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ASSIGNMENT OF PROTON ENDOR RESONANCES OF NITROXYL SPIN-LABELS IN FROZEN SOLUTION

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Spin-label nitroxyl derivatives of tetramethylpyrroline and tetramethylpyrrolidine in frozen solutions of perdeuterated methanol have been characterized by electron nucleus double resonance (ENDOR spectroscopy). With use of selectively deuterated derivatives of 2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxamide, proton ENDOR resonance features have been assigned to the vinylic proton in the five membered pyrrolinyl ring and to the methyl groups. The ENDOR resonance features were analyzed on the basis of their dependence on H_0. Two pairs of resonance features were assigned to the vinylic proton and were shown to correspond to $\parallel$ and $\perp$ hyperfine coupling (hfc) components. Six pairs of resonance features were ascribed to the methyl groups. The proton ENDOR spectra of the 3-carboxylic acid spin-label derivatives of tetramethylpyrroline and of tetramethylpyrrolidine compounds exhibited comparable features with nearly identical line splittings. From the observed ENDOR splittings, we have estimated the isotropic hfc component of the vinylic proton in 2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxamide to be -1.81 ± 0.04 MHz in frozen methanol. On the basis of the anisotropic dipolar hfc components, the electron-to-vinylic proton distance is estimated as 3.78 ± 0.01 Å, in excellent agreement with that of 3.79 Å calculated from X-ray defined coordinates.

KEY WORDS: Electron paramagnetic resonance, electron nuclear double resonance, hyperfine coupling, nitroxides, spin-labels.

INTRODUCTION

The nitroxyl free radical species known as spin-labels are widely employed in biophysical studies as spectroscopic probes of macromolecular structure.[1] With use of these free radical species, we have shown how electron nuclear double resonance (ENDOR) spectroscopy can be applied for determination of structure and conformation of spin-labeled molecules in frozen solutions with an accuracy approaching that afforded by X-ray diffraction methods.[2-4] In such studies the ENDOR absorption features of protons and fluorine substituents not directly attached to the spin-label moiety may overlap with those of the protons intrinsic to the spin-label probe. The use of these free radical species for structure determination requires, therefore, unambiguous assignments of the resonance features of all hydrogen atoms that are directly associated with the spin-label moiety. In this communication, we analyze the proton ENDOR spectra of spin-labels with five-membered 2,2,5,5-tetramethylpyrroline-1-oxyl and 2,2,5,5-tetramethylpyrrolidine-1-oxyl ring structures. Resonance assignments are made on the basis of selectively deuterated derivatives and on the

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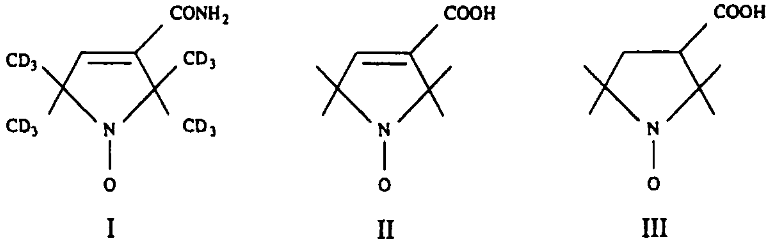

FIGURE 1 Illustration of the chemical bonding structures of 2,2,5,5-($^2H_{12}$)tetramethylpyrroline-1-oxyl-3-carboxamide (I), 2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxylic acid (II), and 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-carboxylic acid (III).

basis of the H_0 dependence of the ENDOR spectra. For the vinylic proton in 2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxamide, our estimate of the isotropic hfc component made on the basis of ENDOR spectra of frozen solution samples agrees well with results of TRIPLE experiments reported by others,[5] and the electron-to-proton distance calculated on the basis of our estimates of the dipolar hfc components is in excellent agreement with X-ray data.[6]

