ELECTRON SPIN RESONANCE ANALYSIS OF THE NITROXIDE SPIN LABEL 2,2,6,6-TETRAMETHYLPIPERIDONE-*N*-OXYL (TEMPONE) IN SINGLE CRYSTALS OF THE REDUCED TEMPONE MATRIX

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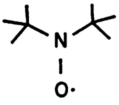
ABSTRACT The nitroxide spin label Tempone (2,2,6,6-tetramethylpiperidone-*N*-oxyl) can be reduced with ascorbic acid to give a nonparamagnetic species. Single crystals of reduced Tempone serve as a suitable host matrix to orient trace quantities of Tempone for ESR analysis. In these crystals the majority of the Tempone molecules are well-oriented, but a smaller fraction of the molecules tumble freely to give an isotropic electron spin resonance (ESR) spectrum. ESR transitions for the oriented molecules are saturated at much lower microwave power levels than for the tumbling molecules. For the oriented molecules, an analysis of the anisotropy of the spectroscopic splitting factor (g) gives principal values of $g_1 = 2.0094$, $g_2 = 2.0061$, $g_3 = 2.0021$. The hyperfine coupling tensor is nearly axially symmetric, with principal values (in gauss) of $A_1 = 6.5$, $A_2 = 6.7$, $A_3 = 33.0$. Within experimental error, the principal axis systems for the g tensor and the hyperfine tensor are identical. Comparison of the average values of g and A with the isotropic values of these parameters for Tempone in solvents of different polarity suggests a method for choosing the most appropriate tensor elements to be used for spin label experiments in various solvent systems.

INTRODUCTION

A large portion of the information content in the electron spin resonance (ESR) spectra of nitroxide spin labels is due to the anisotropies in the spectroscopic splitting factor (g) and the hyperfine coupling (A). The principal values for the g and A tensors do not vary greatly for nitroxides having different molecular structures. Nevertheless, as spin label studies become more sophisticated and more quantitative, accurate principal values of g and A for the basic nitroxide structures become more essential. Analyses that require a knowledge of these principal values include (*a*) determination of rotational correlation times from measurements of the heights and widths of the individual hyperfine lines; (*b*) interpretation of spectra from oriented or partially oriented spin-labeled samples; (*c*) computer simulations for line shape analysis or estimation of rotational correlation times too long to be determined by direct measurements of line width; and (*d*) the qualitative or quantitative treatment of anisotropic motion, where rotation about one axis within the spin-labeled molecule may be much less restricted than rotation about other axes. The observance and accurate interpretation of this latter phenomenon of anisotropic motion is critically dependent upon the relative values of the g and A principal tensor components.

Representative examples of the basic nitroxide structures are shown below.



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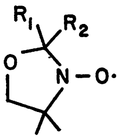

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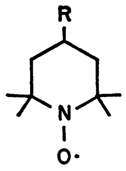

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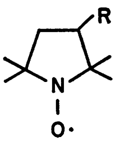

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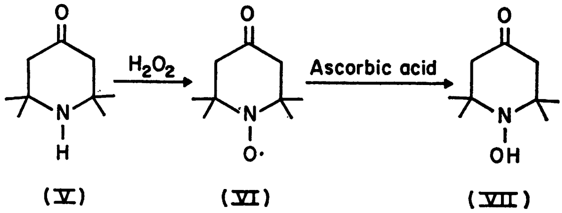
By far the best characterized with regard to the spectral parameters (Libertini and Griffith, 1970), but of limited use as a spin label, is di-*t*-butyl nitroxide (I). Two spin labels having the oxazolidine structure II have been studied (Hubbel and McConnell, 1969; Jost et al., 1971), and have principal values of g and A that are nearly identical, yet significantly different from those of I. The widely used piperidine (III) and pyrrolidine (IV) structures have not been characterized, except for a brief report of their approximate hyperfine principal values (Griffith et al., 1965).

In the work described here we have analyzed the spectral parameters of the spin label Tempone, a representative of the piperidine group, as an impurity in single crystals of the nonparamagnetic reduced Tempone matrix. Principal values of g and A and the direction cosines relating the corresponding principal axes to the external (crystal) axis system are reported. An interesting observation was that a portion of the nitroxide molecules in these crystals are not oriented, but tumble freely to give a nearly isotropic spectrum. We have compared the microwave power saturation properties of the oriented and freely tumbling molecules and found them to be quite different. In addition, we have compared the isotropic components of g and A for Tempone in the crystal with the *g* value and hyperfine coupling of this molecule in olvents of different polarity in order to estimate the best principal values to use ins spin label studies in a given solvent system.

