Comments on the Interpretation of Electron Spin Resonance Spectra of Spin Labels Undergoing Very Anisotropic Rotational Reorientation[1a]

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An analysis is given for motional effects on esr spectra of spin labels undergoing very anisotropic rotational relaxation in isotropic liquids. This analysis examines the spectral consequences for cases when the nitroxide is undergoing rapid rotation about a single bond, while the macromolecule to which it is attached is reorienting slowly. A simplified effective diffusion tensor R is employed such that $R_\parallel$ refers to the bond motion and $R_\perp$ to the effects of overall rotation. This approach is seen to be a useful approximation to developing the motional corrections to the effective time-independent S parameter approach of McConnell, *et al.* Our analysis including motional effects is found to be in good agreement with recent experiments of Wee and Miller on spin-labeled polymers. We show how motional effects may account for certain inconsistencies of results on polymer and membrane studies originally interpreted in terms of the effective time-independent approach, and how motional effects can influence the interpretation of the S parameter.

Wee and Miller[2] (WM) have recently studied esr spectra of solutions of spin-labeled polybenzylglutamate (PBLG) polymer in dimethylformamide (DMF). This system was found to have many of the spectral characteristics of spin label studies in membrane models and membranes.[3-6] We have found their results both intriguing in their similarities to the other work as well as in the reduced ambiguities of the physical–chemical nature of their systems.

WM analyze their spectra in the manner of Hubbell, McFarland, and McConnell,[3-6] who have utilized an effective time-independent spin Hamiltonian to account for rapid anisotropic motion. That is, the spin Hamiltonian for a nitroxide radical, whose motion is so slow as to yield rigid-limit spectra, is

$$\hbar \mathcal{H} = |\beta_e| \mathbf{S} \cdot \mathbf{g} \cdot \mathbf{B}_0 + h \mathbf{S} \cdot \mathbf{A} \cdot \mathbf{I} - g_N \beta_N \mathbf{I} \cdot \mathbf{B}_0 \tag{1}$$

where $\mathbf{B}_0$ is the applied dc field, $\mathbf{g}$ and $\mathbf{A}$ are the g and hyperfine tensors. However, a nitroxide spin label undergoing a complicated but very anisotropic motion is approximated by considering the motion about some molecular axis v as being very fast while the motion perpendicular to that axis is very slow. This leads to an effective time-independent rigid-limit Hamiltonian

$$\hbar \mathcal{H} = |\beta_e| \mathbf{S} \cdot \mathbf{g}' \cdot \mathbf{B}_0 + h \mathbf{S} \cdot \mathbf{A}' \cdot \mathbf{I} - g_N \beta_N \mathbf{I} \cdot \mathbf{B}_0 \tag{2}$$

where the effective $\mathbf{g}'$ and $\mathbf{A}'$ tensors are axially symmetric about v, so one need only specify $g_\parallel'$, $g_\perp'$, $A_\parallel'$, and $A_\perp'$ in the usual notation. The use of such an effective Hamiltonian is based on the assumptions that (1) the motion about v, which may be described by an effective rotational diffusion tensor component[7] $R_\parallel$, is so fast that residual time-dependent effects of the averaging process, which could lead to line broadening, etc. are negligible and (2) the motion perpendicular to v, described by an effective $R_\perp$, is so slow, its effects on the spectrum are negligible.

Thus the effective Hamiltonian of eq 2 corresponds to the limiting case in which $R_\parallel[\tau_{R\parallel} = (6R_\parallel)^{-1}]$ and $R_\perp [\tau_{R\perp} = (6R_\perp)^{-1}]$ are respectively too fast and too slow to appreciably affect the spectrum. The rotational correlation time, $\tau_R = (6R)^{-1}$ where $R = (R_\perp R_\parallel)^{1/2}$ is undefined. It is an objective of this work to show that the results of WM as well as

much of the spin-labeled membrane work show unmistakable motional effects, contrary to their original interpretation in terms of eq 2.

