Appendix: Corrections for Anisotropically Averaged Hyperfine Splittings and Order Parameters from Pseudopowder Electron Paramagnetic Resonance (EPR) Line Shapes

An Update for Slow-Motion Contributions to Lipid Spin Label Spectra from Membranes

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In the first volume of this series (Berliner, 1976), consideration was given to corrections that must be made to extract order parameters from the EPR spectra of lipid spin labels in randomly oriented membrane dispersions (Gaffney, 1976; Griffith and Jost, 1976). A similar analysis leading to a somewhat different correction was also given previously by Hubbell and McConnell (1971). A hybrid of these results, depending on the range of order parameters studied ($0 \leq S_{app} < 0.45$ or 0.45

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$\leq S_{app} \leq 1$) was given later by Marsh (1985), based on results of simulations presented by Griffith and Jost (1976). These corrections were widely used to analyze membrane EPR spectra from doxyl-labeled lipid chains, and these continue to be so. The purpose of this appendix is to update these corrections to account for components of lipid chain motion in fluid membranes that are now known to be in the slow regime of conventional nitroxide EPR spectroscopy (compare Freed, 1976; Schneider and Freed, 1989).

Restricted anisotropic rotation of spin-labeled lipid chain segments in fluid membranes is specified by an order tensor with principal element S_{zz} (relative to the membrane normal). This limited anisotropic reorientation results in the following principal elements of the partially motionally averaged hyperfine tensor (see, e.g., Seelig, 1976; Marsh and Horváth, 1989):

$$A_{\parallel} = a_o + (2/3)\Delta A \cdot S_{zz} \tag{1}$$

$$A_{\perp} = a_o - (1/3)\Delta A \cdot S_{zz} \tag{2}$$

where with a ^{14}N-hyperfine tensor $\boldsymbol{A} = (A_{xx}, A_{yy}, A_{zz})$, the isotropic hyperfine coupling constant is $a_o = (1/3)(A_{xx} + A_{yy} + A_{zz})$ and the maximum extent of the axial hyperfine anisotropy is $\Delta A = A_{zz} - 1/2(A_{xx} + A_{yy})$. Here it is assumed that the z-principal axis of the ^{14}N-hyperfine tensor is oriented parallel to the molecular axis, as for stereospecifically doxyl- or proxyl-labeled lipid chains. It is also assumed that the nonaxiality of the ^{14}N-hyperfine tensor is small, as is found experimentally, such that only the S_{zz} principal element of the ordering tensor is required to describe its anisotropic motional averaging.

For a powder pattern EPR line shape or a partially averaged EPR spectrum resulting from fast anisotropic reorientation in an unoriented membrane suspension, the splitting between the outer hyperfine extrema of the first-derivative spectrum $2A_{max}$ is rigorously equal to $2A_{\parallel}$ (Weil and Hecht, 1963; Hubbell and McConnell, 1971). However the effective splitting of the inner hyperfine extrema A_{min} is complicated by spectral overlap in this region of the (pseudo-)powder line shape. Previous corrections proposed to determine order parameters from randomly oriented samples related this effective inner splitting $2A_{min}$ to the true value of the hyperfine splitting given by $2A_{\perp}$ (Hubbell and McConnell, 1971; Griffith and Jost, 1976; Gaffney, 1976). These empirical corrections were based on the simulation of powder spectra corresponding to the motionally averaged hyperfine tensor $\boldsymbol{A}' = (A_{\perp}, A_{\perp}, A_{\parallel})$. These corrections therefore correspond to fast partial motional averaging of the hyperfine anisotropy. Unlike the situation in soap systems, which exhibit fast motional averaging and high order (Schindler and Seelig, 1971; Seelig, 1976), EPR spectra from chain-labeled lipids in fluid phospholipid membranes contain important features corresponding to motional components that are slow on the conventional nitroxide EPR time scale (Lange *et al.*, 1985; Moser *et al.*, 1989).

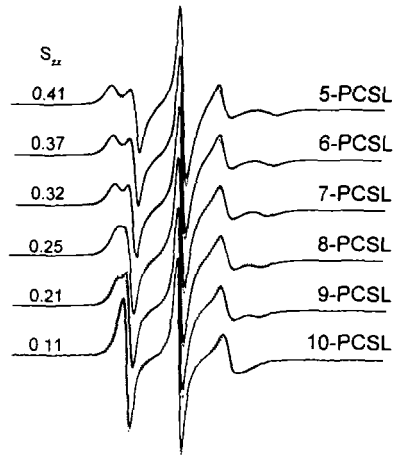

Figure 1. Experimental EPR spectra (*solid lines*) and simulations using the VAR model (*dotted lines*) for different positional isomers of the n-PCSL phosphatidylcholine spin labels in dimyristoyl phosphatidylcholine/dimyristoyl glycerol (58:42) mol/mol bilayers at 60°C. The order parameters S_{zz} used in the simulations are indicated in the figure. Total scan range = 100 G (Schorn and Marsh, 1996).

In the pseudopowder EPR spectra from spin-labeled lipids in fluid phospholipid bilayers or biological membranes, the low-field perpendicular peak is characteristically less deep than that of the central line (see Fig. 1); this feature is reversed in soap systems, and it cannot be reproduced by simulations based on fast motional narrowing theory (Schindler and Seelig, 1973; Seelig, 1976). Furthermore the high-field parallel peak is less deep than the overshoot feature from the high-field perpendicular peak (see experimental spectra in Fig. 1); this feature is not reproduced (for $S_{zz} \leq 0.7$) by previous simulations used to obtain corrections for calculating order parameters (see Griffith and Jost, 1976; Gaffney, 1976).

