

After cooling, the solvents were evaporated, the resulting gum was extracted with 50 ml of diethyl ether, and the residue was dissolved in a minimum volume of methanol. The solution was applied to a silica gel column (2 × 16 cm) which had previously been washed with a 50% chloroform-methanol solution; and the column was eluted with chloroform and then with 50% chloroform-methanol. Material with $R_f^{\text{CHCl}_3-\text{MeOH}, 1:1}$ 0.45 (silica gel tlc) was collected, the solvents were evaporated, and the residue was precipitated from methanol by addition of diethyl ether to give 40 mg (22%) of dAp(CH₃)dA, mp 183° dec.

Samples for optical measurements were further purified by chromatography on a Sephadex G-10 column using water as eluent. The triester eluted immediately after the void volume and was lyophilized from water.

Ethyl Ester of Deoxyadenylyl-(3'-5')-deoxyadenosine [dAp-(C₂H₅)dA]. The fully protected dinucleotide DMTrdA^{Bz}p-(CE)dA^{Bz}DMTr (1.05 g, 0.78 mmol) was converted to the ethyl ester, DMTrdA^{Bz}p(C₂H₅)dA^{Bz}DMTr, by the procedure described above for the methyl ester, except dry ethanol was used instead of

methanol. The resulting DMTrdA^{Bz}p(C₂H₅)dA^{Bz}DMTr weighed 647 mg (63%), mp 126-130°. *Anal.* Calcd for C₇₈H₇₅O₁₄N₁₀P·1H₂O: C, 65.13; H, 5.53; N, 9.99. Found: C, 65.06; H, 5.12; N, 10.43.

The protecting groups were removed from the fully blocked triester (650 mg, 0.49 mmol) in the same manner described above for the methyl ester to yield 232 mg (86%) of dAp(C₂H₅)dA, $R_f^{\text{MeOH}-\text{CHCl}_3, 1:1}$ 0.51 (silica gel tlc), mp 146° dec. Samples for optical measurements were further purified as described for the methyl ester. The resulting triester was lyophilized from water.

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A Spin Label Investigation of the Active Site of an Enzyme. Bovine Carbonic Anhydrase¹

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Abstract: A spin-labeled sulfonamide inhibitor bound to the active site of the enzyme carbonic anhydrase has been studied by electron paramagnetic resonance. It is demonstrated that a 1:1 complex between the label and enzyme exists and that rapid anisotropic motion of the label about one of its bonds is taking place. The data are consistent with the aromatic nucleus of the inhibitor being held rather firmly to the hydrophobic portion of the active site cleft. The value of a_N , the isotropic coupling constant of the nitrogen atom of the paramagnetic nitroxide group, indicates that this portion of the sulfonamide is in a highly polar region.

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1), a metalloenzyme which catalyzes the reversible hydration of CO₂, has been extensively investigated since its discovery in 1932. It is of interest not only for the elucidation of its particular catalytic mechanism but also because it is felt that the mechanism involved may serve as a useful model for the study of enzyme action in general. It is a nearly spherical molecule consisting of a single polypeptide chain of approximately 260 amino acid residues.^{2a,b} In its active form the enzyme contains a zinc(II) atom found near the center of the molecule at the bottom of a crevice. Although the presence of the zinc atom (or cobalt) is necessary in order for the enzyme catalytic activity to be present, both the crevice and the zinc atom constitute the active site for the enzyme.³ It is thought that an OH⁻ ion attached to the zinc attacks the CO₂ molecule which is loosely bound to a hydrophobic surface or cavity near the zinc.⁴ It is also felt that proton transfer plays a role in the mechanism although the proton transfer path is not known in detail.⁵ Pocker and coworkers⁶ have suggested facili-

tation by a strategically located basic imidazole group of the protein.

The enzyme is inactivated by complexation with aromatic sulfonamides.⁷ It is thought that the SO₂NH₂ group (probably as the SO₂NH⁻ anion)⁸ complexes with the zinc, thus competing with the OH⁻ ion. X-Ray studies^{2a,9} indicate that a number of anionic inhibitors occupy the same site in the enzyme, namely, the cavity or crevice at the bottom of which is the zinc atom. Sulfonamide binding is stabilized by hydrophobic interaction involving the sulfonamide ring with the enzyme cleft.⁵ From consideration of the interactions between inhibitors and the enzyme it is concluded that the cleft in the region of the zinc provides a good fit for the inhibitor.^{2a,8}

The present study concerns a spin-label investigation of the active site of the bovine carbonic anhydrase (BCA) enzyme. The need for such "site specific"

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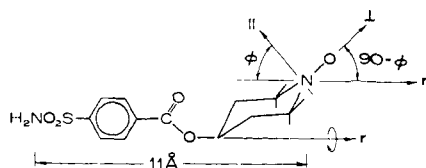


Figure 1. The structure of the sulfonamide spin label showing approximately an 11-Å separation between the terminal nitrogen atoms. The rotation axis (r) is seen to be the bond axis of the ester oxygen and the 4-carbon of the piperidine ring. The relationships of the $2p\pi_z$ orbital of the nitroxide nitrogen (\parallel axis) and the N-O bond axis (\perp axis) to the rotation axis are also indicated.

spin labels has been demonstrated by Kosman, *et al.*,¹⁰ who investigated α -chymotrypsin. We have prepared an aromatic sulfonamide spin label and have demonstrated a 1:1 binding to the enzyme. Our findings are consistent with the idea of a strong interaction between the hydrophobic part of the spin label and the zinc crevice while the magnitude of the isotropic hyperfine interaction from the paramagnetic NO group of the spin label indicates that this portion of the label is in a highly polar environment. In addition, it is found that there is rapid anisotropic rotation of the spin label about the ester linkage in the spin label, further indicating a lower limit to the crevice size near the enzyme surface.

