A Mechanism for the Action of Penicillamine in the Treatment of Wilson's Disease

J. PEISACH[1] AND W. E. BLUMBERG

Departments of Pharmacology and Molecular Biology, Albert Einstein College of Medicine, Yeshiva University, New York, New York 10461, and Bell Telephone Laboratories, Inc., Murray Hill, New Jersey 07974

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SUMMARY

Electron paramagnetic resonance studies were performed with nitrogen and sulfur containing chelators of Cu(II) ranging from a *vic*-dicarbonyl bis-thiosemicarbazone, through butyraldehyde thiosemicarbazone, to penicillamine and cysteine. In the former two cases, stable complexes were formed with a great deal of charge delocalization throughout the chelate ring structures as a result of pseudoaromaticity. In the latter two cases, in which ring electron delocalization is not possible, the unstable Cu(II) complexes are formed, ultimately yielding Cu(I) and oxidized chelator. This reaction, which we term "reductive chelation," is described as a probable mechanism for the mobilization of copper with penicillamine in patients with Wilson's disease.

INTRODUCTION

D-Penicillamine (β,β-dimethyl-D-cysteine) has long been used as the drug of choice in the treatment of Wilson's disease, or hepatolenticular degeneration (1), an inborn metabolic disorder often associated with abnormalities in copper metabolism (2). This disease is recognized in patients by means of typical corneal pigment rings, originally described by Kayser (3) and Fleischer (4). Its clinical features usually include hepatic degeneration and central nervous system disorders, presumably caused by copper toxicity (5). The use of chelating agents such as BAL (2,3-dimercapto-1-propanol) (6) and EDTA (7) in the treatment of Wilson's disease, although unquestionably of benefit to many patients, has proven disappointing clinically, either because of toxic side effects, as in the case of the former, or difficulty in long-term administration of the drug, as in the latter. Penicillamine therapy, however, has clearly proven to be the most effective in mobilizing toxic copper in Wilson's disease patients (5).

Although penicillamine is potentially an effective chelator for copper as well as for other transition metals, its chelative capacity alone cannot be responsible for the mobilization of toxic copper, since EDTA, which has similar or even greater affinity for transition metals, is far less effective in causing the excretion of toxic copper. Clearly, the mobilization of toxic copper by penicillamine in Wilson's disease is a little-understood phenomenon, and we wish to suggest a mechanism of action for this

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drug based on EPR and chemical studies of this compound and its analogues where copper is ligated to sulfur and nitrogen. We have studied various model compounds which are both more and less stable than the complex of Cu(II) and penicillamine. In those which are more stable, we shall show the effect of charge delocalization on molecular stability. For those that are less stable, we shall show that stability is lessened by the reductive effects of ligands.

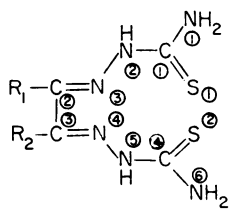
MATERIALS AND METHODS

Electron paramagnetic resonance (EPR) spectra of Cu(II) complexes were obtained at liquid helium temperatures using a spectrometer operating near 9500 Mc/sec. Samples of crystalline 3-ethoxy-2-ketobutyraldehyde bis-thiosemicarbazone and its copper derivative were sent to us by Dr. Harold G. Petering.

Butyraldehyde thiosemicarbazone was prepared by slowly dropping a 20% molar excess of butyraldehyde into a continuously stirred aqueous solution of 0.5 M thiosemicarbazide and 1.5 M sodium acetate. After the addition, the solution was warmed to 50° and cooled at 4° overnight. The crystalline thiosemicarbazone that precipitated was filtered by suction, washed twice with ice water, and dried in a vacuum desiccator. All other reagents were obtained commercially.

RESULTS

When an equimolar quantity of Cu(II) in water or other polar solvent is added to a solution of a *vic*-dicarbonyl bis-thiosemicarbazone, in this instance 3-ethoxy-2-ketobutyraldehyde bis-thiosemicarbazone (Structure I), there is instantaneous formation of a red paramagnetic copper complex and the release of 2 protons.