One may relate the $\mathbf{A}'$ of eq 2 and $\mathbf{A}$ of eq 1 in terms of the direction cosines α_i, $i = x$, y, or z of v in the molecular principal axis system. One takes the principal axes of $\mathbf{A}$ (and of $\mathbf{g}$) such that the z axis is along the $2p$-π orbital of nitrogen. The x axis is along the N–O bond, with the y axis perpendicular to the other two. Then one has

$$A_\parallel' = \sum_{i=x,y,z} \overline{\alpha_i^2} A_i \tag{3}$$

$$A_\perp' = \tfrac{1}{2} \sum_{i=x,y,z} (1 - \overline{\alpha_i^2}) A_i \tag{4}$$

where the superbars imply time averages. Similar equations hold for the elements of $\mathbf{g}$ and $\mathbf{g}'$. In many nitroxides $A_x \approx A_y$, so eq 3 and 4 become

$$A_\parallel' = A_0 + 2/3(A_z - A_x)S \tag{5}$$

$$A_\perp' = \tfrac{1}{2}(3A_0 - A_\parallel') \tag{6}$$

where A_0 is the isotropic hyperfine term

$$A_0 = 1/3 \mathrm{Tr}\,(A) = 1/3 \mathrm{Tr}\,(A') = A_0' \tag{7}$$

and S (by analogy with liquid crystalline spectra) is known as the order parameter and is given by

$$S = \tfrac{1}{2}(3\overline{\alpha_z^2} - 1) = (A_\parallel' - A_\perp')/(A_z - A_x) \tag{8}$$

Thus S is a measure of the mean rotational amplitude leading to $\mathcal{H}'$, such that when $S = 1$, eq 2 becomes identical with eq 1 corresponding to the nitroxide exhibiting no motional averaging; while when $S = 0$, $A_\parallel' = A_\perp' = A_0$ corresponding to isotropic rotational motion with a correlation time less than about 1 nsec.[6] Hubbell, *et al.*,[3-6] have emphasized that for this interpretation in terms of the "pseudoaxial" rigid limit of eq 2 to be valid, one must have $\mathrm{Tr}\,A = \mathrm{Tr}\,A'$ according to eq 7, which follows directly from the rotational invariance of the trace of a tensor. We wish, in this work, however, to show that, while this condition is a necessary one, it is not, in general, sufficient as a result of motional effects. Before we consider our analysis in terms of motional effects we wish to review

some of the relevant experimental results and their original analysis.

Generally, A_0' as determined from spectra based upon the effective spin Hamiltonian of eq 2 have been found to fulfill the criterion of eq 7. However, A_0 for nitroxide radicals is weakly dependent upon solvent polarity, and this has sometimes resulted in uncertainty as to whether the criterion of eq 7 is fulfilled especially if the environment of the spin label is not known. Thus, in the work of Hubbell, et al., variations in A_0' have been interpreted as changes in the polarity of the local environment of the nitroxide group, and a correction has been used to adjust the measured S for the polar-hydrophobic effect.[5] Spectra of the dimethyl-N-oxyloxazolidine (DOXYL) long-chained spin labels in phospholipids and membranes are satisfactorily simulated with an effective $\mathcal{H}'$ of eq 2 with one exception (cf. Figures 2-4 of ref 5). In this case, those computed spectra, which gave the best overall agreement with the observed spectra, still had outer hyperfine extrema that were narrower and of greater amplitude regardless of the line shape assumed. Although the principle source of this discrepancy may well be due to line broadening from heterogeneity of the fatty acid chains of the lecithin host, as suggested by Hubbell and McConnell,[5] we note that the widths of outer hyperfine extrema can be markedly broadened by a rotational uncertainty-in-lifetime effect which can broaden these outer hyperfine extrema without significantly shifting their positions.[8] So it is possible that a residual motion transverse to the symmetry axis ν is responsible for this discrepancy, although an orientation-dependent line width could also adequately account for this discrepancy. The fact that $R_\perp$ may be fast enough that it affects the spectrum has been generally recognized. The rigorous theory of Freed, Bruno, and Polnaszek (FBP)[9] has already been used to simulate spectra where the motion is anisotropic but the motional effects from both $R_\parallel$ and $R_\perp$ have direct observable effects on the spectrum.[10]

We give in Tables I-III some of the published experimental data for anisotropically immobilized spin labels. They include the results of ref 5 (Table I), ref 12 (Table II), and WM (Table III). These data have several trends in common. Hubbell, et al., have noted that as $A_\parallel'$ approaches A_z, S approaches unity and the symmetry axis of $\mathcal{H}'$ must approach the molecular z axis. $\cos^{-1} \overline{(\alpha_z{}^2)}^{1/2}$ is referred to as the mean angular deviation between ν and z. In Tables I-II, an increase in $A_\parallel'$ and the resulting increase in S is accompanied by an increase in A_0'. Most, but not all, of the published data exhibit this phenomenon of an increase in A_0' with an increase in S.[2,5,11,12] In the case of the DOXYL spin labels in aqueous dispersions of natural phospholipids this trend in A_0' is as noted explained as an increase in the polarity of the environment of the nitroxide as it is attached closer to the polar head group. The value of A_0' becomes as great as 15.2 G, which is the value of the isotropic hyperfine splitting of the radical in pure distilled water. Seelig, Limacher, and Bader have attributed this variation in terms of electrostatic interactions between the nitroxide dipole and the dipolar regions of the bilayer.[13] The increase in S and its interpretation as a restriction in the motional amplitude of the DOXYL ring concomitant with an increase in the polarity of the environment as the DOXYL spin label is attached progressively closer to the polar head group is brought into question by our analysis below.