Our present study adds to the knowledge of this important enzyme and is a good illustration of the power of the extremely successful spin labeling technique developed by McConnell.^{11,12}

Experimental Section

a. Synthesis and Material. The sulfonamide spin label (SSL), 4-[(*p*-sulfonamidobenzoyloxy)-2,2,6,6-tetramethylpiperidine 1-oxyl] (see Figure 1), was prepared by treating 1.5 g of *p*-sulfonamidobenzoyl chloride with 1.2 g of 2,2,6,6-tetramethylpiperidin-4-ol 1-oxyl. The paramagnetic alcohol was dissolved in 20 ml of absolute pyridine. The resulting magnetically stirred solution was chilled to 2° in an ice bath. The acid chloride was then slowly added maintaining the temperature at 5° or lower. After the addition was completed, the contents of the reaction vessel were allowed to come to room temperature and react for 5–6 hr. The reaction mixture was then poured, with vigorous stirring, onto 150 g of an ice-water slurry. The orange precipitate was filtered, washed with cold deionized water, and finally recrystallized from a hot (*ca.* 80°) 1:1 methanol-H₂O solution. The resulting orange paramagnetic flakes were found to melt at 176–178°. Infrared analysis (Beckman IR-8) gave COO bands at 1720 and 1268 cm⁻¹, SO₂NH₂ bands at 1351 and 1136 cm⁻¹; para ring substitution was identified by peaks at 1106 and 855 cm⁻¹, and the >NO moiety gave a shoulder at 1340 cm⁻¹. *Anal.* (M-H-W Laboratories) Calcd: C, 54.14; H, 6.33; N, 7.78; S, 9.26. Found (C₁₆H₂₃N₂O₅S): C, 54.08; H, 6.48; N, 7.89; S, 9.01. The preparations of 2,2,6,6-tetramethylpiperidine 1-oxyl and 2,2,6,6-tetramethylpiperidin-4-ol 1-oxyl were identical with those given by Briere.¹³ The *p*-sulfonamidobenzoyl chloride was prepared by allowing 3 g of oven-dried (110–115°) *p*-carboxybenzenesulfonamide, K⁺ salt, to react with a fourfold excess of freshly distilled thionyl chloride in a water bath at 60°. A calcium chloride drying tube was used to protect the reaction mixture from atmospheric moisture. After a 1-hr reaction period, the excess SOCl₂ was removed on a Rotovap and the acid chloride was extracted into 700 ml of hot benzene (*ca.* 75°).

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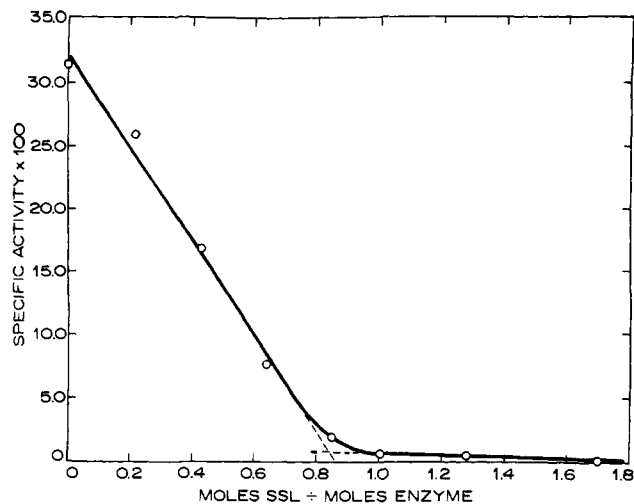


Figure 2. Titration curve for the specific activity of BCA as a function of the ratio of moles of sulfonamide spin label to moles of enzyme.

On cooling the acid chloride crystallized. The product was not hygroscopic and was stable in air. The product was obtained in 2.5 g yield (91%), mp 141–143°.

Bovine carbonic anhydrase was obtained from Worthington Biochemical Corp. and was used without further purification or separation of the isozymes. A solution was prepared by dissolving 100 mg of the material in 10 ml of deionized water. The activity was accurately determined by preparing a 1:20 dilution of the stock solution in 2.5×10^{-2} M Tris-HCl buffer, pH 7.50, and reading the absorbance at 280 m μ , molar absorptivity 5.7×10^4 , mole weight = 3.0×10^4 .^{2b,14} The concentration of the enzyme as determined from absorbance at 280 m μ was thus determined to be 7.0 mg/ml.

The spin labeling of the enzyme was accomplished by placing 0.5 ml of enzyme stock solution along with 0.10 ml of Tris-HCl buffer (0.200 M, pH 7.50) in a polyethylene vial and then adding an equivalent amount of 1.00×10^{-3} M spin label; the pH of the resulting solution was found to be 7.50. The spin label itself was prepared by dissolving a weighed amount of the solid compound in 5 ml of acetone and then diluting to the mark with deionized water in a 50.0-ml volumetric flask.

b. Methods. The specific activity of the carbonic anhydrase was determined with respect to rate of hydrolysis of *p*-nitrophenyl acetate.¹⁵ Values commonly found in the literature range from 0.29 to 0.39 mol of PNPA hydrolyzed/mol of enzyme per sec;^{15,16} the value obtained for the enzyme used in the present study was 0.31.

