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*Review*

# Use of electron spin resonance spectroscopy of spin labels for studying drug-induced membrane perturbation

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**Abstract**: The use of electron spin resonance spectroscopy of spin labels is reviewed in the context of drug-induced membrane perturbation. The correlation between membrane perturbation and biological effects is also considered.

**Keywords**: *ESR spectroscopy; nitroxide spin labels; drug–membrane perturbation.*

## Introduction

Many biological effects of pharmacologically active compounds result from their interactions with membranes. The interactions have various degrees of specificity leading to structural, dynamic, electrical and other physico-chemical changes of the properties of membrane components.

Electron spin resonance (ESR) spectroscopy of nitroxide stable radicals has been found a useful method for the detection of membrane properties and a suitable tool for studying the drug–membrane interaction at molecular or submolecular levels. The nitroxide stable radicals used in membrane studies include either spin probes (molecules that do not form a molecular bond with a host molecule) or spin labels (molecules with the nitroxide group covalently attached, giving spin-labelled lipids, proteins, drugs or other membrane components) [1–5]. However, some molecules may be spin labels in one system and spin probes in another. For simplicity, both spin labels and spin probes will be referred to as spin labels. Spin probes and spin-labelled lipids are primarily applied in lipid or biological membranes for detection of membrane dynamics, order, lipid–protein interaction, potentials, pH gradient, phase behaviour and permeability. Spin-labelled proteins and drugs reflect the behaviour of the proteins and drugs in systems under investigation.

Many reviews dealing with ESR methodology for membrane studies are available [1–3, 6–13]. Therefore the present review does not cover all applications of ESR spectroscopy in membrane research, focusing only on applying the technique of conventional *X*-band (9.5 GHz) ESR spectroscopy of nitroxide spin probes and spin-

labelled lipids for the detection of perturbation effects of drugs on model lipid and biological membranes.

The purpose of the present review is to survey the possibilities of ESR spectroscopy in this field and to summarize some of the results of drug–membrane interactions obtained using this technique.

## ESR Spectroscopy and Spin Labels

ESR spectroscopy is a technique for detection of unpaired electrons. In membranes, organic free radicals and transition metal free radicals possess unpaired electrons. However, the former are usually unstable and the latter cannot be detected at room temperature. In an attempt to utilize many of the benefits of ESR spectroscopy, a number of stable organic free radical spin labels were synthesized in the 1960s.

Spin labels are usually molecules containing the nitroxide moiety which possesses an unpaired electron localized on the nitrogen and oxygen atoms. The adjacent methyl or alkyl groups are necessary to stabilize the free radical. Such molecules are stable in a wide pH range. Typical spin labels used in membrane research are shown in Figs 1 and 2. These labels are specifically incorporated in the lipid or lipid part of biological membranes as shown in Fig. 2. Thus each label reflects properties of a different membrane region.

ESR spectrometers measure an absorption of microwave energy by spin labels in the samples as a function of magnetic field strength. A measurable net absorption of energy by the sample can occur when the unpaired electrons associated with spin labels move from the lower permitted energy state to the higher state. This transition of the electrons, detectable as the ESR spectrum, depends on the quantity, orientation, motion and other

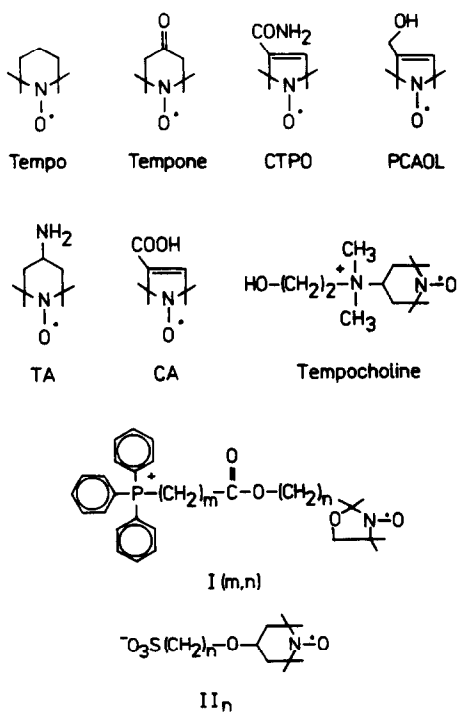


**Figure 1**
The chemical structure of spin labels.

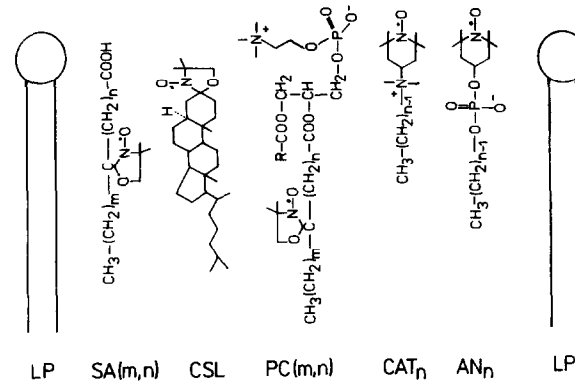


**Figure 2**
Membrane spin labels intercalated into lipid (LP) bilayer. Spin-labelled-stearic acid [SA(*m*,*n*)], -cholesterol (CSL), -lecithin [PC(*m*,n)], -cationic ($CAT_n$) and -anionic ($AN_n$) spin labels.

magnetic properties of the environment of the nitroxide moiety. In practice, ESR spectra are usually recorded as the first derivative of the absorption with respect to magnetic field strength versus the magnetic field. The basic physics of ESR spectroscopy of nitroxide spin labels has been reviewed [1–3, 6, 9–11, 14] and will not be discussed here.

*ESR measurement*

Details of the practical aspect of the ESR measurement were reviewed previously [2, 3, 11, 15].

*Sample cells.* For measurement of membranes primarily in aqueous solution, either commercial quartz flat aqueous sample cells with dimensions 6–15 cm × 0.5–1.0 cm × 0.25–0.8 mm i.d., or capillary tubes 0.2–1.0 mm i.d. are used. Sample volumes, depending on the kind of the experiment, are about 10–100 μl, containing 0.1–10 mg of lipids or membrane proteins. Usually lipid-to-label ratios of 100–500 are used to minimize label perturbation and label–label interaction. However, higher concentrations of spin labels are used to generate spin–spin exchange or dipole–dipole interactions.

*ESR instrumental parameters.* Field modulation amplitude, microwave power, scan time and filter time constant must be properly set to avoid distortion of the spectrum. Parameter settings depend on the physical condition of the spin label. The value for modulation amplitude should be equal to, or less than the ESR linewidth in gauss (G), frequently 0.5–2 G. A microwave power of 1–5 mW appears to be acceptable for near room temperature spin-labelling studies. Scan times of 4–8 min at 100 G are suitable. The time constant must be much shorter than the time required to sweep through the ESR line. Proper adjustment of instrumental parameters can be checked experimentally [2, 3, 11, 15].

*Spin labels*

*Synthesis.* The synthesis of spin labels is described in previous reviews [12, 16–19]. Detailed syntheses of small nitroxides are given by Rozantsev [16] while synthesis of

nitroxide moieties attached to molecules is given by Keane *et al.* [20]. The nitroxide moiety 4′,4′-dimethyloxazolidine-*N*-oxyl for simplicity is referred to as the "doxyl" moiety. The synthesis of a variety of fatty acids containing the doxyl nitroxide group has been reviewed [19]. Examples of the use of doxyl fatty acids for the synthesis of spin-labelled lipids are given for spin-labelled cardiolipin [21], phosphatidylethanolamine [22], phosphatidylcholine [23, 24], gangliosides [25] and sulphatides [26]. Many of these spin labels are commercially available.

