Immobilized Radicals. I. Principal Electron Spin Resonance Parameters of the Benzosemiquinone Radical

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Abstract: ESR spectra of the benzosemiquinone radical and the perdeuteriobenzosemiquinone radical in a rigid methanol glass were recorded at both X-band and Q-band microwave frequencies. The principal g factors were determined to be g^{xx} = 2.0065, g^{yy} = 2.0053, and g^{zz} = 2.0023 while the hyperfine splitting constants were a^{xx} = 0.5 ± 0.4 G, a^{yy} = +3.0 ± 0.3 G, and a^{zz} = 2.4 ± 0.4 G. The axis system was chosen to have the x axis along the oxygen atoms in the plane of the radical and the z axis perpendicular to the plane of the radical. The principal ESR parameters were determined additionally through a calculation on a theoretical model to be g^{xx} = 2.0059, g^{yy} = 2.0037, g^{zz} = 2.0023, a^{xx} = 1.1 G, a^{yy} = 3.1 G, and a^{zz} = 2.9 G.

The utilization of electron spin resonance (ESR) spectroscopy for the observation of paramagnetic species in biological systems has increased enormously in recent years. Several examples[1] of radical signals detected in these systems are: (1) radicals generated in cellular structures by X-ray or ultraviolet radiation; (2) reversible light-induced radical signals in membranes and proteins isolated from photosynthetic cells; (3) signals from metaloproteins from electron transport systems; (4) free-radical forms of quinones, flavin, and flavoproteins. Since the majority of the radicals observed in biological systems are either in slow tumbling structures or in rigid media, the ESR signals observed are best described as powder spectra associated with overlapping spectra of randomly oriented molecules. These powder spectra incorporate the anisotropic contributions of the g factor and nuclear hyperfine splitting which are normally averaged out during rapid tumbling of the radical species. Recently, spin labels[2] have been used to take advantage of this anisotropic property of rigid molecules. Spin labels are stable radicals (often nitroxides) which when incorporated into membrane or protein systems monitor the fluidity of the labeled region. In general, the less fluid the environment the greater the observed anisotropic components in the spectrum of the label. Apropos of this, it seems only logical that radicals indigenous to biological systems could be used as spin labels to monitor structural properties of the environment. Although synthetic spin labels have been invaluable in their aid in the elucidation of numerous questions concerning biological structure, natural labels have several advantages over synthetic probes. First, natural labels, being innately present in biological systems, are not concerned with the complications sometimes encountered when attempting to incorporate synthetic labels into systems. Second, a natural label will not perturb or change its environment from its normal physiological state. Last, ESR spectra of natural labels relate the native environment of these radicals which are often species of biological importance whose surroundings are frequently difficult to study with synthetic labels. This paper commences a series of studies on radicals of biological importance in order to demonstrate how these natural labels can be employed to probe biological structures.

Quinones are ubiquitous in living systems. Their functions in these systems are numerous,[3-6] varying from mediation in electron transport reactions to participation in the reactions involving the regulation of blood clotting and aging. In their role in the mediation of electron transport reactions in both mammalian and photosynthetic cells, quinones have been shown to be one- or two-electron reduced to the semiquinone and quinol forms. Of these two reduced forms the semiquinone is paramagnetic and has been detected with ESR spectroscopy in pigment-protein extracts of living cells. The spectra observed[7-10] are those of immobilized radicals, i.e., powder spectra, thereby supporting the hypothesis that these quinones are associated with large protein or membrane moieties.

The past inability to identify definitively the association of ESR spectra with various in vivo radicals has been due mainly to the frequent lack of sufficient fine structure in powder spectra. Semiquinone powder spectra represent a good example of such broadened spectra. Unfortunately, before a biological radical such as the semiquinone can be used as a natural label, the values of its anisotropic g factors and splitting constants (the principal anisotropic parameters) must be known. Therefore, in order to simplify the identification of immobilized biological quinones and aid in their use as natural labels, this article presents a detailed empirical and theoretical analysis of the powder spectrum of the benzosemiquinone radical anion. The anisotropic parameters of this nonbiological quinone are used to interpret the spectra of the more involved biological quinones such as ubiquinone and plastoquinone. ESR spectra of the protonated and deuterated benzosemiquinone were recorded at X-band (9.5 GHz) and Q-band (35 GHz) microwave frequencies. Anisotropic g factors and hyperfine splitting constants are determined from a computer simulation of the spectra and are shown to compare favorably with values calculated from a theoretical model.

