Electron-Paramagnetic-Resonance Studies on Cobalt(II) Carbonic Anhydrase–Sulphonamide Complexes

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Sulphonamide adducts of three Co(II) carbonic anhydrases were investigated by e.p.r. (electron paramagnetic resonance) at helium temperatures. The highly anisotropic 9 GHz spectra exhibited only three distinct features, with g values between 6.3 and 1.5. Such spectra arise from an electronic state with effective spin $S' = \frac{1}{2}$, indicating that the high-spin ($S = \frac{3}{2}$) ground level is split into two spin doublets differing in energy by an amount large compared with the microwave quantum, but small in relation to thermal energies at ambient temperature. This situation would occur in a tetrahedral system suffering a large rhombic distortion. Calculations based on this model accounted for apparent discrepancies in integrated spectral intensities, and yielded magnetic moments in good agreement with independent measurements, especially in the case of certain small Co(II) complexes resembling the enzyme adducts in their e.p.r. signals. Precise sets of g values, reflecting a particular co-ordination geometry, were found to be representative of each enzyme variant and the type of sulphonamide inhibitor, whether benzocyclic or heterocyclic. A series of substituted benzene sulphonamides bound to the same enzyme gave rise to closely similar spectra despite a wide range of pK_i values. Thus benzocyclic and heterocyclic sulphonamides were evidently held in the active-site cleft in characteristic orientations irrespective of side chains that might considerably influence the total binding strength. Visible absorption spectra of various sulphonamide adducts at room temperature showed a similar pattern of inhibitor dependence to the e.p.r. spectra, suggesting a correspondence between the co-ordination structures in liquid and frozen solution. E.p.r. spectra of the sulphonamide complexes were remarkable not only for their range of g values, but also for their variations in line-width and spin-lattice relaxation behaviour. Addition of glycerol to the medium produced marked enhancement in resolution, owing to the creation of a more homogeneous frozen matrix. The non-uniform spin relaxation was probably a consequence of the large anisotropy in effective g tensor.

Many aromatic and heterocyclic sulphonamides are powerful and specific inhibitors of the zinc metallo-enzyme carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) (Maren, 1967). Since these reagents are not known to form stable metal complexes, their mode of interaction with the active site of carbonic anhydrase has been the subject of numerous investigations (see Lindskog *et al.*, 1971). Although the unsubstituted $-\text{SO}_2\text{NH}_2$ function is required for strong inhibitory action, modifications to the ring system can produce a wide range of binding constants. X-ray crystallo-

graphic data on the human C isoenzyme at 0.2–0.3 nm (2.0–3.0 Å) resolution reveal that the inhibitor effectively fills the inner part of the active-site crevice, displacing an ordered array of solvent molecules present in the native enzyme (Liljas *et al.*, 1972). The sulphonamide group is clearly bonded to the Zn(II) ion, but other interactions with side chains in the wall of the crevice seem probable, depending on precise details of enzyme and inhibitor structure (Bergstén *et al.*, 1972). Many solution studies are in accord with this picture. Thus sulphonamides exert a pronounced

effect on the energy and optical activity of the ligand-sensitive $d-d$ absorption bands in the cobalt(II) enzyme, which implies a close association with the metal ion (e.g. see Lindskog, 1963; Coleman, 1968a). The pH dependence of binding suggests that the $-SO_2NH_2$ moiety is deprotonated in the complex (Kernohan, 1966; Lindskog & Thorslund, 1968), and this is supported by changes in optical properties of the sulphonamide molecule itself (Chen & Kernohan, 1967; King & Burgen, 1970). Evidence for hydrophobic forces between the aromatic ring system and the active-site wall derives from the enhancement in quantum yield when a fluorescent sulphonamide is attached (Chen & Kernohan, 1967). Both n.m.r. (nuclear magnetic resonance) work (Lanir & Navon, 1971) and e.p.r. (electron paramagnetic resonance) studies with spin-labelled inhibitors (e.g. see Mushak & Coleman, 1972; Chignell *et al.*, 1972) show that the sulphonamide molecule is indeed rigidly held within the protein cavity.

The operation of several bonding modes might explain the remarkably strong but structure-dependent affinity of sulphonamides for carbonic anhydrase. Several comparisons have demonstrated that replacement of Zn(II) by Co(II) at the active site has little effect on the equilibrium or rate constants of sulphonamide binding (Lindskog & Thorslund, 1968; Taylor *et al.*, 1970b), although the apoenzyme and other metallo-derivatives possess small affinity (Lindskog, 1963; Coleman, 1967). The present paper describes the use of e.p.r. to investigate the binding of a range of sulphonamides to three Co(II) carbonic anhydrase variants. Since the e.p.r. transitions of the Co(II) ion are sensitive to ligand field strength and symmetry, it should be possible to determine how the co-ordination geometry is influenced by other protein-inhibitor interactions. The results should also be helpful in understanding e.p.r. spectra of the active enzyme, which are likely to be complicated by the greater flexibility of the active-site region. We have previously applied e.p.r. to the study of a unique low-spin ($S = \frac{1}{2}$) cyanide adduct of Co(II) carbonic anhydrase (Cockle, 1973, 1974), but in other situations the metal ion adopts the high-spin ($S = \frac{3}{2}$) configuration (Lindskog & Ehrenberg, 1967). Owing to rapid electron spin relaxation, satisfactory measurements on high-spin Co(II) require temperatures below about 20°K, which in the past has impeded experimentation on molecular species in frozen solution. However, reports have appeared concerning some small Co(II) complexes whose e.p.r. behaviour provides a useful comparison with that of the enzyme-sulphonamide derivatives (Yablokov *et al.*, 1971; Kennedy *et al.*, 1972). Our e.p.r. spectra describe Co(II) in a highly distorted tetrahedral environment which is characteristic of the enzyme variant and the type of sulphonamide. Some pre-

liminary results have been published (Grell & Bray, 1971).

Materials and Methods

Cobalt carbonic anhydrase

Native carbonic anhydrase was isolated from human and bovine erythrocytes by established procedures (Henderson & Henriksson, 1973; Lindskog, 1960; Nilsson & Lindskog, 1967). Some bovine enzyme was also purchased from Miles-Seravac (Pty) Ltd., CH-1006 Lausanne, Switzerland. The cobalt enzymes were prepared as described by Cockle (1974).

