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Evidence for a Ternary Complex Containing Albumin, Copper, and Penicillamine

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SUMMARY

The interaction between penicillamine and albumin-bound copper has been investigated. The presence of a ternary complex involving albumin, copper and penicillamine as an intermediate step in the course of the removal of copper has been demonstrated by gel filtration technique with [^{3}H]penicillamine and ^{64}Cu. Copper is presumed to exist in the complex in both the cupric and cuprous states. These findings suggest a mechanism for the migration of copper from the albumin–copper(II) complex to penicillamine, and explain the unique reductive chelating action of penicillamine in the treatment of Wilson's disease.

INTRODUCTION

Wilson's disease is a hereditary metabolic disorder associated with abnormal storage of copper in liver, brain, and kidneys. The pathological changes commonly found in these organs are related to the toxicity of copper, and the neurological, hepatic, and renal lesions can be reversed once a negative copper balance has been established. This effect is achieved by the oral administration of penicillamine (β,β-dimethylcysteine) (1, 2).

After oral or intravenous administration of ^{64}Cu, the radioactivity is soon present in blood cells and plasma, where copper is bound mainly to albumin and partly to amino acids. Albumin-bound copper is transported to the liver, where some copper is incorporated into serum protein and stored as ceruloplasmin before returning to the circulation. It is known that the concentration of copper is very high in the brains, livers, and kidneys of patients of Wilson's disease, whereas the concentration of ceruloplasmin is deficient, and excessive levels of albumin-bound copper are present in plasma (3–8). It has been suggested that the action of penicillamine is attributable to its ability to render plasma copper more readily available for diffusion across the glomerular membrane, with a consequent fall in plasma copper concentration and rise in the renal clearance of copper (9, 10).

Although penicillamine has been used as a therapeutic agent for Wilson's disease (11–13), little information has been obtained on the relationship between the chelating ability and the pharmacological activity of the drug. We have studied complex formation with penicillamine, with emphasis on its efficacy as a detoxicating agent against poisoning with various heavy metals, and have clarified its mode of coordination with metal ions (14). We have reported that in the reaction with copper(II) an oxidation-reduction reaction takes place in parallel with complex formation to form a red-violet copper(I,II) mixed-valence chelate and a yellow copper(I) chelate, depending upon the reaction conditions. The mixed-valence copper chelate is exceptionally stable in

penicillamine compared to various thiol-containing chelating agents, including cysteine. We have supposed that the extreme effectiveness of penicillamine in promoting the excretion of copper, when compared to other thiol-containing chelating agents, may be due to its ability to form the stable mixed-valence chelate (15). Peisach and Blumberg (16) also suggested, on the basis of an electron spin resonance study, that the activity of penicillamine is due to chelation and to oxidation-reduction reactions with copper. On the other hand, McCall *et al.* (17) have proposed that the increased excretion of copper during penicillamine treatment does not result simply from the formation of a penicillamine-copper chelate, and that the mechanism involved is considerably more complex.

In order to evaluate further the mechanism of the removal of copper with penicillamine, we have now investigated the interaction between penicillamine and albumin-bound copper, which plays an important role in the transport of copper in *vivo* and exists in abnormal excess in patients with Wilson's disease. We have used gel filtration and ultraviolet-visible absorption spectra in this study.

MATERIALS AND METHODS

Materials. DL-Penicillamine and crystalline bovine serum albumin were purchased from Sigma Chemical Company. $^{64}Cu(II)$ with a specific activity of 178 mCi/g of copper was obtained from the Japan Atomic Energy Research Institute and used as a solution of $^{64}CuCl_2$. [^{3}H]Penicillamine, prepared by the contact method with tritium gas, was supplied by Sinloihi, Ltd., and the product was recrystallized from an ethanol–water mixture until its specific activity became constant (4 mCi/g). A solution of Cu(II) prepared from reagent-grade $CuCl_2$ was standardized with EDTA. Michaelis buffer (0.033 M potassium phosphate–0.033 M sodium phosphate) was used to adjust the pH. All other reagents used were commercially available reagent-grade materials. Deionized water was used throughout.