First, the activity of the enzyme (as defined above) was determined for solutions of varying mole ratio of enzyme to spin label. Figure 2 shows the resulting titration curve; the end point is indicated to be 0.85 ± 0.05 mol of spin label per mole of enzyme, indicating that the enzyme preparation is approximately 85% active and that the label is binding in essentially a 1:1 manner as judged by this method. The active enzyme concentration determined by titration¹⁷ with 2-acetyl-amino-1,3,4-thiadiazole-2-sulfonamide (acetazolamide) using *p*-nitrophenyl acetate as substrate was 89% of the protein concentration determined from the absorbance at 280 m μ .

The binding ratio was determined by conducting a titration using the esr spectrometer. The end point was denoted by the appearance of the first trace of unbound spin label in the resonance spectrum (see Figure 3). Varying amounts of 1.00×10^{-3} M spin label solution were added through a microburet to solutions containing 0.50 ml of 7.01 mg/ml of carbonic anhydrase and 0.10 ml of Tris-HCl buffer, pH 7.50. The binding ratio thus determined was 1.0 ± 0.1 mol of spin label per mole of enzyme, in good agreement with the previous result.

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The magnetic resonance work was carried out on a standard Varian 12-in. system previously described.¹⁸ A Varian V-4531 multipurpose rectangular cavity equipped with an aqueous sample cell was employed for the room temperature (ca. 22°) esr studies. A Varian V-4557 variable temperature accessory with an aqueous dewar sample cell was used to facilitate low temperature work. Care was taken to avoid overmodulation, exchange broadening, and saturation by working at low microwave powers (ca. 25 mW), low modulation amplitudes (ca. 0.18 G), and dilute spin label concentrations (ca. 10⁻⁴ M). The visible and ultraviolet absorbance measurements used to determine the concentration of the enzyme solution and to assay the enzyme activity were made using a Cary 15 recording spectrophotometer.

Results and Discussion

The experimental observables in the present study are the isotropic hyperfine coupling constant and the rotational correlation times deduced from the line width anisotropy of the nitroxide free radical esr spectrum. The hyperfine coupling constants for systems of the type under study vary slightly from compound to compound (13–17 G) and also show a dependence on solvent polarity; in systems of high polarity, high polarizability, or systems easily able to form hydrogen bonds the hyperfine coupling constant will tend to be larger while in systems of low polarity, etc., the coupling constant will tend to be reduced.

The asymmetry in the line width of the spectrum results from a cross coupling of the g and hyperfine tensors in the relaxation function. Anisotropies in both these tensors contribute to T_2 , the inverse of the line width. The physical situation corresponds to the electron spin experiencing randomly fluctuating magnetic fields whose frequency distribution may be characterized by a correlation time, τ_c : the inverse of τ_c gives one a measure of the mean fluctuation frequency for the relaxation mechanism involved. Because both the g and hyperfine tensors are tied to the same molecular frame of reference their effects are correlated, leading to a dependence of T_2 on the nuclear quantum number. Explicitly, the line width of the various lines in the hyperfine pattern may exhibit different widths and by determining this line width asymmetry one can deduce the appropriate (in this case, rotational) correlation time. The usefulness of nitroxide radicals in deducing correlation times lies not only in their chemical stability and relative simplicity but also in the fact that, normally, a simple three-line esr spectrum is observed which is easily analyzed. The three lines of equal integrated intensity result from the coupling between the radical odd electron and the nitroxide nitrogen of nuclear spin 1.

The theoretical expression for T_2 as a function of the rotational correlation time and other pertinent parameters has been discussed many times elsewhere.¹¹ Kivelson's¹⁹ expression for T_2 is used with the assumption of axial or near-axial T tensor symmetry²⁰ and correlation times such that $\omega\tau_c \gg 1$. The appropriate expression is given by

$$R(m) = \frac{T_2(0)}{T_2(m)} = 1 - \frac{4}{15}\tau_c b \Delta\gamma H_0 T_2(0) m + \frac{b^2}{8}\tau_c T_2(0) m^2 \quad (1a)$$

(18) J. C. Bailey and D. B. Chesnut, *J. Chem. Phys.*, **51**, 5118 (1969).

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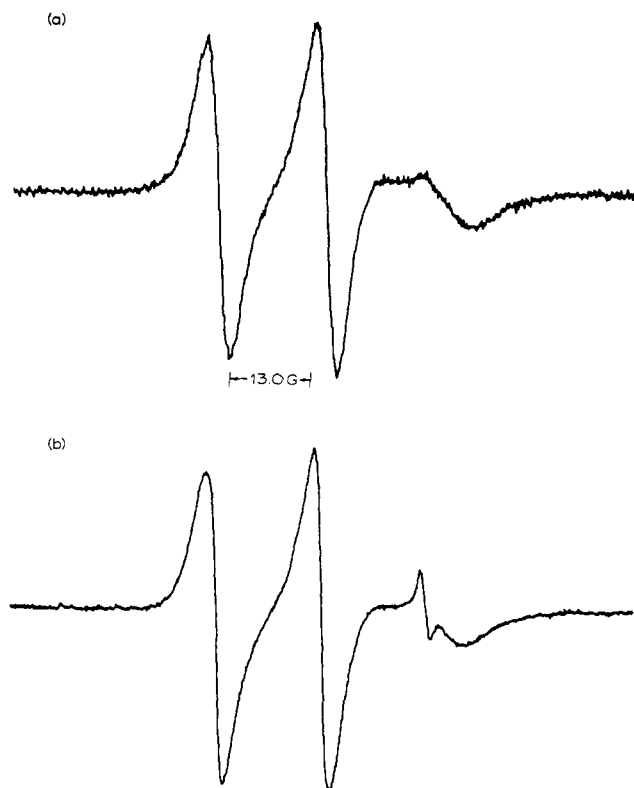


