Colorimetric measurement of cuprous ion formation to detect certain reducing agents as illustrated with cysteine, glutathione, and serum albumin

Recently[1,2] we reported on the reaction of certain reducing agents with cupric ion in the presence of specific cuprous ion complexing reagents (*e.g.*, Cuproin, Neocuproine). This reaction has now been adapted for the quantitative determination of specific reducing agents of biological origin and is illustrated here with cysteine and glutathione. These sulfhydryl compounds are one of the principal groups of substances which undergo oxidation as follows:

$$Cu^{II} + RSH \longrightarrow Cu^{I} + RS\cdot + H^{+}$$

$$\downarrow \text{ Neocuproine [2,9-dimethyl-1,10-phenanthroline]}$$

$$Cu^{I}\text{-Neocuproine complex (Yellow)}$$

Considerable discussion on the determination of sulfhydryl groups, particularily in amino acids and proteins, has appeared in reviews[3,4] and numerous papers of which those of HATA[5], GRUNERT AND PHILLIPS[6], BOYER[7], BENESCH AND BENESCH[8], and FRIDOVICH AND HANDLER[9] may be cited as examples. While the above reaction is not necessarily specific for sulfhydryl groups, cysteine is the only amino acid found in simple proteins which appears to reduce cupric ion under the very mild reaction conditions. It was therefore thought to be of interest to explore this reaction as a possible convenient method for the determination of the sulfhydryl amino acids and the proteins containing them.

The application of this technique to the quantitative determination of cysteine and glutathione is shown in Fig. 1. The cuprous–Neocuproine complex has a maximum absorption around 455 mμ and follows the Beer-Lambert Law at concentrations in the $10^{-4}M$ range[10]. The color appears to be independent of pH over the 5–9 range and of the nature of the buffer ion. It is stable for at least 15 min. However, as shown in Fig. 2, a Neocuproine to cuprous ratio of 5:1 was required for maximum color formation under these experimental conditions. This ratio is high in view of the reported 2:1 ratio in the Neocuproine–cuprous complex[10].

When cysteine and glutathione are made the limiting reducing agents, the data of Fig. 1 are obtained. This graph represents standard curves for the determination of cysteine and glutathione in the absence of other reducing agents. The stoichiometry of the reaction appears to be

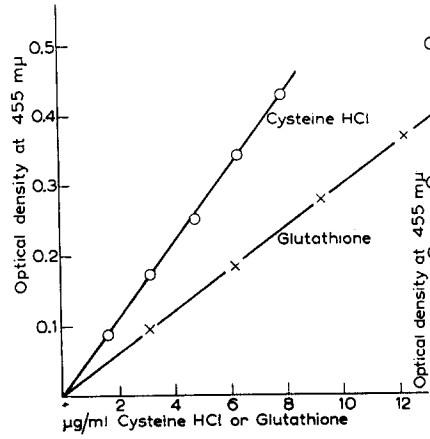

Fig. 1. Determination of cysteine and glutathione by the cupric ion reduction method. Each 4.0 ml of reaction mixture contained $5.0 \cdot 10^{-5}M$ cupric chloride, $2.5 \cdot 10^{-5}M$ Neocuproine (G. Frederick Smith Chem. Co., Columbus, Ohio), 0.01 M phosphate buffer, pH 7.0, and the indicated amount of cysteine or glutathione. The optical density at 455 mμ was measured in a Beckman model B spectrophotometer at 25° C.

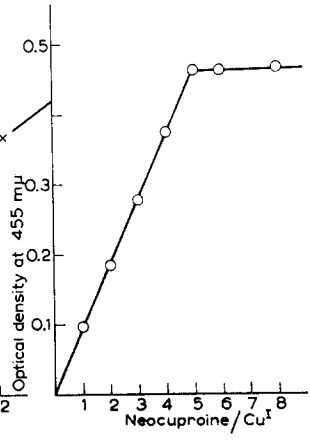

Fig. 2. Ratio of Neocuproine to cuprous ion for maximum color production by the cuprous-Neocuproine complex. Conditions were the same as in Fig. 1, except the Neocuproine concentration was varied from $5.0 \cdot 10^{-5}M$ to $4.0 \cdot 10^{-4}M$. The cuprous ion was generated by the addition of a slight excess of solid ascorbic acid.

one cupric ion for every sulfhydryl group. A recovery of 97–98 % of cysteine (HCl) was obtained. This procedure permits the estimation of 2–8 μg/ml cysteine (HCl) to better than the nearest 0.2 μg/ml. The sensitivity of the method with respect to glutathione is about one-half as great.

It must be emphasized that the method, as described above, is limited to analyses in the absence of such other reducing groups as enediols, polyphenols, hydroxylamines, and certain substituted polyphenols such as thyroxin[2]. Adaptations of the procedure are possible which would permit the determination of sulfhydryl groups in the presence of other reducing substances by measuring the reaction before and after the addition of an organic mercurial reagent such as *p*-chloromercuribenzoate (PCMB)[7–9]. PCMB will eliminate the reducing ability of the sulfhydryl groups and thus allow the estimation of other reducing groups. At $10^{-4}M$ PCMB did not alter the color intensity of the cuprous–Neocuproine complex but did reduce the stability of the color so that readings within 1 min were necessary. It has also been observed that slight modifications of the above procedure can be used for the determination of micro amounts of ascorbic acid, thyroxin, and certain other similar compounds[11].

A spray of a mixture of cupric ion and Neocuproine may also be used to detect cysteine and other reducing agents on filter paper. The reagents should be about ten times more concentrated than used above and it is preferable to mix them immediately before used to prevent extraneous color formation. The spots should be marked as considerable background develops with time.

Evidence has also been obtained for the reduction of cupric ion by proteins such as serum albumin and β-lactoglobulin. These reactions were slower and a temperature of 40° was used to obtain 90 % completion in about 3 h. It was also necessary to increase the cupric ion concentration 5-fold to overcome the competition for cupric ion by the protein. However, the reaction was considerable —0.68 mg/ml serum albumin (Armour bovine) produced an optical density at 455 mμ of 0.25 in 200 min. But only an approximate proportionality between the amount of serum albumin added and the amount of cupric ion reduction was obtained under these conditions. While this reaction shows promise for the quantitative determination of sulfhydryl groups in proteins, it remains to be rigorously proved that this is the only group in proteins capable of reducing cupric ions.

Finally, it should also be noted that the reduction of cupric ion by proteins may have considerable bearing on estimates of the valence state of copper ion in biologically active copper proteins such as hemocyanin[12] and polyphenoloxidase[13]. The release of copper ion from these proteins in the presence of a cuprous ion reagent may be accompanied by the partial or complete reduction of cupric ion to cuprous ion by the protein moiety*. This will result in an erroneous estimate of the valence state of copper ion in the original protein. We have reported earlier[14] that Neocuproine inhibits polyphenoloxidase reversibly and uncompetitively.

I thank Professor K. LINDERSTRØM-LANG and his colleagues at the Carlsberg Laboratorium for their advice and generous hospitality.

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Received October 22nd, 1957

* Similar precautions might be relevant to the evaluation of the valence state of certain other metal ions in proteins, such as iron, cobalt, etc.

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