*Biological effects of membrane spin labels.* In recent years, the use of nitroxide spin labels for studies of biological systems has greatly increased. Among the various biological effects reported for spin labels, reversible blocks to conduction of rat phrenic nerve ($ED_{50}$ = 6.4 mmol $l^{-1}$) [27] and frog sciatic nerve ($ED_{50}$ = 13 mmol $l^{-1}$) were described for Tempo [28]. The spin label 4-hydroxy-Tempo (Tempol) was found to be mutagenic using *Salmonella typhimurium* tester strain TA 104. The nitroxide mutagenicity was increased in the presence of superoxide radical generating system [29]. With the exception of 4-maleimido-Tempo, water soluble spin labels did not inhibit Chinese hamster ovary cell survival at 1 mmol $l^{-1}$ concentration [30]. At concentrations commonly used for spin labelling of cells (30–50 μmol $l^{-1}$) none of the stearic acid SA(12,3), SA(5,10) and SA(1,14) spin labels was found to be cytotoxic [30]. Spin-labelled stearic acids had no effect on growth of bacteria at concentrations of $3 \times 10^6$ labels per cell (label/lipid, 1:25) but at concentrations $2.5 \times 10^8$ labels per cell, the majority of cells were killed within 15 min [31]. Labelled stearic acids stimulated the $Na^+$–$Ca^{2+}$ exchange and passive $Ca^{2+}$ permeability in cardiac sarcolemmal vesicles [32].

*Loss of ESR signal of spin labels.* Loss of the ESR signal of nitroxide spin labels in biological samples can result from reduction of the nitroxide to hydroxylamine by ascorbate, dithionite, hydroxylamine, phenylhydrazine, chlorpromazine, etc. [33–36], reaction with free radicals involved in metabolic processes in cells [37–43], or oxidation of nitroxide by an iron–hydrogen peroxide–amino acid system [44]. Spin label reduction may be reversed upon addition of oxygen [1] or $K_3Fe(CN)_6$ [46–48]. However, studies on the ESR signal loss can yield information about metabolic changes in the cells [41, 42] and have potential utility for investigation of cellular free radical reactions [43].

*Antioxidant activity.* Nitroxide radicals were found to have marked antioxidant activity in unsaturated lipids [49, 51]. The ESR signal of nitroxides was shown to decrease when lipids in membranes were oxidized, as related to the reaction of nitroxides with free radicals produced in the course of lipid oxidation in membranes or during the catalysed decomposition of hydroperoxides. The loss of the ESR signal can be used as a sensitive method to study lipid peroxidation [49, 51].

## ESR Spectral Parameters

Only the spectral parameters of conventional ESR from the *X*-band (9.5 GHz) instrument will be discussed here. Details of the parameters have been presented in several review articles [2, 6–8, 11, 13]. The parameters most often evaluated from the spectra of spin-labelled fatty acids or lipids in membranes are outer ($2A'_{\parallel}$) and inner hyperfine splittings ($2A'_{\perp}$) (Fig. 3C) [13, 52]. In the more dynamic and isotropic parts of membranes the line widths, line heights and isotropic splitting ($a_0$) can be evaluated

(Fig. 4A) [2, 86]. The spin label Tempo in membrane–buffer systems exhibits superposition of two ESR spectra, one coming from the polar aqueous (P) and the other from the non-polar hydrocarbon lipid (H) region (Fig. 3B) [54]. In the slow motion of spin labels full line widths at half-height of the hyperfine extremes and parameter $2A^*_{zz}$ can be evaluated (Fig. 4B) [55, 56]. In order to determine spin label concentration in a sample, it is necessary to integrate the first derivative spectrum twice (Fig. 3A). The height $H_n$ is proportional to the spin concentration [3]. Possible errors of interpretation of the ESR spectral parameters were previously discussed [6, 64].

## ESR Membrane Parameters

Using the spin labels as shown in Figs 1 and 2 different membrane parameters can be measured [1–3, 6–8, 11, 13]. ESR spectroscopy of spin labels has been applied primarily to studying the effect of drugs on membrane dynamics, order and phase transition, whilst

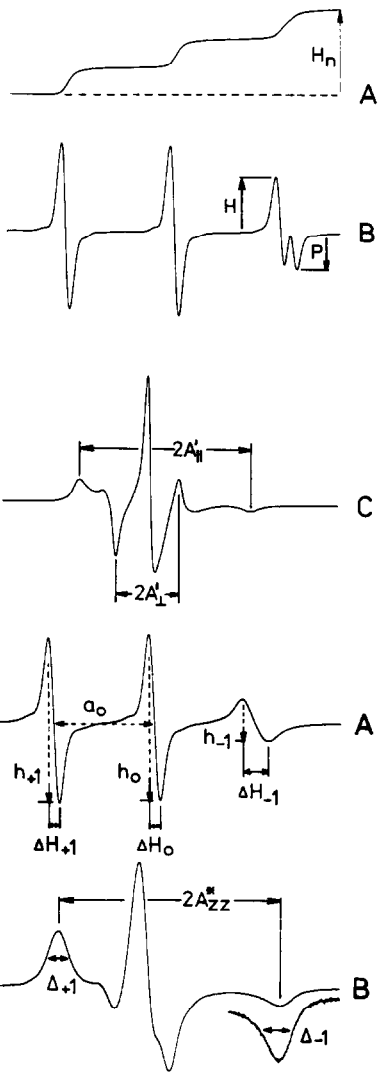


**Figure 3**
ESR spectra of spin labels in lecithin liposomes (5 mg lecithin and 10 μg label in 50 μl buffer (100 mmol $l^{-1}$ KCl, 1 mmol $l^{-1}$ Tris–HCl, 7.4 pH). A, second integral of the spectrum B. B, Tempo in lecithin–buffer liposomes; temperature 25°C; spectrum width 50 G. C, SA(12,3) in lecithin–buffer liposomes; temperature 25°C; spectrum width 100 G.




**Figure 4**
ESR spectra of the spin label SA(1,14) in lecithin liposomes (prepared as in Fig. 3). A, temperature 25°C. $h_0$, $h_{-1}$ and $h_{+1}$ are the peak-to-peak amplitudes of the first derivatives resonance of the central, high- and low-field peaks, respectively. $\Delta H_0$, $\Delta H_{-1}$ and $\Delta H_{+1}$ are the peak-to-peak linewidths of the lines. Spectrum width 50 G. B, temperature −40°C. $2A^*_{zz}$ is the apparent hyperfine splitting. $\Delta_{+1}$ and $\Delta_{-1}$ are the full linewidths at half height of the hyperfine extrema. Spectrum width 100 G.

drug effects on other membrane parameters (see below) have so far been rarely or not at all elucidated.

*Phase transitions*

The ESR spectrum of the label Tempo in a lipid–buffer system was used to measure the endothermic phase transition of lipid membranes [54, 57, 58]. The spectrum is a superposition of two spectra, aqueous (P) and hydrocarbon lipid (H) (Fig. 3B) [54]. The amplitude ratio $f = H/(H + P)$ is proportional to the amount of Tempo in the lipid phase and is a measure of fluid lipid membranes [54]. The change of the $f$ parameter versus temperature in lipid membrane was used to measure the phase transition temperature [57]. Alternatively, the change in Tempo partitioning at the phase transition can be followed either by continuously monitoring the height of the aqueous, P [58], or the line height of the central line $h_0$ of membrane labels. This line height increases with the increase in molecular motion at the phase transition [7, 59]. The phase transition can also be detected from the temperature dependence of the $2A'_{\parallel}$ and $2A'_{\perp}$ parameters [60, 61]. The degree of Tempo partitioning is presumably determined by the amount of free volume in the hydrophobic portion of membranes, which is a consequence of fluidity [7]. The shape of the phase transitions can depend on the method of sample preparation [62] and kinetics of measurement [63]. However, the restricted motion or spatial arrangement of labels in membranes may produce artifacts in the $f$ parameter; for detailed discussion see ref. 64.

*Phase separation.* Binary mixtures of phospholipids undergo gel–liquid phase transition over a wide temperature range, where within the transition solid and fluid lipid coexist within the plane of the bilayer in a state of lateral phase separation. Phase diagrams of lipid mixtures can be obtained by the Tempo partitioning method [7, 54].

*Hexagonal lipid phase.* Certain phospholipids, including phosphatidylethanolamine undergo a phase transition between fluid lamellar and reverse hexagonal liquid crystalline phases. Spin-labelled lipid at the acyl chain, and steroid spin label (CSL) indicate both gel–fluid lamellar and lamellar–hexagonal phase transitions in phosphatidylethanolamine dispersions [65]. The transition to the hexagonal phase was accompanied by an increase in conformational freedom of the acyl chain, more pronounced towards the methyl terminus. On the other hand, the lamellar–hexagonal transition could not be unambiguously assigned from the ESR spectra of spin labels SA(12,3), SA(5,10), and CSL in mixtures or oriented systems of equimolar phosphatidylethanolamine and cholesterol [66].