As already noted, the results of WM have characteristics similar to that of the DOXYL spin labels in membrane

TABLE I[a]

Spin label	$A_\parallel'$, G	$A_\perp'$, G	A_0', G	$g_\parallel' - g_\perp'$	S
IV(10,3)	27.8	9.0	15.2	−0.0036	0.69$_5$
IV(7,6)	26.0	9.5	15.0	−0.0033	0.62
IV(5,10)	21.8	10.3	14.1	−0.0026	0.46

[a] Table I entitled "Resonance Data for Phospholipid Spin Labels IV(m,n) in Egg Lecithin-Cholesterol (2:1 Mole Ratio)" of ref 5.

TABLE II[a]

Probe	T, °C	$A_\parallel'$, G	$A_\perp'$, G	A_0', G	S
4 NS	22	26.6	9.3	15.1	0.65
4 NS	5	29.1	8.0	15.1	0.72
9 NS	5	25.0	9.7	14.8	0.58
12 NS	5	23.3	10.1	14.5	0.52

[a] Table 3 entitled "Behavior of Spin-Probes Incorporated into Aqueous Dispersions of Lipids Extracted from Microsomal Preparations" of ref 12.

models and membranes in that an increase in $A_\parallel'$ results in an increase in S, which is concomitant with an increase in A_0'. However, in this case, the increase in A_0' is clearly not primarily due to solvent effects, because the PBLG polymer has a chemical composition similar to that of the DMF solvent. When we note that A_0 is found to be essentially independent of temperature for similar nitroxides,[10,14] then the changes in the apparent A_0' with temperature in the results of WM appear to be definitely anomalous.

It is this anomaly, most unequivocal in the results of WM, which first prompted us to consider the possibilities that motional effects in these spectra are leading to inaccuracies, if not a break down, in their interpretation in terms of eq 2. That is, what happens when (1) $R_\parallel$ is slow enough and/or (2) $R_\perp$ is fast enough to lead to motional effects? It is already known, in the simpler case of isotropic rotation, that an increase in $A_\parallel'$ may be interpreted in terms of a slowing of the motion of the spin label.[15] Thus, perhaps, in the case of "anisotropically immobilized" spectra, an increase in $A_\parallel'$ may be due to a decrease in $R_\parallel$.[16]

We have developed, for problems of this sort, a slow motional computer program[17] based on the rigorous theory of FBP.[9] It allows the principal axes of $\mathbf{R}$ (i.e., x', y', and z') to be tilted relative to the principal axes of the magnetic tensors. The irreducible tensor components of $\mathbf{A}$ and $\mathbf{g}$ are then expressed in the x', y', z' coordinate system. We take θ to be the angle between z' and z. (Note that ν corresponds to z'.) In our analysis of the results of WM we tilted z' toward x (i.e., the symmetry axis of $\mathbf{R}$ lies in a plane formed by the $2p_\pi$-π orbital and the N-O bond).[18] Thus θ is the same as $\cos^{-1} \overline{(\alpha_z{}^2)}^{1/2}$ of Hubbell, et al., while $\cos^{-1} \overline{(\alpha_x{}^2)}^{1/2}$ is $(\pi/2) - \theta$ and $\cos^{-1} \overline{(\alpha_y{}^2)}^{1/2}$ is $\pi/2$.

We show in Figure 1 a set of simulated spectra designed for comparison with the results of WM, some of which are shown in Figure 2 with an illustration of their spin label in Figure 3. The series of spectra shown in Figure 1 (labeled A-I) were computed with a constant value of $R_\perp = 3.33 \times 10^6$ sec^{-1} or $\tau_{R\perp} = (6R_\perp)^{-1} = 5 \times 10^{-8}$ sec but with $\tau_{R\parallel}$ ranging from 6×10^{-11} sec through 5×10^{-8} sec. Further, we have used a value of $\theta = 41.7°$ corresponding to an $S = 0.336$, and this value of S remains unchanged for all the simulations. The spectra were calculated for spatially isotropic distribution of spin labels, i.e., there is no true ordering. We have also prepared a table (cf. Table IV) in the manner of Tables I-III, in which the simulations have been analyzed as if they were from anisotropically immo-