(I)

The EPR spectrum of this compound in frozen solution is shown in Fig. 1, curve *a*. The analysis of the superhyperfine structure of the spectrum shows the interaction of Cu(II) with ^{14}N-ligand nuclei. The X-ray crystallographic analysis of the copper compound performed by Taylor *et al.* (8, 9) indicates that copper is ligated to 2 nitrogen atoms. A careful analysis of the data of Taylor *et al.* indicates that the carbon-carbon bond (C-2—C-3) has some

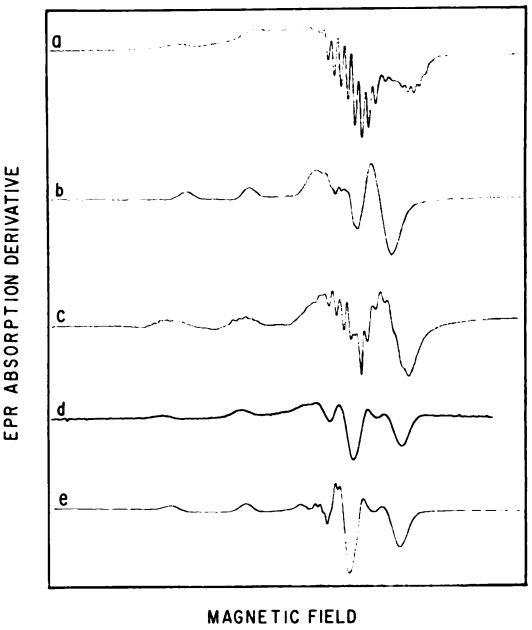
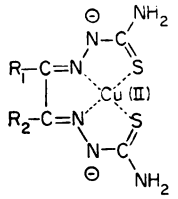


FIG. 1. *EPR spectra of compounds formed by Cu(II) and ligand molecules containing nitrogen and sulfur atoms*

All spectra were taken at approximately 1.4°K. The spectra were taken at different frequencies within the X-band range. As the sweep rates were slightly different for each of these, no common magnetic field scale is given. The spectra are arbitrarily placed so that the $g_\perp$ regions are aligned. *a*, Cu-KTS red complex: crystalline material dissolved in *N,N*-dimethylformamide glass (10^{-3} M). *b*, $Cu(BTS)_2$ green complex: 2 volumes of BTS were added to 1 volume of cupric acetate (each 10^{-3} M in *N,N*-dimethylformamide), and the sample was frozen immediately after stirring. *c*, Same as *b*, except that NaOH (final concentration, 10^{-3} M) was added, and the EPR spectrum was taken 24 hr later. *d*, Cu(II)-bis-cysteine complex, prepared as described in the text. *e*, Cu(II)-bis-penicillamine complex, prepared as described in the text.

double-bond character, since it is shorter than a single bond. Also the C-1—S-1 and C-2—N-3 bonds are longer than double bonds, while C-1—N-2 and N-2—N-3 are shorter than single bonds. Also, a comparison of the infrared spectra of KTS[2] and its Cu(II) derivative indicates that the metal complex has a carbon-carbon double bond (10). One concludes that the unpaired electron of copper has a high degree of delocalization in the three-ring aromatic system in which it is placed. This imparts a great deal of stability to the molecule. Cu-KTS then represents a pseudoaromatic structure with bond delocalization. Some of the possible resonance structures for this compound are II–V.



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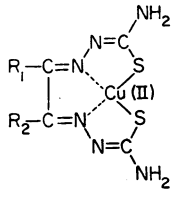

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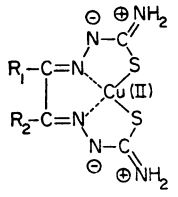

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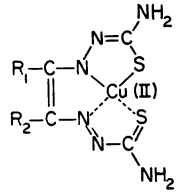
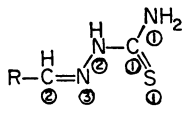


(V)