Experimental Section

The benzosemiquinone anion radicals were generated using one of the three following techniques: (1) air oxidation[11] of the quinol; (2) electrochemical reduction[12] of the quinone; (3) photochemical excitation[13] of the quinone. All reactions took place in methanol. Base (10^{-4} M NaOH) was added to retard the decay process of the anion radical. Since there was no detectable difference in the recorded powder spectrum of the radical generated in any of these techniques, air oxidation of the quinol was used for most experiments. The spectrum was also unaffected by the radical's concentration up to 10^{-2} M. On the other hand, frozen solutions of the radical at these higher concentrations exhibited a strong blue hue in contrast to the typical yellow hue of the anion radical. The reason for this change in hue is not known but is hypothesized to be due to radical complexes in the concentrated systems. Because of this, spectra were recorded only of solutions with radical concentrations less than 10^{-4} M.

Methanol (absolute, Mallinckrodt), methanol-d, and methanol-d_4 (both 99%, Bio-Rad Laboratories) were used without further purification. Benzoquinone and benzoquinol (allied) were purified by double vacuum sublimation. Perdeuteriobenzoquinol was prepared according to the method of Charney and Becker.[14] Powder samples of the semiquinones were obtained by freezing the alcohol-

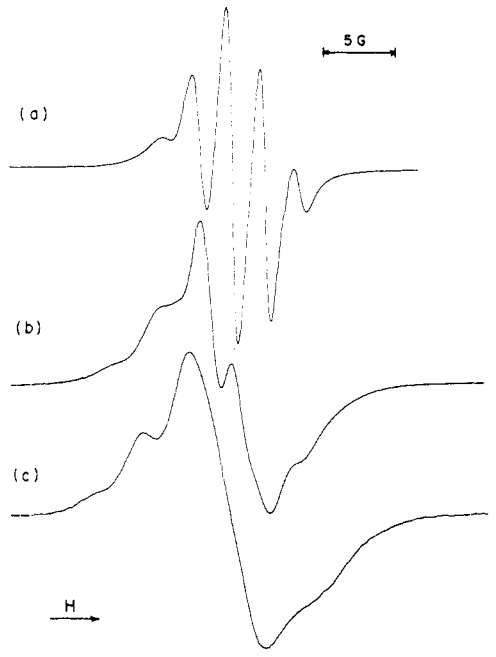


Figure 1. Temperature dependency of the ESR spectrum of the benzosemiquinone radical in methanol at X-band frequencies: (a) −95°, (b) −110°, (c) −160°.

ic radical samples to a glass prior to their introduction into the resonance cavity of the ESR spectrometer.

X-Band spectra were recorded on a JEOL spectrometer (JWS-3BS-X) equipped with a 12 in. magnet, while Q-band spectra were recorded on a modified Varian spectrometer (V-4500). Each spectrometer employed a cylindrical cavity resonating in the TE_{011} mode. Magnetic field intensities were measured with a Magnion Gaussmeter (G-504).

All calculations and spectral simulations were performed on an IBM-360 computer. INDO calculations employed program 141 from the Quantum Chemistry Exchange program. Simulations of powder spectra was accomplished using a program written by Dr. Brian Hoffman and coworkers of Northwestern University; the program was slightly modified to accommodate the calculations of the anisotropic hyperfine splitting constants of five nonaxial nuclei.

