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THE EFFECT OF COPPER ON HUMAN ERYTHROCYTE GLUTATHIONE REDUCTASE

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ABSTRACT

1. The influence of copper on purified human erythrocyte glutathione reductase (E.C. 1.6.4.2) was studied. The holoenzyme was inhibited at low oxidized glutathione (GSSG) concentrations. At a glutathione concentration of 1 mM and higher no inhibition at all was found. The inhibition was independent of the concentration of NADPH.

2. When the holoenzyme was preincubated with copper much more inhibition was found. This inhibition could be prevented by adding oxidized glutathione to the incubation mixture.

3. The recombination of the apoenzyme with FAD was inhibited by copper. This inhibition by copper was competitive with respect to FAD.

Glutathione reductase (NAD(P)H: oxidized glutathione oxidoreductase, E.C. 1.6.4.2) catalyzes the reaction

$$GSSG + NADPH + H^+ \leftrightharpoons 2GSH + NADP^+.$$

Glutathione reductase is a flavoprotein and contains FAD as prosthetic group (Icén, 1967; Staal, Visser & Veeger, 1969).

Raised levels of free serum copper and erythrocyte copper cause hemolytic anaemia, which is seen in Wilson's disease (Deiss, Lee & Cartwright, 1970) and in rare cases of copper poisoning by renal hemodialysis equipment (Manzler & Schreiner, 1970), following ingestion of copper salts or topical application of copper sulphate solutions to denuded skin. Increased copper levels are also found in patients with various haematological disorders (Boulard, Blume & Beutler, 1972). For this reason the influence of copper on several enzymes from the red cell has been studied. Inhibition at nearly physiological levels of copper has been reported for the enzymes hexokinase, phosphofructokinase, phosphoglyceric kinase, 6-phosphogluconate dehydrogenase (Boulard *et al.*, 1972) and pyruvate kinase (Boulard *et al.*, 1972; Willms, Blume & Löhr, 1972). Other investigators reported the inhibition by copper for glucose-6-phosphate dehydrogenase (Fairbanks, 1967) and glutathione reductase (Deiss *et al.*, 1970).

Willms *et al.* (1972) and Boulard *et al.* (1972), however, did not observe any influence of copper on glutathione reductase. Metz & Sagone (1972) reported a decreased level of red cell reduced glutathione, not caused by enzyme inhibition but by catalytic auto-oxidation and oxidative stress on the red cell.

In view of these contradictory reports we studied the effect of copper on purified glutathione reductase isolated from human red cells. We also studied the effect of copper on the recombination of the glutathione reductase apoenzyme with FAD.

MATERIALS AND METHODS

Chemicals

NADPH, FAD and GSSG were obtained from Boehringer and Soehne. DEAE-Sephadex A-50, capacity $3{\cdot}5 \pm 0{\cdot}5$ meq./g., partical size 40–120 μm. and CM-Sephadex C-50, capacity $4{\cdot}5 \pm 0{\cdot}5$ meq./g., partical size 40–120 μm. were purchased from Pharmacia, Uppsala. Human erythrocytes were obtained from healthy donors from the Netherlands Red Cross Blood Transfusion Service. In the copper experiments $CuSO_4$ was used.

Determination of Activity

The assays were carried out, at 25° C, (with a Zeiss spectrophotometer PMQII in combination with a Vitatron UR 401 recorder) by following the change in extinction at 340 nm. due to the oxidation of NADPH with GSSG as acceptor (Staal *et al.*, 1969). NADPH-GSSG reductase activity was determined in 0·2 M Tris-HCl buffer pH 6·8 in a final volume of 2·5 ml. containing 1 mM GSSG, 120 μM NADPH and enzyme, unless stated otherwise. A unit of activity is defined as the amount of enzyme required to oxidize 1 μmole of NADPH per minute under the above conditions. The specific activity is defined as units/mg. protein. The protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline human serum albumin as standard.

Purification of the Enzyme

The purification procedure was carried out according to Staal *et al.* (1969), except that the last three steps were omitted and that the amount of DEAE-Sephadex suspension (step 2 and step 4) was doubled.

Preparation of the Apoenzyme

The apoenzyme was prepared according to Warburg & Christian (1938). The enzyme solution was 55% saturated with $(NH_4)_2SO_4$ and was brought to pH 3·1 with 1 N HCl. After standing for 10 minutes in an ice bath, the apoenzyme was collected by centrifugation and was dissolved in 0·2 M Tris buffer, pH 6·8. This solution had only 8% of its original activity (rest-activity). In all recombination experiments of apoenzyme with FAD, the measured activities were corrected for the rest-activity.