Protein concentrations were determined from measurements of optical absorbance at 280nm. The following molar absorption coefficients were used, based on the molecular weights given in parentheses (see Lindskog *et al.*, 1971): human isoenzyme B (29000) $47\,300 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, human isoenzyme C (30000) $561\,00 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, bovine enzyme (30000) $57\,000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Enzyme activity

Preparations were assayed for esterase activity in the following medium: Co(II) enzyme (0.5–1.5 μM), 4-nitrophenyl acetate (0.40mM) and acetone (1%, v/v), in 0.08M-Tris-sulphate buffer, adjusted to pH 7.60 with H_2SO_4 at 25°C (Whitney *et al.*, 1967). Specific activities (mol of ester hydrolysed/s per mol of enzyme) were: Co(II) human isoenzyme B, 0.31–0.36; Co(II) human isoenzyme C, 0.91–0.99; Co(II) bovine enzyme, 0.37–0.43.

The proportion of active enzyme in these samples was determined by titrating the esterase activity against ethoxzolamide (6-ethoxybenzothiazole-2-sulphonamide; compound 8 in Table 1). Owing to the extremely high binding constant of this inhibitor zero activity is reached at a 1:1 molar equivalence of sulphonamide to active enzyme. Recorded purities were: Co(II) human isoenzyme B, 88–92%; Co(II) human isoenzyme C, 86–100%; Co(II) bovine enzyme, 85–100%, with respect to protein concentration derived from optical absorbance at 280nm.

Sulphonamides

The aromatic and heterocyclic sulphonamides used in this work are listed in Table 1. The non-sulphonamide inhibitor 9 (4-aminophenylthioacetate) was included because its enzyme complex has a very similar e.p.r. spectrum to those of the sulphonamide adducts, in contrast with the behaviour of other simple anions (Grell & Bray, 1971). Compounds 1, 2, 7, 8 and 9 were obtained commercially and twice

Table 1. *Dissociation constants for sulphonamide complexes of Co(II) carbonic anhydrase*

Equilibrium constants K_i were determined from enzyme-sulphonamide titrations by following the inhibition of esterase activity at 25°C. Assay media contained: Co(II) enzyme (0.5–1.5 μM), 4-nitrophenyl acetate (0.40 mM), 1% (v/v) acetone. The buffer system comprised: Tris (0.05 M), Bistris [*NN*-bis-(2-hydroxyethyl)-*N*-tris(hydroxymethyl)methylamine] (0.05 M), Na_2SO_4 (0.15 M), adjusted with H_2SO_4 to pH 7.0 at 25°C. Sulphonamide concentrations were in the range 0–60 μM (0–500 μM in the case of inhibitor 9). The Michaelis constants K_m for ester hydrolysis are considerably larger than the substrate concentration used. Accordingly, the sulphonamide inhibition may be regarded as non-competitive, so that:

$$v_i/v_0 = 1/(1 + [\text{I}]/K_i) = 1 - i$$

where v_i and v_0 are the inhibited and uninhibited reaction velocities, and $[\text{I}]$ is the free substrate concentration; $i = [\text{EI}]/[\text{E}]_0$ is the fractional degree of inhibition. Replacing $[\text{I}]$ by $[\text{I}]_0 - i[\text{E}]_0$ gives:

$$[\text{I}]_0/i = [\text{E}]_0 + K_i/(1 - i)$$

K_i may therefore be derived from the slope of the plot $[\text{I}]_0/i$ versus $1/(1 - i)$ (cf. Lindskog & Thorslund, 1968).

Compound no.	Structure	p <i>K_i</i> at pH 7.0 of Co(II) enzyme		
		Human B	Human C	Bovine
1		4.65	5.35	5.45*
2		5.6	6.3	6.35
3		—	—	6.15
4		—	—	5.6
5		—	—	6.5
6		—	—	7.2
7		6.55	7.95	7.85*
8		(>9)	(>9)	(9.70)†
9		—	—	3.95

* Determined by Lindskog & Thorslund (1968) as described above.

† Determined by Kernohan (1970) for the native zinc enzyme at pH 7.5 by fluorescence quenching techniques.

recrystallized from ethanol before use (compounds 1, 2 and 9 from Fluka AG, CH-9470 Buchs, Switzerland; compound 7 from Lederle Laboratories, Pearl River, N.Y., U.S.A., compound 8 from the Upjohn Co., Kalamazoo, Mich., U.S.A.). Compounds 3, 4 and 5 were kindly provided by Dr. P. W. Taylor, Department of Pharmacology, University of California, La Jolla, Calif., U.S.A.

Compound 6 was included in the list of benzocyclic sulphonamides because its bulky aromatic portion interacts particularly strongly with the protein, thus possibly influencing the metal-ligand bonding. This inhibitor was synthesized from 3-nitro-4-chlorobenzenesulphonate as follows. 3-Nitro-4-chlorobenzenesulphonamide was prepared as described by Kaiser & Lo (1969). This was treated in a closed tube with a saturated solution of NH_3 for 4 h at 150°C to obtain 3-nitrosulphanilamide, which was recrystallized from ethanol-water (m.p. $204\text{--}206^\circ\text{C}$). The product was diazotized in acidic solution with NaNO_2 and added to a cold alkaline solution of sodium 1-hydroxynaphthalene-2-sulphonate. Compound 6 precipitated as the sodium salt on addition of HCl , and was recrystallized from ethanol-water and ethanol. No impurities could be detected by t.l.c. on alumina with ethanol-water (2:1, v/v) as solvent. Elemental analyses were: Calculated for $\text{C}_{16}\text{H}_{11}\text{N}_4\text{S}_2\text{O}_8\text{Na}$: C, 40.5; H, 2.33; N, 11.81%; Found: C, 40.02; H, 2.69; N, 11.46%.

Equilibrium dissociation constants for most of the complexes studied by e.p.r. were obtained from esterase inhibition experiments as described in the legend to Table 1.

