Spectroscopic Investigation of Copper(II) Bovine Carbonic Anhydrase and its Inhibitor Derivatives

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Copper(II) bovine carbonic anhydrase and its derivatives with ligands which act as inhibitors of the native zinc(II) and of the cobalt(II) enzyme have been investigated by n.m.r. and e.s.r. spectroscopy. Measurements of T_1 of the water protons of copper enzyme solutions have shown that in the donor set there is a group containing mobile hydrogens which is not substituted by anionic donors. The derivatives with anionic ligands have been assigned a five-co-ordinate structure, whereas the sulphonamide derivatives have been assigned a pseudo-tetrahedral stereochemistry. For the copper enzyme a five-co-ordinate structure is also suggested, in which the distance between the metal atom and one of the donors is longer than normal.

REPLACEMENT of the zinc(II) ion of the native enzyme carbonic anhydrase with a cobalt(II) ion gives a compound with comparable enzymatic activity.[1,2] However, when the substituent ion is Mn^{II}, Cu^{II}, or Ni^{II} the resulting compounds are not active or show little residual activity,[3] although the metal ions are believed to occupy the same position as the native zinc ion.[4] Any attempt to relate the chemical properties of the various metalloenzymes with their enzymatic activity seems quite important. A large amount of experimental work has allowed extensive chemical characterization.[3,5-9] However, owing to the complexity of the problem, the data obtained from physicochemical measurements could not be safely related to structural information.

Copper carbonic anhydrase is known to undergo an acid–base equilibrium having a pK_a of 8.0.[5] The common inhibitors of the native enzyme bind quite strongly to the copper derivative. The electronic spectra of the copper enzyme and of its inhibitor derivatives have been reported to show a single broad absorption with a maximum at 12 000—14 000 cm^{-1}.[5] As discussed by Rosenberg *et al.*[10] for the copper carboxypeptidase, this type of spectrum could be consistent either with a pseudo-tetrahedral or a five-co-ordinate structure. Planar complexes, especially those containing nitrogen atoms in the donor set, show a broad absorption in the range 15 000—20 000 cm^{-1};[11,12] Jahn–Teller effects[12] rule out the possibility of the copper(II) complexes having an octahedral geometry. Six-co-ordinate complexes commonly have two *trans* donors at a much larger distance from the metal than the four in the plane and are hardly distinguishable from planar complexes.

When a group containing mobile hydrogens is co-ordinated to a paramagnetic metal ion and the protons exchange rapidly with the protons of the solvent water the ^1H n.m.r. parameters are affected by the paramagnetic centre and may provide a method of obtaining structural information.[13] In particular, the dipolar coupling of electron spins with nuclear spins increases both the longitudinal and the transverse nuclear relaxation rates. The dipolar effect on the longitudinal relaxation rate is given[14] by the relation (1) where τ_c

$$\frac{1}{T_1} = \frac{2}{15} \cdot \frac{S(S+1)\gamma_I^2 g^2 \beta^2}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) \quad (1)$$

[1] P. O. Nyman and S. Lindskog, *Biochim. Biophys. Acta*, 1964, **85**, 141.
[2] A. Thorslund and S. Lindskog, *European J. Biochem.*, 1967, **3**, 117.
[3] S. Lindskog and P. O. Nyman, *Biochim. Biophys. Acta*, 1964, **85**, 462.
[4] S. Lindskog, L. E. Enderson, K. K. Kannan, A. Liljas, P. O. Nyman, and B. Strandberg, *Enzymes*, 1971, **5**, 587.
[5] L. Morpurgo, G. Rotilio, A. Finazzi Agrò, and B. Mondovì, *Arch. Biochem. Biophys.*, 1975, **170**, 360.
[6] P. H. Haffner and J. E. Coleman, *J. Biol. Chem.*, 1975, **250**, 996.
[7] A. Lanir, S. Gradstajn, and G. Navon, *Biochemistry*, 1975, **14**, 242.
[8] J. E. Coleman, 'Inorganic Biochemistry,' vol. 1, ed. G. I. Eichhorn, Elsevier, London, 1973, p. 488.
[9] S. Lindskog, *Biochemistry*, 1966, **5**, 2641.
[10] R. C. Rosenberg, C. A. Root, P. K. Bernstein, and H. B. Gray, *J. Amer. Chem. Soc.*, 1975, **97**, 2092.
[11] W. E. Hatfield and R. Whyman, *Transition Metal Chem.*, 1969, **5**, 47.
[12] B. J. Hathaway and D. E. Billing, *Co-ordination Chem. Rev.*, 1969, **5**, 143.
[13] A. S. Mildvan and M. Cohn, *Adv. Enzymol.*, 1970, **33**, 1.
[14] I. Solomon, *Phys. Rev.*, 1955, **99**, 599; N. Bloembergen, *J. Chem. Phys.*, 1957, **27**, 572.