Spectral measurements. Absorption spectra were measured with a Hitachi recording spectrophotometer, model EPS-2, and all absorption measurements were made with a Shimadzu spectrophotometer, model QV-50. The solution of penicillamine (1.0–66 mM, 1.0 ml) was added to the blue-green solution (9.0 ml) obtained by the reaction of serum albumin (0.5 mM) with Cu(II) (0.5–2.0 mM), and the time course of the spectral change was followed. The pH of the solution was measured with a Hitachi-Horiba pH meter, model F-5.

Gel filtration technique. Dry Sephadex G-25 (8 g) was allowed to swell in distilled water, packed in a column measuring 2.0 × 30 cm, and equilibrated with Michaelis buffer (pH 6.2). The column was calibrated isotopically with [^{64}Cu]penicillamine chelate and ^{64}Cu–bovine serum albumin complex. An albumin-copper complex (2.0 ml), prepared by mixing a solution of albumin (0.5–2.0 mM) with Cu(II) (2.0 mM), with or without ^{64}Cu, was added to a solution containing penicillamine (2.75 mM, 2.0 ml), with or without [^{3}H] penicillamine. The reaction mixture was placed on the column and eluted with buffer at pH 6.2. The radioactivity of each 1-ml fraction collected was determined, and the absorbance was measured at 278.5 nm to determine the concentration of serum albumin. ^{3}H radioactivity was measured with a Beckman model LS-233 liquid scintillation counter in a phosphor mixture of toluene and 2-phenylethylamine (18). ^{64}Cu radioactivity was measured with a Fujitsu well-type scintillation counter, model ATS-621.

RESULTS AND DISCUSSION

Time course of spectral changes. The addition of an approximately equimolar concentration of penicillamine to the albumin-copper complex at pH 6.2 produced the spectral changes shown in Fig. 1. Within a few seconds the spectrum revealed maximal absorbance at 420 nm, and the characteristic blue-green color of the albumin-copper complex turned to yellow-brown. The absorbance at 420 nm decreased gradually, and was replaced by an absorption maximum at 520 nm that became apparent with a color change of the solution to red-violet. In this spectral change, no isosbestic point was observed, suggesting the presence of an intermediate. The spectrum of the red-violet complex coincides exactly with that of

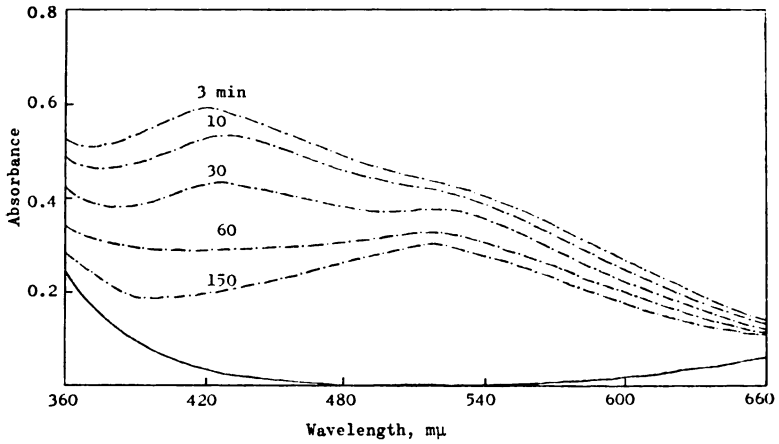

FIG. 1. *Absorption spectra at various times during migration of copper from albumin-copper complex to penicillamine at pH 6.2*

–·–·, albumin concentration, 0.5 mM; copper concentration, 2.0 mM; penicillamine concentration, 2.5 mM. ——, albumin concentration, 0.5 mM; copper concentration, 2.0 mM.

the penicillamine–copper(I,II) mixed-valence chelate previously reported (15). The red-violet color probably arose from charge transfer transitions of the oligomeric forms of copper(I) and copper(II). Blumberg and Peisach (19) indicated that the biscopper complexes of penicillamine and cysteine prepared at pH 9 are virtually colorless compared to the red copper–3-ethoxy-2-oxobutyraldehyde bisthiosemicarbazone complex, which has a pseudoaromatic structure with bond delocalization throughout. Strong absorption due to copper *d-d* electronic transitions from the simple copper complexes of the saturated ligands, such as penicillamine and cysteine, would not be expected. Therefore the spectral change may be interpreted as due to the formation of the copper(I,II) mixed-valence complex through a ternary complex involving albumin, copper, and penicillamine, with an absorption maximum at 420 nm. However, a small amount of the penicillamine–copper(I,II) mixed-valence chelate may have been present from the outset, since the peak at 520 nm was present throughout the time course of the spectral change.