*Interdigitated lipid phase.* When a bilayer is made from lipid having different chain lengths, a mixed interdigitated phase is formed. In this phase the shorter chain of lipid on one side of the bilayer is packed end to end with the longer chain of lipid on the other side of the bilayer. It was found, that the hyperfine splitting, $2A'_{\parallel}$, of the ESR spectrum of SA(1,14), was significantly greater, i.e. the spin label was motionally restricted, in an interdigitated lipid bilayer than in non-interdigitated phases of lipid bilayers [67, 68].

*Membrane order and dynamics*

*Order parameter* S. Details of the theory of the order parameters are covered in the reviews [1–3, 6–11]. Phospholipid molecules in membranes undergo anisotropic motion

as a result of wobbling of the lipid chains and fast rotations around their long molecular axis. Spin-labelled fatty acids and lipids (Fig. 2) have been primarily used to reflect this anisotropy. When molecular motions of the labels in membranes are fast on the ESR time scale (correlation time $\tau_c < 3 \times 10^{-9}$ s), the hyperfine splittings, $A_\parallel = A'_\parallel$ and $A'_\perp$, can be measured from the spectrum (Fig. 3C). The correction for $A'_\perp$ is given by the formula

$$A_\perp = A'_\perp + 1.4\ \{1\text{-}(A_\parallel - A'_\perp)/[A_{zz} - 0.5(A_{xx} + A_{yy})]\},$$

where $A_{xx}$, $A_{yy}$ and $A_{zz}$ are the principal hyperfine splittings (in gauss, G) corresponding to the molecular axes of the spin label [69]. The hyperfine splittings for 2-doxylpropane measured in oriented single crystal hosts are 5.9, 5.4 and 32.9 G for $A_{xx}$, $A_{yy}$ and $A_{zz}$, respectively [70]. The hyperfine splittings for SA(12,3) are 6.3, 5.8 and 33.6 G for $A_{xx}$, $A_{yy}$ and $A_{zz}$, respectively [69]. Since the hyperfine splitting also depends upon the polarity of the nitroxide environment, a polarity normalization term, $a'_0/a_0$, has been introduced into the calculation of the order parameter $S$:

$$S = \frac{A_\parallel - A_\perp}{A_{zz} - 0.5(A_{xx} + A_{yy})} \times \frac{a'_0}{a_0},$$

where $a'_0 = 1/3(A_{xx} + A_{yy} + A_{zz})$, and $a_0 = 1/3(A_\parallel + 2A_\perp)$ [52].

For rigid or fluid ESR spectra of the labels, where only $A'_\parallel$ or $A'_\perp$ splittings can be measured, the following formulae for order parameters have been applied [71, 72]:

$$S_\parallel = 0.5\left[\frac{3(A_\parallel - A_{xx})}{A_{zz} - A_{xx}} - 1\right],$$

$$S_\perp = 0.5\left[\frac{3(A_{zz} + A_{xx} - 2A_\perp)}{A_{zz} - A_{xx}} - 1\right],$$

where values of $A_\perp = A'_\perp + 0.8$, $A_{zz} = 32.4$ and $A_{xx} = 6.1$ G were used [71]. Increasing concentration of the label SA(12,3) in biological membranes decreased both $S$ and $S_\perp$ parameters but exerted no effect on the $S_\parallel$ parameter [73–76]. It was therefore suggested that changes of these order parameters were not due to an increased mobility of the membrane, but were instead the result of enhanced label–label interactions, and thus a label clustering in biological membranes could be measured [75, 76].

Generally, the order parameter refers to the amplitude of molecular motion within a given frequency or time domain. The limits are $S = 1$ for no motion and $S = 0$ for complete isotropic averaging [2, 6, 77]. For molecular motion with slower correlation times ($\tau_c > 3 \times 10^{-9}$ s) the line positions depend on the rate as well as the amplitude of motion. For these motions a spectral simulation is needed to obtain proper ESR spectral parameters [2, 78, 79]. Spectral simulation with the inclusion of one order parameter and two correlation times were suggested [6]. However, in slower motion, the parameters $A'_\parallel$ and $A'_\perp$, $\Delta H_{+1}$ and $\Delta H_0$ can be used as a convenient empirical measure of dynamics, including both amplitude and rate motion [7]. Decrease of the parameters $S$, $S_\parallel$, $S_\perp$, $A'_\parallel$, $\Delta H_{+1}$, $\Delta H_0$ or increase of $A'_\perp$, indicate higher disorder and/or dynamics of the hydrophobic part of membrane [6, 69]. At much slower motion ($10^{-7} \leq \tau_c \leq 10^{-3}$ s) saturation transfer ESR can be applied [10, 80, 81].

Taylor and Smith [82–84] tested the reliability of spin labels by comparison of the data derived for the nitroxides and corresponding deuterium NMR probes in lipid system. They found poor agreement of order parameter changes using stearic acid spin labelled between $C_2$ and $C_{12}$ of the acyl chain. Better agreement was found when the acid was labelled at $C_{14}$ and $C_{16}$ positions. In contrast, good agreement was found in a similar comparison of nitroxide and deuterium-labelled steroids. They concluded that the orientation of the doxyl group of the spin-labelled fatty acid is disturbed in highly ordered membrane regions $C_2$–$C_{12}$ and less perturbed in the more fluid hydrocarbon core. Detailed lineshape simulations have shown that the spectra of lipid spin labels in fluid bilayers contain important contributions from slow molecular motions [78]. Thus the calculated order parameters and the rotational correlation times (described below) can be considered apparent values. These parameters are useful for relative comparisons of propensities of drug effects on membranes and for intercomparison between different membranes [78].

*Rotational correlation time.* The theory of correlation time $\tau_c$ has been reviewed [2, 6, 8, 10, 11, 14]. For fast isotropic motions of spin labels ($\tau_c \sim 10^{-11}$–$10^{-9}$ s) the apparent rotational correlation time of spin labels can be obtained from linewidth and line amplitude measurements (Fig. 4A) using the formulae below:

$$\tau_1 = 6.5 \times 10^{-10}\, \Delta H_0\, [(h_0/h_{-1})^{1/2} - (h_0/h_{+1})^{1/2}]$$

$$\tau_2 = 6.5 \times 10^{-10}\, \Delta H_0\, [(h_0/h_{-1})^{1/2} + (h_0/h_{+1})^{1/2} - 2]$$

$$\tau_3 = 6.6 \times 10^{-10}\, \Delta H_{+1}\, [(h_{+1}/h_{-1})^{1/2} - 1]$$

$$\tau_4 = 6.5 \times 10^{-10}\, \Delta H_0\, [(h_0/h_{-1})^{1/2} - 1]$$

[6, 14, 85–88].

However, the motion of spin labels in membranes or cells is most often not fully isotropic. The increase of the parameters $\tau_{2-1} = \tau_2 - \tau_1$ and EPS $= [(h_0/h_{+1})^{1/2} - 1]/[(h_0/h_{-1})^{1/2} - 1]$, indicates higher motional anisotropy, molecular ordering, or motion in the slow time domain in apparent fast isotropic spectra [6, 10, 86, 89]. The ratio of the amplitudes $h_{-1}/h_0$ and $h_{+1}/h_0$, $\Delta H_{+1}$ and $\Delta H_0$ parameters were shown to correlate with mobility of the environment of spin labels [9, 90–92]. For measuring $\tau_c$ in the time range of $\tau_c \sim 10^{-8}$–$10^{-6}$ s a spectral simulation is needed [2, 55, 56, 93–96].

Goldman *et al.* [93] showed that the $\tau_c$ in the time range of $\tau_c \sim 10^{-8}$–$10^{-7}$ s can be related to the apparent hyperfine splitting $A^*_{zz}$ by the expression

$$\tau_c^{-1} = a_z\, [1 - (A^*_{zz}/A^0_{zz})] b_z,$$

where $a_z$ and $b_z$ are parameters derived from spectral simulation, and $A^0_{zz}$ is the true hyperfine splitting in the absence of motion (i.e. the rigid limit splitting; Fig. 4B) [94]. The slow-motion hyperfine extreme linewidths were also found sensitive to rotational motion over the time range of $\tau_c \sim 10^{-8}$–$10^{-6}$ s by the approximate expression:

$$\tau_c = a_m\, (\Delta m/\Delta m^0 - 1)^{-b_m},\ [55, 94]$$

where $a_m$ and $b_m$ are parameters derived from spectral simulation, and $\Delta m$ is the full line widths at half height of hyperfine extrema (Fig. 4B). The $\Delta m^0$ factor is the linewidth in the rigid limit, while $\Delta m$ is the linewidth in the presence of motion [55, 97].