E.p.r. spectra

The majority of spectra were recorded at 9 GHz on a Varian E-3 spectrometer over the temperature range $4.2\text{--}30^\circ\text{K}$, or on a Varian E-9 between 7 and 40°K . The helium cooling systems used with these instruments have been described (Lundin & Aasa, 1972; Lowe *et al.*, 1972). Sample temperatures were determined from the e.p.r. intensity of a Cu(II)-EDTA standard run at a non-saturating power; the signal amplitude was assumed to be inversely proportional to absolute temperature. The field scan between 100 and 500 mT (1000 and 5000 G) was calibrated by reference to the room-temperature spectrum of Cr(III) doped in Co(III)-EDTA , for which experimental g values are accurately known (Aasa *et al.*, 1966). Double integration was carried out by the method of Wyard (1965), and a correction for the variation of transition probability with g value was applied as noted by Aasa & Vänngård (1970). Spectra of enzyme samples were measured over a wide range of microwave powers (0.1–200 mW) at modulation amplitudes of 1–2 mT (10–20 G).

Some e.p.r. spectra were also obtained at 35 GHz on the E-9 instrument. The sample temperature was about 25°K , and no field-scan calibration was made.

Optical absorption spectra

Visible spectra of the Co(II) enzyme-sulphonamide complexes were run on a Zeiss PRQ20A, a Cary 118 or a Unicam SP. 1800 recording spectrophotometer.

Experiments and Results

The following complexes of Co(II) carbonic anhydrase were examined by e.p.r.: human isoenzyme B with sulphonamides 1, 2, 7 and 8; human isoenzyme C with compounds 1 and 2; bovine enzyme with all compounds 1–9. Solutions contained enzyme (1–4 mM) and sufficient inhibitor to ensure complete formation, as indicated in the legends to Figs. 1–3. Sample tubes were normally closed before freezing and carefully wiped before insertion into the instrument cavity, otherwise complex signals were likely to be seen from oxygen solidified on the inside or outside surfaces.

General features of the e.p.r. spectra

All the adducts examined exhibited highly anisotropic spectra characterized by three well-defined resonances, with experimental g values of g_1 5.9–6.3, g_2 2.2–2.8 and g_3 1.5–1.8. Fig. 1 shows some examples recorded at 10°K . The remarkable variations in line-width are undoubtedly due to a combination of factors, including unresolved anisotropic ^{59}Co hyperfine coupling, anisotropic spin-relaxation behaviour, and inhomogeneity in the population of molecular species. This problem will be pursued below. No conditions could be found under which additional spectral structure was resolved. Spectra were not affected by repeated freezing and thawing of the solutions, nor by extending the time of freezing from around 20 s (tube plunged in liquid N_2) to several minutes (sample chilled in an ice-salt bath). The presence or absence of dissolved O_2 had no effect on the observed line widths. Complexes of Co(II) bovine enzyme with reagents 1 and 2 were shown to be quite insensitive to pH in the range 7–11.

The e.p.r. spectra described here are consistent with single paramagnetic species of low symmetry having effective spin $S' = \frac{1}{2}$. Co(II) concentrations were determined on this basis in a number of cases. Spectra of human isoenzyme B with sulphonamides 2, 7 and 8, and of bovine enzyme with 1 and 7 measured at 10°K represented respectively 85–90% and 75–80% of the active enzyme originally present. Measurements at 35 GHz on two adducts (human isoenzyme B with compound 8, bovine enzyme with compound 1) indicated no appreciable frequency dependence of g value, in accordance with the expected behaviour of

Fig. 1. *E.p.r. spectra of some Co(II) carbonic anhydrase-sulphonamide complexes*

Spectra were recorded at frequency 9.14 GHz and microwave power 100 mW at 10°K, with arbitrary instrument gain. The traces have been slightly displaced in the Figure. Sample compositions were: (a) Co(II) bovine enzyme (2.37 mm), 3-nitro-4-hydroxybenzene sulphonamide (4) (6.7 mm), pH 9.2; (b) Co(II) bovine enzyme (2.37 mm), 4-aminophenylthioacetate (9) (13.3 mm), pH 8.2; (c) Co(II) human isoenzyme B (1.24 mm), ethoxzolamide (8) (1.55 mm), pH 9.7.

a spin one-half system. Unfortunately the limitation on temperature attainable and the excessive widths of the g_2 and g_3 features led to spectra of poor quality, so no other measurements were attempted.

The origin of the spin one-half behaviour in a high-spin Co(II) system and the cause of the low integration values lie in the distortion from cubic symmetry, to be considered in a later section.

Variation of enzyme and inhibitor

Although the metal-ion environment is basically similar in all sulphonamide derivatives, the Co(II) e.p.r. spectra are sensitive both to the enzyme variant and to the type of sulphonamide. Experimental g values for the complexes studied are listed in Table 2. The substituted benzene sulphonamides produce groups of rather similar spectra with each of the Co(II) carbonic anhydrases, although the parameters depend on the enzyme. The two heterocyclic sulphonamides 7 and 8 also give rise to nearly identical spectra with each of two enzyme variants; the g values are again characteristic of the enzyme, and differ appreciably from those typical of corresponding benzene sulphonamide adducts.

Since a set of g values denotes a particular co-ordination geometry, these results illustrate the small conformational variations that might be expected in the active sites of different carbonic anhydrases, as well as some of the factors affecting the orientation of a bound sulphonamide molecule. The non-sulphon-

Table 2. *Experimental g values for complexes of Co(II) carbonic anhydrase*

The g values were identified with the low-field maximum (g_1), the high-field minimum (g_3) and the central cross-over point (g_2).

Co(II) enzyme	Inhibitor	g_1	g_2	g_3
Human B	1	6.04	2.51	1.69
	2	6.05	2.50	1.68
	7	5.91	2.80	1.81
	8	5.91	2.76	1.80
Human C	1	6.14	2.29	1.63
	2	6.08	2.35	1.65
Bovine	1	6.25	2.16	1.54
	2	6.22	2.24	1.59
	3	6.27	2.21	1.59
	4	6.25	2.24	1.58
	5	6.29	2.19	1.55
	6	6.23	2.28	1.62
	7	5.99	2.52	1.71
	8	6.02	2.46	1.70
	9	6.02	2.76	1.80

amide inhibitor 9 evidently produces a similar ligand field strength and symmetry to the heterocyclic derivatives. Such behaviour is not found with other anionic inhibitors tested (Grell & Bray, 1971; S. A. Cockle, S. Lindskog & E. Grell, unpublished work), and suggests the importance of hydrogen bonding and van der Waals interactions in fixing the position of the aromatic part of the molecule.

