[Contribution from the Chemical Laboratory of Northwestern University]

A Mixed-valence Copper Complex with Thiol Compounds

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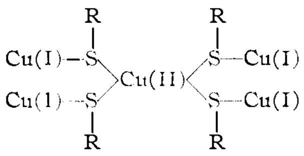
Solutions of copper and certain thiols, in the proper mole ratios, have been observed to give intense violet colors. Polarographic and optical titrations have been carried out with thiomalic acid, RSH and copper, and the results obtained point to a complex, I, containing copper in two valence states, as the substance with the violet color.

Introduction

Among the oxygen-carrying proteins, one, hemocyanin, contains copper, instead of iron, at its active site. The available evidence suggests that the copper is attached to a sulfur ligand from the protein.[1,2] It seemed of interest, therefore, to see if a strongly-absorbing, oxygen-binding complex could be produced from copper ions and simple mercaptans. Experiments with a variety of thiols have uncovered some complexes with an intense absorption band in the visible, but no evidence could be obtained that molecular oxygen participated in these complexes. Polarographic and spectrophotometric methods were used, nevertheless, to establish the stoichiometric relationships in these interactions, and the observations accumulated point to the formula I for these complexes.



I

Experimental

Colorimetric Titrations.—Deaerated solutions of copper(II) chloride and mercaptan were mixed in specially designed two-, or three-, legged tubes, of which Fig. 1 shows a typical example. Each leg had a small sidearm and inlet capillary so that nitrogen could be bubbled through each solution before mixing. The mouths of the main legs were usually placed at a small angle to facilitate filling. In Fig. 1, the mouth of the right leg was placed in direct line so that a Beckman probe glass electrode could be inserted readily. The legs of the tubes in Fig. 1 were of a size which fitted into a Hellige-Diller Photoelectric Colorimeter. All colorimetric readings were made with a 520 mμ filter. Analogous tubes also were made with a one-centimeter square absorption cell at the end for use with the Beckman spectrophotometer. Still other tubes were made to fit a Klett colorimeter.

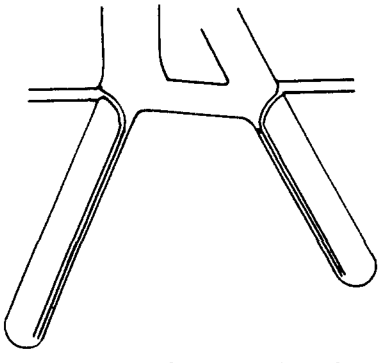


Fig. 1.—Two-legged tube for deaeration of solutions and subsequent mixing.

For the measurements reported here, all reagents were dissolved in a 0.2 *M* acetate buffer, *p*H 6. Temperatures were 25 ± 2°.

Polarography.—Current-voltage curves were determined with a Sargent recording Polarograph Model XXI. All potentials were measured against the saturated calomel electrode. A stream of nitrogen was passed through a bubbler with water and then through the test cell for about 30 minutes. All measurements were made with an atmosphere of nitrogen over the test solution, at a temperature of 25.0 ± 0.2°. Gelatin, at a concentration of 0.0075% or less, was used as a maximum suppressor. The capillary characteristics for the results shown in Fig. 3 were: $m = 2.14$ mg./sec.; $m^{2/3}t^{1/6} = 2.11$ mg.$^{2/3}$ sec.$^{-1/2}$.

All measurements reported are for mercaptan and copper in a 0.2 *M* acetate buffer near *p*H 6. All *p*H's were measured with the Beckman Model G *p*H meter.

Reagents.—Thiomalic, or mercaptosuccinic acid (II), from a commercial source, was extracted from aqueous solution several times with ether. The purified crystalline compound melted at 153–154°; lit.[3,4] 148°, 149–150°. Analyses by amperometric silver titration led to assays of 97–100.4% purity for various samples.

Redistilled thioglycolic acid in aqueous solution was obtained from Toni Laboratories, the purity being listed as 64.7%. An iodine titration gave a purity of 64.3%. β-Mercaptopropionic acid was also a gift of Toni Laboratories. β-Mercaptoethylamine was obtained from Evans Chemetics and Edcan Laboratories, and cysteine hydrochloride was purchased from Nutritional Biochemical Co. The last three mercaptans were used without further purification.