The rotational correlation time $\tau_c$ is related to the viscosity, $\eta$, by the Debye equation $\tau_c = 4\pi r^3 \eta / 3kT$, where $kT$ is thermal energy and $r$ is the radius of the spin label [98].

*Drug disordering propensities.* The disordering propensities or perturbation effect of drugs, detected by spin label, were quantitated as a change in order parameter per unit concentration of the drug [99]. In order to compare the perturbation propensities of drugs in membranes, detected by different spin labels at various membrane depths and temperatures, the following parameters were found useful: $P_s = \Delta S/(\Delta S_0/\Delta T)$, $P_{\parallel} = \Delta A_{\parallel}/(\Delta A_{\parallel 0}/\Delta T)$ and $P_{\perp} = \Delta A_{\perp}/(\Delta A_{\perp 0}/\Delta T)$ [100–102]. $\Delta S$, $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ are changes in the parameters after drug addition. $\Delta S_0/\Delta T$, $\Delta A_{\parallel 0}/\Delta T$ and $\Delta A_{\perp 0}/\Delta T$ are temperature gradients of the control membrane parameters. The parameters $P_s$, $P_{\parallel}$ and $P_{\perp}$ express the temperature effect necessary to reach the same values of the ESR parameters in the control membrane as in the sample with fixed temperature but with drugs. Effects of drugs on other membrane parameters can correspondingly be expressed in the temperature scale.

*Detection of different membrane parts*

Spin labels are usually incorporated specifically into various membrane regions [2, 52, 70, 103]. Depending on the location of the nitroxide group, the spin label reflects the properties of different membrane regions (Fig. 2).

*Membrane surface.* The spin-labelling approach for studying membrane surfaces can be used in different ways. Phospholipids spin-labelled at the polar head group [33, 105] and spin labels $CAT_{12}$ and $CAT_{16}$ incorporated into the lipid phase with nitroxide group exposure on membrane surfaces are most often used [106–110]. In other methods, the selective labelling of sialic acid residues of glycoproteins and glycolipids with nitroxide spin label are utilized to characterize cell-surface phenomena [110–112]. From the ESR spectra, the relative head group mobility in slow motion can be estimated by measuring the hyperfine splitting parameter $2A'_{\parallel}$, and in fast motion by calculation of apparent correlation times [108–110, 112]. The CLS label has the nitroxide group at the polar membrane firmly attached to the steroid nucleus with the acyl chain pointing towards the centre of the bilayer. Thus the ESR spectra reflect the order and motion of the entire steroid nucleus [113–116]. The order and motion of the spin label can be estimated by measuring the $A'_{\perp}$ parameter or by the ratio of amplitudes of the central and low field peaks [6, 114, 116]. Simulation of ESR spectra was applied for obtaining order parameter or correlation times [117, 118]. An androstanol spin analogue in sonicated vesicles was, however, found to experience two opposite orientations in lipid membrane, presumably with a rapid reorientation [113].

*Hydrophobic membrane region.* Spin labels with the doxyl moiety located at different positions on the acyl chain of fatty acids or lipids (Fig. 2) reflect dynamics and order at different membrane depths [2, 3, 6–8, 52, 70]. Depending on membrane samples, dynamic and structural parameters can be evaluated [2, 3, 6–8].

*Asymmetric lipid distribution.* To study this phenomenon spin-labelled lipids with a short β-chain ($C_5$) bearing the doxyl group at the fourth position which were immediately incorporated into biological membranes were used. The orientation of the spin labels was assessed in the bilayer by addition of the non-permeate reducer (ascorbate at 5°C) to the medium or by following spontaneous reduction at 37°C due to the endogenous reducing agents present in the cytosol [119]. This ESR spin-labelling method was applied for evaluating deformability of red blood cells [120–122].

*pH-dependent ESR spectra.* The ESR spectra of the spin labels bearing amino and carboxyl groups depend on the pH of the solution [123]. The ionizable groups of the spin labels affect the isotropic hyperfine splitting ($a_0$) and lower $g$-factor values. The ESR spectra of spin labels SA(12,3) and SA(5,10) were found to be pH-dependent in dispersions of lecithin and lecithin–cholesterol mixtures. Anisotropy of motion of the spin labels increased (order parameter $S$ increased) as the pH values were changed from 4 to 7, and a superposition of two spectra was seen when the temperature of the sample was >30°C [124]. The increase of anisotropy was attributed to a vertical shift of the spin labels in the bilayer, depending on ionization of their carboxylic acid groups [125]. In intact erythrocytes and ghosts the $A_{\parallel}$ values of SA(12,3) remained almost constant over the pH range from 3 to 9. On the other hand, for the spin label SA(5,10), the $A_{\parallel}$ values increased with increasing pH [126]. Similar results were found for the label SA(1,14) in bovine brain lipid membranes, where the parameter $A_{\perp}$ decreased as the pH values increased from 6 to 8 [127].

*Proteolytic activity.* The increase in the ratio of low-field amplitudes of weakly and strongly immobilized signals of ESR spectra of a maleimide spin label bound to erythrocyte membranes was attributed to self-digestion of membrane proteins by endogenous proteinases, and was attenuated by proteinase inhibitors [128]. From this, the method for the assay of proteolytic activity based on the measurement of changes in the weakly and strongly immobilized signals of substrate proteins labelled with the maleimide spin label was proposed [129].

*Membrane electrical potentials*

Spin labels are used in lipid or biological membranes for the estimation of transmembrane potential [130–132], surface potential [7, 133–136], boundary potential [137, 138], changes in dipole potential [139, 140] and transmembrane pH gradient [131, 141–146]. For details see refs 7, 145, 147.

In these techniques, except for pH-gradient determination, spin labels that partition in membranes are used. Thus, in the presence of membranes, there are two equilibrium populations of such spin labels: one bound to the membrane and the other free in solution. In aqueous solution, the motion of spin label is rapid and so gives rise to narrow, intense lines. When associated with the membrane, the motion of the label decreases and the lines in ESR spectrum are broad and low in amplitude [7, 147]. The motional differences in the two populations thus allow resolution of their individual contributions to the overall ESR spectrum, and hence a determination of the number of bound and free labels. The bound/free ratio, depending on experimental approach, is related to different electrical potentials.

*Transmembrane potential.* ESR spectroscopy is used to monitor the distribution of

membrane permeable hydrophobic cation labels (e.g. *I*(*m*,*n*), Fig. 1) between aqueous and membrane phases which depends on transmembrane potential.

*Surface potential.* This potential is a consequence of fixed-charge density associated with lipid or protein at the membrane solution interface [149]. The cationic or anionic spin labels (e.g. $CAT_n$ or $AN_n$, Fig. 2) bind strongly to the membrane surface and are not expected to penetrate the low dielectric interior of membranes. Thus these labels will establish a binding equilibrium with the external membrane surface with bound/free ratio related to surface potential. Except for model lipid membranes this method was used in purple membranes [150, 151], photoreceptor membranes [137, 138], mycoplasma cell membranes [152] and chloroplast thylakoid membranes [143]. However label $CAT_{12}$ was found not suitable for measurement of surface potential in mitochondria [153, 154].

*Transmembrane pH gradient.* In this technique, spin-labelled amines and weak acids (e.g. TA or CA, Fig. 1) which show little binding but high permeability to membranes are primarily used. The method relies upon the assumption that the uncharged species are permeable and the charged species are not. By adding an impermeable paramagnetic broadening agent such as $K_3Fe(CN)_6$ to the external solution, the ESR signal from an externally located spin label becomes extremely broad and of low intensity. In this way it is possible to independently determine the amount of internalized spin labels. Changes in the internal/external label ratio are related to ΔpH. The pH gradients were investigated in photoreceptor [137], thylakoid [143] and cell envelope vesicles of *Halobacterium halobium* [144].