Figure 3. (a) The esr spectrum of the 1:1 complex between the sulfonamide spin label (SSL) and bovine carbonic anhydrase (BCA) exhibiting broad lines due to slowing of the rotational motion of the spin label by its attachment to the enzyme. (b) The spectrum shortly after the end point of the esr titration described in the text. The excess freely rotating SSL is seen as the sharp peak of the high field lines. The magnetic field scans are the same for both spectra while the relative amplitudes are arbitrarily scaled.

where

$$\Delta\gamma = \frac{-\beta}{\hbar} \left[g_{zz} - \frac{1}{2}(g_{xx} + g_{yy}) \right] \quad (1b)$$

and

$$b = \frac{4\pi}{3} \left[T_{zz} - \frac{1}{2}(T_{xx} + T_{yy}) \right] \quad (1c)$$

are the anisotropies in the g tensor and hyperfine tensor (in frequency units), respectively. Other symbols include τ_c , the rotational correlation time, H_0 , the applied magnetic field, and m , the m_z quantum number of the nitrogen-14 nuclear spin. The value of $T_2(0)$ was determined by direct measurement of the appropriate line width. The line-width ratios which determine $R(m)$ were determined by taking the square roots of the respective inverse amplitudes. This procedure is valid when rotation is rapid and the various hyperfine lines do not overlap as is the case in the present study. By taking the experimental results for $m = +1$ and $m = -1$ in eq 1 and by taking the sum and difference of the resulting equations one arrives at two solutions for τ_c

$$\tau_c^m = \frac{15}{2} \left(\frac{R(-1) - R(1)}{4b\Delta\gamma H_0 T_2(0)} \right) \quad (2a)$$

$$\tau_c^{m^2} = 4 \left(\frac{R(-1) + R(+1) - 2}{b^2 T_2(0)} \right) \quad (2b)$$

Table I. T and g Tensor Data. T Values are Expressed in Megacycles per Second

	$T_{ }$	T_{\perp}	$a_N = 1/3 \cdot$ Tr(T)	g_x	g_y	g_z	$g_{iso} = 1/3 \cdot$ Tr(g)
SSL, aqueous solution			47.0				2.0059
SSL-enzyme complex, H ₂ O, frozen	101.1	21.5 ^b	48.1	(2.0093) ^e	2.0068 ^e	2.0016	
SSL-enzyme complex, H ₂ O, rapid anisotropic rotation	73.6 ^c	35.2 ^c	48.1	2.0067 ^c	2.0067 ^c	2.0043 ^c	2.0059
SSL-enzyme complex, H ₂ O-sugar	89.3 ^b	27.5	48.1				
Di- <i>tert</i> -butyl nitroxide ^d	89.0	19.1 ^d	42.4	2.00881	2.00625	2.00271	2.00592

^a Ref 21. ^b Calculated from knowledge of the trace and one component. ^c Primed tensor quantities calculated for $\phi = 36^\circ$. ^d Average of $T_x = 21.5$ and $T_y = 16.7$ Mcps. ^e Best fit values for $\phi = 36^\circ$ and g' values.

The superscripts indicate that one relies on a knowledge of the term linear or quadratic in m in order to deduce the correlation time. Theoretically, of course, the two correlation times must be identical. Any observed differences in these two values gives one a measure of the correctness of the input values for the hyperfine and g tensor anisotropies. One should note, however, that equality of τ_c^m and $\tau_c^{m^2}$ is not sufficient to conclude that one is using the correct values of b and $\Delta\gamma$. The ratio $\tau_c^{m^2}/\tau_c^m$ demands that only the ratio $\Delta\gamma/b$ have a specific value. Accordingly, it is possible to choose many values for both b and $\Delta\gamma$ satisfying the above condition but yielding different absolute correlation times. Additional criteria are necessary to judge the correctness of the b and $\Delta\gamma$ that one uses in a calculation. One such criteria would, of course, be the agreement of the correlation time calculated from the esr spectrum with the correlation time of the same system calculated by one or more independent methods.