In a second case, in which the carbon-carbon bond cannot participate in chelate stability, as in the bis complex of an aldehyde thiosemicarbazone and Cu(II), the chemistry differs only slightly from the case of Cu-KTS. For example, butyraldehyde thiosemicarbazone (Structure VI) forms a

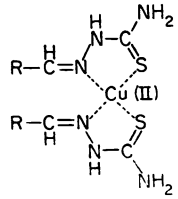


(VI)

2:1 green complex with copper, the EPR spectrum of which is shown in Fig. 1, curve *b*. If this complex were formed from S-1 and N-3, the lack of superhyperfine structure in the spectrum could readily be understood, since the *d*-electron of copper has little tendency for delocalization because the chelate ring containing the metal ion cannot conjugate double bonds until N-2 loses a proton. A similar lack of ^{14}N superhyperfine structure in the EPR spectra of Cu(II) complexes with nitrogen ligands has been reported (11).

The protons from $Cu(BTS)_2$, in contrast to Cu(KTS), are slowly dissociated; indeed, there is a change of color of the copper complex with time from green to orange and the appearance of nitrogen superhyperfine structure in the EPR spectrum (Fig. 1, curve *c*). This release of protons has been followed potentiometrically (10).

We suggest Structure VII for green



(VII)

$Cu(BTS)_2$. In this case, we believe that Cu is ligated to S-1 and N-3 rather than N-1 and N-3, since its magnetic parameters, as

TABLE 1
Magnetic parameters for Cu (II) complexes ligated to nitrogen and sulfur

Cu(II) compound	$g_\parallel$[a]	$g_\perp$[b]	$A_\parallel$	A_N
			Mc/sec	*Mc/sec*
KTS	2.14	2.02	560	33
BTS, orange form	2.14	2.02	560	44
BTS, green form	2.15	2.04	450	<25[c]
Penicillamine	2.13	2.03	570	<25[c]
Cysteine	2.13	2.03	570	<25[c]

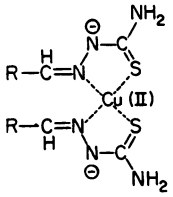
[a] $g_\parallel$ determined from $M_I = +3/2, +1/2$ components, ±0.01.

[b] $g_\perp$ determined from absorption maximum, ±0.02.

[c] Determined from line width of $M_I = +3/2, +1/2$ components of $g_\parallel$ absorption.

[2] The abbreviations used are: KTS, 3-ethoxy-2-ketobutyraldehyde bis-thiosemicarbazone; BTS, butyraldehyde thiosemicarbazone.

given in Table 1 (especially the g values), are similar to those for other complexes discussed in this paper, where copper is clearly ligated to 2 nitrogen and to 2 sulfur atoms. Of course, an ambiguity exists as to whether the ligand molecules are arranged *cis* or *trans* with respect to the copper in the planar complex. The conversion of $Cu(BTS)_2$ from its green to orange form takes place with release of protons from N-2, and these nitrogen atoms become formally negative. Possible resonance forms of this orange complex are given in Structures VIII–X. These structures are



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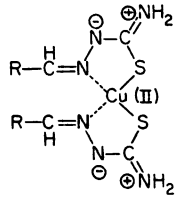

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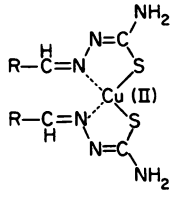
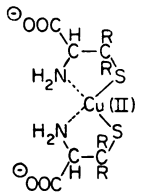


(X)

similar to those written for Cu(KTS), except that there is no possibility for a carbon-carbon double bond.