MATERIALS AND METHODS

Materials

In Figure 1 are illustrated the structural formulae of spin-label compounds I–III employed in this investigation. To prepare spin-label I, we have employed the synthesis method of Rosantzev,[7] beginning with 4-oxo-2,2,6,6-($^2H_{17}$)tetramethylpiperidine (98% deuterated, MSD Isotopes, St. Louis, MO 63116) but using solvents and other reagents of natural abundance isotopic composition. The proton ENDOR spectra of I were then compared to those of 2,2,5,5-($^2H_{12}$)tetramethyl-4-(^{2}H)pyrroline-1-oxyl-3-carboxamide obtained by carrying out the synthesis with use of ($^2H_4$)acetic acid and $N^2H_4O^2H$, as described earlier.[3] We also obtained a sample of I from Dr. Howard J. Halpern of the Department of Radiation Oncology at The University of Chicago, synthesized by a method in which the 3,5-dibromo-2,2,6,6-($^2H_{12}$)tetramethyl-4-oxo-3,5-($^2H_2$)piperidine synthesis intermediate was treated with a solution of hydrobromic and acetic acid to ensure incorporation of a hydrogen atom at the 4-position of the product in high yield.[8]

The spin-label II was obtained by hydrolysis of 2,2,5,5-tetramethylpyrroline-1-oxyl-3-carboxamide (Aldrich Chemical Company, Milwaukee, WI 53223) as described earlier.[3] Compound III was obtained from Aldrich. All other reagents and solvents were as described in earlier studies.[3] ($^2H_4$)methanol was obtained from Cambridge Isotope Laboratorires, Inc. (Woburn, MA 01801).

EPR and ENDOR

EPR and ENDOR spectra were recorded at 40 K with an X-band Bruker ER200D spectrometer equipped with an Oxford Instruments ESR10 liquid helium cryostat and a Bruker digital ENDOR accessory, as previously described.[9,10] ENDOR spectra were recorded in the first derivative absorption mode with 1.28 mW incident microwave

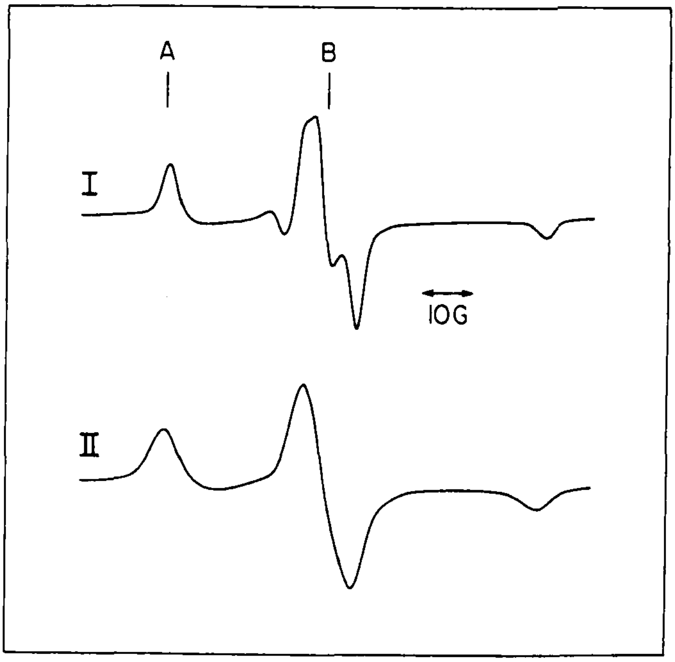

FIGURE 2 First-derivative EPR absorption spectra of **I** (upper) and **II** (lower) in frozen ($^2H_4$)methanol. The spectra were recorded at a sample temperature of 40 K with 64 microwatts of incident microwave power modulated at 12.5 kHz. The modulation amplitude was $0.2\,G_{pp}$. A and B mark the H_0 settings that were saturated for ENDOR. See text.

power, 12.5 kHz frequency modulation of the radiofrequency (rf) field, and 50 watts rf power with 8–10 kHz modulation depth of the rf field. The spin-label compounds were dissolved to a concentration of 5×10^{-3} M in ($^2H_4$)methanol.