is a correlation time determined by whichever of the tumbling time or electron-relaxation time is shorter, r is the distance between the resonating nucleus and the paramagnetic centre, and the other symbols have their usual meanings.

An investigation of the relaxation of water protons in solutions containing 10^{-3} mol dm^{-3} copper bovine carbonic anhydrase has allowed a better understanding of the corresponding electronic and e.s.r. spectra and therefore of the structure of the copper enzyme.

EXPERIMENTAL

Materials.—Bovine carbonic anhydrase (carbonate dehydratase E.C. 4.2.1.1.) was obtained as lyophilized material from Sigma Chemical Co. All the reagents were of the highest commercial purity, and solutions were made up from freshly distilled water. Apocarbonic anhydrase was prepared according to the usual procedure.[15] The copper enzyme was obtained by adding unbuffered copper sulphate solutions to apocarbonic anhydrase in slightly less than the stoicheiometric amount, in order to prevent additional binding of Cu^{II} to extra sites in the protein molecule.[16] The concentration of the native enzyme, as well as of the apo- and copper(II) carbonic anhydrases, was determined from the absorbance at 280 nm, using a molar absorption coefficient of 5.7×10^4 dm^3 mol^{-1} cm^{-1}.[1] Concentration values for the copper derivative, obtained from the absorbances at 770 nm (ε 125 $\pm$ 5 dm^3 mol^{-1} cm^{-1} at pH 5.8) [15] were the same as those obtained at 280 nm within 10% error.

All the measurements were performed on several different preparations as well as on the pure isoenzyme B obtained through chromatography on DEAE cellulose.[17] The measurements on the latter isoenzyme and on the commercial mixture of isoenzymes gave the same results.

The model complex [Cu(salmhpn)] [18] [salmhpn = *NN'*-4-methyl-4-azaheptane-1,7-diylbis(salicylideneiminate)] doped into the zinc analogue was prepared as previously reported. The ligand is the Schiff base between salicylaldehyde and bis(3-aminopropyl)methylamine.

Physical Measurements.—Optical spectra were recorded on a Cary 17 spectrophotometer, in the 0—0.1 absorbance range, using 5×10^{-4}—10^{-3} mol dm^{-3} solutions of the copper enzyme. The electronic spectra of the various derivatives were recorded on samples obtained by mixing unbuffered solutions of the enzyme and inhibitor at the same pH; in each case the inhibitor was added until the electronic spectrum showed no further change.

Proton-relaxation measurements on water solutions were carried out on a Varian CFT 20 spectrometer at 15 °C. The enzyme concentration was varied from 1×10^{-3} to 3×10^{-3} mol dm^{-3}; ligands were added until the complete formation of the adduct was reached as judged from the electronic spectra. The pH was adjusted to ranges in which the ligands show a large affinity for the copper enzyme. Measurements of T_1 were performed using the inversion recovery method; T_2 values were obtained from the equation $T_2 = (\pi\Delta\nu)^{-1}$ where $\Delta\nu$ is the linewidth measured at half-peak height. Signals for T_2 measurements were obtained by the Fourier-transform method from the free induction decay without any weighting function.

E.s.r. spectra were recorded on a Varian E-9 spectrometer. The field was calibrated using crystalline diphenylpicrylhydrazyl (g 2.003 6). All the spectra, reported as the first derivative of the absorption curve, were recorded at liquid-nitrogen temperature, using solutions *ca.* 10^{-3} mol dm^{-3} in copper enzyme. The spectra of the enzyme are interpreted in terms of an axial Hamiltonian, although slight rhombic components were often observed.

