasing function of τ_c and computer simulation with the first method yields

$$\tau_c = a\,(1 - S)^b \tag{29}$$

– to within 3% for Brownian diffusion with $a = 5.4 \cdot 10^{-10}$ s, $b = -1.36$
– to within 2% for jump diffusion with $a = 2.55 \cdot 10^{-9}$ s, $b = 0.615$.
Also, the inward shift ΔH of the highfield hyperfine line with respect to complete immobilization is closely related to S [186]. It is found by computer calculations based on modified Bloch equations that

$$\Delta H \propto \tau_c^{-2/3} \quad \text{or} \quad \Delta H \propto \left(\frac{T}{\eta}\right)^{2/3}$$

for τ_c up to 10^{-7} s.

Values of $(12 \pm 2)\ 10^{-9}$ s at 20° C are reported for spin labeled α-chymotrypsin in aqueous solution [62] and of $1.5 \cdot 10^{-7}$ s for Tanol in sec-butylbenzene at −97.5° C [156].

A slightly different method is based on a calibration as a function of τ_c of the relative change in width Δ of the outer hyperfine extrema [188], it yields τ_c even up to 10^{-6} s by

$$\tau_c = a(W - 1)^{-b} \quad \text{where} \quad W = \frac{\Delta_i'}{\Delta_i} \quad i = h, l \quad \text{(see Fig. 4)} \tag{31}$$

with $a = 1.31 \cdot 10^{-8}$ s, $b = 1.033$ for free diffusion
and $a = 1.16 \cdot 10^{-8}$ s, $b = 0.943$ for Brownian diffusion.

This method is expected to be more sensitive to changes in τ_c when the motion is very slow for, when τ_c decreases from its rigid limit value, "lines begin to broaden before they shift".

c. Slow Motion $\tau_c > 10^{-7}$ s

For the description of molecular motion in this region of τ_c values, where the linear response spectrum is insensitive to changes in τ_c ("completely immobilized spectrum") the use of non linear ESR techniques is developing rapidly and seems very promising. Here again the interesting dynamical parameter is τ_c.

In a study of the system $3 \cdot 10^{-3}$ M Tanol in sec-butylbenzene (SBB) [156], for which the viscosity η as a function of temperature is known, the dispersion signal is recorded (see III.1) for different $\tau_c s$, also with different modulation frequencies ω_m. τ_c can be calculated from the tabulated values of η by inversion of (23). Furthermore, when τ_c overlaps the intermediate motion region, it is measured from the linear response spectrum with the calibration of ref. [186]. The shapes of the dispersion signals for constant $\omega_m \tau_c$ are almost the same, as predicted by the theory. The spectra of this well defined model system can then be used as reference spectra to obtain τ_c with other systems.

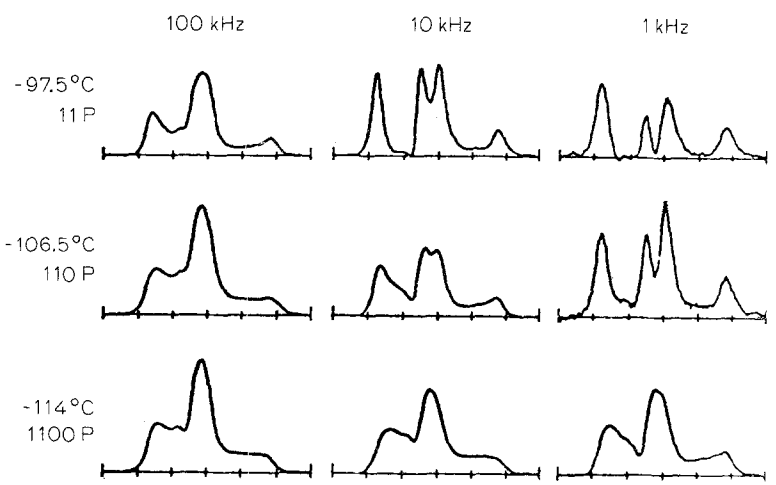


Fig. 5. Out of phase dispersion non linear EPR spectra for Tanol in supercooled sec-butylbenzene. The abscissa ticks are 20 G intervals. ω_m (in KHz), η (Poise) and temperature are shown on the figure. With τ_c proportional to η, note the similarity of the spectra for constant $\omega_m \tau_c$ (from ref. [156], with permission)