TABLE III[a]

Spectrum index	v_z^b	T, °C	$A_{||}'$, G	$A_\perp'$, G	$\Delta A'$, G	A_0'	S	$\cos^{-1} \overline{(\alpha_z{}^2)}^{1/2}$, deg
F	0.42	Rt[c]	22.5	11.2	11.3	15.0	0.452	37.2
	0.30	Rt	21.3	11.2	10.1	14.6	0.404	39.1
E	0.20	Rt	21.2	12.0	9.2	15.1	0.368	40.5
		4	22.8	11.6	11.2	15.3	0.448	37.3
		−43	25.8	10.8	15.0	15.8	0.600	31.1
	0.187	Rt	21.1	11.9	9.2	15.0	0.368	40.5
D	0.148	Rt	21.2	11.9	9.3	15.0	0.272	40.3
		−43	26.4	10.8	15.6	16.0	0.624	30.1
C	0.128	Rt	20.6	11.6	9.0	14.6	0.360	40.8
B	0.092	Rt	20.2	11.5	8.7	14.4	0.348	41.2
A	0.008	Rt	19.8	11.7	8.1	14.4	0.324	42.2
		−43	25.8	10.9	14.9	15.9	0.596	31.2

[a] Data from Table I of Wee and Miller (ref 1). [b] The polymer concentration (volume fraction) in DMF. [c] Rt denotes room temperature.

TABLE IV

Spectrum index	$R_{||} \times 10^8$, sec^{-1}	$\tau_{R||}$, nsec	$A_{||}'$, G	$A_\perp'$, G	$\Delta A'$, G	$A_0'^f$	$\cos^{-1} \overline{(\alpha_z{}^2)}^{1/2}$, deg^e	$g_{||}' - g_\perp'$	S^e	$g_{||}'$	$g_\perp'$	$g_0'^g$	$\cos^{-1} \overline{(\alpha_x{}^2)}^{1/2}$, deg^h
A[a]	27.8	0.06	18.9^j	11.5	7.4	14.0	43.2	−0.0008	0.296	2.0051	2.0059	2.0056	55[i]
B[a]	16.7	0.10	18.9	11.3	7.6	13.8	42.9	−0.0008	0.304	2.0051	2.0059	2.0056	55
C[a]	8.33	0.20	19.2	11.1	8.1	13.8	42.2	−0.0010	0.324	2.0050	2.0060	2.0057	57
D[a]	4.17	0.40	20.2	10.7	9.5	13.9	40.0	−0.0014	0.380	2.0047	2.0061	2.0056	60
E[a]	2.50	0.67	22.6	10.3_5	12.2_5	14.4	35.7	−0.0025	0.490	2.0037	2.0062	2.0054	80
F[a]	1.67	1.00	24.1	10.9	13.2	15.3	34.1	−0.0017	0.528	2.0033	2.0058	2.0050	47
G[a]	0.278	6.00	27.3	c							2.0030	c		
H[b]	0.167	10.0	27.7	c							2.0028	c		
I[b]	0.033	50.0	29.7_5	c							2.0025	c		
J[b]	0^d	∞^{d}	30.8	c							2.0021	c		

[a] A peak-to-peak residual derivative width of 1.0 G was used. [b] A peak-to-peak residual derivative width of 3.0 G was used. [c] The inner hyperfine extrema are not resolved. [d] $R_\perp$ and $R_{||}$ are 0. [e] S and α_z are defined here by eq 8. [f] A_0' is defined by eq 7. [g] g_0' is defined as $1/3(g_x' + g_y' + g_z')$. [h] From construction $\cos^{-1} \overline{(\alpha_y{}^2)}^{1/2} = \pi/2$. [i] If $S = 0.336$ is used $\cos^{-1} \overline{(\alpha_x{}^2)}^{1/2}$ equals 50°, see text. [j] If (30.8 G − 29.75 G) is added to $A_{||}'$ then $S = 0.338$.[22]

bilized spin labels, and we note it exhibits the typical aforementioned trends of the actual experimental results.

Our choice for $\tau_{R\perp}$ is somewhat arbitrary, but we note that τ_R values of about 1.5×10^{-5} and 7.5×10^{-8} sec were estimated by WM for relaxation of and about the helix axis, respectively, of their helical polypeptide (with a weight average molecular weight of 122,000 consisting of an average of 560 monomeric units) from standard equations given by Perrin.[2,7,19] Note, however that even an isotropic τ_R of 7.5×10^{-8} sec will not significantly decrease the separation of the outer hyperfine extrema below that of the rigid limit (i.e., $A_z \geq A_{||}' \geq 0.96 A_z$).[15] Thus a comparison of spectra I and J in Figure 1 for an isotropic $\tau_R = 5 \times 10^{-8}$ sec and the rigid limit, respectively, shows that the spectra are not substantially different except for a somewhat broader appearance for spectrum I. The simulation Figure 1I is in good agreement with spectrum Figure 2G of WM, which is for the solid end-labeled polymer near the rigid limit.