*Internal volume in liposomes and cells*

In the following method, membrane permeable, yet water soluble labels (e.g. Tempone) are used. The method consists of quantitating the label signal in the internal volume when the signal from the external compartment is removed by using broadening ($NiCl_2$, $K_3Fe(CN)_6$, $K_3Cr(C_2O_4)_3$) or reducing (ascorbate) agents [7, 33, 131, 154, 155]. This method can also be applied for detection of fusion of lipid vesicles [155] or liposome with cells [156]. The major shortcoming of applying this method in the cells is that cell organelles can reduce nitroxide labels to their non-paramagnetic hydroxylamine derivatives. This complication can be avoided in some cases by using a low concentration of $K_3Fe(CN)_6$ (1 mmol $l^{-1}$) to maintain nitroxide in its oxidized state [46–48].

*Membrane permeability*

Using suitable spin labels (e.g. Tempocholine) the permeability of liposomes or biological membranes for the label, broadening or reducing agents, or ions can be measured [7, 38, 103, 156, 158]. The permeability for the spin label can be determined by adding water soluble label to the liposome or cell suspension, removing the external signal by broadening or reducing agents, and by measuring the kinetics of appearance of the internal ESR signal [159]. However, the impermeability of the broadening or reducing agents and reduction of the label in the cell should be controlled. The decay or broadening of the ESR signal of water soluble label within the liposome or cells can indicate the permeability of the membrane for the broadening or reducing agents. The permeability profile of a lipid bilayer to ascorbate can be determined by the reduction of the membrane spin labels by ascorbate [103].

*Transmembrane currents.* The time-dependent ratio of free and membrane bound ESR signals of labels [e.g. $I(m,n)$] rapidly mixed with lipid vesicles was related to transmembrane transport of the labels. This method can be used to investigate time-dependent electrical phenomena in membranes and to estimate transmembrane currents of the labels [160] or other ions (e.g. $H^+/OH^-$) [139, 140, 161].

*Incorporation of lipids into membranes.* The ESR spectra of spin-labelled lipids distinguish the labelled lipid which is integrated into the membrane from the unincorporated or peripherally associated label. Spectral subtraction or relative peak intensities of the two lipid populations have been used to quantitate the degree of incorporation of the labelled lipids into lipid or biological membranes [162–165]. Spin-labelled phospholipids at polar or hydrophobic regions were used to measure the transfer of phospholipids from virus envelopes to target cell membranes [166] and Tempocholine was used to measure its release out of preloaded virus particles upon interaction with cells [167].

*Polarity profile of membranes.* The isotropic splitting parameter, $a_0 = 1/3 \times (A_{\parallel} + 2A_{\perp})$, reflects the polarity of the local surroundings of the nitroxide group of spin labels and it increases with increasing the polarity [11, 72]. The polarity profile across the bilayer can be measured by using spin-labelled fatty acids or lipids [2, 7].

*Lateral diffusion*

The lateral diffusion of membrane spin labels, within the plane of the membrane bilayer, leads to a collision between the labels resulting in spin–spin exchange and dipolar interactions. In weak exchange these interactions broaden the ESR lines. Using moderately high concentrations of the labels, provided the dipolar interaction is minimized, line width of the ESR spectrum is related to this lateral diffusion [7, 11, 168]. Sachse *et al.* [169] and King *et al.* [170] introduced a method using the spin–spin broadening of the nitroxide spin labels at low concentrations (0.2–2.0% labels in lipids) to determine lipid translational diffusion coefficients in lipid membranes. Time dependence changes of the ESR spectra of a highly concentrated region of spin-labelled lipid in lipid bilayers was also used for calculating lateral diffusion [171]. Steady-state continuous wave electron–electron double resonance [172, 173] and short-pulse saturation recovery ESR [174] were applied to measure lateral diffusion of labels in membranes. Feix *et al.* [175] introduced two stearic acids spin labelled at different positions on the acyl chain. One of these contained the $^{14}N$-isotope and one the $^{15}N$-isotope. On applying this approach they could measure "vertical fluctuations" of the acyl chains. Application of spin-labelled lipid at the head group plus ascorbate allowed measurement of the probability of spin label passage from the external monolayer to the internal monolayer (flip-flop) in lipid vesicles [105] or excitable membrane vesicles [176].

*Lipid–protein interaction*

In membranes, integral proteins are embedded in the lipid bilayer. Lipids at the protein–lipid interface have different physical properties, compared with the bulk lipids. ESR spectroscopy of lipid spin labels was found useful in the study of lipid interaction with the integral membrane protein [19, 177]. ESR spectra of the labels in lipid–protein membrane systems revealed two separate components [19, 177–181]. One component corresponded to the fluid bilayer and the second, with a higher degree of motional

restriction, was attributed to the lipids interacting directly with the intramembrane surface of the integral membrane proteins. The two components may be separated by digital subtraction, which permits the investigation of the number of motionally restricted lipids per protein, lipid specificity, molecular conformation, mobility, and exchange of the lipids at the protein surface. The benzaldehyde lipid labels selectively attached to protein by covalent bonds [179], and photo-spin-labelled lipids [180] reacting with protein upon photolysis were also used to prove the origin of the motionally restricted component of the ESR spectra.

*Oxygen concentration*

Line width of the ESR spectrum of spin labels is sensitive to its spin exchange with paramagnetic oxygen molecules. To determine the oxygen concentration the exchange broadening in the spectra of labels was utilized. In the method introduced by Backer *et al.* [182] the resolution of the superhyperfine coupling from the protons of the labels (e.g. CTPO) which depends on the bimolecular collision rate with oxygen was used. This method was applied for the detection of mitochondrial respiration [182], the rate of oxygen uptake per cell [183], the determination of the partition coefficient of oxygen in hydrocarbon regions of the lipid bilayer [184] and for the detection of oxygen consumption in lipid peroxidation [185].

Changes in amplitude of the low field line of the ESR spectrum of Tempone were used for monitoring the oxygen concentration in photosynthetic thylakoid membranes [186]. With rising oxygen concentration the amplitude of the line decreases or increases at 1 or 100 mW of microwave power, respectively. This method measures oxygen at the level of 0.1 nmol in a 40 μl sample containing 25 ng of chlorophyll. The width of the midfield line in the ESR spectrum of the membrane permeable spin label 2,2,5,5-tetramethyl-3-methanol-pyrroline-*N*-oxyl (PCAOL) was applied to measure oxygen concentration up to 5 mmol $l^{-1}$ [187]. Using the impermeable spin broadening agent, $Na_2MnEDTA$, together with the spin label, it is possible to measure intracellular oxygen concentration [187]. Kusumi *et al.* [188] applied the saturation-lattice relaxation time of spin labels for detection of the translational diffusion of oxygen in lipid membranes. A technique was described [189] by which the oxygen concentration in a liquid ESR sample can be controlled using a polytetrafluoroethylene tube which is permeable to oxygen and nitrogen.

## Spin Labelling in Membranes

*Lipid membranes*

Different lipid membranes are used for studying drug–membrane interactions, i.e. unilamellar or multilamellar liposomes, macroscopically oriented multibilayers, and lipid–protein vesicles. Classical liposome preparations are made by mechanical shaking (multilamellar liposomes) or by sonication of a lipid suspension (small unilamellar liposomes). Care must be taken to avoid lipid peroxidation [190], which can occur during lipid isolation, sample preparation [191] and sonication [192]. Titanic particles should be removed by centrifugation from samples after sonication.

Recently more sophisticated methods have been introduced. A transient increase in pH of lipid mixtures containing phosphatidic acid led to the formation of unilamellar liposomes [193], where size was characterized by gel filtration, quasi-elastic light scattering and electron microscopy [194]. Giant liposomes, uni- and oligolamellar, with a

diameter in the range of 10–20 μm were generated with a freeze–thaw step and removal of sodium trichloracetate, guanidine–HCl, or urea by dialysis from a solution of egg phospholipids [195], or by dialysis of lipid and indifferent solute in a water-miscible organic solvent, against an aqueous buffer [196, 197]. Using Bio-Beads SM-2®, with the detergent octylglucoside removed by dialysis, unilamellar phospholipid–cholesterol liposomes (1 μm dia.) can be formed [198, 199]. Methods utilizing low boiling solvents or a reversed-phase evaporation process [200] were used to prepare large, unilamellar lipid liposomes at physiological temperature [201]. Hydration of single or mixed phospholipids or lipid–protein mixtures at low ionic strength resulted in the formation of solvent free unilamellar liposomes with 0.1–300 μm dia. [202]. Mixing aqueous suspensions of long-chain lecithins with small amounts of micellar synthetic short-chain lecithins resulted in spontaneous formation of stable unilamellar liposomes [203]. Preparation of multivesicular liposomes using evaporation of organic solvents from chloroform–ether spherules suspended in water was reported [204]. Carbohydrates and sugars were found useful to prevent fusion and leakage in freeze-dried liposomes [205–207].