MATERIALS AND METHODS

Preparation of Crystals

The spin label Tempone (VI) was prepared by oxidation of 2,2,6,6-tetramethylpiperidone (V) with H_2O_2 and purified by preparative thin layer chromatography (Williams et al., 1972). A 50-fold excess of ascorbic acid was added to Tempone in water to produce reduced Tempone, whose structure is presumed but not proven to be that of VII below.



This latter reaction is not complete, and leaves trace quantities of the spin label in solution. The reaction mixture was partitioned between diethyl ether and water. Three repeated ether extracts were combined and evaporated to dryness under vacuum in a rotary evaporator. The white solid residue was dissolved in sodium-dried diethyl ether and hexane was added to about 50% by volume or until cloudiness occurred. The preparation was gently heated until optically clear and then stored at about −20°C for the growth of single crystals. The crystals obtained by this procedure contained Tempone in quantities small enough for suitable ESR studies.

Fig. 1 shows the typical morphology of crystals obtained in this manner. The external axis system shown in Fig. 1 was chosen as a reference frame for taking ESR measurements. This orthogonal *abc* axis system is not necessarily the true crystallographic axis system of the unit cell, but serves our purpose for evaluation of ESR tensor parameters.

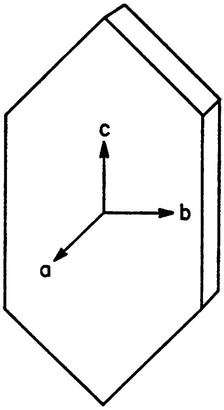


FIGURE 1 Typical external morphology of crystals used in this study. Data were taken with respect to the orthogonal *abc* axis system shown.

ESR Spectroscopy

A Varian X-band spectrometer (Varian Associates, Palo Alto, Calif.) with a 300 mW klystron and a rotating cavity was used for most ESR measurements. Some data were taken at a frequency of 15.6 GHz with a microwave bridge constructed in our laboratory. In each case the spectrometer frequency was measured with a Hewlett-Packard wavemeter (Hewlett-Packard Co., Palo Alto, Calif.). Hyperfine couplings were measured by comparison with a standard marker of Mn^{++} in MgO. The separation between the central hyperfine lines ($M_I = \pm\frac{1}{2}$) for this reference marker is 90.6 G, as determined with a proton magnetic resonance probe. Diphenylpicrylhydrazyl (DPPH), for which $g = 2.0036$, was used as a standard for determining g values.

RESULTS AND DISCUSSION

Analysis of Spectra

With the external magnetic field (H) of the spectrometer oriented along any of the three axes of Fig. 1, the ESR spectrum consisted of two triplets having, in general, different hyperfine couplings and different g values. The spectral characteristics of these two triplets were quite dissimilar, and different crystals gave spectra with different relative contributions of the two triplets. One triplet had narrower absorption lines and has a hyperfine coupling and g value that were independent of crystal orientation in the magnetic field. In all respects this isotropic triplet appears to arise from nitroxide molecules that are tumbling freely within the crystal. This triplet from freely tumbling molecules is indicated by arrows in the second-derivative ESR spectra of Fig. 2 taken with H along the c axis. The lines of the freely tumbling molecules are

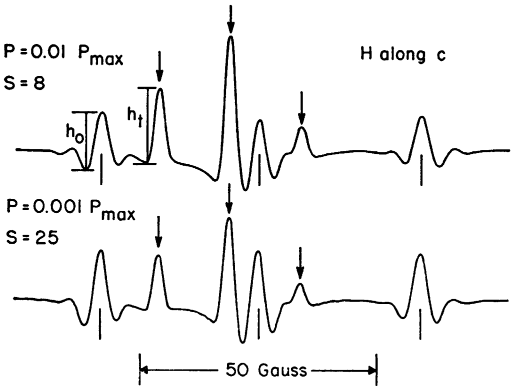


FIGURE 2 Second-derivative ESR spectra taken with the external magnetic field parallel to the c axis. The relative power level (P) and spectrometer sensitivity (S) are given for each spectrum. Arrows pointing downward indicate the field position for the freely tumbling nitroxide molecules and bars beneath the spectra correspond to the oriented molecules. Line height parameters h_o and h_t are used subsequently to compare the power saturation properties of the oriented and tumbling molecules. In these and all other ESR spectra, the magnetic field intensity increases from left to right.

broadened somewhat beyond their minimum width, because the modulation amplitude was chosen to optimize detection of the remaining portion of the spectrum.