RESULTS

Purification of the Enzyme

Starting from 300 ml. of erythrocytes with a specific activity in the haemolysate of $5{\cdot}2 \times 10^{-3}$ units/mg. protein, an enzyme solution was obtained with a specific activity of 44 units/mg. protein. This means that the enzyme preparation was purified 8500 times.

Influence of Copper on the Holoenzyme

Fig. 1 shows the influence of copper on the holoenzyme at three different GSSG concentrations. The inhibition of the enzyme by copper ions apparently depends on the GSSG

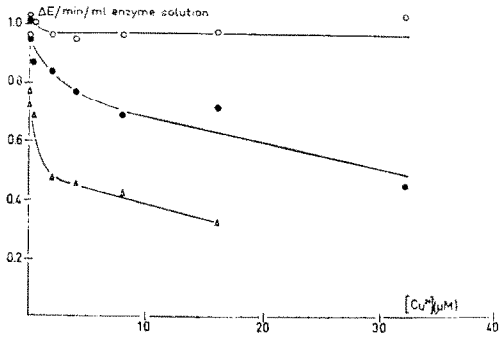

Fig. 1.—The influence of increasing Cu^{2+} concentration on glutathione reductase at different GSSG concentrations. ○, 1 mM GSSG; ●, 0·2 mM GSSG; △, 0·1 mM GSSG. The reaction was started by adding enzyme solution to the cuvette. For further conditions see Methods.

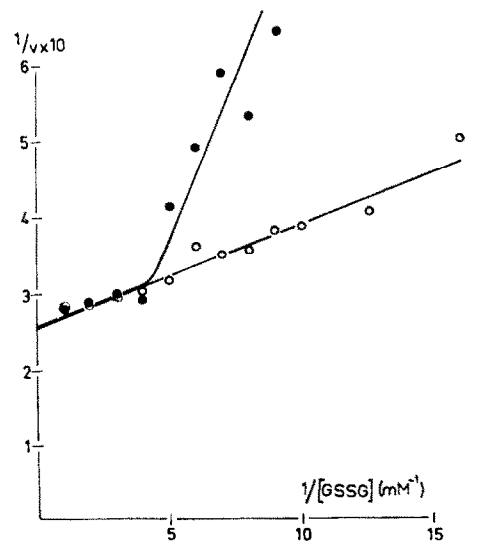

Fig. 2.—$1/v$ against 1/[GSSG] plot with (●) and without (○) copper. $[Cu^{2+}] = 2 \times 10^{-6}$ M. The reaction was started by adding enzyme solution to the cuvette. v is expressed as ΔE/minute/ml. enzyme solution. For further conditions see Methods.

concentration. Increasing the copper concentrations up to $3{\cdot}2 \times 10^{-5}$ M no inhibition could be observed at all at a GSSG concentration of 1 mM. However, at lower GSSG concentrations an inhibition by copper was found.

FIG. 2 is obtained by plotting $1/v$ against 1/[GSSG]; again it can be observed that the inhibition by copper started at a certain GSSG concentration. But it is quite difficult to conclude which mechanism of inhibition is involved.

FIG. 3 shows the $1/v$ against 1/[NADPH]

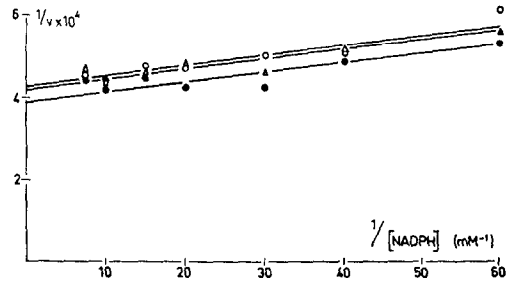

FIG. 3.—$1/v$ against 1/[NADPH] plot at two different Cu^{2+} concentrations. ○, without copper; ●, 10^{-6} M Cu^{2+}; △, $2{\cdot}5 \times 10^{-6}$ M Cu^{2+}. The reaction was started by adding enzyme solution to the cuvette. v is expressed as ΔE/minute/ml. enzyme solution. For further conditions see Methods.