RESULTS AND DISCUSSION

Proton T_1^{-1} values of water solutions containing 10^{-3} mol dm^{-3} copper enzyme are one order of magnitude larger than those obtained in the presence of the diamagnetic native enzyme (Figure 1). This enhancement is interpreted as due to the presence of a donor group having mobile hydrogens which are coupled to a paramagnetic centre.[19,20] On the other hand, the effect on T_1^{-1} of 10^{-3} mol dm^{-3} copper(II) sulphate is one order of magnitude smaller than that of the copper enzyme. This can be accounted for by recalling that typical values of the electronic relaxation time for copper(II) compounds are *ca.* 10^{-9} s.* In the case of the aqua-complex the cor-

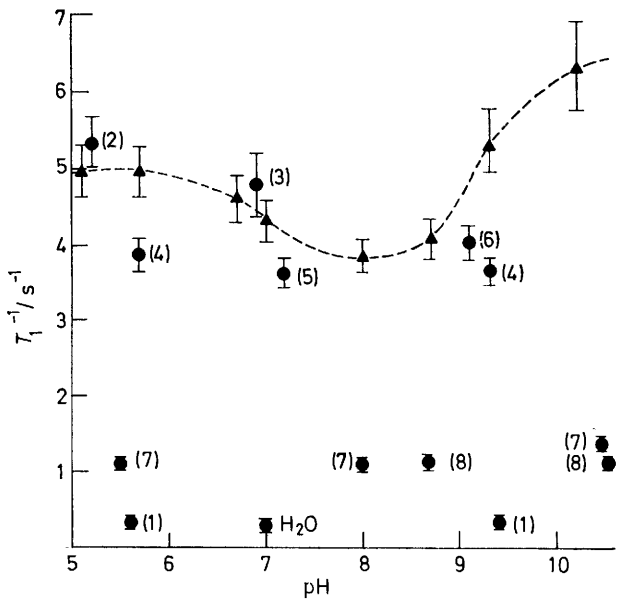

FIGURE 1 pH Dependence of T_1^{-1} values (▲) of the ^1H signal of unbuffered water solutions of 1.0×10^{-3} mol dm^{-3} copper(II) bovine carbonic anhydrase. Values for solutions containing the native zinc enzyme (1), the copper enzyme (10^{-3} mol dm^{-3}) and excess of acetate (2), iodide (3), azide (4), cyanate (5), cyanide (6), oxalate (7), and toluene-*p*-sulphonamide (8) are also reported

* From the e.s.r. linewidth a T_2 value of 2×10^{-9} s has been estimated from the relation $T_2 = (\pi\Delta\nu)^{-1}$.

[15] S. Lindskog and B. G. Malmström, *J. Biol. Chem.*, 1962, **237**, 1129.
[16] J. S. Taylor and J. E. Coleman, *J. Biol. Chem.*, 1973, **248**, 749.
[17] S. Lindskog, *Biochim. Biophys. Acta*, 1960, **39**, 218.
[18] L. Sacconi and I. Bertini, *J. Amer. Chem. Soc.*, 1966, **88**, 5180.
[19] S. H. Koenig and R. D. Brown, *Ann. New York Acad. Sci.*, 1973, **222**, 752.
[20] I. Bertini, G. Canti, C. Luchinat, and A. Scozzafava, *Inorg. Chim. Acta*, 1977, **23**, L15.
[21] P. Poupko and Z. Luz, *J. Chem. Phys.*, 1971, **57**, 3311.
[22] A. S. Mildvan and J. L. Engle, *Methods Enzymol.*, 1972, **26**, 654.

relation time τ_c is determined by the rotational correlation time (*ca.* 10^{-11} s),[21,22] whereas in the copper enzyme it is probably determined by the electronic relaxation time, the rotational time being *ca.* 10^{-8} s as estimated from the Stokes–Einstein model.[23]

Also interesting is the pH dependence of the T_1^{-1} values (Figure 1). Outside any experimental uncertainty, there is a larger copper–hydrogen interaction in the ranges pH 5—7 and 9—11. The relaxation times are almost constant between pH 7 and 9, *i.e.* in the pH range in which the enzyme goes from 90% in the acidic form to 90% in the basic form (pK_a 8.0). At higher and