The other triplet, indicated by vertical bars beneath the spectra in Fig. 2, is due to nitroxide molecules that are oriented in the crystal. An analysis of the spectral anisotropies of these oriented molecules is the major concern of this paper. We observed with some initial confusion that the transitions of the oriented molecules are quite easily power saturated, in contrast to those of the freely tumbling molecules. This is evident in the spectra of Fig. 2 taken at two different power levels, and is illustrated quantitatively in Fig. 3. A spectrometer power attenuation of 25 dB was used for studies of the oriented molecules.

Two observations lead us to conclude that both the oriented and freely tumbling molecules are Tempone. First, the spectrum of a sample prepared by dissolving a crystal in water shows a single nitroxide triplet with spectral characteristics identical to those of Tempone in water. Second, an individual crystal frequently showed different relative contributions of the oriented and freely tumbling spectral lines, depending on whether it had been stored at room temperature or at 4°C. It appears that Tempone can be trapped in two distinct ways in a reduced Tempone matrix, one giving oriented molecules that are readily saturated by microwave power absorption and another giving freely tumbling molecules with more rapid spin relaxation properties.

The oriented nitroxide molecules gave a triplet absorption with H along any of the crystal axes or anywhere in the *ab* or *bc* planes. In the *ca* plane, this pattern was further split into two distinct triplets due to the existence of two magnetically distinct

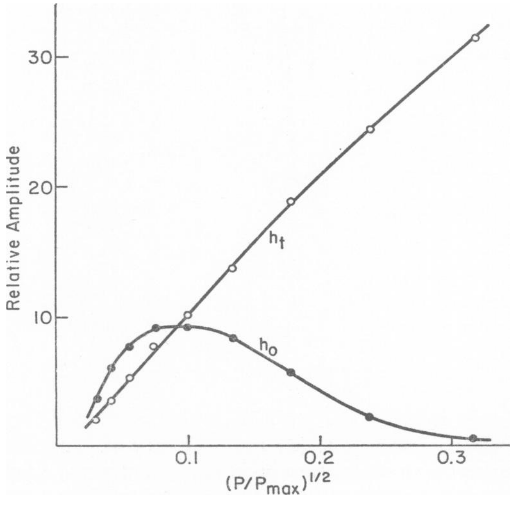


FIGURE 3 Comparison of the power saturation properties of the oriented and freely tumbling molecules in a single crystal. Data were taken with the external magnetic field along the *c* axis of Fig. 1.

sets of molecules with *H* at these orientations. Solid bars and dashed bars beneath the middle spectrum of Fig. 4 indicate these two magnetically distinct triplets for the oriented molecules. Observations at 9.4 GHz and 15.6 GHz confirmed this interpretation of spectra in the *ca* plane. This site splitting was the major factor which led us to analyze data taken in planes of crystal symmetry rather than searching empirically for the nitroxide principal axis system.

Weak satellite lines on either side of the main hyperfine lines of the oriented molecules were observed for many crystal orientations and are evident in the spectra of Fig. 2. These satellite lines arise from simultaneous changes in the spin state of the unpaired electron and the spin state of a single nearby proton. Trammel et al. (1958) have discussed the theory of these transitions which are induced by a weak dipole-dipole coupling between the electron magnetic moment and the magnetic moment