We note that the spectrum Figure 2B of WM closely resembles the computed spectra A and B of Figure 1. Apparently the rotational motion about the symmetry axis is very rapid and very unlikely attributable to the much slower motions of the polymer itself where $\tau_R \geq 10^{-7}$ sec. The rotational rate $R_{||}$ is typical of that for motion about single covalent bonds (see below). WM have argued that the nitroxide spin label of this end-labeled polymer is only free to rotate about the single covalent bond joining the piperdine ring to the terminal peptide nitrogen (NH–CH bond), and these simulations support their arguments, because it is reasonable that $R_{||}$ be taken as the rotational rate about this single bond (e.g., Brevard, et al., determined a correlation time of 1.1×10^{-10} sec for rotation

about a sterically hindered single bond[20]). Although the internal motion of the nitroxide radical relative to the polymer was considered by WM, they did not appréciate that the changes in the progression of their spectra in Figure 2 were likely due to the slowing of the internal motion of the spin label.

The outer and inner pairs of extrema in Figures 1 and 2 are clearly not spaced about the same field position. This noticeable g factor asymmetry is consistent with a tilt toward the x axis; a corresponding tilt toward the y axis would result in noticeably less g factor asymmetry. The assignment of $R_{||}$ to motion about the covalent bond linking the polymer to the piperidine ring is consistent with the rotational symmetry axis being in the xz plane (i.e., in the plane formed by the N–O bond and the 2p–π orbital of the nitrogen in Figure 3).

As motion about the symmetry axis slows (i.e., $R_{||}$ decreases), an increase in the apparent ordering parameter S is seen in the data of Table IV. The increase in S results from an increase in $A_{||}'$, which is eventually not compensated for by a corresponding decrease in $A_\perp'$ required by the rotational invariance of A_0'. An increase in the apparent A_0' parallels an increase in apparent S much as in the case of the investigations of membranes and membrane models with the DOXYL radicals (cf. Tables I and II). However, for the data of WM (cf. Table III), the increase in A_0' which accompanies the increase in S as the temperature is lowered can be taken as a clear indication that the limiting case of a fast $\tau_{R||}$ is no longer applicable.