The values for the T and g tensors as recently reported by Libertini and Griffith²¹ from the single-crystal study of di-*tert*-butyl nitroxide in doped tetramethyl-1,3-cyclobutanedione represent some of the more accurate values presently known for these parameters. Since the experimental parameters do not vary very much from system to system it might be thought possible to employ their values for the present study. However, their value for a_N , the isotropic hyperfine coupling constant, is 42.4 Mc while for the sulfonamide spin label (SSL) used in this investigation the observed value of a_N in aqueous solution is 47.0 Mc and is 48.1 Mc in the enzyme sulfonamide complex. The increase in a_N reflects an increase in the spin density on the nitrogen atom. Not only does this affect the isotropic coupling constant but it will also affect the individual components of the T tensor, and, accordingly, the anisotropy in the T tensor. Often it is possible to deduce one or both of the values of $T_{||}$ and T_{\perp} for an axial system from the powder esr spectrum.¹² Accordingly, the sulfonamide-enzyme complex system was frozen and its spectrum as a rigid glass was studied at -95° . The resulting spectrum indicated that $T_{||}$ was 101.1 Mc; T_{\perp} could not be determined directly and was calculated from $T_{||}$ and a knowledge of a_N for that system, yielding a value of 21.5 Mc. Comparing the values of a_N , $T_{||}$, and T_{\perp} for the rigid glass spectrum of the complex sulfonamide spin label with the values of Libertini and Griffith for their nitroxide radical one can see that the increases in all quantities are in approximately the same ratio. This is precisely what one would expect on the basis that the spin density is

determining the relative magnitudes of all these quantities. In a like manner values for the g tensor were also observed and calculated from the rigid glass spectrum. These data as well as other data used in this study are shown in Table I.

Using the above parameters appropriate to SSL, correlation times were calculated from the solution esr spectrum of the SSL-enzyme 1:1 complex. The values obtained were $\tau_c^m = 2.3 \times 10^{-9}$ sec and $\tau_c^{m^2} = 2.8 \times 10^{-9}$ sec. These times seemed somewhat short, indicative of faster rotational motion than one might expect to be present in this system. Although the BCA crystal structure is unknown, that of the human variety is. While they are not necessarily identical in structure the similarity in molecular weight, amino acid composition, zinc binding affinity, and activity indicate that they should be sufficiently similar to allow us to treat the bovine enzyme under study here in terms of the structural data of the human variety. The enzyme in question is very nearly a spherical molecule, with molecular radii of approximately 27.5, 22.5, and 20 Å.^{2a} It is sufficiently large that one should well expect the Stokes' law correlation time for the rotation of the enzyme to be valid. The Stokes' law correlation time²²

$$\tau_c^{\text{Stokes}} = \eta V/kT \quad (3)$$

requires knowledge of the molecular volume, V , and the solution viscosity, η . The molecular volume of the enzyme was calculated for an oblate spheroid of major semiaxis 27.5 Å and minor semiaxis 21.25 Å. The viscosity of the enzyme solution was determined by comparing the esr spectra of 2,2,6,6-tetramethylpiperidine-1-oxyl in deionized water ($\eta = 0.01$ P) with that obtained by placing the same nonbinding radical in the enzyme solution. The two spectra were identical, allowing us to use the viscosity of 0.01 P in our calculation. Using for the temperature 300°K, a value of τ_c^{Stokes} of 1.26×10^{-8} sec was obtained. Our value of the Stokes' law correlation time is somewhat different from that obtained by Chen and Kernohan who used a molecular radius of 25.5 Å and modeled their calculation after a sphere. Chen and Kernohan²³ investigated the rotational correlation times from fluorescence depolarization studies for the carbonic anhydrase-5-dimethylaminonaphthalene-1-sulfonamide complex and obtained a value of 2.89×10^{-8} sec.

There is clearly an order of magnitude difference between the Stokes' law correlation time and that calculated from the present esr studies, indicating that there must be an additional motion of the nitroxide

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moiety independent of the bulk enzyme motion which the Stokes' law time represents. There are two general classes of additional motion that one might consider. In the first case, one might have rapid isotropic motion of SSL relative to the enzyme. In such a case the inverse of the rotational correlation time, the rotational correlation frequency, should be a sum of the correlation frequency of the enzyme motion plus the correlation frequency of the motion of the nitroxide with respect to the enzyme. Were this the case in the present study it would require a rotational frequency of the nitroxide relative to the enzyme of approximately 10^{+9} cps; this is a value for rotational frequencies found in not very viscous solutions and seems to be completely unreasonable in the present case. Accordingly, we focus on the other alternatives.

The second possibility concerns rapid anisotropic motion of the spin label molecule itself. McConnell and Hubbell^{12,24,25} have discussed the topic of anisotropic motion and have shown that such rotation leads to a new effective spin Hamiltonian reflecting axial symmetry about the axis of rotation. This means that the elements of the g and T tensors must be replaced by suitable averages g' and T' . The elements of g' and T' are related to the components of g and T by the time averages of the squares of the direction cosines of the angles between r , the rotation axis, and x , y and z principle axes of the tensor involved

$$T_{\parallel}' = \overline{\alpha^2}T_x + \overline{\beta^2}T_y + \overline{\gamma^2}T_z \quad (4a)$$

$$T_{\perp}' = \frac{1}{2}(1 - \overline{\alpha^2})T_x +$$

$$\frac{1}{2}(1 - \overline{\beta^2})T_y + \frac{1}{2}(1 - \overline{\gamma^2})T_z \quad (4b)$$

where $\overline{\alpha^2}$, $\overline{\beta^2}$, and $\overline{\gamma^2}$ represent the time-averaged squares of the direction cosines mentioned above. Identical equations hold for the components of the g tensor. g and T may not originally be axially symmetric but will become axially symmetric under the rapid rotation. It is clear that if one has knowledge of the true rotational correlation time for such a system, thus allowing calculation of the g' and T' quantities, one can then deduce an angle or angles relating the rotation axis to the molecular axes provided the geometry is sufficiently simple. We feel we have been able to make such a calculation in the present study based on the knowledge of the molecular structure of SSL and upon previously known data concerning binding of such sulfonamide moieties to carbonic anhydrase.