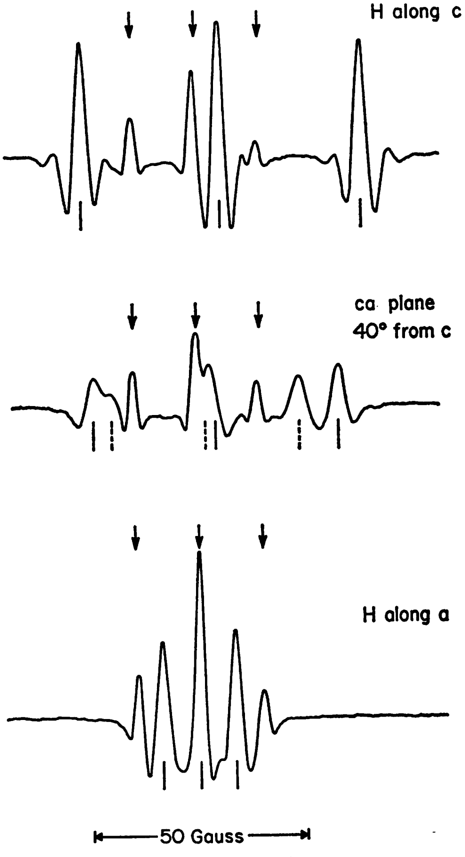


FIGURE 4 Second-derivative ESR spectra taken with the external magnetic field at three different orientations in the ca plane. Arrows above and bars beneath the spectra indicate absorption lines of the freely tumbling and oriented molecules, respectively. Solid and dashed bars for the middle spectrum correspond to the two magnetically distinct molecules in the unit cell.

of the neighboring proton. In accordance with theory, spectra taken at 15.6 GHz showed a reduced intensity and a greater separation for these satellite lines in comparison to spectra taken at 9.4 GHz. These second-order transitions and other higher order transitions (Snipes and Bernhard, 1965) have been observed for a variety of free radicals trapped in solid matrices, and potentially contain important information regarding the distances of nearby protons.

Determination of Tensor Elements

Schonland (1959) has outlined a method for determining the square of the **g** tensor of a free radical in an arbitrary orthogonal or monoclinic laboratory axis system. It involves fitting data from each plane of this axis system to the curve

$$g^2 = \alpha + \beta \cos 2\theta + \gamma \sin 2\theta \tag{1}$$

where α, β, and γ are related to the elements of the square of the **g** tensor and θ is the angle of rotation in each plane. We followed this procedure to determine g^2 for the oriented Tempone molecules relative to the abc axis system. Fig. 5 shows a comparison between experimentally measured g values and the theoretical curve calculated from Eq. 1 for each crystal plane. In the *ca* plane, two sets of g values are shown, corresponding to the two magnetically distinct sets of molecules in this plane. The g value variation for each set of molecules is identical in the *ca* plane, but the variation of one is shifted in phase by approximately 20° from that of the

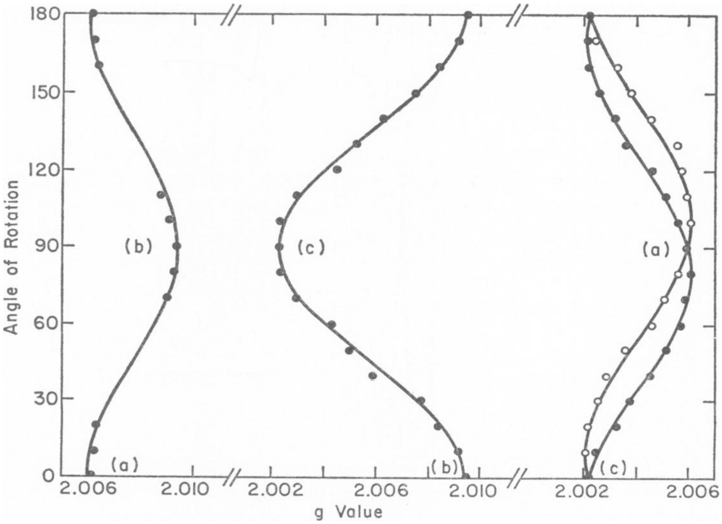


FIGURE 5 Plot of the g value in the three orthogonal planes of the *abc* axis system. Solid theoretical lines are based on the tensor parameters of Table I. Circles are experimental values. Open and closed circles for the *ca* plane correspond to magnetically inequivalent molecules in the unit cell.

other. This gives rise to two sets of α, β, γ, and therefore two sets of elements for g^2 in the laboratory reference frame, that are identical in magnitude but have some elements differing in sign.