Results

Empirical *g* Factors. Attainment of the principal anisotropic parameters of a free radical is best accomplished through a study of the radical oriented in a single crystal. When it is difficult or impossible to orient the radical in a crystal, the radical's power spectrum must be used. This is the case for most radicals observed in biological samples. Unfortunately, with the aid of only a powder spectrum it is often difficult to assign unambiguously values to the principal anisotropic parameters; there is frequent overlapping of structural lines, and also frequent difficulty in determining whether the *g* factors or hyperfine splittings are the major contributors to the spectrum's anisotropic structure. However, three ways exist which aid in assigning values to the principal parameters from powder spectra. (1) Use isotopic substitution on the radical's magnetic nuclei; changes in a spectrum after isotopic substitution occur because of changes in the hyperfine splitting constants and not changes in the *g* factors. (2) Record the ESR spectrum at different microwave frequencies; differences in a spectrum recorded at different microwave frequencies are related to the magnitudes of the principal *g* factors. (3) Compute values of the principal anisotropic parameters; a calculation, although only approximate for organic free radicals, of the principal *g* factors and hyperfine splitting constants will yield supporting evidence for magnitude and relative intensities of empirically determined principal parameters. All three of these techniques will be used to determine the principal parameters of the benzosemiquinone anion.

Figure 1 shows the effect of freezing on the ESR spectrum of the benzosemiquinone anion. In order to simulate the powder spectrum of the anion radical (Figure 1c) seven distinct parameters must be used, i.e., the three principal *g* factors (g^{xx}, g^{yy}, g^{zz}), the hyperfine splitting constants (a^{xx}, a^{yy}, a^{zz}), and the spectral line width. Hyperfine splitting constants are often expressed as the sum of an isotropic (a_{iso}) and an anisotropic (t) component, e.g., $a^{xx} = a_{iso} + t^{xx}$. The isotropic splitting arises from Fermi contact interaction of the unpaired electron with the radicals magnetic nuclei while the anisotropic splitting is related to magnetic dipole-dipole interaction. Both the principal *g* factors and the anisotropic hyperfine splitting constants satisfy the relationships.

$$g_{iso} = {}^1/_3(g^{xx} + g^{yy} + g^{zz}) \quad (1)$$

$$0 = t^{xx} + t^{yy} + t^{zz} \quad (2)$$

The values of the isotropic *g* factor (g_{iso}) and a_{iso} are often solvent dependent while the sum (eq 2) is equal to zero because the anisotropic hyperfine splitting tensor is traceless. For the benzosemiquinone anion g_{iso} ranges[15] from 2.00466 to 2.00518 and a_{iso} ranges[16,17] from 2.368 to 2.419 G, the magnitude of each term being solvent dependent. In methanol at room temperatures[18] g_{iso} = 2.00468 and [19]a_{iso} = 2.368.

For a radical such as benzosemiquinone where the major hyperfine splitting is due to electron–proton interactions, the ESR spectrum can be simplified through deuterium substitution. Figure 2 shows the ESR spectrum of the perdeuteriobenzosemiquinone anion radical in methanol-*d* recorded at X-band and Q-band frequencies. Computer simulations of these spectra (Figure 2) yield the *g* factors

$$g^{xx} = 2.0065$$
$$g^{yy} = 2.0053$$
$$g^{zz} = 2.0023$$

where *x*, *y*, and *z* factors have been assigned arbitrarily to the spectral inflection points in order of increasing magnetic field. It should be noted that the determination of the values of these factors has been greatly aided through the combined use of deuterium substitution and spectral recording at Q-band frequencies. The uncertainty in each factor is ±0.0001. This small uncertainty can be stated since shifts in the *g* factors by this amount cause spectral shifts greater than 0.6 G at Q-band which are easily detected. The isotropic *g* factor calculated from the average of these quantities is g_{iso} = 2.0047 ± 0.0002 which is well within the experimental error of the empirical value for the anion in alcoholic solvents.

Empirical Hyperfine Splitting Constants. After the principal *g* factors have been determined the hyperfine splitting constants can be deduced more easily from the spectrum of the protonated quinone with the use of eq 2. Again, a computer simulation of the powder spectrum (Figure 3) was used to obtain the principal values

$$a^{xx} = 0.5 \pm 0.4 \text{ G}$$
$$a^{yy} = 3.0 \pm 0.3 \text{ G}$$
$$a^{zz} = 2.7 \pm 0.4 \text{ G}$$

As with the principal *g* factors, the *x*, *y*, and *z* directions have been assigned to hyperfine splitting constants according to their spectral position in order of increasing magnetic field.