RESULTS AND DISCUSSION

In Figure 2 the EPR absorption spectra of **I** and **II** are compared. Two positions are marked as settings A and B that correspond to the low-field resonance feature and to the more intense central feature, respectively. The low-field feature arises from molecules with $m_I = +1$ of the nitroxyl ^{14}N nucleus while the central feature arises predominantly from molecules with $m_I = 0$. For **I**, the weaker hyperfine interactions of nearby deuterons result in partial resolution of the g_{xx} and g_{yy} components at the low-field edge of feature B and of the g_{zz} component at the high-field edge. Microwave saturation of setting A for ENDOR selects molecules such that H_0 is oriented perpendicularly to the plane of the pyrrolinyl ring while saturation at setting B selects molecules of all orientations. We have shown previously how selective saturation of these two features of the EPR spectrum of spin-labels leads to a complete assignment of the ENDOR absorptions of nearby protons.[2-4]

Figure 3 illustrates the proton ENDOR spectra of **I** for settings A and B of the static laboratory magnetic field. For setting B there are two pairs of resonance features symmetrically spaced with respect to the proton Larmor frequency, and for setting A

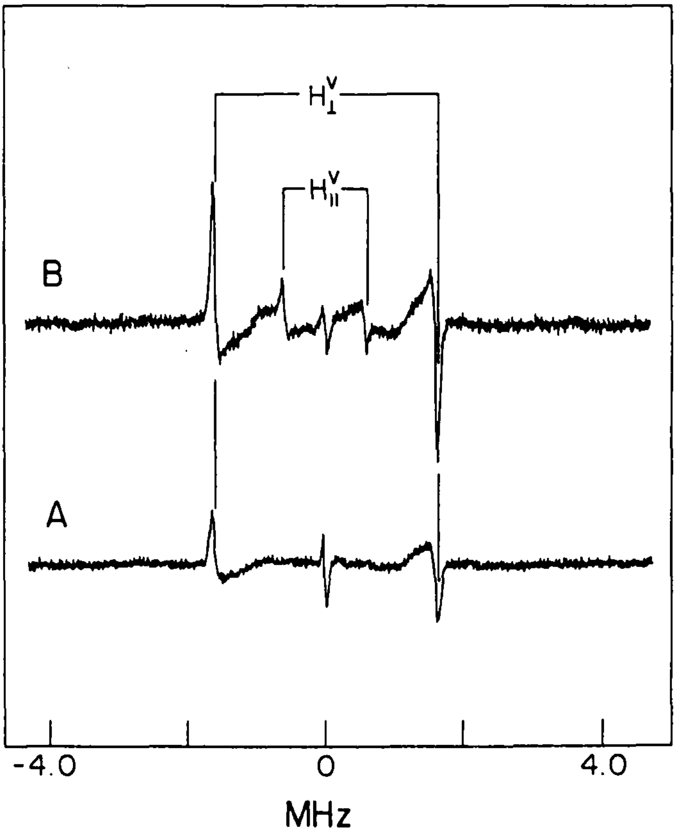

FIGURE 3 Proton ENDOR spectra of **I** in ($^2H_4$)methanol. Letters A and B correspond to H_0 settings at the low-field edge and central region of the EPR spectrum, respectively, that were saturated for ENDOR. Two ENDOR line pairs are identified in the stick diagram and are assigned to the ∥ and ⊥ hfc components of the vinylic proton and are labeled as $H_\parallel^v$ and $H_\perp^v$, respectively. The abscissa indicates the ENDOR shift (measured ENDOR frequency minus free proton frequency). An uncertainty of 0.020 MHz in the ∥ hfc component due to the line width of the ∥ ENDOR resonance features yields an error of ± 0.01 Å in the estimated electron-to-nucleus distance.

there is only one pair of resonances. Since amide protons exchange rapidly with solvent under these conditions,[3] these resonance features can be ascribed to only the vinylic proton in the pyrrolinyl ring. We have shown previously that ENDOR features observed under setting A correspond to the ⊥ hfc component of a proton in the molecular plane while setting B gives rise to both ∥ and ⊥ hfc components.[2-4] On this basis the outermost pair of resonances is assigned to $A_\perp$ while the innermost pair is assigned to $A_\parallel$ of the vinylic proton.