The g^2 tensor obtained by this procedure was diagonalized with the aid of an IBM 370 computer, giving the principal values of g^2 and the direction cosines relating the corresponding principal axes to the *abc* axis system. Both g^2 and g are diagonalized by the same similarity transformation (Pryce, 1950; Schonland, 1959) so the direction cosines obtained in this fashion are also those relating the principal axes of the diagonalized g tensor to the *abc* axis system. The principal values of g are the square roots of the elements of the diagonalized g^2 tensor. Results of these calculations are tabulated in Table I.

A procedure similar to that of Schonland has been described for analysis of the hyperfine coupling tensor by Lund and Vänngård (1965). We used that procedure to determine the principal values of A and the direction cosines given in Table I. Fig. 6 shows experimental values of the hyperfine coupling in the three crystal planes and theoretical plots based on the tensor components of Table I. We note that, within experimental error, the principal axis systems of g and A coincide. This observation is not unanticipated for a molecule with the symmetry properties of Tempone, but is different from the results obtained for di-*t*-butyl nitroxide (Libertini and Griffith, 1970). For this latter molecule, the best agreement between experimental data and theory was obtained by assuming a 6° rotation of the two principal axis systems relative to each other with this rotation taking place about the molecular *z* axis. It should be pointed out that, in the case of Tempone, the near axial symmetry of A would make the observance of any noncoincidence of the two principal axis systems quite difficult.

TABLE I

PRINCIPAL VALUES OF THE SPECTROSCOPIC SPLITTING FACTOR (*g*) AND THE NITROGEN HYPERFINE COUPLING CONSTANT (*A*), AND THE DIRECTION COSINES RELATING THE CORRESPONDING PRINCIPAL AXES TO THE EXTERNAL COORDINATE SYSTEM OF FIG. 1

	Principal values	Direction cosines		
		a	*b*	*c*
g_1	2.0094	0	1.000	0
g_2	2.0061	∓0.985	0	0.173
g_3	2.0021	0.173	0	±0.985
A_1	6.5	0	1.000	0
A_2	6.7	∓0.985	0	0.174
A_3	33.0	0.174	0	±0.985

Plus and minus signs for the direction cosines refer to the two magnetically distinct nitroxide orientations within the crystal.

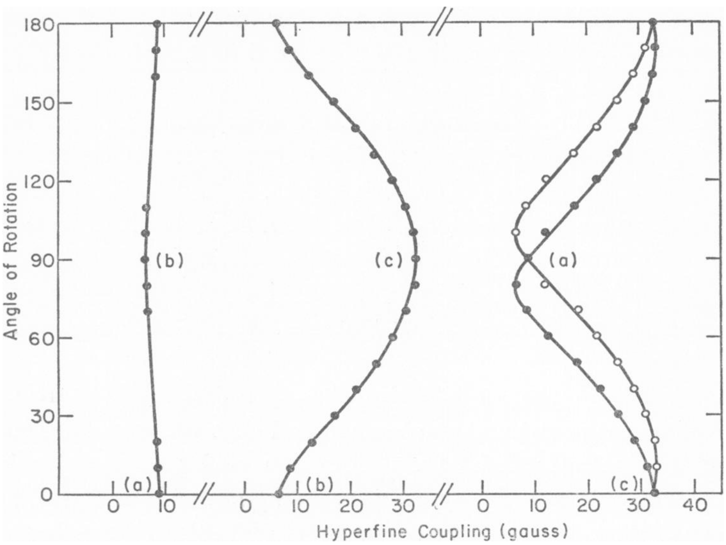


Figure 6 Plot of the hyperfine coupling in the three planes of the *abc* axis system. Details as for Fig. 5.

The crystal structure of reduced Tempone and the orientation of the Tempone molecules in the crystal are unknown. Some inferences can be made, nonetheless, regarding the directions of the principal axes of **g** and **A** relative to the molecular geometry. The minimum g value and the maximum hyperfine coupling are expected to occur with H along the axis of the $2p_z$ orbital of the nitrogen atom. Thus, in the usual terminology, $g_3 = g_z$ and $A_3 = A_z$. The largest principal g value should occur along the N—O bond, by analogy with di-*t*-butyl nitroxide (Libertini and Griffith, 1970). In the spin label literature this is the x direction, so $g_1 = g_x$. The remaining principal value $g_2 = g_y$ should lie in a direction perpendicular to both the nitrogen $2p_z$ orbital and the N—O bond.