Calculated *g* Factors. Until recently, calculations of an-

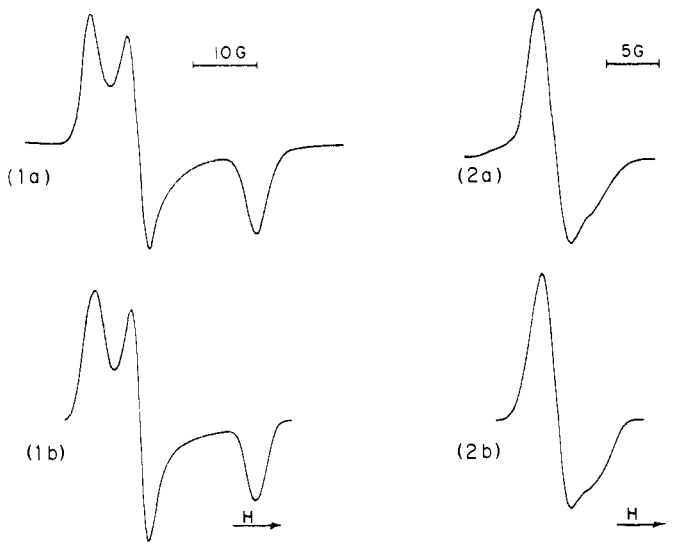


Figure 2. ESR spectra of the perdeuteriobenzosemiquinone radical in a rigid methanol-*d* glass at −196°: (1) Q-band, (2) X-band; (a) experimental, (b) computer simulation. Microwave power 0.1 mW.

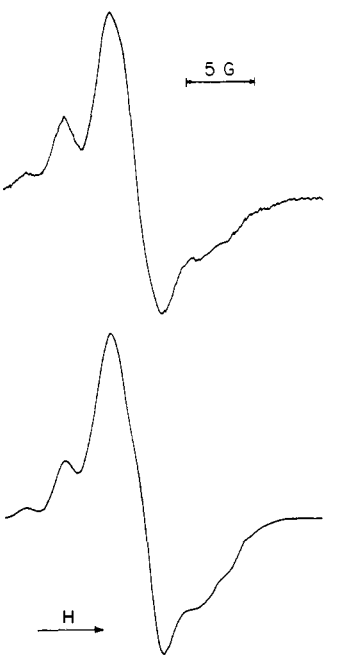


Figure 3. Experiment (top) and computer simulation (bottom) of X-band spectrum of the benzosemiquinone radical in methanol-*d* at −196°. Microwave power 0.1 mW. See text for principal parameters used in computer simulation.

isotropic g factors have been confined almost completely to transition metal compounds. One reason for this is that most organic free radicals (except those that contain halogens) have isotropic g factors which differ by less than 0.1% from that of the free electron[20] ($g_e = 2.00232$) thereby adding little incentive for their calculation. Small deviations of the isotropic g factor from the free electron are generally interpreted[21-24] to be due to spin-orbit mixing of the ground paramagnetic state with the molecule's remaining electronic states. By assuming the spin-orbit mixing small relative to the major Zeeman interaction perturbation theory can be used[22] to calculate the principal g factors. As such, the first-order approximation of the principal g factor in the i direction is

$$g^{ii} = g_e + \Delta g^{ii} \tag{3}$$

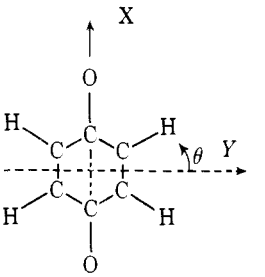
where

$$\Delta g^{ii} = 2 \sum_{m \neq u} \sum_{k} \frac{\langle \Psi_u | \hat{l}_k{}^i | \Psi_m \rangle \langle \Psi_m | \zeta_k \hat{l}_k{}^i | \Psi_u \rangle}{\epsilon_u - \epsilon_m} \tag{4}$$

In eq 4, Ψ_u is the wave function of the molecular orbit containing the unpaired electron and having an energy ϵ_u, while Ψ_m are the wave functions of the set of m remaining nondegenerate states of energies ϵ_m. $\Delta_g{}^{ii}$ therefore represents the magnitude of the mixing of Ψ_u with Ψ_m through the atomic angular momentum operator $\hat{l}_k$, and summed over the set of k different atoms in the radical. The magnitude of the coupling on each atom is expressed in the spin-orbit coupling constant ζ_k. The values[25] of ζ used in (4) for carbon and oxygen were 28 and 152 cm^{-1}, respectively.