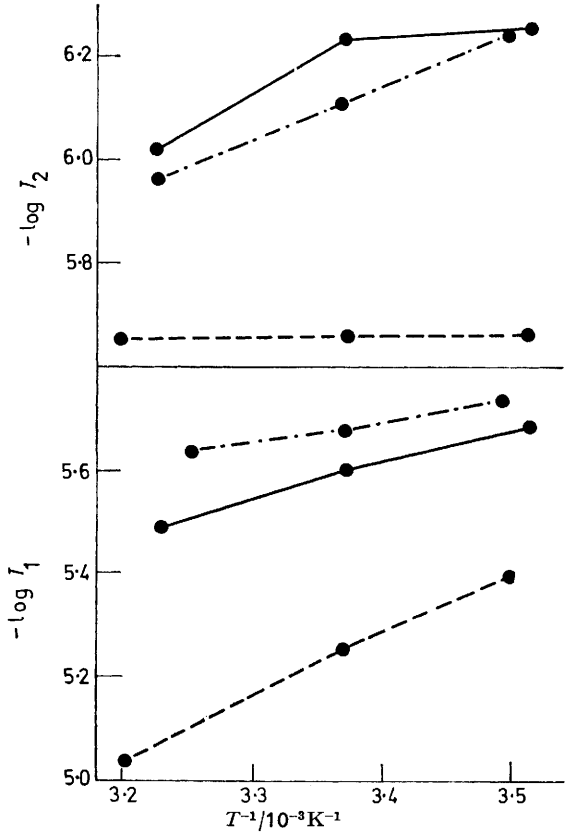

FIGURE 2 Temperature dependence of $-\log T_1$ and $-\log T_2$ of the ^1H signal for water solutions containing 9.5×10^{-4} mol dm^{-3} copper(II) bovine carbonic anhydrase alone (——), and in the presence of large excesses of azide (— · — · —) and oxalate (— — —). The pH of all the samples was 8.3

lower pH values the T_1^{-1} values increase indicating that $[OH]^-$ and H^+ ions may interact with the protein part in the proximity of the metal ion.

The temperature dependence of T_1 and T_2 has been measured for the copper enzyme and for the inhibitor derivatives (Figure 2). Both times increase with increasing temperature in an almost parallel fashion. This indicates [22,24] that the process of water exchange is fast and that T_1 and T_2 are not significantly affected by the residence time of water in the co-ordination sphere. Although such data indicate substantial dipolar contributions to the relaxation times, they do not allow a correct determination of the number of exchangeable protons. If, as is generally believed also for copper complexes,[25] the scalar contribution to T_1 is negligible, it is possible to evaluate the number of protons bound to the metal. For example, if it is assumed that the copper–proton distance is 270 pm and τ_c, as determined from the electronic relaxation times,† is 2×10^{-9} s, the calculated number of protons is 1.9 at pH 7. This value could be consistent with the presence of a water molecule bound to the metal as previously suggested.[19] Since the relaxation data are constant between pH 7 and 9 it could also be inferred that the water molecule is present over this pH interval. However, from inhibition data it is known that copper carbonic anhydrase displays an acid–base equilibrium (pK_a 8.0).[5] Therefore, either this equilibrium does not involve water or the $Cu(OH_2) \rightleftharpoons Cu(OH)$ equilibrium gives an accidentally equal overall relaxation time owing to the other interacting protons. In the former case, deprotonation might involve any group inside the active cavity or even the unco-ordinated nitrogen of a co-ordinated histidine.[26,27] The electronic and e.s.r. spectra of copper carbonic anhydrase are practically independent of pH (Table), the apparent affinity constant of inhibitors being the major pH-dependent property.