Table II gives, for purposes of comparison, the principal tensor elements for all the spin labels that have been reported, including estimated errors. In the present case errors were estimated by fitting the data of Figs. 5 and 6 to theoretical plots with different values of α, β, and γ. The value $g_1 = 2.0094$ for Tempone is the largest g value for any of the nitroxides, but the estimated errors do not preclude larger g values for the oxazolidine derivatives.

Matrix Effects in Spin Label Analyses

Our major purpose for determining the tensor elements of Tempone was the potential use of these tensor elements in spin label studies. We should now consider how appropriate these values obtained from a single crystal analysis are for the analysis of data on other systems, and what modifications of these values, if any, might be made. In particular, the polarity of the matrix surrounding a nitroxide in-

TABLE II

PRINCIPAL VALUES OF g AND A FOR FOUR NITROXIDES

	di-*t*-butylnitroxide*	2N3‡ (oxazolidine)	3NC§ (oxazolidine)	Tempone‖ (piperidine)
A_x	7.59 ± 0.05	5.9 ± 0.5	5.8 ± 0.5	6.5 ± 0.2
A_y	5.95 ± 0.05	5.4 ± 0.5	5.8 ± 0.5	6.7 ± 0.2
A_z	31.78 ± 0.05	32.9 ± 0.5	30.8 ± 0.5	33.0 ± 0.2
g_x	2.00881 ± 0.00005	2.0088 ± 0.0005	2.0089 ± 0.001	2.0094 ± 0.0002
g_y	2.00625 ± 0.00005	2.0058 ± 0.0005	2.0058 ± 0.001	2.0061 ± 0.0002
g_z	2.00271 ± 0.00005	2.0022 ± 0.0005	2.0021 ± 0.001	2.0021 ± 0.0002

* Libertini and Griffith, 1970.

‡ *N*-oxyl-4′,4′-dimethyloxazolidine derivative of propane-2-one; Jost et al., 1971.

§ *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5α-cholestan-3-one; Hubbell and McConnell, 1969.

‖ This work.

fluences both the g value and the hyperfine coupling, and some adjustment of the principal values of Table I (and Table II) for this effect could be made.

The influence of solvent polarity on the isotropic g value and hyperfine coupling of Tempone is evident from the data of Table III. These measurements were made from the isotropic spectra of Tempone in solution, where the anisotropic contributions to **g** and **A** are effectively averaged to zero by the rapid molecular tumbling. These isotropic values are related to the tensor elements by:

$$g_{\text{iso}} = \tfrac{1}{3}(g_x + g_y + g_z) \qquad (2)$$

$$A_{\text{iso}} = \tfrac{1}{3}(A_x + A_y + A_z). \qquad (3)$$

At the bottom of Table III the isotropic values of Tempone in the reduced Tempone crystal are given for comparison.

A shift in the values of g_{iso} and A_{iso} with matrix polarity must arise from a dependency of the principal values of **g** and **A** on matrix polarity. If g_{iso} and A_{iso} can be determined in a particular spin label study, then the principal values can be adjusted accordingly. In the case of the hyperfine coupling, the increased values of A_{iso} in polar media most likely arises from increased unpaired electron spin density on the nitrogen atom and should affect A_x, A_y, and A_z proportionately. This assumes that the change in spin density does not alter the admixture of *s* and *p* states in the nitrogen configuration. Under these assumptions, the most appropriate choice of principal values of $\mathbf{A}^{(m)}$ for a particular matrix would be:

$$A_x^{(m)} = A_x(A_{\text{iso}}^{(m)}/A_{\text{iso}}) \qquad (4)$$

$$A_y^{(m)} = A_y(A_{\text{iso}}^{(m)}/A_{\text{iso}}) \qquad (5)$$

$$A_z^{(m)} = A_z(A_{iso}^{(m)}/A_{iso}) \tag{6}$$

where A_x, A_y, and A_z are the values from Table I (or Table II), A_{iso} is calculated from Eq. 3, and $A_{iso}^{(m)}$ is the isotropic hyperfine coupling measured in the matrix under study.