α,β-Dimercaptosuccinic acid was prepared by Dr. J. Y. C. Ho; m.p. 190–191° (dec.); lit.[5] 191–192°.

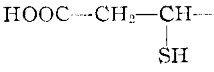
$CuCl_2 \cdot 2H_2O$ and the components of the acetate buffers were reagent grade materials.

The nitrogen used in the colorimetric experiments was a "prepurified" grade of the Matheson Co. with an oxygen content warranted to be no greater than 0.001%. In the polarographic experiments, Linde "high purity dry" nitrogen, was used and was guaranteed to contain less than 0.01% oxygen.

N,N,N′,N′-(β-Aminoethyl)-ethylenediamine was a research sample of high purity given to us by Dr. W. Gauss of the Leverkusen Works of I. G. Farbenindustrie. 8-Quinolinol-5-sulfonic acid was a commercial sample.

Results and Discussion

Optical Titrations.—A very strong violet color is obtained when Cu(II) is mixed with any one of several thiols, the intensity depending on the metal: mercaptan ratio. The most detailed studies were carried out with thiomalic acid (II). Rough



$$\underset{\displaystyle SH}{HOOC—CH_2—CH—COOH}$$

II

experiments[6] indicated that the violet color appeared at a Cu(II)/thiomalate ratio near 0.5. To

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[1] I. M. Klotz, J. M. Urquhart and H. A. Fiess, This Journal, **74**, 553 (1952).

[2] I. M. Klotz and T. A. Klotz, *Science*, **121**, 477 (1955).

[3] E. Biilmann, *Ann.*, **339**, 351 (1905).

[4] B. Holmberg, *Arkiv Kemi, Mineral Geol.*, **6**, 1 (1916).

[5] L. N. Owen and M. U. S. Sultanbawa, *J. Chem. Soc.*, 3109 (1949).

[6] We are indebted to Mrs. Jean Urquhart Dunham, to Miss Janet Ayers and to Dr. J. Y. C. Ho for this preliminary work.

establish the stoichiometry more precisely, extensive measurements were carried out anaerobically in the two-legged (and three-legged) mixing tubes shown in Fig. 1.

A typical set of results is assembled in Fig. 2. Each point represents the absorption at 520 mμ about 1 hr. after deaerated solutions of copper(II) ion and mercaptan had been mixed. In every solution there was ultimately some decrease of color with time. The reading used for each point in Fig. 2 is the plateau maximum in a graph of optical density *versus* time. As the figure implies, there is no (persistent) color at Cu(II)/RSH ratios below 0.5. Slightly above this ratio a persistent color develops and the intensity rises very sharply until the metal/thiol ratio reaches about 0.63, at which point it levels off.

Similar experiments were carried out in unbuffered solutions in which the thiomalic acid and copper(II) produced a *p*H near 3.2. A curve similar to Fig. 2 was obtained but was much less sharply defined. The initial rise in absorption occurred slightly above 0.4, and the plateau above 0.6 in metal/thiol ratio was reached more gradually. In general also, the reaction proceeded more slowly than in acetate buffer. The addition of 0.2 *M* potassium nitrate to the unbuffered solutions produced no difference in behavior, so that it seems evident that it is not the ionic strength, but the *p*H, which produces the sharper curve in acetate.

The course of the optical titration curve of Fig. 2 can be accounted for in terms of the following reactions in which RSH represents the thiomalic acid. Upon initial addition of copper(II) ion to thiol[7]

$$\mathrm{RSH + Cu(II) = {}^1\!/_2RSSR + Cu(I) + H^+} \quad (1)$$

$$\mathrm{Cu(I) + RSH = RS{-}Cu(I) + H^+} \quad (2)$$

so that the net result is

$$\mathrm{2RSH + Cu(II) = RS{-}Cu(I) + {}^1\!/_2RSSR + H^+} \quad (3)$$

where RSSR represents α,α'-dithiomalic acid. On this basis the absence of any color below a ratio of 0.5 for Cu(II)/RSH is reasonable since only univalent copper is present, and that in the RS-Cu(I) complex, which we have shown (see below) is colorless. Since color develops when the total Cu(II) added exceeds the mole ratio 0.5 and reaches a maximum intensity near 0.625, we may write[8]

$$\mathrm{4RS{-}Cu(I) + Cu(II) = Cu(II)[RS{-}Cu(I)]_4} \quad (4)$$

to account for the stoichiometry of the optical titration.