The observation of only two pairs of resonance features belonging to the vinylic proton requires that the principal hfc components are axially symmetric. Since $(A_\parallel + 2A_\perp) = 3A_{iso}$ under this condition, the observed splittings in the spectra of Figure 3 yield values of 1.148 MHz for $A_\parallel$, -3.291 MHz for $A_\perp$, and -1.81 MHz for A_{iso} with $A_\parallel > 0 > A_\perp$. Kirste *et al.* have determined the sign and magnitude of the isotropic hfc of the vinylic proton in the methyl ester of **II** in toluene by ENDOR and

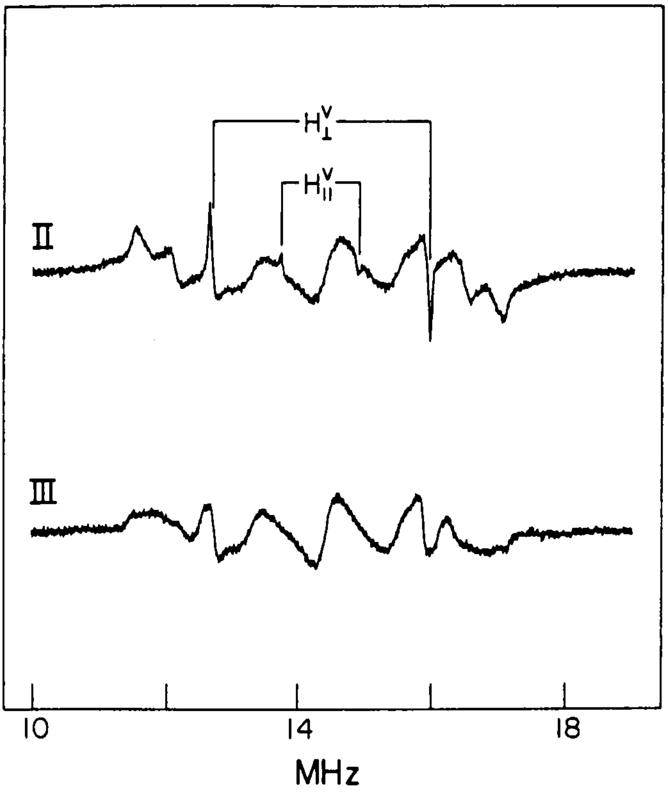

FIGURE 4 Proton ENDOR spectra of II (upper) and III (lower) in $(^2H_4)$methanol with H_0 at setting B of the EPR spectrum. The princpal hfc components of the vinylic proton are labeled with identical line splittings as seen in Figure 3. Other absorption features are ascribed to methyl protons. See text.

TRIPLE resonance methods.[5] Their value of −1.46 MHz confirms our estimate of −1.81 MHz. Hyde and Subczynski[11] have estimated the magnitude of the isotropic coupling of the vinylic proton of the natural abundance form of **I** in water by EPR and find a temperature dependent value ranging from 1.29 MHz at 80 C to 1.51 MHz at 1 C. Application of the temperature dependence of the isotropic coupling reported by Hyde and Subczynski[11] predicts a value of ~ 1.80 MHz at −100 C, the freezing point of methanol. This coincides exactly with the magnitude of our estimate of A_{iso} for **I** in frozen methanol.

The observed values of $A_\parallel$ and $A_\perp$ yield estimates of 2.959 MHz and −1.480MHz for the $\parallel$ and $\perp$ dipolar hfc components of the vinylic proton in **I**. Under the point-dipole approximation as applied earlier for **II** in methanol,[2-4] this yields an electron-to-proton separation of 3.78 ± 0.01 Å. Application of a C–H bond distance of 1.045 Å for an sp^2 hybridized carbon[12] with parameters for idealized valence bonding geometry to the X-ray determined coordinates[6] of the natural abundance form of **I** yields a value of 3.79 Å, in excellent agreement.