Hubbell, et al., have implied that the first observable effect of incomplete averaging by motion about the symmetry axis is an overall broadening of the spectrum, and the interpretation in terms of $\mathcal{K}'$ of eq 2 and the ordering
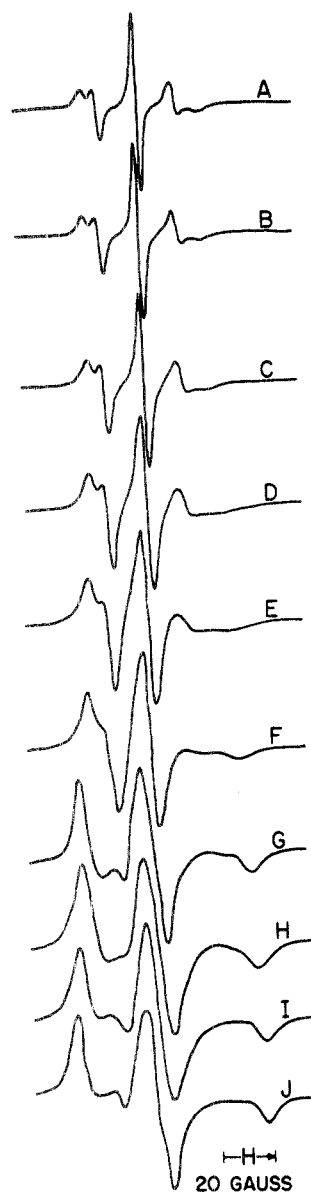
Figure 1. These simulations were computed with the magnetic parameters of the DOXYL spin label where $A_z = 30.8$ G, $A_x = A_y = 5.8$ G, $g_x = 2.0089$, $g_y = 2.0058$, and $g_z = 2.0021$.[5] The symmetry axis of the rotational–diffusion tensor is defined in the molecular axis system by the angles $\cos^{-1} (\overline{\alpha_x^2})^{1/2} = 48.3°$, $\cos^{-1} (\overline{\alpha_y^2})^{1/2} = 90.0°$, and $\cos^{-1} (\overline{\alpha_z^2})^{1/2} = 41.7°$. $\tau_{R\perp}$ is 5.0×10^{-8} sec and only $\tau_{R\parallel}$ was varied in this series of simulations. In these spectra the resonant magnetic field for $g = 2.0056$ occurs at 3235 G. See Table IV for values of $\tau_{R\parallel}$ and residual derivative width used in the simulations.
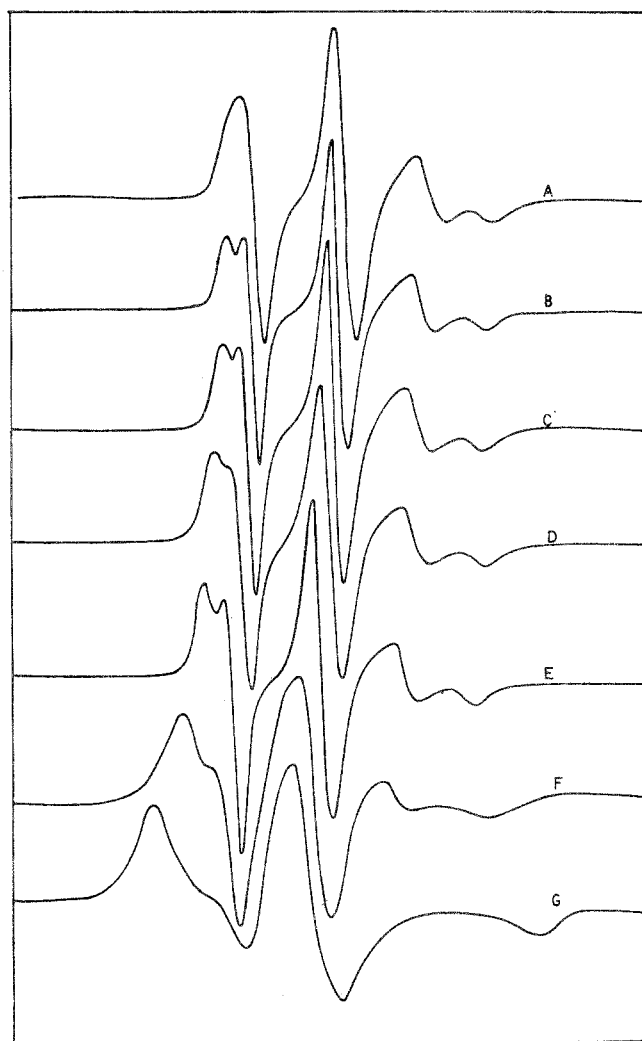

Figure 2. Esr spectra of polybenzylglutamate labeled with 2,2,6,6-tetramethyl-4-aminopiperidine 1-oxyl in dimethylformamide solutions of varying concentrations at room temperature. The polymer concentration (volume fraction) was (A) 0.008, (B) 0.0917, (C) 0.128, (D) 0.148, (E) 0.200, (F) 0.42 (0.5 weight fraction), and (G) 1.0 (solid polymer). (From ref 1, with permission).

parameter remains unchanged.[3b] It is clear from our series of stimulations, that, if $\tau_{R\parallel}$ is slow enough to broaden the spectrum, a change in the apparent S will also result. Note that given our analysis, the internal motion of the piperidine ring is only slightly dependent on the bulk viscosity η (or alternatively on polymer concentration). In the series of room temperature spectra Figure 2A through 2F of WM, η has increased considerably more than the order of magnitude that $R_\parallel$ has decreased, while the spectra at $-43°$ are all quite similar.[2] The rotational rate $R_\parallel$, if identified with the motion of the piperidine ring about the NH–CH bond, implies that θ be identified as the angle between the 2p–π orbital of nitrogen (molecular z axis) and the linking covalent bond. It appears that this

tilt angle remains essentially unchanged, through the series of experiments of WM (as assumed in the simulations), which is expected if the tilt angle depends only on the geometry of the piperidine ring. This is supported by the absence of significant changes in the observed spectra due to phase transitions (i.e., transitions between isotropic, biphasic, or liquid crystal phases), plus the fact that the overall motion of the polymer is slow enough so as not to affect the spectrum.