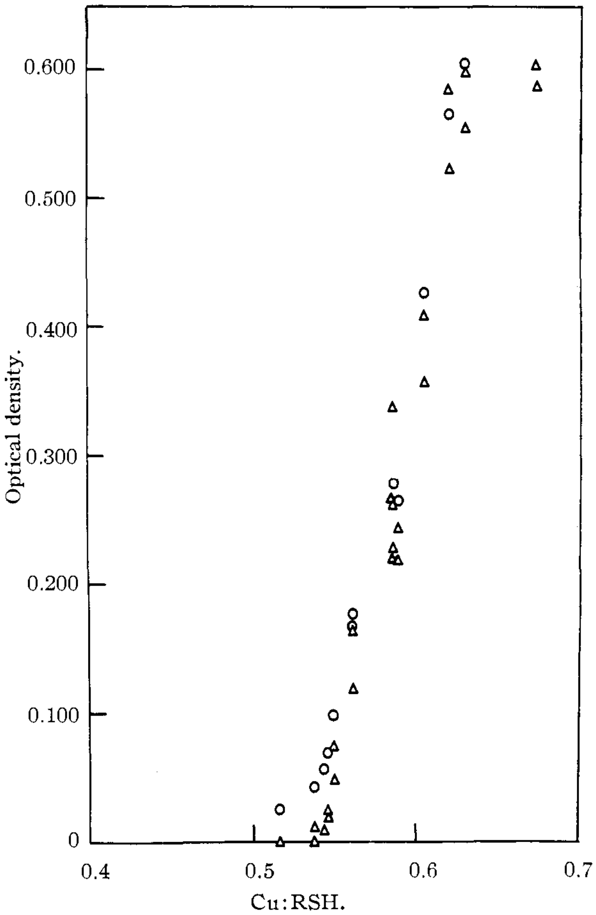

Fig. 2.—Changes in color intensity when Cu(II) is added in increasing amounts to an 0.0005 *M* thiomalic acid solution. All reagents in a 0.2 *M* acetate buffer, *p*H 5.9, 26°. Solutions deaerated with a stream of nitrogen before they were mixed. Hellige–Diller colorimeter used with filter set at 520 mμ.

Polarographic Experiments.—In view of the presence of readily reducible and oxidizable species, it seemed likely that the scheme of equations 1–4 could be tested readily by polarographic analysis[7] of solutions (under nitrogen) with various copper(II) ion/mercaptan ratios. At low ratios, two waves were observed, an anodic one (A) with a half-wave potential of -0.30 volt (*vs.* saturated calomel electrode) and a second, cathodic wave (B) at $E_{1/2}$ of -0.53 volt. With increasing quantities of added copper(II) ion, wave A decreased in height, wave B increased (see Fig. 3). At a Cu(II)/RSH ratio slightly over 0.5 wave A disappeared. At a ratio near 0.625, a third wave (C), with $E_{1/2}$ near zero and clearly due to free Cu^{++} ion, appeared, and its diffusion current continued to increase with further additions of copper.

The anodic wave, A, was shown readily to be due to free mercaptosuccinic acid by polarography of a buffer solution containing only this mercaptan. Wave B was suspected to be due to a 1:1 complex

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[7] Reactions corresponding to (1) and (2) have been observed also with cysteine as mercaptan; see I. M. Kolthoff and W. Stricks, THIS JOURNAL, **73**, 1728 (1951).