In Figure 4 we have compared the proton ENDOR spectra of **II** and **III**. In separate experiments we have established that the spectrum of **II** is identical to the spectrum of the natural abundance form of **I**. In the spectrum of **II** there are two pairs of ENDOR resonance features identical in line shape and in splitting with those in Figure 3, identifying these features arising from the vinylic proton. Consequently the other ENDOR resonance features in the spectrum of **II** must arise from the four nearby methyl groups attached to the pyrrolinyl ring. With H_0 at setting A we have identified three pairs of ENDOR resonances features with hf splittings of ~ 1.23, 3.97, and 6.20 MHz. With H_0 at setting *B*, three additional ENDOR line pairs with splittings of 4.51, 5.51, and 10.36 MHz were identified. The observation of six line pairs can be rationalized in terms of four equivalent methyl groups with respect to the nitroxyl group such that the three protons within each methyl group occupy nonequivalent positions. We did not make further attempts to assign corresponding sets of $\|$ and $\perp$ hfc components, and, therefore, we cannot estimate the isotropic hf splitting and corresponding electron-to-nucleus separations. By TRIPLE experiments Kirste *et al.* have determined a value of -0.66 MHz for A_{iso} at 185 K for the four methyl groups in the methyl ester of **II** in toluene.[5] The value of this isotropic hfc exhibits no temperature dependence.[11]

In Figure 4 we have also shown the proton ENDOR spectrum of **III**. The resonance features corresponding to the $\perp$ hfc component of the methylene protons are broadened in comparison to those for the single vinylic proton of **II** while the features belonging to the $\|$ hfc component are too broad to be directly recognized. Similarly the prominent ENDOR resonance features belonging to methyl groups exhibit comparable splittings but are also broadened. This broadening is a likely consequence of the non-planar structure of the five-membered pyrrolidinyl ring and the nonequivalent positions of the four methyl groups.[13]

Aliphatic nitroxyl radicals in polycrystalline or disordered matrices have been employed for ENDOR studies by other investigators.[14,15] Their ENDOR spectra are comparable to those illustrated in Figure 4 and exhibit, in particular, a pair of sharp resonance features with an approximate 3.1 MHz hf splitting. Since the resonance features with the 3.1 MHz splittings are the most prominent of all features in the spectrum, their intensity has been attributed alternatively to the methyl groups undergoing rotation[14] or to the combined absorption of both methyl and methylene protons.[15] Our results, as illustrated in Figures 3 and 4, require that these sharp features arise from the $\perp$ hfc components of either the vinylic or the methylene protons in **II** or **III**, respectively. The spectroscopic assignment presented in this communication is based on the use of a site specifically synthesized 2,2,5,5-($^2H_{12}$)tetramethyl-4-(^1H) = pyrrolinyl-1-oxyl-3-carboxamide derivative and the unambiguous H_0 dependence of the ENDOR spectra identifying the sharp resonance features as belonging to the $\perp$ hfc component.

In Figure 3 it is seen that the pair of $\perp$ resonance features exhibits a larger ENDOR splitting than the pair of $\|$ resonances. This relationship is opposite to the relative magnitudes of the $\|$ and $\perp$ hfc components observed under conditions of very small or negligible isotropic coupling.[2-4] In those studies, application of the point-dipole approximation to estimate electron-to-nucleus distances is made under the conditions $A_{\|}$ and $A_{\perp} >> A_{iso}$ and $A_{\|}^{D} > 0 > A_{\perp}^{D}$. With $A_{\|} = 1.148$ MHz, $A_{\perp} = 3.291$ MHz, and $A_{iso} = -1.81$ MHz, as estimated above for the vinylic proton, the agreement of the ENDOR determined electron-to-nucleus distance of 3.78 ± 0.01 Å with that of 3.79 Å obtained on the basis of X-ray structural parameters[6,12] shows that the principal hfc components have been correctly assigned. These relationships probably obtain rigorously because of the close adherence of the principal hfc components of the vinylic proton to axial symmetry, the apparent near coincidence of principal nitrogen hyperfine axes with those of the g matrix of the nitroxyl group,[16] and the nearly complete localization of the unpaired electron to the nitroxyl group. In fact, our estimate of A_{iso} for the vinylic proton corresponds to less than 2.5% of the unpaired electron density at the C(4) position of the pyrrolinyl ring.