Spectra A and B of Figure 1 are representative of the case where $R_\parallel$ is large enough to completely average the "time-dependent" part of the Hamiltonian (i.e., $\mathcal{K} - \mathcal{K}'$) and $R_\perp$ is too small to significantly shift the positions of the outer hyperfine extrema. Thus they effectively represent the limiting case of Hubbell, et al. One may confirm this by determining the pseudo-axial A' from the computer simulations of Figure 1A and 1B. One then obtains (in the manner of Seelig[21]) an order parameter in good agreement with the value of θ used in the simulations. The discrepancy of $1.5°$ between θ and the one obtained from S of our simulations probably results from our assumptions of motion due to $R_\perp$.[22]
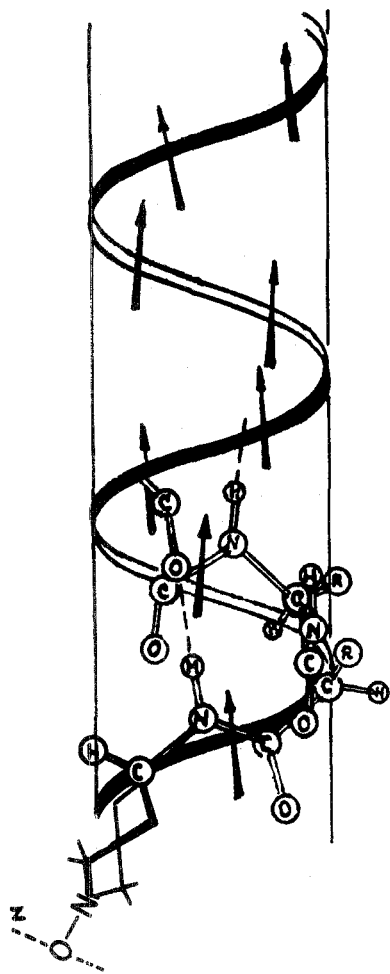
Figure 3. End-labeled polybenzylglutamate, right-handed α-helix. (From ref 1, with permission).

When $R_{\parallel}$ is no longer fast enough to average out the "time-dependent" part of $\mathcal{K}$, then an increase in the *apparent S* results, as well as eventually an increase in the *apparent A_0'*. This is clearly seen in Figure 1 and Table IV. This increase in the *apparent A_0'* is clearly observed in the work of WM. We again emphasize that θ, hence the true S, is unchanged in our simulations.

If $R_{\parallel}$ is the rotational rate of the piperidine ring about the covalent bond attaching the spin label to the polymer, an Arrhenius-type dependence of $R_{\parallel}$ is expected.[23,24] The similarities between the spectrum of Figure 2B of WM and Figure 1B imply a $R_{\parallel}$ for the piperidine ring of about 1.67×10^9 sec^{-1} at room temperature and a volume fraction of 0.0917. Likewise, the similarities between Figure 2F of WM and Figure 1F imply a $R_{\parallel}$ of about 1.67×10^8 sec^{-1}. Note that Figure 2F of WM resembles all their spectra at $-43°$.[2] Thus one may use the same value of $R_{\parallel}$ for $-43°$. One then estimates an activation energy of 1.9 kcal/mol from these results, which is a reasonable value for the rotational barrier about a single bond.[20] WM do not provide enough temperature-dependent data to otherwise confirm an Arrhenius-type behavior.

There is one aspect of our computer simulations which differs somewhat from the experimental spectra of WM. That is, our simulations show somewhat excessive width and related reduced amplitude of the outer hyperfine extrema. This clearly indicates that a $\tau_{R\perp}$ of 5.0×10^{-8} sec is too short; a longer value of τ_R would narrow these ex-

trema and improve agreement with observed amplitudes. Also, as already noted, the positions of the hyperfine extrema would not be significantly shifted. We have already called attention to the opposite discrepancy in comparison with results of Hubbell and McConnell, and this could mean a $\tau_{R\perp}$ on the order of 5.0×10^{-8} sec in their systems.

We wish to emphasize that from our above analysis, even if eq 7 is fulfilled, our simulations Figure 1A–E and Table IV show that substantial changes in apparent S can occur without a real change in θ as a result of a slowing of $R_{\parallel}$. It is clear, then, that changes in apparent S can result from a slowing of the motion about v as well as a change in $\cos^{-1} \overline{(\alpha_z^2)}^{1/2}$ (*i.e.*, the mean angular deviation between v and z). Therefore the invariance of Tr A is a necessary, but not sufficient, condition that motion about v be sufficiently rapid to permit the spectrum to be analyzed in terms of the effective static Hamiltonian of eq 2. It would appear that the rotational rates expected for covalent single bonds at room temperature are not, in general, fast enough to fulfill this condition.