Effects of temperature and microwave power

Spectra were examined as a function of temperature and power to check for the existence of resonances previously undetected owing to saturation or relaxation effects. No new peaks were detected under any conditions, and the existing features remained unchanged in position. All lines grew broader with increasing temperature, until only the g_1 peak was clearly discernible above 40°K. Lines continued to sharpen down to the lowest temperature attainable; at 4.2°K, the width at half-height for g_1 was typically 6–7 mT (60–70 G). Some spectra of Co(II) human isoenzyme B-sulphanilamide (compound 1) are shown in Fig. 2.

Saturation studies were attempted to investigate possible causes of the unequal line widths associated with the three g values. A temperature of 4.2°K was required to observe an appreciable effect on all three components. Fig. 3 contains a series of spectra for Co(II) human isoenzyme C-sulphanilamide (1). Although each resonance peak behaves differently, the signal saturates without distortion of the line shape, as expected when inhomogeneous broadening is predominant. In the present case, the

Fig. 2. E.p.r. spectra of Co(II) human carbonic anhydrase B-sulphanilamide at various temperatures

The sample composition was: Co(II) enzyme (4mM) and sulphanilamide (1) (5mM) in 0.05M-Tris-sulphate buffer, pH 7.60 at 25°C. Spectra were recorded at frequency 9.18GHz and microwave power 5mW, with arbitrary instrument gain. Cavity temperatures were: (a) 29°K, (b) 13°K and (c) 9°K. The small signal at 330mT (3300G) is due to a trace of enzyme-bound Cu(II).

Fig. 3. E.p.r. spectra of Co(II) human carbonic anhydrase C-sulphanilamide at various powers

The sample composition was: Co(II) enzyme (1mM) and sulphanilamide (1) (1.25mM) in 0.05M-Tris-sulphate buffer, pH 7.60 at 25°C. Spectra were recorded at frequency 9.17GHz at 4.2°K, with arbitrary instrument gain. Response-time and scan rate were optimized for the 'sharp' and 'broad' regions of the spectrum separately. Microwave powers were: (a) 1.15mW, (b) 25mW and (c) 200mW. The small signal at 155mT (1550G) is due to a trace of enzyme-bound Fe(III).

most obvious source of such broadening is unresolved cobalt hyperfine structure. The experiment is illustrated graphically in Fig. 4. The saturation curves for the three g components show increasing curvature

Fig. 4. Saturation curves for Co(II) human carbonic anhydrase C-sulphanilamide

The experiment is summarized in the legend to Fig. 3. Data are plotted in the form $\log(A/P^{1/2})$ versus $\log P$, where A is the signal amplitude divided by gain setting and P is the microwave power (Beinert & Orme-Johnson, 1967). Amplitudes were measured for each of the principal g values: $\bullet$, g_1 ; $\blacksquare$, g_2 ; $\blacktriangle$, g_3 . Owing to the overlap of g_2 and g_3 , the amplitude for g_2 was represented by the height of the positive portion of the derivative signal. The curves are arbitrarily spaced on the ordinate. When no saturation occurs, A is proportional to $P^{1/2}$, and the lines are parallel to the abscissa; they bend towards the abscissa with progressive saturation. (I) and (H) are idealized curves for inhomogeneous and homogeneous broadening respectively, plotted according to the equations: (I), $A/P^{1/2} = (1 + P/P_4)^{-1/2}$; (H), $A/P^{1/2} = (1 + P/P_4)^{-1/4}$ (Beinert & Orme-Johnson, 1967). The power at half saturation (P_4), given by the intersect of the tangents (----), is 10mW in these examples. The ordinate is fixed so that $A/P^{1/2} = 100$ in the absence of saturation. Estimated $P^{1/2}$ values for the experimental curves are: g_1 , 100mW; g_2 , 10mW; g_3 , 13mW.

in the order $g_1 < g_3 < g_2$. This is also the order of increasing ease of saturation, as indicated by the corresponding P_4 values. Since the broad g_2 and g_3 signals are more readily saturated than the sharp g_1 signal, and so are presumably associated with longer spin-relaxation times, we conclude that unresolved hyperfine structure is a major cause of the line-width anisotropy. However, the g_1 saturation curve closely approaches the ideal case of inhomogeneous broadening as far as measurements permit, whereas those for g_2 and g_3 lie between the two theoretical curves. Thus the latter components of the spectrum are influenced by additional line-broadening effects, perhaps arising from a variability in the precise co-ordination geometry induced by discontinuities in the frozen medium

(cf. Beinert & Orme-Johnson, 1967). A final practical conclusion is that saturation studies may be of little help in identifying related features to assist the analysis of more complex anisotropic spectra.

Effect of solvent on spectral resolution

E.p.r. spectra of small metal-ion complexes in frozen aqueous solution are often extensively broadened as a result of aggregation of solute molecules and general strains and irregularities in the packing, which promote rapid spin-spin relaxation and distortions in the co-ordination sphere of each paramagnetic centre (Ross, 1965; Cockle *et al.*, 1972). A remarkable enhancement in resolution is commonly achieved by introducing high concentrations of alcohols, salts or other inert polar materials, which induce the solution to freeze to a vitreous rather than a polycrystalline medium. To test for such effects on the Co(II) enzyme-sulphonamide spectra, samples were prepared in solvent mixtures containing various polyhydric alcohols. Unlike simple alcohols, these reagents show little tendency to disturb the native conformation of proteins (Tanford, 1968) and do not appear to inhibit carbonic anhydrase (Pocker & Stone, 1968).

Fig. 5. E.p.r. spectra of Co(II) human carbonic anhydrase B-4-carboxybenzene sulphonamide in the absence and presence of glycerol

Spectra were recorded at frequency 9.13 GHz and microwave power 5 mW, at 10°K, with arbitrary instrument gain. The samples comprised: (a) Co(II) enzyme (1.34 mM) and sulphonamide (2) (1.68 mM) in 0.05 M-Tris-sulphate buffer, pH 7.60 at 25°C; (b) as in (a) but with the addition of 10% (v/v) glycerol. The peak-to-peak widths of the g_2 component in the two spectra are: (a) 61 mT (610 G) and (b) 37 mT (370 G). The broad signal underlying the g_1 peak is due to denatured enzyme.