The set of wave functions and their respective energies were approximated from an open shell INDO calculation on the benzosemiquinone radical. Fortunately, calculation of the principal g factors from eq 3 and 4 need not involve all the wave functions generated in the INDO calculation but can be simplified through symmetry considerations. The benzosemiquinone radical is a member of the point group D_{2h} in which Ψ_u has symmetry B_{2g}.

Using the molecular-fixed coordinate system shown below where the Z direction is out of the plane of the molecule, it is easy to show that $\hat{l}^X$, $\hat{l}^Y$, and $\hat{l}Z$ transform as B_{3g}, B_{2g}, and B_{1g}, respectively. Therefore, Δg^{XX}, Δg^{YY}, and Δg^{ZZ} will be nonzero only for wave functions Ψ_m of symmetry B_{1u}, A_g, B_{3u}, respectively. Since none of the wave functions of the benzosemiquinone radical transform as B_{3u}, g^{ZZ} equals g_e. Furthermore, only the p_y orbitals on benzosemiquinone transform as A_g while only the p_y orbitals transform as B_{1u}. In others words, the variation Δg^{XX} is caused mainly by the spin-orbit coupling on the unpaired electron in Ψ_u with the p_y orbitals of the remaining wave functions. This statement can be shown to be generally true and is in agreement with the findings of Stone.[23,24]



Using these symmetry considerations along with the wave functions generated from INDO calculations, the molecular-fixed axis g factors can be calculated from eq 3 and 4 to be

$$g^{XX} = 2.0059$$
$$g^{YY} = 2.0037$$
$$g^{ZZ} = 2.0023$$

Stone has shown[23,24] that a similar technique can be used to calculate isotropic g factors ($g_{iso}{}^{(S)}$) of aromatic free radicals. Using a semiempirical procedure, he derived the formula

$$g_{iso}{}^{(S)} = g_e + (p_C + p_O) + \lambda(q_C + q_O) \tag{5}$$

In this equation the constants p_C, p_O, q_C, and q_O are empirically derived from radicals containing carbon and oxygen atoms and $\epsilon_u = \alpha + \lambda\beta$ is the Hückel molecular orbital energy of orbital Ψ_u. Although not derived by Stone for anisotropic g factors, eq 5 can be rederived for such calculations. Again, symmetry considerations dictate that $g_{(S)}{}^{ZZ}$

equals g_e. As in the above derivation, the deviation of $g_{(S)}{}^{XX}$ and $g_{(S)}{}^{YY}$ from g_e can be broken down into their spin-orbit contributions from the carbon and oxygen atoms of the quinone. These contributions depend on the angle that each bond in the radical makes with the X and Y axes as well as the spin density on each carbon and oxygen atom. Therefore, using the molecular-fixed coordinate system described above, the anisotropic g factors can be calculated from the following modified forms of Stone's equation:

$$g_{(S)}{}^{XX} = g_e + 12|\rho_C{}^{\pi}|(p_C + \lambda q_C)\cos\theta + 6\gamma_n|\rho_O{}^{\pi}|(p_O + \lambda q_O)$$

$$g_{(S)}{}^{YY} = g_e + |\rho_C{}^{\pi}|(p_C + \lambda q_C)(6 + 12\sin\theta) + 6\gamma_s|\rho_O{}^{\pi}|(p_O + \lambda q_O) \quad (6)$$

$$g^{ZZ} = g_e$$

$\rho_i{}^{\pi}$ is the unpaired electron density on atom i and is approximately equal to $-a_i{}^2$, the square of the atomic orbital cooeficient of atom i in Ψ_u. For simplicity, $\rho_C{}^{\pi}$ has been assumed to be the same for all carbon atoms in the quinone. Even though this assumption may lead to as much as a 50% error in the calculation of this term, this approximation can be used since the magnitude of the carbon split-orbit coupling term is small compared to the oxygen term. θ is the angle between each bond in the molecule and the Y axis. γ_n and γ_s represent the amount of mixing of the unpaired electron in the oxygen $2p_z$ orbital with the oxygen's nonbonding and σ bonding orbitals, respectively. These fractions can be approximated from their relative effects in the calculation of eq 4 above to yield $\gamma_n = 0.78$ and $\gamma_s = 0.22$. Using the empirically derived[18,23,24] constants

$$p_C = 3.2 \times 10^{-4}$$
$$p_O = 5.7 \times 10^{-3}$$
$$q_C = -1.7 \times 10^{-4}$$
$$q_O = 6.4 \times 10^{-3}$$

the principal g factors can be calculated from eq 6 to be:

$$g_{(S)}{}^{XX} = 2.0063$$
$$g_{(S)}{}^{YY} = 2.0038$$
$$g_{(S)}{}^{ZZ} = 2.0023$$