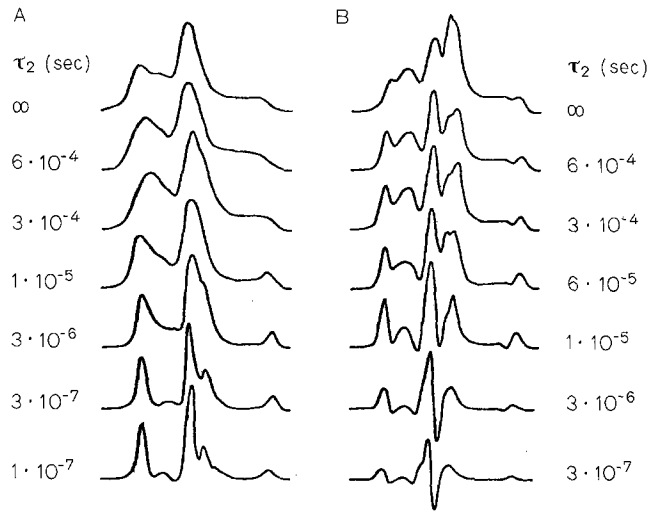


Fig. 6. Out of phase non linear ESR spectra, A: dispersion at the first harmonic of the modulation, B: absorption at the second, for maleimide labeled human oxyhemoglobin in glycerol/water mixtures. The τ_2 (or τ_c) values are calculated with a Debye formula from temperature and viscosity data, using a radius of 29 Å for hemoglobin (from ref. [159] with permission)

For maleimide labeled hemoglobin [157], the viscosity of the aqueous solvent was changed by the addition of glycerol, again τ_c was calculated from (23). From a qualitative comparison of the spectra with those obtained for Tanol in SBB, consistency of the estimated correlation times could be established. In both cases, the values of τ_c obtained range from $2 \cdot 10^{-7}$ s to $2 \cdot 10^{-5}$ s. But the best way of obtaining τ_c is to compare experimental spectra with computer calculated spectra.

Two procedures are, at present, available [216]. One is based on the already mentioned stochastic Liouville treatment of the spin density matrix equation modified to include the effects of saturation and of the modulation field [189, 190]. The other is a numerical solution of the Bloch equations in which the effects of the modulation field as well as the effects of Brownian diffusion are taken into account [186].

With both methods, sets of reference spectra can be obtained from chosen values of H_1, H_m, ω_m, τ_c and the molecular tensors [189]. Since isotropic diffusion is used in all these calculations, the simulation is poorer in the central region of the spectra than in the wings, if there is anisotropic motion in the sample. Yet, since the outer wings are sensitive to the reorientation of the principal axis z of the nitroxide tensors an accurate simulation of this region can yield a good value of τ_c for this axis. In the study of fragments and supramolecular complexes of muscle proteins, in solvents of varying viscosity, using the iodoacetamide and N-ethyl maleimide spin labels, the set of reference spectra was obtained from the Bloch equations calculations. τ_c was then determined by comparison of the outer experimental wings with these spectra, and when possible using the calibration of the inward shift ΔH on the linear response spectrum recorded at the same time. It could be shown that τ_c is essentially determined by solvent viscosity; values of τ_c ranging from 10^{-7} s to $3 \cdot 10^{-4}$ s are reported [191].

Investigation of molecular dynamics in the time range 10^{-7} s-10^{-4} s is now possible, and may be very useful in the future for the study of highly organized systems.

3. Interacting Spins. When the molar concentration c of a monoradical label increase, there is increasing probability that a given free electron "meets" another one, hence interacts with it.

a. The exchange interaction described by the hamiltonian

$$h\mathscr{H}_e = -J\,\vec{S}_1 \cdot \vec{S}_2\,, \tag{32}$$

results in the exchange of the two spin states (for instance $\alpha_1\beta_2$ becomes $\alpha_2\beta_1$) via the overlapping of their wavefunctions during collisions. It is clear that exchange is governed by the number of possible collisions between the labels, hence depends on both the viscosity of the medium and the diffusional characteristics of the probe [193, 194].

If the exchange interaction (J) is large enough, one electron spin will interact preferentially with the field created by the other electron during their collision, and this interrupts its interaction with the main magnetic field $\vec{H}_0$. If this interruption rate ν_e is sufficient, the coherence between the spins is lost, the line is broadened. On further increase of ν_e, when $\nu_e \sim a_N$ the hyperfine structure blurs, then gradually disappears into a single line [192].

b. The dipolar interaction between the electrons

$$h\mathscr{H}_D = \frac{g^2\beta_e^2}{r_{12}^3}\left(\vec{S}_1 \cdot \vec{S}_2 - 3\,\frac{(\vec{S}_1 \cdot \vec{r}_1)\,(\vec{S}_2 \cdot \vec{r}_2)}{r_{12}^2}\right) \tag{33}$$

depends on the orientation with respect to $\vec{H}_0$ as well as on the length of the distance vector $\vec{r}_{12}$ of the two electrons. Increasing molecular motion tends to average it out.

In the cases usually encountered with spin labels the conjugation of these two spin-spin interactions results in a line broadening which increases with c when $c > 0.05$.