In the case of the g value, it seems unlikely that all three principal values will be altered proportionately by solvent effects. Large contributions to the anisotropy of **g** may arise from unpaired spin density on the oxygen atom, and spin density shifts may affect one principal g value more than another. In particular, the value of g_z is essentially the free spin value (2.0023), and should be nearly independent of matrix effects. If this is assumed, and if g_x and g_y are assumed to be affected proportionately, then principal value of $\mathbf{g}^{(m)}$ would be:

$$g_x^{(m)} = (g_x)\frac{3g_{iso}^{(m)} - g_z}{g_x + g_y} \tag{7}$$

$$g_y^{(m)} = (g_y)\frac{3g_{iso}^{(m)} - g_z}{g_x + g_y} \tag{8}$$

$$g_z^{(m)} = g_z \tag{9}$$

where g_x, g_y, g_z are the values in Table II (or III), and $g_{iso}^{(m)}$ is the isotropic g value in the system of interest.

Eqs. 4–9 suggest one method for adjusting the principal values of **g** and **A** obtained from single crystals to more appropriate values for a given spin label matrix system. These equations require knowledge of the isotropic values of **g** and **A** in the system being investigated. If in addition a spectrum can be obtained under conditions where the spin label is immobilized, a further check on the values of A_z and g_z is available.

TABLE III

ISOTROPIC COMPONENT OF THE NITROGEN HYPERFINE COUPLING AND *g* VALUE FOR TEMPONE IN SOLVENTS OF DIFFERENT POLARITY AND IN A CRYSTAL OF REDUCED TEMPONE

Solvent	Dielectric constant	Hyperfine coupling	*g* value
n-hexane	1.9	14.5±0.1	2.0060±0.0002
Ethyl ether	4.3	14.6	2.0060
t-butanol	10.9	15.2	2.0058
Ethanol	24.3	15.4	2.0057
Ethylene glycol	37.7	16.1	2.0055
Water	78.5	16.3	2.0054
Crystal (tumbling)		14.9	2.0060
Crystal (oriented)		15.4	2.0059

On a first-derivative tracing, these parameters can be determined (with limited accuracy) from the low-field and high-field peaks corresponding to points of inflection in the absorption spectrum. The close correspondence between these spectral components and the absorption lines for H along z is illustrated in Fig. 7. The first-derivative spectrum is that of a ground-up crystal of Tempone in reduced Tempone. While the values of g_z and A_z estimated in this way are not as accurate as those from single crystal studies, they do reflect the polarity of the spin label environment and provide a useful check of the values assumed for analytical purposes. The value of A_z obtained from the spectrum of Fig. 7 is approximately 0.7 G larger than that in Table II, and the values of g_z are, within experimental error, identical.

The accuracy with which tensor parameters must be known is strongly dependent upon the type of spectral analysis being undertaken, and a full discussion of this aspect of spin label work is not appropriate here. It is worth noting, however, that the ability to detect anisotropic molecular motion is strongly dependent upon the relative principal values of g and A. In particular, some values allow one to recognize enhanced tumbling about the x axis quite readily from line height ratios, and other values do not allow this qualitative observation even though enhanced motion about the x-principal axis might exist. Care should be taken in making conclusions about anisotropic motion unless the tensor elements are known with some degree of accuracy.

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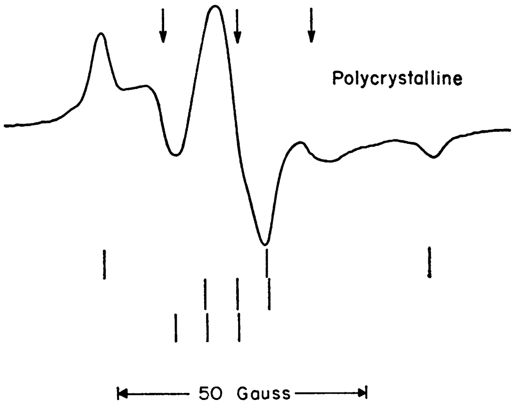

FIGURE 7 First-derivative ESR spectrum of a polycrystalline sample obtained by grinding a single crystal to a fine powder. Bars beneath the spectrum, from top to bottom, give the field position of the absorption lines when the external magnetic field is along the z, y, and x principal axes, respectively. Arrows show the expected position of absorption lines for the freely tumbling molecules.

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