To account for slow-motional contributions with spin-labeled lipids in membranes, spectral simulations were made using the very anisotropic reorientation (VAR) model (see Meirovitch *et al.,* 1984). This is the very simplest of the slow motional models that emulates, although it does not describe exactly, anisotropic local ordering in macroscopically unoriented samples. Nevertheless it was found to be very successful in systematically representing spin label EPR spectra from a variety of different lipid spin labels in bilayer membranes over a wide range of temperature and membrane lipid compositions (Schorn and Marsh, 1996). Figure 1 shows representative simulations of experimental spectra from different positional isomers of the 1-acyl-2-(n-doxyl stearoyl)-phosphatidylcholines (n-PCSL) in fluid lipid membranes. Unlike the fast-motional analysis, experimental spectra can be fitted with a high degree of precision by simulations using the VAR model. Further refinement of the model is not justified by the relatively low resolution obtained in experimental spectra from nonaligned membrane samples.

The EPR spectra were simulated for ^{14}N-nitroxide spin labels at a microwave frequency of 9.0 GHz for the full range of molecular order parameters. $S_{zz} = 0–1$

by using the VAR model (Schorn and Marsh, 1997). The spin Hamiltonian parameters used were **A** = (5.9 G, 5.4 G, 32.9 G) and **g** = (2.0076, 2.0072, 2.0021) (Schorn and Marsh, 1996). This g-tensor differs slightly from single-crystal values for a doxyl nitroxide (Jost *et al.*, 1971) because it has reduced nonaxiality to account in part for some of the effects of rapid *trans-gauche* isomerism not explicitly included in the model. Dynamic parameters and intrinsic line widths were chosen to reproduce experimental spectra typical of n-PCSL spin labels in membranes (see Schorn and Marsh, 1996; compare Fig. 1). Splittings between the outer peaks ($2A_{max}$) and between the inner peaks ($2A_{min}$) in simulated spectra were measured to establish empirical corrections that must be made to obtain the true hyperfine splittings $2A_{\|}$ and $2A_{\perp}$, respectively, which are used to determine the order parameter S_{zz} from Eqs. (1) and (2).

Values of $2A_{max}$ measured from the simulated spectra vary linearly with S_{zz} from $2A_{max} \approx 2a_o$ for $S_{zz} = 0$ to $2A_{max} \approx 2A_{zz}$ for $S_{zz} = 1$ (Schorn and Marsh, 1997), as would be predicted by Eq. (1) from motional narrowing theory. For spectra that contain contributions from slow motional components however, this is neither an evitable nor necessarily expected result. It arises from a balance of the effects parallel and perpendicular components of the diffusion tensor have on the outer spectral line positions. Although somewhat fortuitous, this simple result corresponds best to experimental spectra in this model (Schorn and Marsh, 1996; see Fig. 1); it has the advantage that the relationship $A_{\|} = A_{max}$ normally used may be retained to a reasonable degree of approximation.

Values of the inner splittings $2A_{min}$ obtained from the simulated spectra do not correspond to Eq. (2) nor to those obtained previously from fast motional narrowing simulations. The dependence of A_{min} on the order parameter is linear, at least over the range $S_{zz} = 0.05$–0.8 (for values outside this range, the inner splitting is difficult to resolve). This leads to the following relation for the $A_{\perp}$ motionally averaged tensor element (Schorn and Marsh, 1997):

$$A_{\perp} = A_{min} + 1.29\ \text{G} - 1.75\ \text{G} \cdot S_{zz} \tag{3}$$

This correction is similar in form to that introduced previously from motional narrowing simulations (Gaffney, 1976) except that in the latter case, both numerical terms had a value of 1.4 G.

Finally, the order parameter may be expressed in terms of the experimentally accessible parameter $S_{app} = (A_{max} - A_{min})/\Delta A$ by using Eqs. (1)–(3) with $A_{\|} = A_{max}$:

$$S_{zz} = 1.069\ S_{app} - 0.051 \tag{4}$$

where $\Delta A = 27.2$ G. This very simple form allows immediate estimation of S_{zz} by using values of A_{max} and A_{min} obtained from experimental spectra. If necessary a further correction may be applied to account for differences in spin label environ-

ment polarity by using the ratio of the isotropic hyperfine coupling constants (Hubbell and McConnell, 1979; Gaffney, 1976; Jost and Griffith, 1976). The hyperfine tensor used here to determine the corrections corresponds to a value of $a_o = 14.7$ G, which is reasonably close to the mean value for doxyl labels in the acyl chain regions of lipid membranes (Marsh and Watts, 1981).

Including the polarity correction, which corresponds to $a'_o = (1/3)(2A_\perp + A_{||})$, gives rise to a quadratic equation for S_{zz}. The solution of this may be approximated by:

$$S_{zz} = \tfrac{1}{7}(A_{\max} + 2A_{\min}) - \sqrt{[\tfrac{1}{7}(A_{\max} + 2A_{\min})]^2 - 0.46(A_{\max} - A_{\min}) + 0.6} \quad (5)$$

where $A_{\max}$ and $A_{\min}$ are expressed in gauss. Values for the order parameter obtained in this way agree quite well with those obtained by direct simulations, such as are given in Fig. 1 (Schorn and Marsh, 1997). The method may therefore be used with experimental spectra to yield order parameters that closely approximate those obtained by spectral simulations using the VAR slow motional model.

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