E.s.r. parameters,[a] and electronic absorption maxima, of some copper carbonic anhydrase derivatives [b]

Derivative	$g_\parallel$	$g_\perp$	$10^4 A_\parallel$/ cm^{-1}	*d–d* transition (10^3 cm^{-1}) [c]
Pure enzyme	2.31	2.06	131	13.0 (125)
	2.31	2.06	131 [d]	13.3 [d] (125)
Adducts				
Acetate	2.31	2.03	131	13.3 (125)
Benzoate	2.26	2.02	165	13.3 (125)
Oxalate	2.29	2.04	150	14.1 (125)
Aniline	2.30	2.02	131	13.4 (125)
Nitrite	2.30	2.03	134	13.7 (150)
Imidazole	2.27	2.04	164 [e]	14.0 [e] (130)
Azide	2.26	2.04	124	13.6 [f] (330)
Cyanate	2.29	2.05	128	13.6 [f] (160)
Cyanide	2.24	2.02, 2.09	122 [g]	14.5 [f] (190)
Dicyanide	2.20	2.05	190 [h]	19.4 [i] (230)
Toluene-*p*-sulphonamide		*j*		12.5 [k] (115)
[(Cu,Zn)(salmhpn)]	2.24	2.05	147	12.3

[a] E.s.r. spectra were recorded at liquid-nitrogen temperature. [b] Electronic and e.s.r. spectra were recorded at pH 5.6 unless otherwise specified. [c] Molar absorption coefficients (ε/dm^3 mol^{-1} cm^{-1}) are given in parentheses. [d] pH 9.2. [e] pH 7.0. [f] Ref. 5, pH *ca.* 6. [g] Ref. 6; the data are referred to the monocyano-adduct of the ^{65}Cu derivative of human carbonic anhydrase B at pH 8.0. [h] Ref. 6; the data are referred to ^{65}Cu bovine carbonic anhydrase at pH 8.0. [i] Ref. 6; the data are referred to human carbonic anhydrase B at 77 K and pH 8.0. [j] See spectrum in Figure 3. [k] pH 9.5.

† Note on p. 1270.

[23] A. Lanir and G. Navon, *Biochemistry*, 1971, **10**, 1024.

[24] T. J. Swift, in ' NMR of Paramagnetic Molecules,' eds. G. N. La Mar, W. DeW. Horrocks, jun., and R. H. Holm, Academic Press, New York, 1973, p. 53.

[25] W. G. Espersen and R. B. Martin, *J. Amer. Chem. Soc.*, 1976, **98**, 40.

[26] D. W. Appleton and B. Sarkar, *Proc. Nat. Acad. Sci., U.S.A.*, 1974, **71**, 1686.

[27] J. M. Pesando, *Biochemistry*, 1975, **14**, 675.

When monoanionic ligands are added to the solution containing the copper enzyme they bind to the metal with affinity constants larger than those displayed for the native or cobalt enzyme. The relaxation times, however, are only slightly affected, which can simply be attributed to variations in the relaxation parameters due to the different complexes (Figure 3). This means that

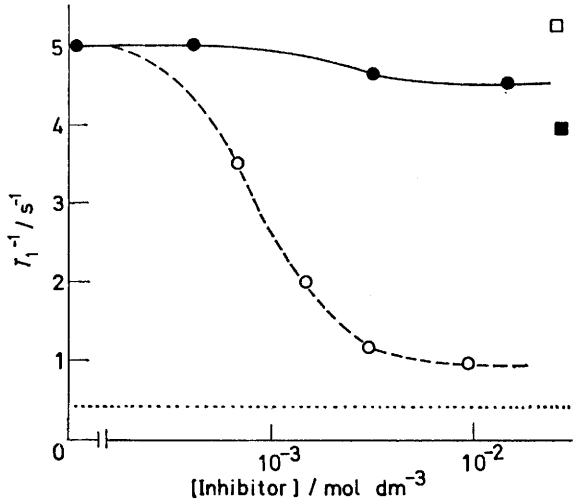

FIGURE 3 T_1^{-1} values of the ^{1}H signal of unbuffered aqueous solutions of copper(II) bovine carbonic anhydrase (1.0×10^{-3} mol dm^{-3}) containing increasing amounts of toluene-*p*-sulphonamide (○), cyanide (●) (both at pH 9.2), acetate (□) (pH 5.3), and azide (■) (pH 5.7). The baseline indicates the relaxation rate of a diamagnetic solution containing 1.0×10^{-3} mol dm^{-3} zinc bovine carbonic anhydrase