Even in DOXYL spin label membrane systems where A_0' is independent of S[13] and/or temperature,[12] the quantitative interpretation of the variations in S based upon eq 2-8 is uncertain, because the condition that trace (A) be invariant is a necessary, but not sufficient, condition. In any case, a change in S can arise from a real change in the angle between v and z or a *change in the rotational rate* about v as is the predominant phenomenon in the remaining spectra of WM. In general, these two phenomena cannot be distinguished unless the rotational rate about v slows to where A_0' is clearly anomalous, and/or the slowed motion manifests itself in the other spectral characteristics illustrated in Figure 1. Note the difference in the spectra A and B of Figure 2 has been shown by computations to arise from a shorter $\tau_{R\perp}$ of about 3.0×10^{-8} sec for spectrum A. Apparently at infinite dilution the motion of the polymer is significant. Calculations of a statistical mechanical variety of S itself are not always fully justified, since, in general, it cannot be proved that changes in S did not arise from a change in the rotational rate about v.[25] However, the alternative of careful analysis of the complete spectrum holds open the possibilities of obtaining considerable information of interest.

At this point it should be emphasized that the widely used interpretation of S as a qualitative indication of the viscosity or the "fluidity" of the environment of the spin label is not changed.

One should, of course, recognize that our above analysis in terms of a single $R_{\parallel}$ and $R_\perp$ represents a considerable simplification of the complex dynamics of polymer motion as well as localized bond motions, including internal rotations. A somewhat more complete analysis would, for example, include the coupling of the internal rotation of the piperidine C–N bond to the overall anisotropic rotation of the helical polymer requiring the specification of R for the polymer, a τ_R for the bond rotation as well as the tilt angle θ.[26,27] However, as long as the internal rotation is much faster than the overall motion, it can be treated as uncoupled from the latter. Also, to the extent that the overall motion is only showing marginal spectral effects, it would be difficult to obtain anything more precise than an effective $R_\perp$, as we have done above, as opposed to an actual R tensor. Of course, in principle, there are the helical flexing modes of motion, which are also slow, and which would be contributing to that quantity we refer to as an effective $R_\perp$. Finally, in this regard, we note the impor-

tance of localized cooperative modes of relaxation for the polymer motions even in isotropic polymer solutions of moderate concentration, and it becomes even more important in liquid–crystalline phases (cf. ref 17, 24, and 28). We also note that our analysis implies the internal rotation is Brownian, while a more complete analysis would include potential barriers and the possibilities of reorientation by jumps of substantial angle. Thus our analysis given above may be regarded as the first approximation to the motional corrections to the effective time-independent approach of Hubbell, et al.

In conclusion, we call attention to the fact that spectra of the "anisotropically immobilized" type do exhibit interesting dependencies on the molecular dynamics of the spin label, and phenomena related to motion about the symmetry axis of the rotational–diffusion tensor and even transverse to the symmetry axis have been observed experimentally but not generally recognized as such.

References and Notes

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(16) McConnell, *et al.*, note that the lower limit on $R_{||}$ required for complete averaging depends markedly upon $\cos^{-1}(\alpha_z^2)^{1/2}$. If $v_{||}z$, then $\cos^{-1}(\alpha_z^2)^{1/2} = 0$, and for $A_x = A_y$, the only anisotropy to be averaged to produce axial symmetry is simply due to g. Thus $R_{||} \gg \hbar^{-1}|(g_x - g_y)\beta|B_0 \sim 1.4 \times 10^7$ sec^{-1}. While, if $v_{\perp}z$, then $\cos^{-1}(\alpha_x^2)^{1/2} = \pi/2$ and one requires $R \gg 7 \times 10^7$ sec^{-1}.

(17) C. F. Polnaszek, Thesis, Cornell University, 1974. One may take advantage of the large anisotropy in R to truncate the eigenfunction expansions for the simulations in the "quantum number K." Truncation at $K = 2$ is nearly equivalent to treating the time-dependent fluctuations of $\{\mathcal{H}' - \mathcal{H}\}$ by perturbation theory (see ref 6).

(18) Although a tilt of R toward the x axis (*i.e.*, the N–O bond) can be distinguished from the tilt toward the y axis, in practice $g_{||}'$ and $g_{\perp}'$ are not as useful as $A_{||}'$ and $A_{\perp}'$ because the anisotropy is not as great for g.

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(22) The apparent A_z (or $A_{||}'$) is reduced by 1.05 G from its rigid limit value by an isotropic $\tau_R = 5 \times 10^{-8}$ sec.[14] If 1.05 G is added to $A_{||}$, the calculated S becomes 0.338 in near perfect agreement with the expected value of 0.336 for $\theta = 41.7°$. Note, α_z^2, and therefore S are most sensitive to changes in angles when $\cos^{-1}(\alpha_z^2)^{1/2}$ is near 45°.

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