It is conceivable that the molecule might be rotating rapidly about its long molecular axis (with the molecule in its extended conformation; see Figure 1). Not only does this type of motion minimize the viscous drag but it would be consistent with the sulfonamide being coordinated to the zinc atom. It will, however, be argued shortly that the aromatic nucleus is fairly tightly bound to the cleft. In addition, such long axis rotation might also be expected to occur in, say, a glycerine solution of the label. It was observed, however, that the behavior of SSL in glycerine compared to its behavior in the enzyme complex is qualitatively dif-

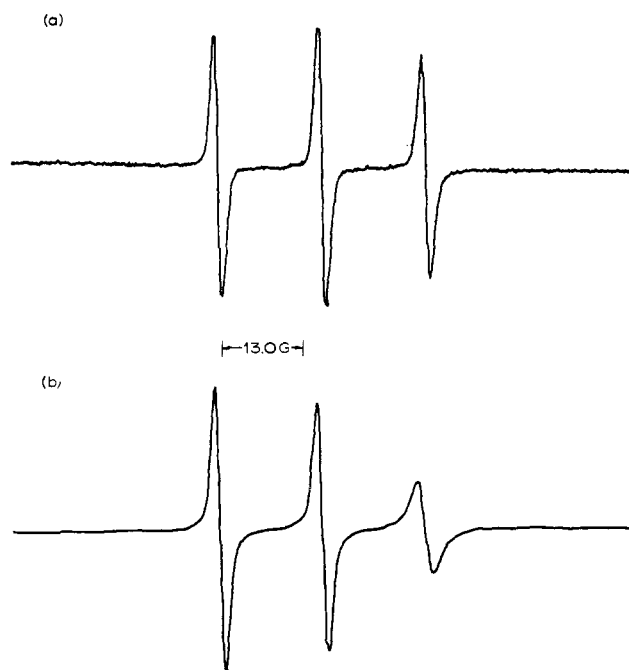


Figure 4. The esr spectrum of 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-ol (a) and the sulfonamide spin label (b) in 60% by volume glycerin-H₂O solution at identical concentrations [of 1.0×10^{-4} M]. The magnetic field scan is the same for both spectra while the relative amplitudes are arbitrarily scaled.

ferent (see Figure 4). The fact that the forms of the spectra are so different helps to rule out axial rotation in the enzyme.

The additional motion within the spin label must then result from rotations about one or more of the bonds present in the label. Motion arising from piperidine ring boat-chair interconversions is not considered to be important since it has been shown that this system exists predominantly in the chair form in solution and that the activation energy required for interconversion is high (about 10–15 kcal/mol).¹³ Analysis of the rotational motion begins with Strandberg's X-ray crystallographic study cited previously and the electronic triplet-triplet exchange between *m*-acetobenzenesulfonamide (triplet donor) and the tryptophan residue (triplet acceptor) situated on the surface of the enzyme cleft adjacent to the aromatic nucleus of the sulfonamide, a study carried out by Galley and Stryer.²⁶ Galley and Stryer, by virtue of the high value obtained for the exchange efficiency in their triplet-triplet exchange studies, corroborated Strandberg's belief that the aromatic nucleus of the sulfonamide inhibitor is securely situated within the hydrophobic region of the enzyme cleft. The agreement between the Stokes' law correlation time and the correlation time obtained from Chen and Kernohan's²³ fluorescence depolarization studies of the naphthalene inhibitor adds further confirmation to the hypothesis. Had the naphthalene nucleus possessed a high degree of motional freedom within the cleft the value calculated for the correlation time would not have been comparable to the Stokes' law value. Furthermore, the kinetics of binding and the high binding constants^{5,6,27} obtained for sulfonamide inhibitors are

(24) W. L. Hubbell and H. M. McConnell, *Proc. Nat. Acad. Sci. U. S.*, **63**, 16 (1969).

(25) W. L. Hubbell and H. M. McConnell, *ibid.*, **64**, 20 (1969).

(26) W. C. Galley and L. Stryer, *ibid.*, **60**, 108 (1968).

(27) W. H. Müller, A. M. Dessert, and R. O. Roblin, Jr., *J. Amer. Chem. Soc.*, **72**, 4893 (1950).

thought to arise first from the cooperative or chelating effect involving the interaction of the aromatic nucleus with the hydrophobic enzyme cleft as well as interaction of the metal ion through the sulfonamide. It seems likely, therefore, that rotation of the aromatic ring is severely hindered. This leads us to consider rotations about the ester linkage of the molecule.

Rotation of the entire carboxylate group with respect to the aromatic ring can be discounted because of the considerable conjugation of the carboxylic group with the aromatic nucleus. One is left then to consider rotation about the 4-carbon of the piperidine moiety and the ester oxygen. Provided that the piperidine ring itself does not interact strongly with the enzyme this motion should be relatively unhindered and, therefore, should make a significant contribution to the independent motion of the nitroxide. The bond in question, the rotation axis, and the principle axes of the nitroxide hyperfine and g tensors are indicated in Figure 1. It has been assumed that the rotation axis is in the plane containing the N-O bond axis and the $2p\pi$ orbital axis of the nitrogen atom. There is, thus, only one direction cosine to be determined.