Variable spectral changes took place when ethylene glycol or polyethylene glycol solutions were used, but consistent results were obtained with glycerol (10–15%, v/v). For the six benzene sulphonamide complexes examined, all resonances were noticeably sharpened, particularly the broad g_2 and g_3 features. The effect was most marked for Co(II) human isoenzyme B (see Fig. 5) and least pronounced with the bovine enzyme. Only minor deviations were found in the measured g values, indicating the absence of gross co-ordination changes; as noted above, access to the metal site is effectively blocked by the sulphonamide inhibitor. Since the Co(II) ion is so deeply embedded in the globular protein, spin-spin relaxation between adjacent molecules must always be minimal. Accordingly, studies below 6°K revealed no variations in the ease of saturation, despite the enhanced resolution. However, the saturation curves

Fig. 6. Visible absorption spectra of some Co(II) bovine carbonic anhydrase-sulphonamide complexes

Spectra were recorded at 25°C on the absorbance range 0–0.2 with a path-length of 1 cm. Sample (a) comprised Co(II) enzyme (0.415 mM) and Na_2SO_4 (0.05 M) in 0.1 M-Tris–0.1 M-Bistris-sulphate buffer, pH 7.05 at 25°C. Sample (b) contained in addition 4-aminophenylthioacetate (9) (3.5 mM). The other solutions each contained sulphonamide at 0.5 mM: (c) sulphanilamide (1); (d) 3-amino-4-carboxybenzene sulphonamide (3); (e) 4-carboxybenzene sulphonamide (2); (f) 4-carboxy-3,5-dinitrobenzene sulphonamide (5). All reference solutions contained equivalent concentrations of inhibitor. Co(II) $d-d$ spectra could not be recorded for adducts of inhibitors 4 and 6, since the visible region is dominated by $\pi-\pi^*$ bands from the aromatic portions of these sulphonamides.

for g_2 and g_3 now resembled more closely the ideal curve for inhomogeneous broadening. This behaviour in comparison with that observed in the absence of glycerol suggests that the action of the mixed solvent is to create a more uniform medium in which conformational stresses imposed on the enzyme by solvent crystallites are decreased. If such forces are sensed in the immediate environment of the Co(II) ion, then a sharpening of the e.p.r. signals can be understood.

Optical spectra of Co(II) enzyme-sulphonamide complexes

Absorption spectra associated with transitions of the Co(II) d electrons occur in the visible and near-i.r. regions. Visible spectra were recorded for most of the complexes studied by e.p.r. and some examples for the bovine enzyme are shown in Fig. 6. The near-identity in optical absorption of the benzene sulphonamide complexes corresponds with the close similarity in their e.p.r. signals. On the other hand, the 4-aminophenylthioacetate (9) derivative has a rather different absorption-band profile and a distinct set of g values. In so far as both physical properties depend on the ligand field strength and symmetry (see below), such parallel behaviour is to be expected.

Theory and Interpretation

Experience has shown that the geometry of high-spin Co(II) complexes can be predicted reasonably well from the optical properties associated with the d electrons (absorption spectra, circular dichroism and magnetic circular dichroism). Such analysis has suggested an irregular tetrahedral co-ordination for inhibitor complexes of Co(II) carbonic anhydrase (Lindskog & Ehrenberg, 1967; Coleman & Coleman, 1972). This conclusion has been confirmed by X-ray crystallographic studies on the native human C isoenzyme, at least in the case of sulphonamide adducts (Bergstén *et al.*, 1972). It is necessary to demonstrate that the e.p.r. spectra of the Co(II) enzyme are also compatible with a distorted tetrahedral geometry, and if possible, to relate the measured parameters to the nature of the distortion.

Unfortunately, previous e.p.r. work on high-spin Co(II) has been largely restricted to single crystal studies of binary compounds approaching cubic symmetry. Thus tetrahedral Co(II) is characterized by an isotropic g value close to 2.3 (e.g. see McGarvey, 1966). However, a few reports have appeared on high-spin Co(II) complexes of irregular geometry, which should prove useful in understanding the metal-ion environment in Co(II)-substituted zinc metallo-enzymes (Yablokov *et al.*, 1971; Kennedy *et al.*, 1972). These authors have observed powder e.p.r. spectra which are strikingly similar to the frozen-

solution spectra of the enzyme complexes described herein. They arise from Co(II) doped in a number of compounds Py_2ZnX_2 , where Py is a pyridine base and X is chloride or bromide; some data are summarized in Table 3(a). These materials crystallize in molecular lattices, and a distorted tetrahedral structure has been confirmed by X-ray work on $(\text{C}_5\text{H}_5\text{N})_2\text{ZnCl}_2$ (Porai-Koshits *et al.*, 1960). Independent experimental evidence therefore implies that the observed e.p.r. behaviour of both enzyme and model complexes is characteristic of a particular kind of co-ordination geometry.

Ligand field treatment

The usual approach in describing a system of low symmetry is to start from an ideal cubic field and apply successive axial and rhombic distortions as perturbations. The free Co^{2+} ion has a 4F ground state with sevenfold orbital degeneracy; in a tetrahedral field this is split into a lower orbital singlet 4A_2 and two excited triplets 4T_2 and 4T_1 , as shown in Fig. 7(a). Lower symmetry components of the field lift the orbital degeneracy of the T states. As a result of spin-orbit coupling between 4A_2 and 4T_2 the fourfold spin degeneracy is also removed under these conditions; the 4A_2 level becomes two Kramers doublets, which may be assigned effective spin wave-functions $|\pm\frac{1}{2}'\rangle$ and $|\pm\frac{3}{2}'\rangle$. The separation between these levels is the zero-field splitting δ (Fig. 7b).

A spin Hamiltonian ($\mathcal{H}$) appropriate to this situation is:

$$\mathcal{H}_1 = D[S_z^2 - \frac{1}{3}S(S+1)] + E(S_x^2 - S_y^2) \quad (1)$$

where S is the value of the total spin (i.e. $\frac{3}{2}$); D and E are empirical parameters pertaining to the axial and rhombic components of the ligand field in a convenient co-ordinate frame. Evaluation of the interaction matrix yields the two doubly degenerate eigenvalues ($\mathcal{E}$):

$$\mathcal{E}_{\pm\frac{1}{2}} = -(D^2 + 3E^2)^{\frac{1}{2}} \quad (2)$$

$$\mathcal{E}_{\pm\frac{3}{2}} = +(D^2 + 3E^2)^{\frac{1}{2}} \quad (3)$$

Hence:

$$\begin{aligned} \delta &= \mathcal{E}_{\pm\frac{3}{2}} - \mathcal{E}_{\pm\frac{1}{2}} \\ &= 2(D^2 + 3E^2)^{\frac{1}{2}} \end{aligned} \quad (4)$$

In practice either doublet could be the ground state, according to the sign of $(D^2 + 3E^2)^{\frac{1}{2}}$.