Separation of ternary complex by gel filtration. The presence of a ternary complex involving albumin, copper, and penicillamine was confirmed by gel filtration. The eluents obtained from the albumin–copper (II)–penicillamine system showed distinct peaks of high and low molecular weights when absorbance was measured at 420 nm. In an attempt to confirm the presence of the ternary complex directly, [^{3}H]penicillamine and ^{64}Cu were applied to the gel filtration column. When the albumin–copper(II) complex and [^{3}H]penicillamine were mixed, the activity due to [^{3}H]penicillamine appeared in the high molecular weight fraction, as shown in Fig. 2, and the activity based on [^{3}H]penicillamine in the albumin fraction decreased with time. In the system containing albumin and [^{3}H]penicillamine without copper(II), the activity in the fraction of albumin was negligible.

Similarly, the pattern of radioactivity in the system consisting of albumin, copper, and penicillamine was compared with those obtained with the albumin-^{64}Cu and penicillamine-^{64}Cu systems. It is clear that the low molecular weight copper chelate and the albumin-bound copper are resolved from each other by this technique (Fig. 3). The chromatography on Sephadex was carried out 2 min after mixing the reactants in the experiments shown in Figs. 2 and 3. When chromatography was carried out 20, 60, and 120 min after mixing the reactants in the system containing the albumin-^{64}Cu complex and penicillamine, the activity based on ^{64}Cu with the large molecular weight fraction

moved to the low molecular weight fraction with time. However, with the system containing albumin-^{64}Cu without penicillamine, the activity of ^{64}Cu was retained in the albumin fraction.

These results demonstrate the existence of a ternary complex of albumin, copper, and penicillamine. This ternary complex may be regarded as an intermediate in the removal of copper from the albumin-copper

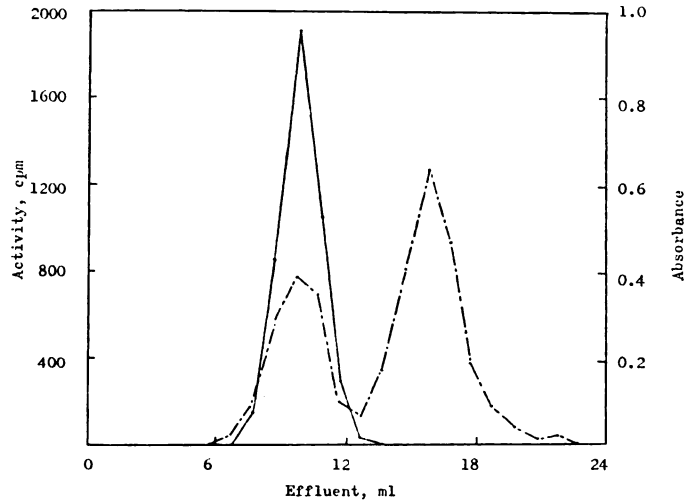

FIG. 2. *Elution pattern of radioactivity of [^{3}H]penicillamine mixed with albumin-bound copper* (-·-·) *on Sephadex G-25*

The elution pattern of albumin (——) was measured spectrophotometrically at 278.5 mM. The albumin concentration was 0.5 mM; copper concentration, 2.0 mM; penicillamine concentration, 2.75 mM.