An analysis of the EPR spectrum for orange $Cu(BTS)_2$ suggests, as in the case of Cu-KTS, electronic delocalization of copper *d*-electrons within the chelate and a superhyperfine pattern attributable to nitrogen atoms. It should be noted that the magnetic parameters for Cu-KTS and $Cu(BTS)_2$ are almost identical (Table 1), and these parameters may be used as a signature for nitrogen and sulfur chelation of Cu(II). That this is so may be seen from a plot of $A_\parallel$ and $g_\parallel$ for various complexes of Cu(II) (Fig. 2). Here we have chosen Cu(II)-bis-diethyldithiocarbamate (12) as an example of 4-sulfur ligation, Cu(II)-phthalocyanine (8) as an example of a resonance-stabilized 4-nitrogen complex, and Cu(II)-bis-glycylglycylglycine at pH 11 (13) as a 4-nitrogen complex arising from a peptide chain. It can be seen that the copper complexes of KTS and BTS all lie within the range represented by these cases, indicating that Cu(II) is bound to the same ligands, namely, 2 nitrogen atoms and 2 sulfur atoms. The values for the green $Cu(BTS)_2$ complex may deviate from the others because this molecule has 2 positive charges whereas the others are neutral.

Other Cu(II) complexes containing nitrogen and sulfur ligands which have biological significance are penicillamine (R = CH_3) and cysteine (R = H) (Structure XI). Here, however, all bonds of the chelated structure are saturated, and, because of *d*-electron localization, these Cu(II)-bis complexes are unstable. It is possible to prepare these chelates, but not in stoichiometric quantities.



(XI)

When $CuSO_4$ in 6 M NH_3 is added to a solution of cysteine, there is an immediate darkening in color to intensely blue, almost black, which subsequently bleaches to a light blue in a few seconds. If a large excess of cysteine is added to the already bleached light blue solution, it quickly turns light brown, and the EPR signal of this material, shown in Fig. 1, curve *d*, is attributed to Cu(II)-bis-cysteine on the basis of magnetic parameters (Table 1 and Fig. 2).

The bis-penicillamine complex is prepared by adding a copper sulfate solution to a cold slurry of D-penicillamine in glycerol–water (4:1) raised to pH 9 with NH_3–water, and quickly freezing it to prevent reduction of copper. The EPR of this material is shown in Fig. 1, curve *e*. At low pH (near 5) only the EPR spectrum of the copper-glycerol complex is observed for this mixture. We had no success in preparing Cu(II)-bis-cysteine with this technique, as

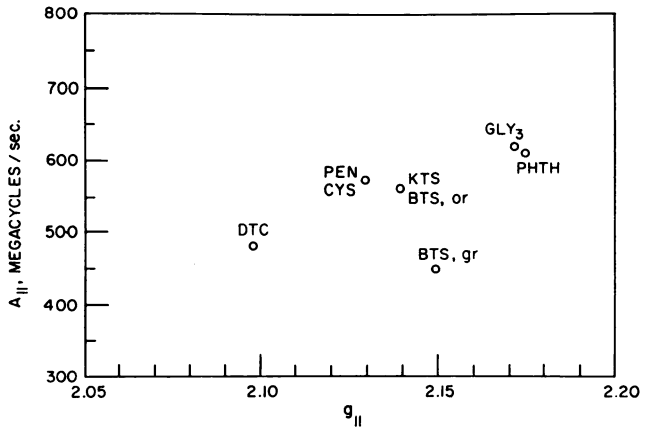


FIG. 2. *A plot of magnetic parameters in the parallel direction for sulfur- and nitrogen-ligated Cu(II) complexes*

DTC, Cu(II)-bis-diethyldithiocarbamate; GLY$_3$, Cu(II)-bis-glycylglycylglycine, pH 11; PHTH, Cu(II)-phthalocyanine. PEN, CYS, KTS, and BTS are the Cu(II) complexes of penicillamine, cysteine, KTS, and orange (or) and green (gr) BTS discussed in the text.

we invariably observed an EPR spectrum attributable to a Cu(II)-glycerol complex under these conditions. This difference was not due to any difference in solubility of the ligand molecules, as both are freely soluble in water and the concentrations *in solution* were many times in excess of the Cu(II) concentration.