Calculation of Hyperfine Splittings Constants. Unlike the anisotropic g factors, the anisotropic hyperfine interactions in organic free radicals have been studied extensively. It is well known that the orientation dependency of this interaction arises from dipole-dipole interactions of the free electron with the magnetic nuclei in the molecule. Specifically, for π radicals this is typically the interaction of the molecules' magnetic nuclei with the nearest p_z orbital of the π system, which for most organic radicals is the carbon $2p_z$ orbital. Using this interaction, McConnell and Strathdee[26] derived the expression

$$a^{ii} = -(7.69\rho_C{}^{\pi} + 6.03\rho_C{}^{\sigma})(1 - 3\cos^2\alpha_{(i)}) - 5.95(\rho_C{}^{\pi} - \rho_C{}^{\sigma})(\cos^2\alpha_{(i)} - 1)\cos 2\phi_{(i)} + a_{iso} \quad (7)$$

for the anisotropic hyperfine splitting constant a^{ii} in the i direction. For the benzosemiquinone anion $\rho_C{}^{\pi}$ and $\rho_C{}^{\sigma}$ are the spin densities in the carbon $2p_z$ and σ atomic orbitals, respectively. $\alpha_{(i)}$ is the angle between the C-H bond and the i axis while $\phi_{(i)}$ is the angle between the i axis and the carbon $2p_z$ orbital. For the molecular-fixed axes described above, these angles are:

i	$\alpha(i)$	$\phi(i)$
X	30°	90°
Y	120°	90°
Z	90°	0°

Using these values along with $a_{iso} = 2.4$ G in eq 7 yields

$$a^{XX} = 8.1\rho_C{}^{\pi} + 76.9\rho_C{}^{\sigma} + 2.4$$
$$a^{YY} = -6.4\rho_C{}^{\pi} - 10.6\rho_C{}^{\sigma} + 2.4 \quad (8)$$
$$a^{ZZ} = -1.7\rho_C{}^{\pi} - 66.3\rho_C{}^{\sigma} + 2.4$$

$\rho_C{}^{\pi}$ can be approximated again from the INDO calculation on the benzosemiquinone to obtain $\rho_C{}^{\pi} = -0.104$; $\rho_C{}^{\sigma}$ is much more difficult to determine but is typically 5% of $\rho_C{}^{\sigma}$. Substituting these values into eq 8 yields

$$a^{XX} = 1.1 \text{ G}$$
$$a^{YY} = 3.1 \text{ G}$$
$$a^{ZZ} = 2.9 \text{ G}$$

Discussion

As stated in the introductory section, many of the ESR spectra observed in biological systems are those associated with immobilized radicals. This paper commences a series of studies aimed at demonstrating how these natural radicals can be used to probe the structure of living systems. Before a radical can be used as a probe, however, the values of its principal ESR parameters must be known. Obtaining these values from powder spectra is often difficult. On the other hand, being confined to the use of powder spectra for interpretation does not a priori mean that the radical's principal ESR parameters are hopelessly buried in what is often an amorphous spectrum. As demonstrated above, the proper combination of isotope substitution, spectral recording at different frequencies, theoretical calculations, and eventual computer simulation of spectra can aid in the determination of the principal parameters. In the case of the benzosemiquinone radical, this analysis technique not only helped to determine the principal parameters but also showed the coincidence of the spectrally labeled x, y, and z directions with the molecular fixed X, Y, and Z axes.