For example, the spectrum of pure vesicles of spin labeled phospholipids (see Table 1) is a single broad line. In most experimental situations, the differences between a "concentration broadened" spectrum and a dilute spectrum (sharper triplet) can be easily detected by eye. This difference has been used without further analysis in studies of vesicle-vesicle and vesicle-membrane fusion: when vesicles of pure II are incubated with Acholeplasma laidlawii [195] or sarcoplasmic reticulum membrane [196] vesicles, the gradual appearance of a sharper triplet superimposed on the broad single line suggest that (1) the labeled vesicles have fused with the membrane; (2) the labeled lipids undergo fast lateral diffusion in the membrane. The value $D_{tr} = 6 \cdot 10^{-8}$ cm^2/s is reported for the lateral diffusion coefficient of II in sarcoplasmic reticulum membranes [196]. In an analogous fashion, if a highly concentrated patch of spin labeled lecithin III is included in oriented lecithin bilayers the study of the time dependance of the spectral changes also allows a measurement of the lateral

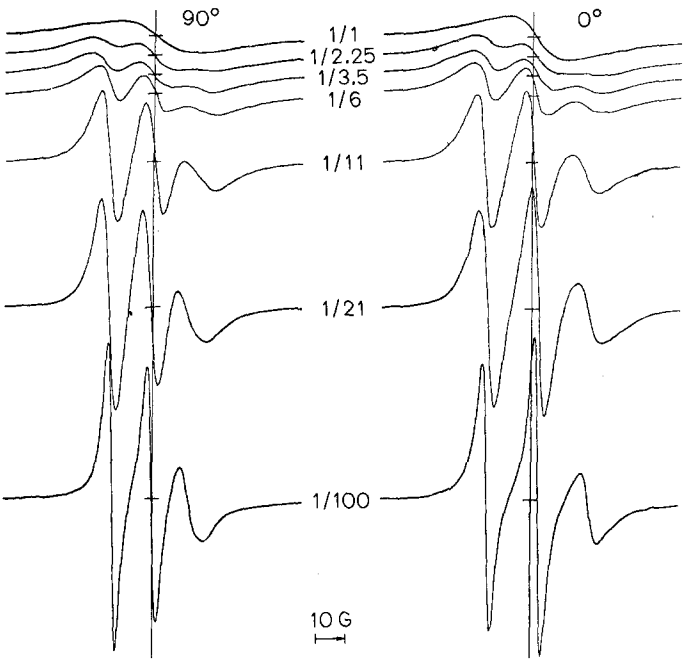


Fig. 7. Normalized linear response ESR spectra of multilayers of homogeneously mixed III and didihydrosterculoyl phosphatidylcholine. The molar ratio label/lipid is shown on the figure, the applied field $\vec{H}_0$ is perpendicular (90°) or parallel (0°) to the planes of the bilayers (from ref. [197], with permission)

diffusion coefficient D_{tr} of the spin labeled lipids. The value $D_{tr} = (1.8 \pm 0.6)\ 10^{-8}$ cm^2/s is reported at room temperature [197]. Also, the interaction between clustered labels is an evidence of lateral phase separation in phospholipids bilayers, for $t < 20°$ C and $c > 0.05$.

The published spectra of interacting labels have till now been obtained by linear response ESR. Calculation of these spectra has been achieved using a set of Bloch equations modified by the addition of an exchange and a dipolar term, both angular independant [198]. The agreement between simulated and experimental spectra is good, especially for labels having a weakly anisotropic motion (e.g. II(1, 14) or androstanol, see Table 1) and when exchange prevails rather than dipolar interaction, that is at higher temperatures. The exchange frequency $W_{ex} = \nu_e$ can then be obtained. Its concentration dependance yields the value $D_{tr} = 10^{-8}$ cm^2/s in vesicles of dipalmitoyl lecithin [199] in good agreement with independant measurements.

This method allows a detailed description of lipid model and biological membranes below and above their phase transitions [199].

IV. Reliability of the Spin Labeling Technique

The answer to our question: "how reliable are the answers we get with the spin labeling technique?" depends not only on the design and quality of the samples, on the now possible accuracy of our analysis of the ESR spectrum but also on the clear

knowledge of the properties of the labeled molecule used, of its location, anchoring, and of its eventual effects on the host system.

The presence on a given chemical species of the cycle bearing the paramagnetic NO group can confer to it some new physico-chemical properties or behaviour. We shall extend a little on the example of stearic acid I(5, 10) (also called 12 NS) and cholestane spin probes (see Table 1). The cholestane SL forms monolayers in which it is anchored to water via the oxazolidine ring. This is again an evidence for the polarity of the NO group (here the dipole form is stabilized by the hydrophilic environment). In mixed films with myristic acid, it brings a condensation effect, being very similar in this to cholesterol [200].