The four spin wave-functions corresponding to the above eigenvalues take the form:

$$|-\frac{1}{2}'\rangle = A|-\frac{1}{2}\rangle + B|+\frac{3}{2}\rangle \quad (5)$$

$$|+\frac{1}{2}'\rangle = A|+\frac{1}{2}\rangle + B|-\frac{3}{2}\rangle \quad (6)$$

$$|-\frac{3}{2}'\rangle = A|-\frac{3}{2}\rangle - B|+\frac{1}{2}\rangle \quad (7)$$

$$|+\frac{3}{2}'\rangle = A|+\frac{3}{2}\rangle - B|-\frac{1}{2}\rangle \quad (8)$$

Table 3. *Experimental and calculated data for the complexes (C₅H₅N)₂CoCl₂ and (C₅H₅N)₂CoBr₂*

The calculated results in (b) and (c) are based on the average of the experimental g values in (a). The parameters are defined in the text.

(a) Experimental results

Complex	g_1	g_2	g_3	$\mu_{\text{eff.}}$ (Bohr magnetons)†
(C ₅ H ₅ N) ₂ CoCl ₂	6.09*	2.68*	1.79*	4.42
	6.16†	2.65†	1.79†	
(C ₅ H ₅ N) ₂ CoBr ₂	5.48*	3.74*	2.12*	4.50
	5.45†	3.77†	2.10†	

(b) Calculated g values

Complex	A^2	E/D	g_x	g_y	g_z	$g_{\text{av.}}$
(C ₅ H ₅ N) ₂ CoCl ₂	0.95	-0.27	2.32	2.27	2.20	2.27
(C ₅ H ₅ N) ₂ CoBr ₂	0.99	-0.12	2.34	2.31	2.20	2.29

(c) Magnetic moment and zero-field splitting parameters

Complex	$\mu_{\text{eff.}}$ (Bohr magnetons)	$\Delta\mathcal{S}$ (cm ⁻¹)	$\lambda'_{\parallel}$ (cm ⁻¹)	D (cm ⁻¹)	E (cm ⁻¹)	δ (cm ⁻¹)
(C ₅ H ₅ N) ₂ CoCl ₂	4.39	4500	-150	7	-2	15
(C ₅ H ₅ N) ₂ CoBr ₂	4.43	4500	-160	10	-1	20

* Yablokov *et al.* (1971).

† Kennedy *et al.* (1972)

‡ Gill *et al.* (1961).

§ The electronic transition corresponding to the separation Δ has rarely been observed, but Δ can be determined from the positions of the higher energy bands by using the Tanabe-Sugano equations (see Carlin, 1965); spectral data were from Katzin & Gebert (1950, 1953) and Ferguson (1960).

|| For the free Co²⁺ ion $\lambda = -178$ cm⁻¹. Values for λ' are likely to be smaller than this owing to covalent contributions to the bonding.

Fig. 7. *Energy-level diagrams for the Co²⁺ ion*

(a) Splitting of the 4F level of the free ion in a tetrahedral ligand field. (b) Splitting of the 4A_2 state into two Kramers doublets by the combined action of spin-orbit coupling and a low-symmetry field. The effect of an applied magnetic field H is shown formally to the right. The energy scales give an approximate guide to the magnitudes involved; an e.p.r. transition at 35 GHz is marked between the $|+\frac{1}{2}\rangle'$ and $|-\frac{1}{2}\rangle'$ levels.

The coefficients A and B are such that $A^2 + B^2 = 1$; we also expect $A^2 \gg B^2$, since B tends to 0 as the rhombic perturbation to the ligand field is removed. The following general relationship between A and

B and the parameters D and E is derived from the spin interaction matrix:

$$\frac{E}{D} = \frac{2}{\sqrt{3}} \left(\frac{AB}{B^2 - A^2} \right) \quad (9)$$

Analysis of the g values

The remaining spin degeneracies are raised by an external magnetic field, and an extra term appears in the spin Hamiltonian:

$$\mathcal{H}_s = \mathcal{H}_1 + \beta \mathbf{H} \cdot \mathbf{g} \cdot \mathbf{S} \quad (10)$$

This is the Zeeman term, in which β is the Bohr magneton, $\mathbf{H}$ and $\mathbf{S}$ are the magnetic field and spin vectors respectively and $\mathbf{g}$ is the g tensor. Three e.p.r. transitions are possible with $\Delta M_s = \pm 1$, of which only one ($|-\frac{1}{2}\rangle' \leftrightarrow |+\frac{1}{2}\rangle'$) is independent of δ . If the zero-field splitting is too large to be spanned by the available microwave quanta, this transition alone is observed; the system has effective spin $S' = \frac{1}{2}$, and an alternative spin Hamiltonian may be used:

$$\mathcal{H}_s = \beta \mathbf{H} \cdot \mathbf{g}' \cdot \mathbf{S}' \quad (11)$$

where $\mathbf{g}'$ is an effective g tensor. This situation prevails for all the Co(II) carbonic anhydrase-sulphonamide adducts, as only one transition is detectable even at 35 GHz. Since the spectra become more intense at

lower temperatures, we infer that $|\pm\frac{1}{2}\rangle'$ is indeed the ground state, as depicted in Fig. 7(b); if the doublets were reversed, the e.p.r. active state would become progressively depopulated with decreasing temperature.

When the effect of the magnetic field is small compared with the zero-field splitting (cf. Fig. 7b), the Zeeman term may be treated as a perturbation to $\mathcal{H}_1$. The principal components of g' and g are then related by the following expressions:

$$g'_x = 2g_x(A^2 + \sqrt{3}AB) \quad (12)$$

$$g'_y = 2g_y(A^2 - \sqrt{3}AB) \quad (13)$$

$$g'_z = g_z(A^2 - 3B^2) \quad (14)$$

The 'intrinsic' g factors are likely to have values in the range 2.1–2.4; if the co-ordinate frame is chosen so that A and B are both positive, the coefficients in eqns. (12)–(14) indicate that the experimental parameters g_1 , g_2 , g_3 correspond to g'_x , g'_y , g'_z respectively.