We assume, therefore, that there is rapid anisotropic rotation about the ester linkage so noted above. We further assume that this is the extent of the additional motion of the spin label, *i.e.*, that the aromatic nucleus is bound rigidly (on the time scale involved) to the hydrophobic region of the active site cleft. With these two assumptions one would expect to calculate an angle between the $T_{||}$ (or $g_{||}$) major axis and the rotation axis of slightly over 35° , based on ideal molecular structure models. Employing the experimental values for the anisotropies in this study and *requiring* that the experimental correlation times be equal to our Stokes' law correlation time allow us to calculate effective anisotropies in both the T and g tensors. By eq 4a and 4b we then calculate an angle ϕ between the rotation axis and the parallel axes in question, obtaining a result of 36.0° based on data from the T tensor, and a value of 40° based on the g tensor data. One should note that these two values are independent calculations. This calculation may be questioned on the grounds that, strictly speaking, the conditions under which the correlation time eq 1a is valid are beginning to break down;^{11,19} in employing it we presume its extension under the present conditions. It should also be pointed out that the calculations involved using the g tensor data are probably somewhat in error in that, of necessity, one must assume axial symmetry for the g tensor (not the g' tensor, which *must* be axially symmetric) whereas it is most likely not axially symmetric in the present study. One can show, however, that a best fit of the g tensor data to the 36° angle as calculated from the T tensor data leads to values for g_x , g_y , and g_z which are in reasonably good agreement with those of Libertini and Griffith (Table I). Accordingly, we feel that both calculations are complementary and lead to a value of the angle which is immensely plausible.

It should also be pointed out that the calculations involving the T' values above yield results which are consistent with the observed form of the spectra. From eq 1a it is seen that one can write

$$R(m) = 1 - \tau_c(Am - Bm^2)$$

where A and B are the coefficients of the linear and quadratic terms. By looking at the equations defining $R(1)$ and $R(-1)$, one can see that the relative amplitudes of the three lines are determined. One can readily classify the observed spectra into six separate groups according to the sign and relative magnitude of A and B ($B > 0$ always), *independent* of the absolute magnitude of τ_c . Libertini and Griffiths' data as well as the present data for both the frozen and fluid (rotating label) enzyme-label complex systems predict spectra characterized by the inequality $B > A > 0$. On the other hand, for SSL in a glycerine-water solution the observed spectrum indicates $A > B$ (see Figure 4). This is part of our basis for characterizing SSL in the enzyme as firmly bound as opposed to the type of motion that might be expected in an isotropic fluid. Figure 4 shows both SSL and TMPOO (2,2,6,6-tetramethylpiperidin-4-ol *N*-oxyl) in glycerine-water solutions. The TMPOO molecule—which basically constitutes the "head" part of SSL (see Figure 1)—is characterized by $B > A > 0$, as might be expected for isotropic rotation. Clearly, however, the motion involved for SSL in glycerine-water is more complicated. The use of these "spectra classifying" inequalities seems to have been overlooked before. It is a method which with a minimum amount of information can point out the existence of unusual motion.

Unfortunately, there is no experimental evidence regarding the angle discussed above, and what structure data are available does not provide a consistent answer. For example, the crystal structure of 2,2,6,6-tetramethylpiperidin-4-ol 1-oxyl performed by Lajzerowicz-Bonneteau²⁸ shows large distortion of the cyclic six-membered ring. In addition, it was observed that the N-O bond lies 21° out of the C-N-C plane. It is often assumed by those engaged in spin labeling studies that this bond lies in the C-N-C plane. Crystal structures of di-*p*-anisyl nitroxide²⁹ and caryophyllene iodinitroside³⁰ also show significant deviations from coplanarity although the C-N-C bond angles in these studies approach a more nearly ideal tetrahedral value. The crystal and molecular structure of the potassium salt of 2,2,5,5-tetramethyl-3-carboxypyrroline 1-oxyl, on the other hand, shows no deviation from coplanarity within experimental error.³¹ By the same token the electron diffraction studies of Andersen and Andersen³² on gaseous di-*tert*-butyl nitroxide indicate no evidence to suggest any deviation from coplanarity. The significant out-of-plane displacement of the piperidine species as compared with the pyrroline analog may arise partially as a consequence of hydrogen bonding in the former case. The alcohol proton of one molecule in the unit cell can be imagined to hydrogen bond with the nitroxide oxygen of the second molecule in the same unit cell. In the pyrroline case, however, the acid proton has been replaced by a potassium ion, thus eliminating a similar possibility for this species. Because the T and g tensor axes are assumed coincident

(28) P. J. Lajzerowicz-Bonneteau, *Acta Crystallogr., Sect. B*, **24**, 196 (1968).

(29) A. W. Hansen, *ibid.*, **6**, 32 (1953).

(30) D. Hawley, G. Ferguson, and J. M. Robertson, *J. Chem. Soc. B*, 1255 (1968).

(31) G. Kruger and J. Boeyens, *Proc. Nat. Acad. Sci. U. S.*, **61**, 422 (1968).

(32) B. Andersen and P. Andersen, *Acta Chem. Scand.*, **20**, 2728 (1966).

with the molecular axis system this question of coplanarity is an important one. However, we do not feel that the question is readily resolved. From the above considerations, it appears as though deviations from coplanarity may arise as a result of crystal packing forces and other crystal-associated forces not found in the gas and liquid phases. On the other hand angular departures from ideal tetrahedral values are not uncommon in strained ring systems such as the tetramethylpiperidines. In the present case observed deviations from ideal angle values may result from the fact that the piperidine ring is not in a perfect chair conformation but rather a chair-twist or skew form. We believe, however, that our treatment is consistent in many respects with not only a plausible molecular geometry but also with prior data concerning binding of sulfonamides to carbonic anhydrase.