monoanionic ligands do not replace co-ordinated water, and if it is assumed that they do not even replace any of the three histidyl nitrogen donors it follows that the complexes are five-co-ordinate. The oxalate ligand is bidentate and, accordingly, abolishes almost completely the T_1^{-1} enhancement indicating that it replaces co-ordinated water. Since electronic spectroscopy shows that oxalate also competes with monoanionic inhibitors, it follows that the co-ordination number is still five and that the donor set is N_3O_2. The e.s.r. spectra (Table and Figure 4) of the oxalate adduct are quite similar to those of the adducts of the monoanionic inhibitors. The five-co-ordinate inorganic complex [Cu(salmhpn)] (chromophore CuN_3O_2), doped into the zinc analogue, gives similar e.s.r. parameters to the present copper enzyme derivatives.

Sulphonamides also bind to copper carbonic anhydrase and reduce the T_1^{-1} enhancement, indicating that they replace co-ordinated water. A contribution to the reduction in T_1 for protons would be expected since sulphonamides have at least one mobile proton bound to the nitrogen donor. However, linewidth measurements on the aromatic ring protons of all the investigated sulphonamide derivatives up to 45 °C show that the exchange between free and bound inhibitor is slow on the n.m.r. time scale, thus eliminating any contribution of these ligands to the water proton relaxation. Slow exchange of sulphonamides was previously observed also for zinc [27] and manganese [28] carbonic anhydrases. The electronic spectra show that sulphonamides also displace co-ordinated monoanionic ligands. Since sulphonamides are expected to bind through the nitrogen atom, four-co-ordination with an N_4 donor set is consistent with the present data. The e.s.r. spectra at pH 9.5 of both toluene-*p*-sulphonamide and sulphanilamide are different from those of the copper enzyme and of the adducts with monoanionic ligands, as well as from those reported for the human enzyme under the same experimental conditions.[29]

In order to check that the spectrum obtained was not due to the overlap of species, only some of which contain sulphonamides, small amounts of inhibitor were added to a solution of the pure enzyme at pH 9.6. The typical hyperfine signals appeared in the low-field region and the definitive spectrum of Figure 4 was obtained at a 1 : 1 enzyme : inhibitor ratio. The small hyperfine coupling constants in the low-field region are related to the symmetry of the co-ordination polyhedron; however, they have been tentatively related also to the co-ordination number.[30] In particular, small hyperfine coupling

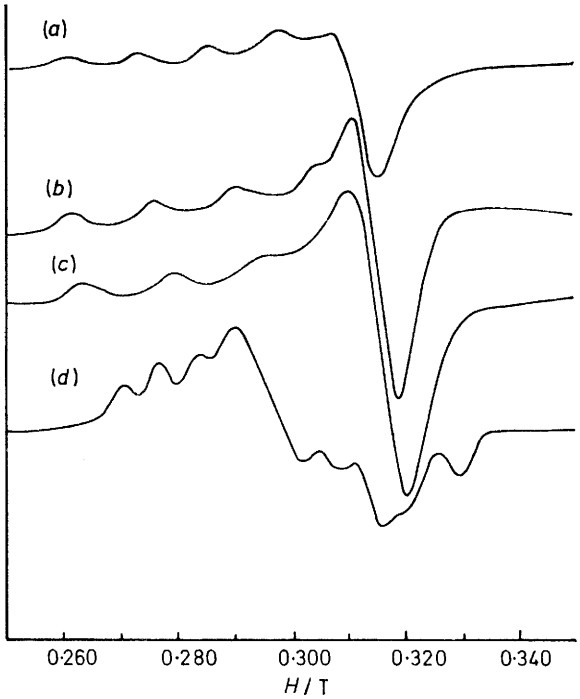

FIGURE 4 E.s.r. spectra of copper(II) bovine carbonic anhydrase at unbuffered pH 5.5 (*a*), and of its adducts with oxalate (*b*) (pH 5.5), benzoate (*c*) (pH 5.5), and toluene-*p*-sulphonamide (*d*) (pH 9.5)

constants have been observed in pseudo-tetrahedral complexes.[10] For example, the tetrahedral complex $Cs_2[CuCl_4]$ [31] doped in the analogous zinc derivative