As can be seen from the EPR spectra and magnetic parameters (Table 1 and Fig. 2), the bis copper complexes of penicillamine and cysteine exhibit EPR spectra not unlike those seen with Cu-KTS and Cu(BTS)$_2$, except that no ^{14}N superhyperfine structure is observed in the parallel direction or on the folding peak. [The small and uninterpretable structure in the perpendicular direction for the penicillamine complex may not arise from Cu(II)-bis-penicillamine.] The 5-membered rings in these complexes are completely saturated, and there is no tendency for the spin of the copper electron to be ring-delocalized. Both these copper complexes are virtually colorless compared to Cu-KTS and Cu(BTS)$_2$, as the copper chromophore of saturated ligand-copper complexes is very weak.

Although ligated to the same atoms as in Cu(II)-bis-penicillamine and in Cu(II)-bis-cysteine, Cu-KTS and orange Cu(BTS)$_2$ are both stable; that is, there is no tendency for reduction of Cu(II) by the sulfur ligands to which the metal is attached. The Cu(II) complexes of both penicillamine and cysteine, however, are unstable, presumably because of the transfer of electron from ligand sulfur to Cu(II) represented by the following equation.

$$\text{Cu(II)}\text{—}^{-}\text{S}\text{—R} \rightarrow \text{Cu(I)} + \cdot\text{S}\text{—R} \qquad (1)$$

As stated before, the EPR of Cu-bis-penicillamine can be observed in the presence of glycerol, while that for the bis-cysteine complex cannot be seen under the same conditions. In a glycerol–water mixture, the EPR spectrum of Cu(II)-glycerol is never observed with Cu-KTS. This suggests that the stability constant for Cu(II)-bis-penicillamine is greater than that for Cu(II)-glycerol, while the stability constant for Cu(II)-glycerol is greater than that for Cu(II)-bis-cysteine. In other words, the stability constant of Cu(II)-bis-cysteine is small, and, since the mobilization of Cu(II) bound to protein *in vivo* is based partially on chelate competition, cysteine is virtually ineffective in this operation. However, EDTA, a good Cu(II) chelator (14), is not as effective as penicillamine in mobilizing protein-bound copper in patients with Wilson's disease. Therefore, it is not the chelation capacity

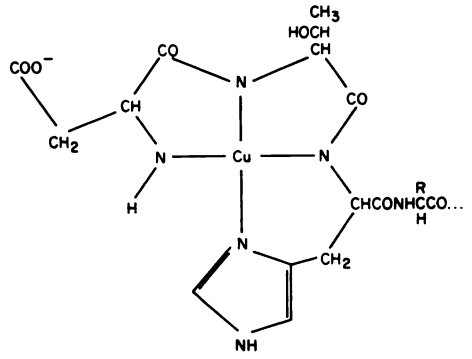
alone that makes penicillamine an effective drug.

MECHANISM

Copper does not exist as free cupric ion in physiological systems, but appears as an integral component of specific proteins. Free copper, as cupric salts, administered intravenously, is quickly carried throughout the body (5). Within a few minutes it is accumulated by the liver, whence it is either incorporated into ceruloplasmin or distributed to other parts of the body for specific incorporation into proteins such as cytochrome *c* oxidase. In the case of oral ingestion of copper, the same mechanism is operative except that liver accumulation requires about 2 hr. Liver-bound copper thus essentially represents the body pool for the free metal ion.

Copper not specifically incorporated into proteins is excreted by normal individuals. In Wilson's disease the accumulation of copper by the liver does not take place, and copper remains nonspecifically[3] bound to various organs, such as the liver, the kidneys, the various components of the central nervous system, and the eyes (5).

Copper as Cu(II), nonspecifically bound to protein, exists in a square planar configuration (13, 15). Its possible ligand atoms are nitrogen, oxygen, and sulfur. In general, polydentate metal complexes are more stable than monodentate complexes. Tissue proteins contain a large number of polydentate systems, and copper, in a square planar environment, binds to protein through them. In general, nitrogenous ligands bind more strongly than oxygenous ligands, and it seems likely that copper, for the most part, is bound to nitrogen atoms which are part of proteins. These nonspecific copper sites have been studied extensively in myoglobin (13) and in serum albumin (15). Although sulfur, as free sulfhydryl, is a potential ligand for Cu(II), its relative abundance in proteins is much lower than that of nitrogen or oxygen. Also, no multidentate copper-chelating site containing a single sulfur atom has been found in proteins. An example of a model for the nonspecific copper-binding site in serum albumin (15) is shown in Structure XII.