For spin labelling of liposomes, spin labels and drugs can be added to the liposomes, either (i) simply in buffer solution or by depositing on the tube wall in a solvent which is evaporated prior to addition of the liposomes, or (ii) lipids, spin labels and drugs are dissolved in a solvent which is evaporated under vacuum, and then the dry lipids are hydrated. Oxyethylene–oxypropylene copolymers were found useful for insertion of water-insoluble drugs into membranes [208]. To reach an equilibrium for drugs in the samples, repeated freeze–thaw cycles were used [209].

*Macroscopically oriented multibilayers*

Several techniques for preparing oriented multibilayers have been developed. Glass slides treated with a polymerizable surfactant were used to obtain thick monodomain bilayer arrays [79, 210]. Oriented lipid multilayers can be formed in ESR quartz flat cells [78, 114, 211], or by depositing on quartz surfaces [212]. The method of preparing oriented multibilayers between silver-coated glass suitable for ESR study of the effect of an electric field on the bilayers of up to $10^5$ V $cm^{-1}$ was reported [213]. For spin labelling, a lipid-label mixture is used for the multibilayers preparation.

*Lipid–protein vesicles*

To obtain lipid–protein vesicles, either (i) protein in buffer can be added to dry lipids followed by vortexing, (ii) the lipid dispersion and protein in buffer are sonicated, or (iii) lipids and proteins dissolved in a solvent are dialysed against buffer [214–216]. The phenomenon of insertion of proteins into preformed bilayers was recently reviewed [217]. The natural detergents lysophosphatidylcholine or lysophosphatidic acid were used for reconstitution of a protein into liposomes. The method is based on fusion of protein–lysophospholipid micelles with liposomes [218]. Removal of detergent from mixed vesicles with protein and octyl glucoside led to formation of lipid–protein vesicles [219]. Short-chain lecithin diheptanoylphosphatidylcholine (20% of total lipid) was found useful in reconstitution of protein into vesicles [220]. Lipid–protein vesicles enhanced incorporation of additional integral membrane proteins [221]. For spin labelling, lipids containing spin labels are used. In order to pellet the liposomes by centrifugation a freeze–thaw technique which increases the size of the liposomes was found useful [222].

*Biological membranes*

Spin-labelling procedures depend on the membranes and on the labels used [11]. Most often membranes at a concentration of 0.5–5.0 mg of protein/100 μl of buffer are vortexed in the tube containing a dry film of spin label. Labelling levels of 1 or 2 mol% to membrane phospholipids are utilized. The labelled membrane suspension, transferred to a 20–100 μl glass capillary, can be centrifuged to increase the signal/noise ratio. For label incorporation into membranes, spin labels in ethanol [215, 216, 223] or in sonicated buffer [224] were used. The spin label PC(12,3) was found to exhibit spin–spin interaction in erythrocytes even at low label concentrations (>1:2250 = label/lipid or >0.22 = μg label/mg protein) [75, 76] yielding lower values of the order parameters $S$ and $S_{\perp}$ in comparison with the $S_{\parallel}$ parameter.

## Drug-induced Membrane Perturbation

A drug-induced change in any of the membrane parameters leads to the perturbation of membrane properties which might be responsible for a biological effect. In considering the involvement of the drug–membrane perturbation in biological effects, three issues should be discussed: (i) detection of the drug-induced changes of membrane parameters; (ii) estimation of the drug perturbation at pharmacological concentrations; and (iii) correlation between membrane perturbation and the biological effect. Some of the drug–membrane perturbation effects were reviewed [225, 226]. Since the effect of drugs on membrane dynamics and order have been primarily studied, only these two membrane parameters are discussed below.

*Detection*

Since the majority of drugs are supposed to interact specifically with membrane proteins, lipids and lipid–protein interface, the ESR detection of the perturbation depends on the spin label used, the membrane composition and on the physical condition of the membrane.

*Spin labels.* Using different spin labels one can obtain disordering, ordering or no perturbation effect from the same drug on membranes. Fluidization effect of butanol, halothane and ketamine on synaptic membranes was observed using SA(1,14) but no disordering effect was seen when the SA(12,3) label was used [227]. Interaction of filipin and amphotericin *B* with sterols in phospholipid–cholesterol liposomes was studied with PC(5,10) and PC(12,3). Filipin disordered the membrane near the centre at the 12th carbon depth while it ordered the membrane near the polar surface. On the other hand, on using these labels amphotericin *B* did not apparently affect the membrane order [228]. Insulin decreased the dynamics of liver plasma membranes detected by CSL and spin-labelled androstane, but not by SA(12,3) [229]. The disordering effect of ethanol on lecithin–cholesterol multilayers was higher when detected by SA(5,10) and SA(1,14), in comparison with SA(12,3) [230]. Quinidine increased the order parameter of membranes prepared from acidic phospholipids in the region close to polar surface, detected by SA(12,3), and decreased the order parameter in its hydrocarbon core, detected by SA(1,14), [231]. Oral administration of $CCl_4$ decreased the apparent rotational correlation time of SA(5,10) in rat liver microsome membranes, while the order parameter of SA(12,3) in the membranes was not changed [232]. Butanethiol was more effective in perturbing erythrocyte membranes when detected by SA(5,10) than by

$CAT_{11}$, whereas the relative effectiveness was reversed for *n*-butanol [233]. Cannabinol, chlorpromazine and pentobarbital increased the order parameter (*S*) in liposomes prepared from lecithin and 4% phosphatidic acid where the ordering effect was higher when detected by SA(12,3) than by PC(7,6) [234].

Because of different temperature gradients of the ESR membrane parameters, the perturbation effect of drugs expressed at the given temperature scale was used to compare their perturbation propensities detected by different spin labels [100–102, 235]. Using this approach, the perturbation effects of local anaesthetics and beta-adrenoceptor blocking drugs were compared at different membrane depths and expressed at the temperature scale. The disordering effect of the local anaesthetics lidocaine, tetracaine, dibucaine, heptacaine and carbisocaine in the rat total lipid and synaptosomal membranes [100–102, 236] and the effect of beta-adrenoceptor blocking drugs oxprenolol, doberol, pronethanol, metipranolol, alprenolol, propranolol, exaprolol and Kö-1124 in total lipid membranes have been found to be about 3–10 times higher at the 16th carbon membrane depth, detected by SA(1,14), than at the 5th depth, detected by SA(12,3) [235, 237]. The disordering effect of tetracaine was 1.5 times higher than that of dibucaine at the same synaptosomal membrane concentration as detected by SA(12,3). However, at the hydrocarbon membrane core detected by SA(1,14), the disordering effect of dibucaine was 2.5 times higher in comparison with tetracaine [102]. From the disordering dependence of local anaesthetics and beta-adrenoceptor blocking drugs on membrane depth, the structural incorporation of the lipophilic drugs in membranes was suggested. The polarizable part of the amphiphile drug is located in the polar portion of the membrane, with the apolar tail penetrating into the hydrocarbon core parallel to the lipid acyl chains [100–102, 235, 238, 239]. It was supposed that such drug incorporation may create "free volume" in the hydrophobic membrane portion leading to changes in membrane elastic energy and destabilization of the lamellar membrane structure [100–102, 235].

In conclusion, drugs specifically incorporated in membranes influence different membrane portions with various propensities, and there is no correlation between the membrane perturbation parameters and "membrane fluidity". The term "membrane fluidity" has been widely used as a membrane parameter. However, this term is valid for isotropic solvents and is not suitable for judging the physical membrane state [6, 240]. Since the detection of drug perturbation effects depends on the spin label used, many confusing conclusions on these effects were obtained from experiments using a limited number of spin labels.