[8] In view of the presence of RSSR in the solution, as a product of equation 1, it is conceivable that it is a constituent of the colored complex too. However, this possibility was ruled out in a separate semi-quantitative experiment under nitrogen in a three-legged tube. One leg contained Cu(I), prepared either by reduction with glucose in 1 *M* NH_3 or by reaction of metallic copper and copper(II) ion in hydrochloric acid, the second contained thiomalic acid and the third copper(II) ion. The contents of the legs were mixed in the order listed: 1 plus 2 gave merely a yellow solution; the addition of 3, however, produced the characteristic violet color. No RSSR is produced when the Cu(I) in leg 1 is mixed with the RSH of leg 2, and hence the disulfide produced in equation 1 cannot be a participant in the violet complex.

A separate experiment (under nitrogen) also was carried out in which 0.01 *M* solutions of Cu(I), thiomalic acid and α,α'-dithiomalic acid were mixed. Again only a light yellow color was obtained. Similar results were obtained in 0.001 *M* solutions. It is clear, therefore, that the violet-colored complex is not a mixed complex of Cu(I) with RSH and RSSR.

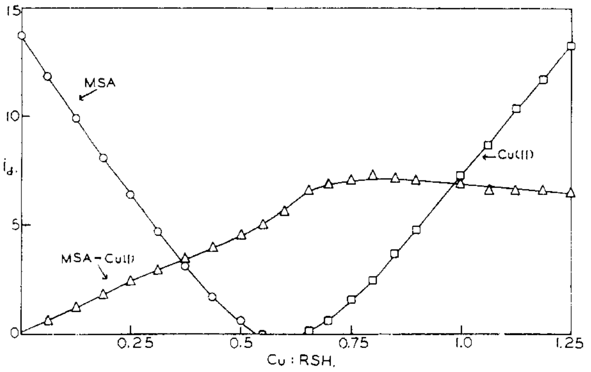

Fig. 3.—Polarographic titration under nitrogen of 0.004 *M* thiomalic acid with Cu(II) in 0.2 *M* acetate buffer, *p*H 5.5 and 25°. MSA represents the free mercapto succinic (thiomalic) acid and MSA-Cu(I) its copper(I) complex.

between copper(I) ion and mercaptosuccinic acid, that is the RS–Cu(I) formed in equation 2, since it appeared only after some copper was added to the solution but before any color (other than pale yellow) was evident. This interpretation was confirmed in a separate polarographic reduction of a solution containing only Cu(I), prepared by reduction with glucose of cupric copper in 1 *M* NH_3, to which mercaptosuccinic acid was added; a yellow solution was obtained with a wave at −0.53 volt. Wave C was confirmed as due to free Cu(II) by separate experiments with only dipositive copper ion in the acetate buffer solution, in which $E_{1/2}$ was at −0.02 volt.

Thus the polarographic observations, summarized in Fig. 3, fit essentially quantitatively the reaction steps outlined in equations 1–4. As required by equation 1, mercaptan decreases in concentration as copper(II) ion is added. In parallel fashion, RS–Cu(I) increases in concentration, as described by equation 2. If these reactions were the only reactions, the RSH wave should disappear completely at a copper(II)/mercaptan ratio of exactly 0.50. Actually the diffusion current of this wave drops to zero at a slightly higher ratio, 0.52–0.54. This discrepancy may be due to a small amount of reaction (5). Such a reaction has been shown to occur when cysteine is the mercaptan.[7]

$$\text{RS—Cu(I)} + \text{Cu(II)} = {}^1/_2\text{RSSR} + 2\text{Cu(I)} \quad (5)$$

With further addition of copper beyond the ratio of 0.52–0.54 reaction 4 begins to contribute on a large scale to the removal of Cu(II). Consequently free Cu(II) is not detectable[9] in Fig. 3 in the range of 0.53 to about 0.65. Above the latter ratio free Cu(II) becomes readily evident and increases rapidly upon further additions of copper. Such behavior would be expected from reaction 4 since it can approach completion when the moles of additional Cu(II) equal one-fourth of the moles of RS–Cu(I) present at the completion of reactions 1 and 2.