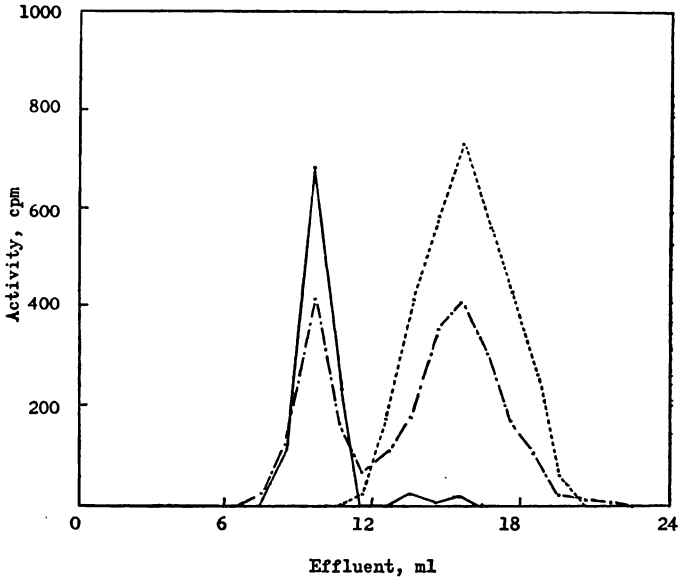

FIG. 3. *Elution pattern of radioactivity of penicillamine mixed with albumin-bound ^{64}Cu* (-·-·) on *Sephadex G-25*

The elution patterns of albumin-^{64}Cu (——) and penicillamine-^{64}Cu (-----) are superimposed for comparison. The concentrations of reactants were the same as those for Fig. 2.

complex by penicillamine. It may be assumed that the copper in the ternary complex acts as a bridge between albumin and penicillamine, because the elution pattern of radioactivity from Sephadex with [^{3}H] penicillamine was quite similar to that obtained with ^{64}Cu with the albumin-copper-penicillamine system. Klotz and several other investigators (20–22) have demonstrated that metal ions may mediate the binding of small molecules to proteins. The hypothesis of a bridge structure involving enzyme, metal, and substrate has been proposed to explain the mechanism of action of many enzymes. Recently Mildvan *et al.* (23, 24) have directly investigated the mode of interaction between enzyme-bound metal and substrate and postulated a bridge structure by the use of proton and fluorine magnetic resonance techniques. Ternary complexes of this type have also been proposed for serum albumin–copper(II)–histidine (25), transferrin–iron(III)–citrate (26), and hemoglobin–mercury(II)–cysteine systems (27) in the course of studies on the transport mechanisms for copper, iron, and mercury, respectively. Therefore ternary complexes may play important roles, not only in the action of enzymes, but also in the transport of metal ions *in vivo*.

Proposed reaction mechanism. A proposal for the reaction mechanism for the migration

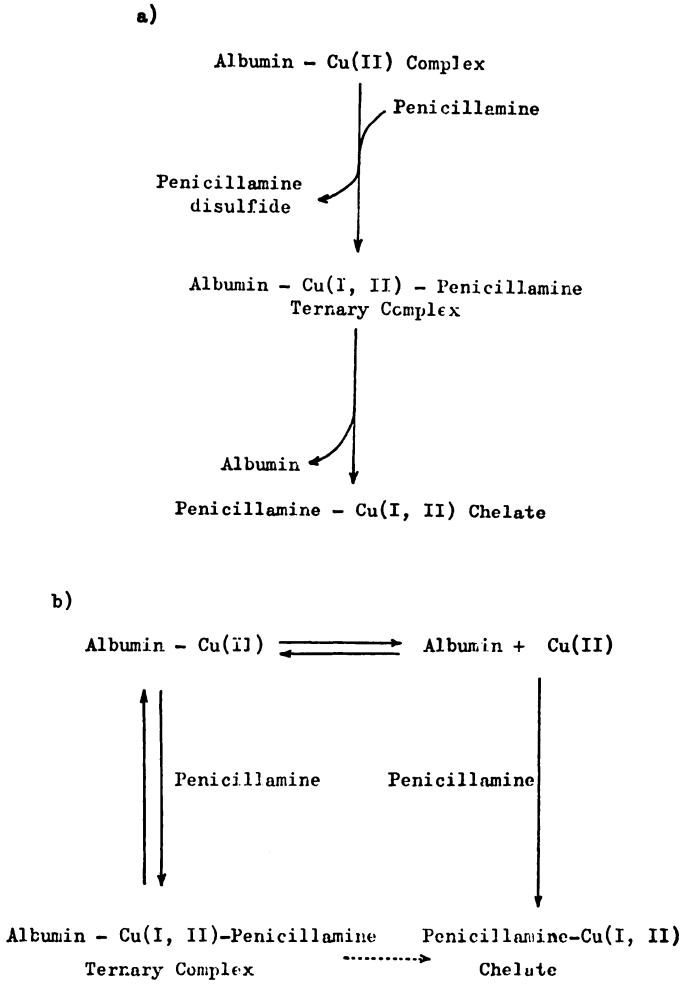

FIG. 4. *Reaction scheme for migration of copper from albumin-copper complex to penicillamine*