The g_i ($i = x, y, z$) are expected to deviate from the free-electron value $g_e = 2.00$ because spin-orbit coupling introduces an element of orbital angular momentum into the 4A_2 ground state. For high-spin Co(II) the dominant interaction is with the low-lying excited states arising from the tetrahedral 4T_2 level, and other contributions may be neglected. By first-order perturbation theory (see McGarvey, 1966):

$$g_{av.} = g_e - \frac{8\lambda'}{\Delta} \quad (15)$$

$$\text{where } g_{av.} = \frac{1}{3}(g_x^2 + g_y^2 + g_z^2) \quad (16)$$

$$D = \frac{1}{2}\lambda'[g_z - \frac{1}{2}(g_x + g_y)] \quad (17)$$

$$E = \frac{1}{4}\lambda'(g_x - g_y) \quad (18)$$

Here λ' is the effective spin-orbit coupling constant appropriate to the Co(II) complex, and Δ is the mean energy separation between 4A_2 and the excited 4T_2 states. From eqns. (17) and (18):

$$\frac{E}{D} = \frac{g_x - g_y}{2g_z - (g_x + g_y)} \quad (19)$$

The five eqns. (9), (12)–(14) and (19) may now be solved to yield values for the g_i and other parameters A^2 and E/D from a set of experimental results g'_i . This procedure was tested first on the published data for the model complexes $(C_5H_5N)_2CoCl_2$ and $(C_5H_5N)_2CoBr_2$. The calculations were carried out by computer and the outcome is summarized in Table 3(b). The A^2 values are close to unity, as required if the ground Kramers doublet consists predominantly of the $|\pm\frac{1}{2}\rangle$ spin states, and the $g_{av.}$ are typical of tetrahedral Co(II). The $g_{av.}$ may be used to obtain the effective magnetic moment $\mu_{eff.}$ from the relation:

$$\begin{aligned} \mu_{eff.} &= \beta g_{av.}[S(S+1)]^{\frac{1}{2}} \\ &= 1.94 g_{av.} \text{ Bohr magnetons} \end{aligned} \quad (20)$$

which is sufficiently accurate at ambient temperatures (see Carlin, 1965). These results (Table 3c) are in excellent agreement with the experimental data of Gill *et al.* (1961); the latter also include contributions from temperature-independent paramagnetism, which probably amount to 0.10–0.15 Bohr magnetons (see Cotton *et al.*, 1961).

To calculate the zero-field splitting parameters D and E , hence δ itself, it is necessary to know λ' [eqns. (17), (18) and (4)]. This is obtained from eqn. (15), since Δ may be estimated from absorption spectral data. Table 3(c) contains these additional results. In the chosen co-ordinate frame, D is considerably larger than E , and the axial distortion to the ligand field is somewhat greater in the bromide than the chloride. The values of δ also greatly exceed the energy of the instrumental microwave quanta (0.3cm^{-1} at 9 GHz, 1.2cm^{-1} at 35 GHz), thereby justifying the perturbation approach that produced eqns. (12)–(14).

Co(II) carbonic anhydrase-sulphonamide complexes

The e.p.r. data reported in the Experiments and Results section were treated as described above for the two model compounds, and some of the results are given in Table 4. Variations among the g_i are small and produce a narrow range of $g_{av.}$ and $\mu_{eff.}$ values. Such uniformity in magnetic moment is expected, since the Co(II) co-ordination sphere remains the same in all adducts and only the geometry is presumed to change. However, the calculated values are considerably smaller than that obtained experimentally for Co(II) bovine enzyme-sulphanilamide, though they agree well with results on other inhibitor complexes after allowing for temperature-independent paramagnetism (Lindskog, 1970). In view of this fact and the success gained with the model compounds, it seems possible that the high value determined for the sulphonamide adduct is in error. More extensive and careful susceptibility measurements are therefore necessary to provide a critical comparison for the calculations.

The examples in Table 4 represent the various enzyme-sulphonamide combinations referred to in the Experiments and Results section. Each category is associated with particular values of A^2 and E/D , which are a measure of the relative magnitude of rhombic and axial distortions to the tetrahedral ligand field. In the chosen co-ordinate frame, the ratio E/D reaches a maximum and a minimum in the respective combinations Co(II) bovine enzyme-benzocyclic sulphonamide and Co(II) human iso-enzyme B-heterocyclic sulphonamide. These two groups also exhibit the greatest and least anisotropy

Table 4. *Calculated data for Co(II) carbonic anhydrase-sulphonamide complexes*(a) Calculated g values

Co(II) enzyme	Inhibitor	A^2	E/D	g_x	g_y	g_z	$g_{av.}$
Human B	1	0.95	-0.28	2.27	2.20	2.12	2.20
	7	0.96	-0.24	2.28	2.23	2.14	2.22
Human C	1	0.94	-0.32	2.26	2.22	2.18	2.22
Bovine	1	0.93	-0.34	2.28	2.20	2.13	2.21
	7	0.95	-0.28	2.25	2.20	2.14	2.20
	9	0.96	-0.25	2.31	2.25	2.16	2.24

(b) Magnetic moment and zero-field splitting parameters

Co(II) enzyme	Inhibitor	$\mu_{eff.}$ (Bohr magnetons)*	$D^\dagger$ (cm^{-1})	$E^\dagger$ (cm^{-1})	δ (cm^{-1})
Human B	1	4.26	8	-2	18
	7	4.29	8	-2	17
Human C	1	4.30	4	-1	10
Bovine	1	4.27	7	-2	17
	7	4.26	6	-2	13
	9	4.34	8	-2	18

* Experimental $\mu_{eff.}$ values reported are 4.72 Bohr magnetons for Co(II) bovine enzyme-sulphanilamide (Lindskog & Ehrenberg, 1967), and 4.30 Bohr magnetons for Co(II) bovine enzyme-cyanate after correction for temperature-independent paramagnetism (Lindskog, 1970).

$^\dagger D$ and E are based on $\Delta = 5300\text{cm}^{-1}$ and $\lambda' = -135\text{cm}^{-1}$, as estimated from absorption spectra of Co(II) bovine enzyme-sulphanilamide (Lindskog & Ehrenberg, 1967; Lindskog, 1970).

in g' . As before we may proceed further if estimates are available for the ligand field splitting Δ . A full examination of visible and near-i.r. absorption bands has been made only for the complex of Co(II) bovine enzyme with sulphanilamide (Lindskog & Ehrenberg, 1967; Lindskog, 1970), but since the derivation of Δ and λ' is necessarily approximate, constant values have been assumed for the entries in Table 4(b). Accordingly, no precision is claimed for the calculated D , E and δ parameters, although the values for the Co(II) human isoenzyme C adduct appear significantly smaller than for other cases.