Beside showing that a radical's principal parameters can be extracted from powder spectra, the above results bring forth several other noteworthy facts. For example, although the benzoquinone structure can be approximated as being axial for the purpose of calculating several different spectroscopic parameters, it would be erroneous to do so in the calculation of the ESR parameters. The differences in the X and Y parameters of both the g factor and the hyperfine splitting are large enough to introduce noticeable errors while attempting to simulate the benzosemiquinone powder spectrum on the assumption of axial symmetry. Unfortunately, many ESR spectra have been published in which little or no anisotrophy is assumed in attempting to simulate the powder spectrum of an organic radical. Therefore, it should be remembered that often a large portion of the broadness of a powder spectrum is due to anisotropic effects in the principal parameters rather than just line broadening effects.

Deviation of the organic radical's g factor from that of the free electron is attributed to be due mainly to the mixing of energy levels through spin-orbit coupling. For example, in the benzosemiquinone radical the g factors corresponding to the x and y axes differ from that of the free electron because of the large spin-orbit coupling of the unpaired electron on the para oxygen atoms. Calculations of the magnitude of this deviation show that both the x and y axes lie in the plane of the radical and that the coupling of

the unpaired electron with the oxygen's x orbitals contributes to the magnitude of g^{yy} while the oxygen's y orbitals similarly contribute to g^{xx}. This direction dependency of the g factors has been of great assistance in the interpretation[27] of semiquinone-solvent interactions[28] and the anomalous saturation behavior[29] exhibited by semiquinones in rigid media.

The g factors calculated both from perturbation theory (eq 4) using INDO wave functions and energy levels and from the modified form of Stone's semiempirical equation (eq 6) give results in close agreement with the experimentally determined g factors. On the other hand, both perturbation theory and the modified Stone's equation yield g^{YY} noticeably smaller than the experimental g^{yy}. The reason for the difference is not known. One possible explanation, however, is that the oxygen's $2p_x$ orbitals are significantly perturbed (possibly by the solvent[27]) to cause a mixing of these orbitals with the $2p_y$ orbitals on oxygen. The result of this mixing would be a relative increase in the value of g^{YY} and possibly g^{XX}. Such a prediction is consistent with the above results.

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Carbon-13 Magnetic Resonance Study of Structural and Dynamical Features in Carbamylated Insulins

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Abstract: Carbon-13 NMR Fourier transform spectra were recorded and assigned and nuclear Overhauser enhancement parameters and spin–lattice relaxation times, T_1, were measured for 90% ^{13}C enriched carbamyl groups attached to the A1 glycine, B1 phenylalanine, and the B29 lysine in zinc insulin and nickel insulin. Similar measurements were made for metal-free insulin in which only the two N-terminal amino acids were carbamylated. All measurements were made with the derivatives in water solution at pH 7.8. The results indicate that the derivatives contain the A1 glycine in two different environments suggesting that the insulin dimers have an imperfect twofold axis. The correlation times for the overall tumbling of the derivatives were estimated which led to evaluation of the state of aggregation of the derivatives. Likewise, information was obtained as to the relative mobility of the location in the insulin molecules to which the carbamyl groups were attached, via an estimation of the correlation times for the internal rotation of the carbamyl groups. Finally, the effective distance between the Ni^{2+} ion in B1 carbamylated nickel insulin and the ^{13}C nucleus in the carbamyl group was estimated to be 10.5 Å or less.

Natural abundance carbon-13 magnetic resonance (^{13}C NMR) spectra of biopolymers have recently been shown[2-9] to contain valuable information on both the structural conformation and the segmental motion present in these molecules. However, the detectability of ^{13}C signals in natural abundance for such large molecules, even with Fourier transform techniques, and the complexity of the spectra resulting from the overlapping of a large number of ^{13}C resonance lines combine to limit an extensive study of these structural and dynamical characteristics.

These difficulties can be overcome in part through ^{13}C enrichment of specific positions of the molecules, thereby increasing the signal to noise level of the labeled position beyond the background signals due to the unenriched parts of the molecule which contain ^{13}C at the 1.1% natural abundance. Reliable data for the enriched position at biologically significant concentrations have been obtained in this manner as recently illustrated[10] with ^{13}C enriched histidine residues incorporated, in vivo, into a native enzyme. This interesting approach in general is expensive and time consuming, and at this stage a more practicable way in which ^{13}C isotopes can be incorporated is through highly