The diffusion current of the wave at −0.53 volt behaves in a simple linear fashion for total copper to mercaptan ratios up to 0.52–0.54, as would be expected for increasing quantities of RS–Cu(I). Above this ratio, i_d tends to rise at a somewhat faster rate[10] but levels off quickly as the ratio exceeds 0.63. It seems likely that the increased slope is due to the reduction of the $Cu^{II}[RS\text{–}Cu^{I}]_4$ being formed, for when the plateau is reached, i_d is greater than the value observed at a copper/mercaptan ratio of 0.5, where the concentration of free RS–Cu(I) is at its maximum. After the plateau has been attained, a slight drop in i_d is observed if further copper is added. Slow drops in i_d are also found merely as a function of time, even without more copper. Simultaneously, the violet color of the solution decreases slowly in intensity. It seems likely that these changes are due to reaction 5, occurring at a slow rate. For our purposes, however, this is a side-reaction, and hence it was not pursued further.

Detection of Cu(II) in Complex.—The optical titration and polarographic data thus both point to the mixed-valence complex, I, formed by reaction 4, as the substance with the intense violet color. In this complex, 20% of the copper is in the +2 state. It seemed of interest, therefore, to see if cupric copper could be removed by a competing strong chelating agent, under anaerobic conditions so that none of the copper(I) ion could be oxidized.

Several chelating agents were tried but "penten," N,N,N′,N′-tetrakis-(β-aminoethyl)-ethylenediamine proved most convenient. A three-legged mixing tube (similar to Fig. 1) was filled with 0.00625 *M* Cu(II), 0.0100 *M* thiomalic acid and 0.0100 *M* penten, respectively, in each leg. Each substance was dissolved in 0.2 *M* acetate buffer. After deaeration, copper and mercapto-acid were mixed, giving the usual deep violet color. After 0.5 hr. penten was added. The violet color disappeared slowly and was replaced by the familiar blue of copper(II) complexes. After 1 hr. no further change in absorbancy occurred. The optical density at 660 mμ was taken and compared with a calibration curve of known Cu(II) concentrations. The Cu(II) extracted from the violet complex corresponded to 19% of the total copper present.

As a check, 8-quinolinol-5-sulfonic acid was used to capture the Cu(II). With initial concentrations of 2.5×10^{-4} *M* copper, 4.0×10^{-4} *M* thiomalic acid and 3.7×10^{-4} *M* quinolinol, all in acetate (*p*H 5.94), approximately 23% of the copper in the violet complex was in the +2 form. Readings with quinolinol were taken at 390 mμ hence are less reliable because RS–Cu(I) also absorbs slightly at this wave length.

Spectrum of the Complex.—The spectrum of $Cu(II)[RS\text{-}Cu(I)]_4$, where RS corresponds to thiomalic acid, is shown in Fig. 4. The molecular extinction coefficients given have been computed in terms of total moles of copper present; in terms of moles of compound I, the ordinate readings would be five times higher. In either presentation the observed intensities are 20–100 times greater than those of common copper(II) complexes. The copper(I) complex of thiomalic acid, as well as of other thiols, shows no absorption peaks in the

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[9] Experiments carried out in the absence of a maximum suppressor showed very small amounts of free Cu(II) in this range; *i*d however remained practically constant until the added copper exceeded 0.65.

[10] This rise in slope is accentuated in polarograms carried out in the absence of a maximum suppressor.

visible, and only a slight yellow color due to the tail of the ultraviolet absorption. The presence of the metal in two valence states in the same molecule thus produces an enormous intensification in absorption, presumably due to a charge transfer mechanism. Similar behavior has been observed in Cu(II)–Cl–Cu(I) complexes but to a much lesser degree.[11,12] Evidently the presence of a sulfide between the Cu(II) and Cu(I) in (I) facilitates the electron transfer greatly.

Comparison of Mercaptans.—Mixing of Cu(II) with several other thiols also produced a violet-colored complex, but not every mercaptan worked. Thioglycolic acid, β-mercaptoethylamine and α,β-dimercaptosuccinic acid formed violet solutions, β-mercaptopropionic acid and cysteine did not. With thioglycolic acid, spectra and polarographic measurements also were recorded. The color intensity was weaker in this case, and the peak at 520 mμ not nearly as strong as in Fig. 4. Correspondingly in the polarographic titration, i_d for the RSH wave followed a course very similar to that for thiomalic acid shown in Fig. 3. However, in contrast to Fig. 3, the Cu(II) wave appeared immediately after the disappearance of the RSH one. Clearly Cu(II)[RS–Cu(I)]$_4$ is a much weaker complex, *i.e.*, its dissociation is much greater, when RS is thioglycolic acid instead of thiomalic. Likewise the complex with β-mercaptoethylamine must also be much weaker for its absorption peak, although at 520 mμ, was much lower than that for thiomalic acid. Thus it is clear that copper forms mixed valence complexes with a variety of thiols and that these complexes are characterized by much more intense absorption spectra than are customarily found in cupric or cuprous complexes.