[28] A. Lanir and G. Navon, *Biochemistry*, 1972, **11**, 3536.
[29] J. S. Taylor and J. E. Coleman, *J. Biol. Chem.*, 1971, **246**, 7058.
[30] H. Kozłowski, *Chem. Phys. Letters*, 1977, **46**, 519.
[31] M. Sharnoff, *J. Chem. Phys.*, 1964, **42**, 3383.

shows three A values (10^{-4} cm^{-1}) of 25, 51, and 46, and Cu^{2+} in $[Zn(py)_2(NCS)_2]$ [32] (py = pyridine) displays an $A_{\|}$ value of 69×10^{-4} cm^{-1}. The electronic spectra are also consistent with the present interpretation since sulphonamides cause a decrease in the energy of the absorption maximum, contrary to what is expected from the spectrochemical series if the stereochemistry is the same as for the other inhibitor adducts (Table).*

The dicyano-adduct [6] of copper(II) human isozyme C is of relevance here. Its electronic spectrum shows an absorption band at 19 500 cm^{-1} which has been suggested to be caused by an increase in co-ordination number from four to five.[6] In the context of the present results, the increase in co-ordination number might be from five to six, the absorption maximum falling in the range expected for tetragonal complexes. The e.s.r. spectra are also consistent with such a geometry, the $A_{\|}$ values being of the order of 200×10^{-4} cm^{-1}.

The copper enzyme itself could either be tetrahedral with three histidyl nitrogens and the group containing the mobile hydrogens, or five-co-ordinate with a further donor group at the fifth position. In the former case the anionic inhibitors would simply add to the enzyme, whereas in the latter they would replace the fifth donor group. The minimal sensitivity of the electronic and e.s.r. spectra to the binding of inhibitors suggests that the enzyme itself is also five-co-ordinate. The histidyl nitrogens and the group containing mobile hydrogens could be regarded as a cage resembling a distorted flattened tetrahedron, a fifth donor group being co-ordinated at a distance somewhat larger than normal. This sort of semi-co-ordination is quite common in copper complexes.[12] The inhibitor, replacing this latter group and leaving the cage unaltered, would not be expected to greatly affect the electronic properties of the complexes.

For comparison, it is recalled that the active cobalt-substituted carbonic anhydrase has been assigned a pseudo-tetrahedral geometry [33-36] with a water molecule in the donor set [19,37] and has been proposed to give five-co-ordinate derivatives in the presence of only some inhibitors.[38-41]

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* *Note added in proof.* Through discussions with Dr. K. K. Kannan of the University of Uppsala our attention was drawn to the possibility, at least for the zinc enzyme, that sulphonamides might have a second donor group located at a larger distance from the metal atom than the first donor (K. K. Kannan, I. Vaara, B. Notstrand, S. Lövgren, A. Borell, K. Fridborg, and M. Petef, in ' Proceedings Symposium Drug Action at the Molecular Level,' ed. G. C. K. Roberts, Macmillan Press, 1976, p. 73). If this were the case also for the copper derivative, then the situation would be somewhat similar to that of the oxalate; however, the peculiarity of the e.s.r. spectra supports the hypothesis of a pseudotetrahedral chromophore with large covalency factors.

[32] U. K. Voronka, M. M. Zapirov, Y. V. Yablomov, A. V. Ablov, and M. A. Ablova, *Doklady Akad. Nauk S.S.S.R.*, 1975, **220**, 623.
[33] J. E. Coleman and R. V. Coleman, *J. Biol. Chem.*, 1972, **247**, 4718.
[34] B. Holmquist, T. A. Kaden, and B. L. Vallee, *Biochemistry*, 1975, **14**, 1454.
[35] S. Lindskog and A. Ehrenberg, *J. Mol. Biol.*, 1967, **24**, 133.
[36] I. Bertini, C. Luchinat, and A. Scozzafava, *Inorg. Chim. Acta*, 1977, **22**, L23.
[37] I. Bertini, G. Canti, C. Luchinat, and A. Scozzafava, *Biochem. Biophys. Res. Comm.*, 1977, **78**, 158.
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[39] I. Bertini, C. Luchinat, and A. Scozzafava, *J. Amer. Chem. Soc.*, 1977, **99**, 581.
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