The estimated zero-field splittings in the range $10\text{--}18\text{cm}^{-1}$ are small in comparison with thermal energies at ambient temperatures, so there is little effect on magnetic properties measured under these conditions. Thus the magnetic moment reflects the high-spin state with $S = \frac{3}{2}$. At helium temperatures the majority of molecules occupy the lower Kramers doublet, but δ is still small enough to allow a substantial population of the upper e.p.r.-inactive state ($kT = 7\text{cm}^{-1}$ at 10°K). The integrated e.p.r. intensity is therefore expected to be less than the theoretical maximum, as observed in practice. When allowance was made for the Boltzmann distribution over upper and lower doublets by using the values of δ obtained herein, spectra of Co(II) human isoenzyme B with sulphonamides 2, 7 and 8, and of Co(II) bovine enzyme with 1 and 7 were found to represent 85–97% of the active enzyme originally present. The remaining discrepancies are not unreasonable in view of

the uncertainties arising from the comparison of a broad Co(II) signal with a narrow Cu(II) standard.

The anisotropy in experimental g values accommodated by the present theory is accompanied by anisotropic spin relaxation, which contributes to the line-width variation and leads to the saturation behaviour described previously. Spin excitation energy is mainly dissipated as thermal energy through spin-lattice coupling; vibrational modes of the surroundings modulate the ligand field, which interacts with the orbital motion of the electrons, and hence with electron spin by spin-orbit coupling. The latter is of crucial importance since the Co(II) ground state is an orbital singlet in purely tetrahedral symmetry. The effect of spin-orbit interaction depends on the direction of the applied magnetic field (cf. the anisotropy in g), so anisotropic spin relaxation is not unexpected. The magnetic field also influences the relaxation behaviour by mixing into the ground Kramers doublet varying elements of the upper doublet according to orientation. However, the current experiments provide no information on the precise mechanisms involved.

Discussion

Sulphonamide binding to carbonic anhydrase

The e.p.r. spectra of the sulphonamide adducts are clearly consistent with the kind of distorted tetrahedral metal-ion co-ordination revealed by X-ray crystallography. Since the zero-field splitting para-

mers are probably similar to those of $(C_5H_5N)_2CoCl_2$, the extent of the deviation from regular symmetry can be judged by reference to the bond angles of $100-110^\circ$ and 126° reported for $(C_5H_5N)_2ZnCl_2$ (Porai-Koshits *et al.*, 1960). However, examination of the sets of g' values or the calculated E/D ratios indicates that the precise geometry depends on the enzyme variant and on the gross structure of the sulphonamide.

Variations in e.p.r. parameters observed among the three carbonic anhydrases are due to compositional and conformational differences in the active-site region, which might affect the disposition of protein histidine ligands as well as the binding of an inhibitor. Such subtle differences are also well illustrated by the optical activity induced in the aromatic portion of bound sulphonamides (Coleman, 1968*a,b*). Numerous studies have shown that human isoenzyme C, a high-activity form, often resembles the high-activity bovine enzyme more than it does the low-activity human B isoenzyme. Thus the human C and bovine enzymes have a similar specificity for a series of benzocyclic sulphonamides, which differs markedly from that of human isoenzyme B [cf. the dissociation constants in Table 1 and those in Taylor *et al.* (1970*a*)]. The e.p.r. parameters (Tables 2 and 4) generally follow the same trend, although the g' values and E/D ratios for Co(II) human isoenzyme C lie intermediate between those for the human B and bovine enzymes.

The variations in ligand geometry associated with benzocyclic and heterocyclic sulphonamides imply that the aromatic portions of these inhibitors are held in distinct orientations in the active-site cleft. Other structural modifications, tested most fully in the case of substituted benzene sulphonamides bound to Co(II) bovine carbonic anhydrase, appear to exert little further effect at the metal site. Crystallographic studies on derivatives of native human isoenzyme C have indeed established that two benzocyclic sulphonamides bind in a similar fashion, whereas acetazolamide (inhibitor 7) takes up a separate position, with other contacts to the protein (Bergstén *et al.*, 1972). The e.p.r. results suggest that the actual strength of the metal-sulphonamide interaction is largely insensitive to changes in the substituents on the inhibitor or to changes in pH values. The dissociation rate constants show a corresponding invariance to such adjustments (Taylor *et al.*, 1970*a*, 1970*b*). However, the total free energy of binding as reflected in the pK_i values can vary over a wide range, and obviously depends on other factors which are structure- and pH-sensitive. These include hydrogen-bonding and van der Waals interactions between enzyme and inhibitor, displacement of ordered water molecules from the active-site cavity, and proton dissociation from the $-SO_2NH_2$ group. The pK_a difference is probably of particular significance in accounting for the more powerful

inhibition of carbonic anhydrase by acetazolamide and ethoxzolamide (8) than by the benzene sulphonamides used herein (see Lindskog & Thorslund, 1968).

The visible absorption spectra of the Co(II) enzyme-sulphonamide adducts at room temperature show the same pattern of inhibitor dependence as the e.p.r. spectra. Thus complexes of Co(II) bovine carbonic anhydrase with various benzocyclic sulphonamides have nearly identical absorption spectra (Fig. 6), but the binding of acetazolamide (see Lindskog, 1970) or the non-sulphonamide inhibitor 9 causes appreciable changes in band structure. The agreement between e.p.r. and crystallographic results has been mentioned above. It therefore seems likely that the metal-ion environment is similar in the frozen and liquid solution and crystalline phases. However, a complete correspondence of structure cannot be assumed. In the room-temperature solution, it is possible that an equilibrium exists between various forms of the enzyme-sulphonamide complex differing slightly in the arrangement of the inhibitor molecule in the active site. Evidence is available from temperature variation and temperature-jump relaxation studies of the Co(II) absorption spectrum that at least two states of the bound sulphonamide are present under these conditions (E. Grell, unpublished work). Since the distribution of molecules among these states is temperature-dependent, their population in a frozen matrix at $10^\circ K$ is certainly quite different.

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