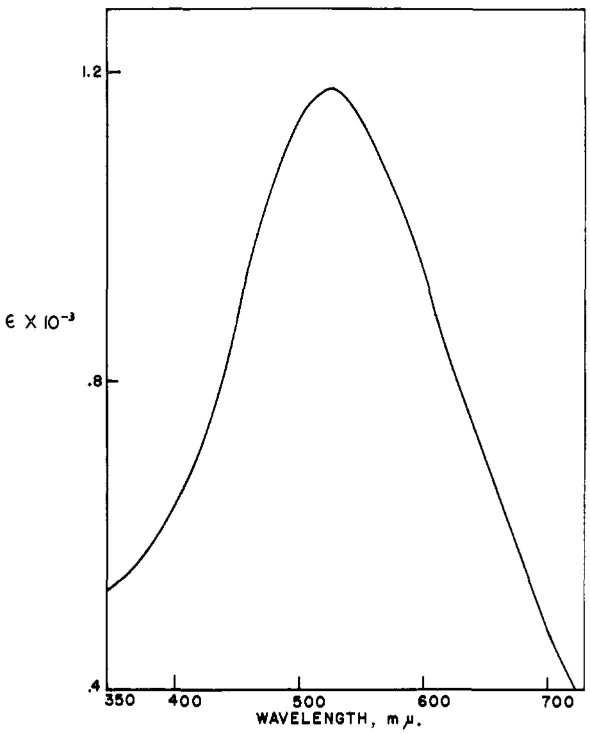

Fig. 4.—Absorption spectrum of the violet complex, Cu(II)[Cu(I)SR]$_4$: Cu(II) total concentration, 0.0010 *M;* thiomalic acid, 0.0016 *M*. In acetate buffer, under nitrogen, at *p*H 5.8, 25°.

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(11) H. Diehl, P. A. Carlson, D. Christian, E. H. Dewel, M. R. Emerson, F. K. Heumann and H. W. Standage, *Proc. Iowa Acad. Sci.*, **55**, 241 (1948).

(12) H. M. McConnell and N. Davidson, This Journal, **72**, 3168 (1950).

[Contribution from Department of Chemistry, University of Oregon]

Osmotic Pressure, Protein Solutions and Active Transport. II[1]

By Terrell L. Hill

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This paper discusses some of the statistical thermodynamic relations between two solutions separated by a membrane with properties such that two species, though present on both sides of the membrane, are not in equilibrium across the membrane while the remaining species are in equilibrium. This situation might arise, for example, in so-called "active transport" across biological membranes (the two non-equilibrium species in this case might be two small ions or one small ion and one protein molecule). The osmotic pressure and activity coefficient ratios are given particular attention. A special case is treated by a modification of the method of Donnan and also by the McMillan–Mayer method.

I. Introduction

In an earlier paper[2] we used the McMillan–Mayer solution theory to discuss the pressure difference across a membrane when one species of ion, though present on both sides, has different electrochemical potentials on the two sides of the membrane, either (1) because the species cannot pass through the membrane or (2) because the membrane does work (active transport) to maintain the electrochemical potential difference. Each solution was assumed to be in an equilibrium state though the solutions are not in complete equilibrium with each other. Since the discussion concerned statistical thermodynamic properties of the solutions only, we could ignore the (possibly non-equilibrium) processes occurring in the membrane itself.

In the present paper we extend the theory to the case of two ionic species with different electrochemical potentials on the two sides. Systems of this type are undoubtedly very important in active

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(1) This investigation was supported by a research grant from the Heart Institute, Public Health Service.

(2) T. L. Hill, This Journal, **78**, 4281 (1956). Hereafter denoted by I.