(XII)

As can be seen, the copper is bound to 4 nitrogen atoms, 2 derived from peptide linkages, 1 from a nitrogen atom of a histidine imidazole ring, and the last from the terminal amino group of the protein. The stability constant for the copper complex is larger than the products of the stability constants of copper bound to each of the four ligands individually. This is suggested from the data given in Table 2 for a series of nitrogenous ligands for Cu(II). As shown, polydentate nitrogen complexes bind many orders of magnitude more strongly than mono- or even bidentate

TABLE 2

Log stability constants of Cu(II) with polydentate nitrogenous ligands

The log K_1, or logarithm of the association constants, given in this table are for the reaction

$$\text{Cu(II)(H}_2\text{N—R)}_3 + \text{H}_2\text{N—R} \overset{\text{aqueous}}{\rightleftarrows} \text{Cu(II)(H}_2\text{N—R)}_4$$

By increasing the multidentate nature of a Cu(II) chelator, the first dissociation of a single nitrogen atom from the fully nitrogen-coordinated Cu(II) becomes increasingly less likely.

Ligand	Log K_1
Ammonia	4.27
Ethylenediamine	10.75
Diethylenetriamine	16.11
Triethylenetetramine	20.62

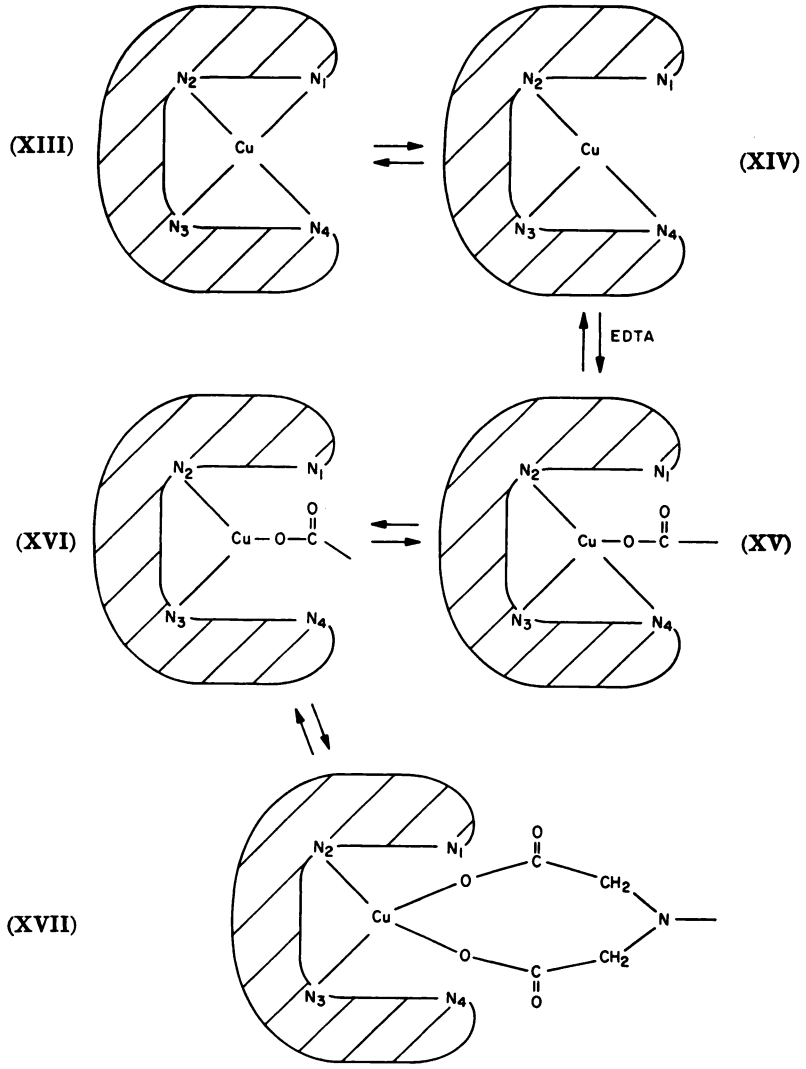
[3] By "specific binding sites" we mean those copper binding sites in which copper is known or presumed to have a physiological function.