On the contrary, while stearic acid melts at 70° C, I(5, 10) melts at ~ 13° C. It also forms monolayers, in which the molecules are in a much more expanded state than in monolayers of pure stearic acid, which are liquid condensed or solid condensed. At high lateral pressure (~ 20 dyne/cm) I(5, 10) is erected normal to the surface, yet, at lower pressures it can adopt a bent conformation with both the acidic and NO groups anchored in the water surface which accounts for an extra-ordinary behaviour of its pressure versus area isotherms [201]. Since such a bent conformation with double binding to the polar interface has also been reported for I(5, 10) in dispersions of H. Cutirubrum lipids, this phenomenon can be a source of artefacts [202]. I(5, 10) (or 12 NS) mixes preferentially with liquid expanded monomolecular films and is still partially miscible with liquid condensed but not with solid condensed films [201]. Consequently, if label I(*m, n*) is used to investigate a two phase mixed bilayer system, or more generally, a heterogeneous system, its concentration will be higher in the more fluid region, and the spectra will not accurately reflect the fluidity of the entire system [203].

Magnetic resonance studies of deuterated lipid bilayers yield order parameters S_3 which are different from those obtained with spin labeling in identical situations; also, no tilted chains are found [204–207]. This last result together with theoretical considerations [211] has led to a questioning of the existence of cooperative tilting – or long range order – in the chain system, that had been deduced from spin label experiments [167].

For small surfaces per polar head, the deuterium order parameter curve shows a plateau of constant S_3, then falls to zero close to the center of the bilayer, whereas a rapid decrease all along the chain is found with spin labels. A general interpretation of this discrepancy in term of the different time scales of the two resonance techniques has been proposed [148], but it cannot be retained for soap systems [204, 214]. Also, other experimental findings indicate that a perturbing effect due to the presence of the oxazolidine ring cannot be ruled out.

Indeed, mixed films of I(5, 10) (or 12-nitroxide stearic acid) and myristic acid show positive deviations from ideality at low probe concentrations. The impurity effect is then represented by a 2–5° C increase in the host hydrocarbon chain mobility [208, 215], since even with dilute samples only the immediate environment of the probe is seen in the ESR spin labeling experiment while surface pressure measurements with monolayers yield averaged macroscopic quantities. Mixed films of lipids with 12-(9 anthroyl)-stearic acid, [where the fluorescent group is located at the same position on the chain as the oxazolidine ring on I(5, 10)] show an opposite behaviour: a condensation effect (negative deviation from ideality) is obtained [209]. Since

the anthroyl group is much more bulky than the oxazolidine ring, this data clearly shows that one mechanism of perturbation by amphiphilic spin labels in membrane like systems is a *breaking of the Van der Waals interactions by the relatively polar nitroxide.* Clearly this perturbing effect will not be the same for different oxazolidine positions on the chain, it will also depend on the surface pressure existing in the investigated bilayer.

No other systematic measurements which could lead to a general calibration of this perturbing effect seem to have been tried up to now, although good theoretical models are available for lipid bilayers [210]. Yet this would be precious for biological membranes studies, because of the unparalleled sensitivity of the spin labeling technique.

It has been shown that the place of anchoring of $I(m, n)$ to a bilayer vesicle system containing zwitterionic phospholipids (e.g. lecithin) is pH dependant [212, 213], hence also the measured order parameters. This phenomenon can effect in meaningless results if the system is not adequately buffered.

In protein studies, the label is generally chosen to have a specific interaction with its target (enzyme and substrate, antibody and hapten label, etc., see Table 1) so that its location is well defined. In the case of covalent labeling it must be ascertained that the label is rigidly bound [62], that is undergoes little or no motion relative to the tertiary structure, if one is interested in a faithful reflection of its dynamical environment. The perturbing effect can be estimated by checking on the biological function. These studies beeing often done in aqueous media, the problem now can be steric hindrance due to the cycle bearing the nitroxide, but this effect would become critical only when the whole label and the host molecules have comparable sizes.

Finally, that the main results obtained with spin labeling are semiquantitatively correct is now beyond doubt.

As compared with other techniques, spin labeling has two unparalleled advantages:

– its sensitivity. The signal to noise possibilities are now even improved by the recent technical advances in the ESR equipment, and the use of spectral accumulation,

– the spectra detect and can be used to describe motions on a uniquely broad range of time scales 10^{-11}–10^{-4} s.

In view of this, spin labeling appears to be a precious tool for comparative studies, also whenever the effects are big enough so that a certain error can be disregarded, that is especially in biological studies.

Acknowledgements. The author is grateful to Professor Ph. Devaux for critically reading the manuscript together with Dr. A. Bienvenue, and acknowledges stimulating discussions with Drs. J. Charvolin, J. L. Monge and M. Schott. She thanks Professor L. R. Dalton and Professor J. Seelig for sending a preprint of their review.

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Received July 6, 1976