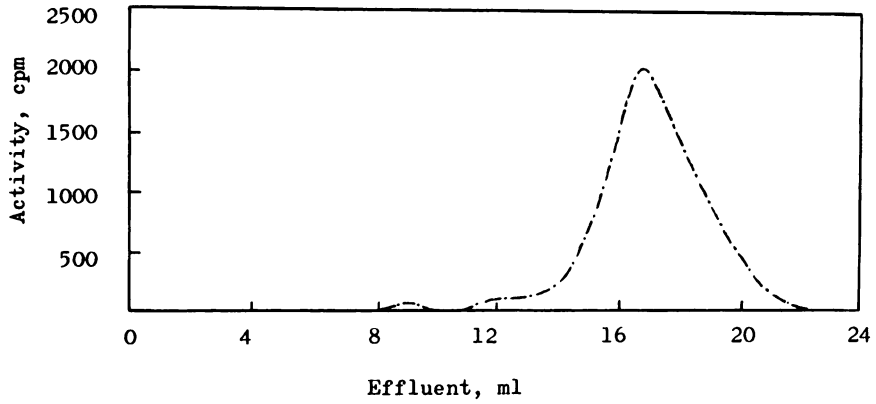

FIG. 5. *Elution pattern of radioactivity of a large excess of penicillamine mixed with albumin-bound $^{64}Cu(II)$ on Sephadex G-25*

The albumin concentration was 0.5 mM; copper concentration, 2.0 mM; penicillamine concentration, 66 mM.

of copper from the albumin–copper(II) complex to the penicillamine chelate, based on the results presented here, is shown in Fig. 4a. Penicillamine rapidly reacts with the albumin-copper complex to form the ternary complex as an intermediate. In this stage of the reaction, oxidation-reduction is assumed to occur on the basis of the spectral change along with the complexing between copper (II) and penicillamine. High molar extinction at 420 nm may be evidence for the formation of the mixed-valence copper complex, as was previously reported (28–30). The existence of univalent copper in the reaction mixture at this stage was indicated by the formation of a pink complex upon the addition of cuproin, a specific reagent for copper(I). Copper then dissociates from this intermediate mixed-valence ternary complex to form the mixed-valence penicillamine-copper chelate. The dissociation reaction of the ternary complex is assumed to be the rate-limiting step in the over-all process, similar to the iron(III) exchange reaction between a chelating agent and transferrin (26). However, the possibility of an alternative pathway (shown in Fig. 4b) cannot be excluded, since penicillamine can combine with copper directly to form the binary copper-penicillamine complex. On the other hand, when a large excess of penicillamine was added to the albumin–copper(II) complex, a yellow complex was immediately formed and its spectrum agreed with that of the penicillamine–copper(I) chelate reported previously (15). Formation of the ternary complex was not detected. Gel filtration of the system containing penicillamine and copper(II) in a molar ratio of 33:1 indicates that a large portion of copper bound to albumin moves to the penicillamine fraction (Fig. 5).

The results presented here support the proposal (15, 16) that the action of penicillamine in the treatment of Wilson's disease may be attributed to its unique reductive-chelating ability with copper(II). In these experiments, however, the concentration of copper was very high compared to those *in vivo*. In addition, formation of the mixed-valence intermediate may be enhanced under the experimental conditions of this study. The problem of whether the ternary complex involving albumin, copper, and penicillamine is an obligatory intermediate in the removal of copper from the albumin-copper complex cannot be solved by the results presented here. Nor is it clear whether the penicillamine-copper chelate is the final form in which the copper is excreted. Precise kinetic studies are needed to solve these problems and to determine the contribution to the transport mechanism of the copper complexes demonstrated in this investigation.

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