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References

1. Berliner, L.J. Ed. *Spin Labelling: Theory and Applications.* Academic Press, New York, pp. 592, (1976).
2. Wells, G.B. and Makinen, M.W. ENDOR determined molecular geometries of spin-labeled fluoroanilides in frozen solution. *J. Am. Chem. Soc.*, **110,** 6343–6352, (1988).
3. Mustafi, D., Sachleben, J.R., Wells, G.B. and Makinen, M.W. Structure and conformation of spin-labeled amino acids in frozen solutions determined by electron nuclear double resonance. I. Methyl N-(2,2,5,5-tetramethylpyrrolinyl-1-oxyl-3-carboxyl)-*L*-alanate, a molecule with a single preferred conformation. *J. Am. Chem. Soc.*, in press (1990).
4. Wells, G.B., Mustafi, D. and Makinen, M.W. Structure and conformation of spin-labeled amino acids in frozen solutions determined by electron nuclear double resonance. II. Methyl N-(2,2,5,5-tetramethylpyrrolinyl-1-oxyl-3-carboxyl)-*L*-tryptophanate, a molecule with multiple conformations. *J. Am. Chem. Soc.*, in press (1990).
5. Kirste, B., Krüger, A. and Kurreck, H. ESR and ENDOR investigations of spin exchange in mixed galvinoxyl/nitroxide biradicals. Syntheses. *J. Am. Chem. Soc.*, **104,** 3850–3858, (1982).
6. Turley, J.W. and Boer, F.P. The crystal structure of the nitroxide free radical 2,2,5,5-tetramethyl-3-carbamidopyrroline-1-oxyl. *Acta Crystallog.*, **B28,** 1641–1644, (1972).
7. Rozantsev, E.G. *Free Nitroxyl Radicals.* Plenum Press, New York Chapter 9, pp. 203–206, (1970).
8. Halpern, H.J., Teicher, B.A., Bowman, M.K., Peric, M., Lin, Y.K., Nguyen, T.D. and Spencer, D.P. T_2 spin label oxymetry: Selective isotopic labeling of a nitroxide spin-label to enhance sensitivity, manuscript in preparation, (1989).
9. Yim, M.B. and Makinen, M.W. ENDOR study of Gd^{3+} complexes in frozen solutions. *J. Mag. Reson.*, **70,** 89–105, (1986).
10. Mustafi, D. and Makinen, M.W. ENDOR determined solvation structure of VO^{2+} in frozen solutions. *Inorg. Chem.*, **27,** 3360–3368, (1988).
11. Hyde, J.S. and Subczynski, W.K. Simulation of ESR spectra of the oxygen sensitive spin-label probe CTPO. *J. Mag. Reson.*, **56,** 125–130, (1984).
12. Coppens, P. Comparative X-ray and neutron diffraction study of bonding effects in *s*-triazine. *Science*, **158,** 1577–1579, (1967).
13. Ament, S., Wetherington, J., Moncrief, J., Flohr, K., Mochizuki, M. and Kaiser, E.T. Determination of the absolute configuration of (+)-3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy. *J. Am. Chem. Soc.*, **95,** 7896–7897, (1973).
14. Brustolon, M., Maniero, A.L. and Segre, U. ENDOR spectra of nitroxide radicals in disordered matrices. *Mol. Phys.*, **55,** 713–721, (1985).
15. Kotake, Y., Tomita, Y. and Kuwata, K. Proton hyperfine splitting and slow motion of spin-labels in frozen glycerol as detected by ENDOR. *J. Phys. Chem.*, **89,** 207–209, (1985).
16. Griffith, O.H., Cornell, D.W. and McConnell, H.M. Nitrogen hyperfine tensor and g tensor of nitroxide radicals. *J. Chem. Phys.*, **43,** 2909–2910, (1965).

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