*Membrane composition.* Using different membrane compositions, disordering, ordering or no perturbation effect of the same drug can be obtained on the membrane. The local anaesthetics procaine, tetracaine, phenacaine, butacaine and lidocaine disordered oriented multibilayers prepared from ox brain lipids, as detected by CSL. Whereas in membranes having low cholesterol contents, the anaesthetics promoted the formation of ordered multilamellar bilayers [114]. Tetracaine, promazine, chlorpromazine, pentobarbitone and mepivacaine increased the degree of order in brain lipid multibilayers containing 5% cholesterol (studied by CSL), but did not induce the same high degree of order found at high concentrations of cholesterol. The ordering effect of tetracaine was potentiated by calcium [115]. Cannabinol, chlorpromazine and pentobarbital increased the order parameter, *S*, of SA(12,3) and PC(7,6) in lecithin membranes containing 4% of phosphatidic acid. The ordering effect of these drugs became smaller when increasing

amounts of cholesterol were incorporated into the phospholipid membranes until a disordering effect was finally observed [234]. On the other hand, the disordering effect of ethanol detected by SA($m$,$n$) on lecithin–cholesterol multibilayers was reduced by the addition of cholesterol [230]. Local anaesthetics were found to stabilize or disrupt lipid bilayers, depending upon the cholesterol content of the lipid membranes. Quinidine strongly perturbed the molecular organization of membranes prepared from acidic phospholipids (phosphatidylserine or phosphatidic acid) and had only a slight effect on neutral lecithin membranes [231]. Propranolol exerted marked ordering effects on bilayers prepared from acidic phospholipids but did not change the order parameter of phosphatidylcholine membranes [241].

Rat brain synaptosomes, liver mitochondria and liposomes prepared from isolated lipids after long term consumption of ethanol by the rat were resistant to structural disordering by ethanol and halothane as detected by SA($m$,$n$) [224, 242, 243]. Thymol and procaine increased partitioning of 5-doxyldecanoic acid methylester into fragmented sarcoplasmic reticulum, whereas no appreciable change in the label partitioning was induced by the drugs into liposomes prepared from the phospholipids extracted from the sarcoplasmic reticulum [244].

The disordering effect of chlorpromazine, verapamil, propranolol and carbisocaine on lipid membranes was found to depend on the ratio of lecithin and rat brain total lipids in the membranes. The drugs had a negligible disordering or ordering effect on the lecithin membrane but the disordering effect was increased with the increase of the total lipid/lecithin ratio in the membrane. The highest effect was found in rat brain total lipid membranes and was comparable to those found in synaptosomal membranes (Ondriaš *et al.*, unpublished results).

The results indicate that drug perturbation effects depend on the combination of different physical forces (electrostatic, hydrophobic, hydrogen-bonds, etc.), resulting from different lipid compositions. The perturbation effects of many drugs could be higher in natural membranes compared with the membranes prepared from single phospholipids.

*Membrane conditions.* Drug perturbation effects also depend on membrane conditions. Since at physiological pH many drugs can exist in neutral or positively charged form, their interaction with the membrane depends on pH. The disordering effect of drugs is usually increased at higher pH, where basic drugs are in the neutral form [114, 245, 246]. However, the disordering effect of the very lipophilic anaesthetic carbisocaine was found to decrease with increasing pH whilst the opposite pH-dependent trend was found for its hydrophilic derivative Ka-O [61, 127]. The disordering effect of ethanol in lecithin–cholesterol membranes, of beta-adrenoceptor blocking drugs in brain total lipid liposomes, and of local anaesthetics in synaptosomal membranes increased with increasing temperature as studied by CSL and SA($m$,$n$) [102, 230, 235]. The effect of the polyene antibiotic amphotericin *B* on lipid membranes, studied by CSL and SA(12,3), depended on the sample preparation. When the antibiotic was added to the aqueous phase, no spectral effect occurred. However, when the antibiotic was incorporated during membrane preparation, changes in spectral parameters suggested the appearance of a new phase [247].

*Biphasic effect.* Some drugs were found to exhibit biphasic perturbation effects. Low concentrations of halothane (0.16 and 0.32 mmol $l^{-1}$) increased the order parameter in

palmitoyllauroyl lecithin [248] and in synaptic plasma or myelin membranes (0.25 and 0.5 mmol $l^{-1}$) [249], whereas at higher anaesthetic concentrations halothane decreased the order parameter as detected by SA(12,3). A similar biphasic effect was found for lidocaine in synaptic plasma membranes [249]. The order of pigeon erythrocyte membranes, detected by SA(*m*,*n*), was decreased at an intermediate range of concentrations of chlorpromazine, methochlorpromazine, tetracaine, octanol and octanoic acid. At higher concentrations they re-increased the order before eventually destroying the membrane structure [250, 251].

*Drug–membrane perturbation effects at "pharmacological concentrations"*

The concentrations of drugs, used in pharmacological *in vitro* experiments are commonly expressed as the drug concentration in a buffer system. On the other hand, in ESR studies the concentrations of the drugs are most often given per sample. Therefore, the drug perturbation effects have usually been studied without determination of the final drug concentration in the buffer after equilibration with the membrane. Owing to high membrane partition coefficients of some drugs and high membrane/buffer ratios in the ESR samples, the drug buffer concentration after equilibration with the membrane usually decreases. For example, when the drug volume partition coefficient is 1000, the membrane/buffer volume ratio in the sample is 0.2 and the drug sample concentration is 1 mmol $l^{-1}$, the drug buffer concentration will decrease after equilibration with the membrane to 5 μmol $l^{-1}$. The drug apparent partition coefficient, depending on the membrane system [241, 252–254], drug concentration [253, 254], temperature [254], and pH [252, 253] is usually unknown. Therefore, in ESR studies, either the drug buffer concentration after equilibration with the membrane should be measured experimentally or such a high buffer/membrane volume ratio should be used in which the buffer concentration of the drug does not decrease significantly after the membrane is saturated by the drug [102].

The lowest membrane disordering concentrations in total lipid membranes for dibucaine and propranolol were found at a lipid/drug molar ratio of about 30 [235, 255]. Using the approximate volume partition coefficient of 1000 for both drugs [241, 256], the lowest buffer calculated disordering concentration is 43 μmol $l^{-1}$. In the synaptosomal membranes the disordering effect was found at buffer concentrations higher than 0.01 and 0.1 mmol $l^{-1}$ for dibucaine and tetracaine, respectively [102]. However, these concentrations are higher in comparison to the beta-adrenergic receptor blocking concentrations of propranolol and to dibucaine concentrations used for blocking the action potential of nerves, but they are in the same order at which the drugs influence several other biological membrane activities [102, 235].

It can be supposed that at low drug membrane concentrations, the drug perturbation effect is a non-cooperative phenomenon, i.e. the drug induces local perturbation in the membrane, leaving the bulk of the membrane unperturbed. Since the ESR signal is a summary of the signals of all membrane spin labels, the negligible amount of label reflecting the perturbated membrane region can be covered by signals from labels incorporated in the unperturbed membrane bulk, resulting in non-significant changes of the ESR spectral parameters.

*Correlation between membrane perturbation and biological effects*

Different membrane perturbation propensities of drugs were compared with their biological effects. However, it must be taken into consideration that the drug

perturbation propensities depend on the spin label used, membrane composition and other physical conditions of the measured sample. Thus the correlations show only a comparison of drug biological potency with their single particular perturbation effect.

The lack of correlation between anaesthetics (halothane, chloroform, diethyl ether, benzyl alcohol, Tempo) and pressure effects on order parameters of lecithin–cholesterol membranes, studied by SA($m$,$n$), failed to support the lipid fluidization hypothesis of anaesthesia [28, 257]. The effect of morphine, naloxone, levorphanol and dextrophan on the phase transitions of phospholipids was studied using Tempo. No correlation was observed between the fluidizing and analgesic effects [258]. A decrease in the order parameter of PC(7,6) in lecithin–cholesterol membranes was found after application of ethanol, butanol, trichloroethanol, α-chloralose, urethane, pentobarbital, thiopental and ketamine. The disordering effect correlated fairly with their general anaesthetic and nerve-blocking potency [99]. The effects of $n$-alkanols on lecithin–cholesterol membranes was studied using PC(1,14). The $n$-alkanols with a chain length of $<10$ carbons increased membrane dynamics, whereas those with a chain length $>10$ carbons decreased it. No direct correlation was found between the effects of the $n$-alkanols on membrane dynamics or on phase transition and their ability to block the conduction of nerve impulses [90]. A slow decline in disordering ability of $n$-alkanols from decanol to arachidyl alcohol on lecithin–cholesterol membranes was not completely consistent with the sharp loss of anaesthetic potency, which was explained by the limited membrane solubility of the higher alkanols [259]. Fluidity changes detected by SA($m$,$n$) induced by chlorpromazine, methochloropromazine, tetracaine, octanol and octanoic acid were found to strictly correlate with those of stimulated adenylate cyclase activity [250, 251]. Steroid anaesthetics were found to disorder lecithin–cholesterol membranes, detected by PC(6,7), whereas a non-anaesthetic steroid produced much less disordering of liposomes [260]. Efficiency of the local anaesthetics lidocaine, tetracaine, dibucaine, heptacaine and carbisocaine in decreasing the phase transition temperature of lipid membranes roughly correlated with their anaesthetic potency [261].