complexes. This inherent stability of polydentate systems puts a restriction on a mechanism of ligand substitution based on the microscopic reversibility of each copper ligand bond. Consider Cu(II) bound to 4 atoms within a polydentate complex such as a protein; for the sake of illustration, 4 nitrogen atoms. At any given point in time, all 4 nitrogen atoms of the copper complex may be bound to the copper, but 1 or more of them may be released. Since all the ligands are joined through a series of covalent bonds of the protein structure (the ligand system is polydentate), the probability that a ligand atom which has released the copper will bond again to the same copper atom is nearly unity. Thus the probability that 2 ligand atoms will simultaneously release the copper is very small, and for 3 and 4 ligand atoms, even less. The great stability of tetradentate chelation is easily understood.

How, then, does a chelating agent such as EDTA remove a metal from a binding site such as the one illustrated above? To react with a metal ion already strongly covalently bonded, a reagent such as EDTA must do so through a series of reversible displacement reactions. Consider the hypothetical copper complex CuN_4 (Structure XIII). For the copper atom to be mobilized from its ligands by a chelator such as EDTA, the EDTA molecule must bind to copper at that instant when the metal ion is microscopically dissociated from one of its original ligands. This means that in order for EDTA to react, it must do so when copper is bonded to only 3 ligands (Structure XIV). Once a carboxyl group of the EDTA molecule is attached to the complex, replacing one of the original ligands (N-1) (Structure XV), the reaction can proceed via one of two pathways. The first, and the more probable, is that EDTA departs from the copper and is replaced by N-1. The other is that there is a microscopic dissociation of N-2 or N-4 (Structure XVI), at which point another carboxyl of EDTA can bind to copper (Structure XVII). The ensuing microscopic reaction proceeds as before, except that the probability of EDTA binding increases with the number of its ligands that are attached to the copper. This also means that with increased EDTA binding, there is a decreased probability that the still-ligated nitrogens will remain bound. Throughout this series of reactions, the individual affinities of each of the protein ligands (N-1, N-2, N-3, and N-4) for the copper remain unchanged. Thus, one can see that under the conditions of treatment of patients with Wilson's disease with EDTA in order to mobilize copper, the process is slow and inefficient.

Another mechanism operative for some metal ions, whereby a chelator can competitively bind to a metal ion already chelated by a different molecule, has been discussed by Margerum *et al.* (16, 17). In this mechanism, Cu(II) is penta-coordinated during the ligand exchange reaction, requiring a pseudorotation of the original ligand arrangement. In proteins, as illustrated, for example, by the Cu(II)-binding site in albumin (Structure XII), the fifth and sixth ligand positions cannot be occupied by nitrogen atoms capable of making as strong a bond as the peptide and histidine nitrogen atoms shown. Thus the stereochemistry of Cu(II) binding to the peptide chain of proteins precludes pseudorotation during the mobilization of the metal ion.

As stated before, copper mobilization with penicillamine is more effective than with EDTA. Although an excellent chelator, EDTA differs in reaction from penicillamine with nonspecifically bound copper. Clearly both chelators react with Cu(II), but only the latter can participate in an oxidation-reduction reaction with the metal ion. EPR evidence shows that penicillamine initially produces a complex with Cu(II) not dissimilar from the ones produced with KTS or with BTS, in which nitrogen and sulfur are the ligand atoms. In the case of penicillamine or cysteine, in which electrons are not delocalized within a chelate structure, electrons are transferred from the sulfur ligand to the metal ion, and a series of different products are formed. Solutions of Cu(II) with excess penicillamine, for example, turn intensely blue, almost black, but on standing ultimately become virtually colorless. These dark solutions arise



from the formation of oligomeric forms of Cu(I) and Cu(II), first described by Klotz *et al.* for cysteine (18). These compounds are intermediates between oxidized Cu(II)-bis-penicillamine and Cu(I)-bis-penicillamine and can be represented by the resonance forms in Equation 2.