Direct experimental observation of the components of T' and g' for the rotating label would, of course, provide additional evidence for our model. An experiment was tried in which the label-enzyme complex in water was added to a highly viscous aqueous solution of sugar. The presumption in this experiment was that the molecular size of the sucrose disaccharide in high concentration would prevent the bulk rotation of the enzyme but would be too large to enter the active site cleft and interfere with the independent motion of the nitroxide. A rigid glass-like spectrum was observed which yielded values of T_{\parallel} and T_{\perp} of 89.3 and 27.5 Mcps, respectively. These are intermediate between the values obtained for the frozen solution (where all motion is restricted) and those obtained from the enzyme-label complex where rapid anisotropic rotation is occurring. It seems likely that although the freedom of motion of the enzyme has been restricted, one has also affected the rotational freedom of the label itself about the ester linkage. A preferable experiment would be one in which the enzyme-label complex acts as a substrate on an immobile surface. Preliminary work along this line is now under way.

The salient feature behind the rotational analysis is that the piperidine ring is relatively free to rotate while the aromatic nucleus is not. Since Strandberg's results indicate a tight fit of the aromatic nucleus within the enzyme cleft, the only feasible explanations of the rotation of the piperidine ring are (1) the cleft "opens

up" so that the nitroxide experiences a wider, less restricting protein environment than does the aromatic nucleus, or (2) the piperidine ring projects out beyond the enzyme surface and experiences essentially an aqueous buffer environment. The length of the extended conformation of the SSL is about³³ 11 Å. Since evidence exists indicating that the sulfonamide group is within the coordination sphere of the zinc atom situated near the center of the enzyme, it appears unlikely that this projection beyond the enzyme surface occurs. Experimental confirmation of this is provided in the value of the isotropic hyperfine coupling constant, a_N . The value of a_N for the bound label is 17.2 G, while the value of a_N for the label in an aqueous Tris-HCl buffer solution is 16.8 G. Briere, *et al.*,¹³ have demonstrated that various solvents perturb the nitroxide moiety causing a redistribution of the spin density on both the nitrogen and oxygen atoms. The more polar the solvent the greater is the spin density on the nitrogen. The shift of density toward the nitrogen arises because of the hydrogen bonding of the oxygen with the solvent molecules. These authors show that the hyperfine splitting may vary by as much as 1.7 G on going from benzene (virtually no ability to form hydrogen bonds) to water (strongly hydrogen bonded). Thus, a difference of 0.4 G in our studies is approaching significance. We conclude that the nitroxide does not see a purely aqueous environment and is therefore situated inside the protein cleft. Accordingly, while many studies^{4,23,34} characterize the cleft as hydrophobic we are here mapping a region of the cleft that is highly polar. Use of dilute enzyme solutions (*ca.* 10^{-4} – 10^{-5} M) virtually negates the possibility of the nitroxide moiety bound to one enzyme molecule interacting with a neighboring enzyme molecule. Our results show nevertheless that the nitroxide is influenced appreciably by a protein structure of hydrogen bonding capacity. Our results would not be inconsistent with the presence of NH- and NH₂-containing amino acid residues (histidine and tryptophan) in the vicinity of the NO group.

(33) This length is derived based on X-ray crystallographic work of B. H. O'Connor and E. N. Maslen, *Acta Crystallogr.*, **18**, 363 (1965), on sulfonanilide, G. A. Sim, J. M. Robertson, and T. H. Goodwin, *ibid.*, **8**, 157 (1955), on benzoic acid, and work by P. J. Lazerowicz-Bonneteau, cited previously, on 2,2,6,6-tetramethylpiperidine-1-oxyl-4-ol.

(34) R. W. Henkens, G. D. Watt, and J. M. Sturtevant, *Biochemistry*, **8**, 1874 (1969).

Communications to the Editor

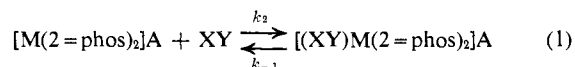
Oxygenation and Related Addition Reactions of Isostructural d⁸ Complexes of Cobalt, Rhodium, and Iridium. A Quantitative Assessment of the Role of the Metal

Sir:

We have synthesized a new series of univalent cationic complexes, $[M(2=\text{phos})_2]A$ ($M = \text{Co, Rh, and Ir}$; $2=\text{phos} = \text{cis}-(\text{C}_6\text{H}_5)_2\text{PCH}_2\text{CH}_2\text{P}(\text{C}_6\text{H}_5)_2$;¹ $A = \text{Cl, I, BF}_4$,

(1) This bidentate ligand, *cis*-1,2-bis(diphenylphosphino)ethylene, is

and/or $\text{B}(\text{C}_6\text{H}_5)_4$), whose reactions with covalent molecules (XY, eq 1) provide the first direct comparison of



the reactivities of planar d⁸ complexes of *three different*

abbreviated as 2=phos throughout this paper. Occasional references are made also to a closely related ligand, 1,2-bis(diphenylphosphino)ethane, abbreviated as 2-phos.