Disordering propensities of local anaesthetics and beta-adrenoceptor blocking drugs were found to vary with the depth of the total lipid membranes [100–102, 235]. The disordering efficiency of lidocaine, tetracaine, dibucaine and heptacaine did not correlate with their anaesthetic potency at the 5th carbon membrane depth in brain total lipid liposomes but qualitatively corresponded to their anaesthetic potency at the hydrocarbon membrane core [100, 102]. Similarly the disordering propensities of the beta-adrenoceptor blocking drugs (atenolol, practolol, pindolol, doberol, oxprenolol, metipranolol, pronethanol, propranolol, Kö-1124, alprenolol, exaprolol) in brain total lipid liposomes and platelet membranes at the 16th carbon membrane depth, corresponded fairly well to some of their biological membrane activities. These include aggregation and displacement of calcium from binding sites in isolated platelets [237], as well as inhibition of serotonin uptake by human platelets and noradrenaline uptake in synaptosomes, depression of transmission velocity in muscle strips of heart, depression of action potential and myocardial contractility [235].

The correlations between drug perturbation and biological effects suggest that some of the biological membrane activities may be mediated, at least in part, through their perturbation effect.

*Perturbation of membrane parameters*

Various kinds of drugs incorporated into membranes, some of which have been

mentioned in the previous section, have been found to influence membrane parameters. Some of the other drug perturbation effects detected by spin labels are summarized below.

The inhalation anaesthetics methyoxyflurane and halothane decreased the order parameter and polarity of lecithin membranes [262]. Etidocaine and bupivacaine disordered lipid and synaptic plasma membranes [263]. Derivatives of glycine esters, as dependent on hydrophobic chains, disordered lecithin liposomes [264]. Propranolol, diazepam, chlorpromazine and pluronic F 68 decreased thermally-induced structural transitions in human erythrocyte membranes [265] and propranolol induced alternations in organization of membrane proteins and decreased order of membrane lipids [266]. The ethanol-induced perturbation of erythrocyte ghosts from blood of long-sleep and short-sleep mice was compared. It was found that the membranes of mice who were especially sensitive to ethanol (long-sleep mice) were more affected by ethanol, than the membranes of short-sleep mice [267]. Adrenaline increased the order parameter in erythrocyte ghosts [268]. The order parameter was increased in ADP activated platelets when compared to intact platelets [269]. Membrane dynamics of human erythrocytes were increased after their transformation to stomatocytes or when the transformation was induced by chlorpromazine, tetracaine, chloroquine, primaquine, Triton X-100, lowering the pH or depleting membrane cholesterol [270]. Chlorpromazine, imipramine, benzyl alcohol, sodium oleate, sodium benzenesulphonate and cholesterol removal induced dose–response decreases in lipid order in human erythrocytes [271]. Benzyl alcohol decreased the thermostability of fluoride-stimulated adenylate cyclase activity and disordered rat liver plasma membranes [272]. The order parameter decreased in hepatocyte membranes isolated from rats chronically treated with hexachlorobenzene and ethanol [273]. Isaxonine at 1% molar concentration in dipalmitoyl–lecithin ordered the membrane in a gel state and disordered it at 30% molar concentration [274].

Anisodamine [275] and propranolol lowered the phase transition temperature in dipalmitoyllecithin membranes and α-tocopherol broadened the transition [276]. The effect of long-chain alcohols, $C_{14:0}$, *cis* and *trans*- $C_{14:1}$, $C_{16:0}$ and *cis*- and *trans*- $C_{16:1}$, on the phase transition of dipalmitoyllecithin membranes depended on the degree of saturation of the alcohols. Saturated alcohols produced a concentration-dependent elevation, the *trans*-unsaturated alcohols a smaller elevation, while the *cis*-unsaturated alcohols produced a substantial depression of the transition. All alcohols broadened the transition [277]. *Cis*- and *trans*-hexadecenol (33 mol%) ordered the lipid membrane slightly above the phase transition, whereas below the transition, the *cis*-isomer disordered, whilst the *trans*-isomer expelled the spin label PC(7,6) from the lipid bilayers [277].

Tetracaine at high concentrations in lecithin liposomes induced a phase separation, indicating the formation of tetracaine–lecithin mixed micelles [278]. Tetracaine and dibucaine in cardiolipin membranes prevented the formation of a phase separation induced by cytochrome *c* [279]. The inhalation anaesthetic methoxyflurane shifted the fluid–gel equilibrium phase diagrams for aqueous dispersions of the binary systems of dimyristoyl- and dipalmitoyllecithin to lower temperatures [280]. The antibiotic polymyxin (4 mol%) created a phase separation in a phosphatidic acid bilayer. The part of the membrane that melted between 30–50°C was the condensed phase, in which polymyxin bound tightly to the phosphatidic acid head groups, and that portion of the bilayer that melted between 50–55°C was the remainder of the phosphatidic acid bilayer that did not interact with polymyxin [281]. Concavalin *A*, a lectin, was found to

agglutinate lecithin–dicetyl phosphate (10:1 molar) liposomes preincubated with spin-labelled glycolipid [282].

Some drugs were found to influence lipid–protein interactions in biological membranes. The immobilized signal from SA(*m*,*n*) was observed in human erythrocytes treated with oxidizing agents such as glutaraldehyde, hydrogen peroxide, phenylhydrazine, copper-*ortho*-phenanthroline [157] and chlorpromazine [148], and in synaptosomal membranes induced by tetracaine, dibucaine [255], propranolol and verapamil (Ondriaš and Staško unpublished results). The effect of halothane, lidocaine and tetracaine was studied on membrane proteins and lipids of frog sciatic nerves. The anaesthetics disordered the lipid regions and the perturbation was transferred to the spin-labelled membrane proteins via lipid–protein interaction [53]. Glycerol and polymyxin *B* induced inter-digitated phases in lipid bilayers [67, 45]. Apparent p*K* values of tetracaine in the presence of lecithin membranes decreased with increasing membrane concentration [246].

The effect of monosodium urate, dimethyl sulphoxide and amphotericin *B* on the permeability of lecithin–cholestane liposomes was studied using the label Tempocholine with ascorbate. Monosodium urate did not affect the permeability, dimethyl sulphoxide promoted, and amphotericin *B* had a pronounced effect on the permeability of the liposomes [158]. Propranolol increased permeation of ascorbate into dipalmitoyllecithin vesicles, whereas α-tocopherol had no significant effect [276]. Residual traces of chloroform, halothane and lipid oxidation increased the $H^+/OH^-$ flux across the lecithin vesicles [161]. Entrapment of the label Tempocholine in human erythrocytes after a sudden hyposmolar stress was decreased by chlorpromazine, trifluoperazine, nicardipine, amperozide and haloperidol, where the exclusion of $Ca^{2+}$ and $Mg^{2+}$ ions from the solutions increased the action of chlorpromazine [104]. The reduction of spin labels in respiring rat liver mitochondria, mitoplasts, and submitochondrial preparations was inhibited by rotenone but was relatively insensitive to antimycin *A* and KCN [38].

## Conclusion

ESR spectroscopy of nitroxide stable radicals has been successfully applied for detection of various membrane parameters. Primarily, membrane order and dynamics have been investigated in these drug–membrane interaction studies, whereas drug effects on other membrane parameters have so far scarcely been evaluated. Since drugs were found to specifically perturb membranes, different spin labels can be applied to advantage in investigating the specific drug–membrane interaction.

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