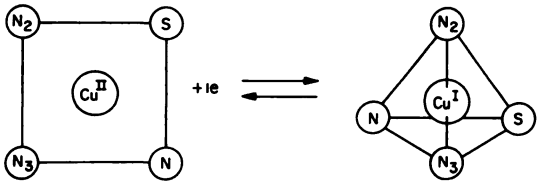
$$R—S^-—Cu(II) \rightleftarrows R—S\cdot—Cu(I) \qquad (2)$$

As written, this equation implies that a complete electron transfer from ligand sulfur to Cu(II) takes place, or that the unpaired electron in the deeply colored Klotz complex, originally written as Cu(II) [Cu(I)—RS]$_4$, can sometimes reside on sulfur and *not* on the copper to which it is linked.

This means, then, that when a molecule such as penicillamine reacts with Cu(II) bonded nonspecifically to a protein, it does so through a microscopically reversible addition of a single ligand atom, presumably nitrogen, to the metal ion. The addition of the second ligand atom, sulfur, however, is quickly accompanied by an electron transfer reaction whereby the metal ion is reduced to Cu(I) and the ligand sulfur is oxidized to a sulfur free radical. It is not clear whether the sulfur radicals formed by this process are capable of propagating free

radical reactions through hydrogen abstraction, or whether they terminate by reacting either with other sulfur radicals formed the same way or with free radicals produced physiologically. It is interesting to conjecture that temporary disorders, such as optic neuritis (19), occasionally observed after the onset of penicillamine therapy, are in fact manifestations resulting from a local production of sulfur radicals.

Once the reduction of Cu(II) to Cu(I) has taken place, the most stable stereochemical arrangement of the 4 ligands for copper changes from a square planar (Structure XVIII) to a tetrahedral (Structure XIX)



(XVIII) (XIX)

configuration; that is, the ligands are arranged at the apices of a tetrahedron with the metal ion at the center. Other configurations are possible with fewer ligands, i.e., trigonal, pyramidal, or linear, but these would require a further step in the dissociation from the protein. If one considers the condition before reduction, with copper bound to 2 protein nitrogen atoms, N-2 and N-3 (see Structure XIII), and also bound to the nitrogen and sulfur atoms of penicillamine, electron transfer to copper immediately situates the metal in an unstable square planar ligand environment, and the ligand geometry around the copper must change. If the protein to which copper is bound is viewed as a fairly rigid molecule, the arrangement of the remaining ligands, N-2 and N-3, is inappropriate for Cu(I), and their bonding to the metal is weaker. That is, it is more probable that at any single moment in time the Cu(I)—N-2 or Cu(I)—N-3 bond will be broken than the Cu(II)—N-2 or Cu(II)—N-3 bond. Copper which is reduced in place on the protein is bound less strongly than before reduction for two reasons: the stereochemical requirement of Cu(I) is different, and, since the charge is less, the ionic contribution to the bonding is smaller. This reduced copper, now less strongly bound to the protein, is more accessible for attachment by another penicillamine molecule, and in this way copper is ultimately mobilized for excretion by the body.

In this described mechanism, we purposely remain vague concerning the molecule of penicillamine initially responsible for the reduction of Cu(II). In order for this same molecule to remain ligated, it requires an electron abstraction, either from another penicillamine molecule or from a physiological reductant, or even surrounding proteins. If this reduction does not take place while the oxidized penicillamine is attached to Cu(I), it may be replaced by another penicillamine molecule by the microscopically reversible process outlined above. Whichever mechanism is operative, the over-all mechanism, which we may call "reductive chelation," remains unchanged. Thus there is a delicate balance between the ability to chelate and the ability to reduce Cu(II). Those agents which can bind strongly but do not reduce, and those which reduce but do not bind strongly, are less effective in mobilizing physiologically bound copper than is penicillamine, which can both bind and reduce.

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