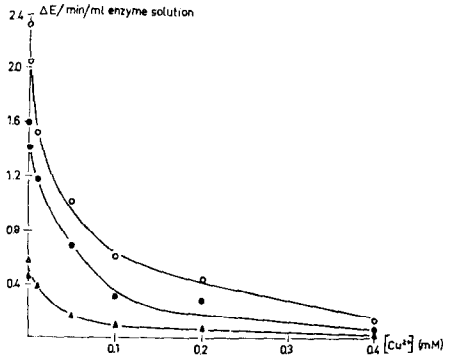

FIG. 4.—The influence of increasing Cu^{2+} concentration on the recombination of apoenzyme with FAD at three different concentrations. ○, 10^{-5} M FAD; ●, 2×10^{-6} M FAD; △, 5×10^{-7} M FAD. [GSSG] = 1 mM. The concentrations of Cu^{2+} and FAD in the preincubation mixture are as indicated. The reaction was started by adding 0·1 ml. preincubation mixture to the cuvette. For further conditions see Methods.

plot with and without copper. From this plot it can be concluded that the copper inhibition is not influenced by the NADPH concentration. This is in contrast with reported observations (Fairbanks, 1967).

Influence of Copper on the Recombination of Apoenzyme with FAD

FIG. 4 shows the effect of copper on the recombination of the apoenzyme with three different FAD concentrations after 10 minutes' preincubation. It is clear that the inhibition of the recombination depends on the FAD concentration. From the $1/v$ against 1/[FAD] plot with and without copper (FIG. 5) it can be concluded that copper is a competitive inhibitor towards FAD.

Preincubation of Holoenzyme with Copper

In the recombination experiments we measured the enzyme activity after 10 minutes preincubation of apoenzyme with FAD (with and without copper). Therefore, we also investigated the influence of copper on the holoenzyme by preincubating holoenzyme with copper. The results are given in FIG. 6. From these experiments it can be concluded that there is also an effect

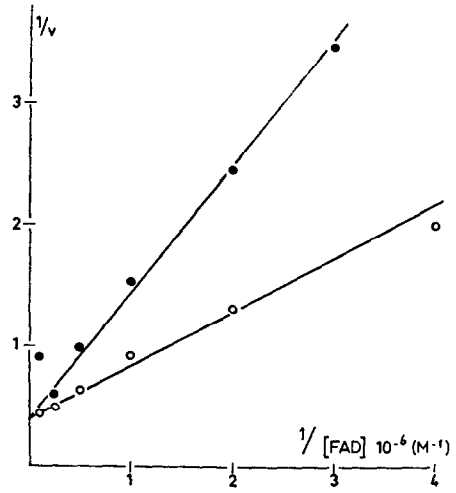

FIG. 5.—$1/v$ against 1/[FAD] plot with (●) and without (○) Cu^{2+}. $[Cu^{2+}] = 10^{-5}$ M; [GSSG] = 1 mM. v is expressed as ΔE/minute/ml. enzyme solution. For further conditions see FIG. 4.

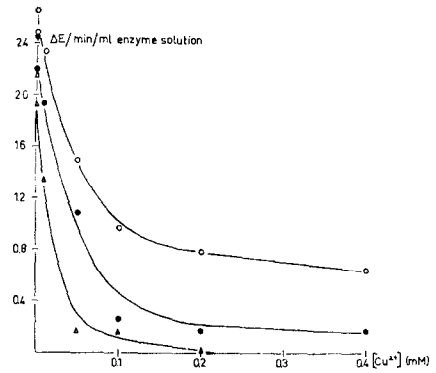

FIG. 6.—Influence of increasing Cu^{2+} concentration on holoenzyme preincubated with the indicated Cu^{2+} concentration at three different concentrations of GSSG. ○, 1·0 mM GSSG; ●, 0·2 mM GSSG; △, 0·1 mM GSSG. For further conditions see FIG. 4.

of copper on the holoenzyme during preincubation. However, when in the preincubation mixture besides holoenzyme and copper GSSG is added in a concentration of 1 mM, it was found that the inhibition by copper was prevented (not shown). This means that the inhibitory effects of copper both on the recombination of apoenzyme with FAD and on the holoenzyme are dependent on the concentration of GSSG.

DISCUSSION

The results described in this paper clearly demonstrate that the influence of Cu^{2+} on glutathione reductase is strongly dependent on the GSSG concentration. This may explain the reason why Boulard *et al.* (1972) and Willms *et al.* (1972) did not find any inhibitory effect of copper on glutathione reductase, because their GSSG concentration was too high (3·3 mM). Under these conditions the enzyme is saturated with GSSG substrate, because the K_m for GSSG is about 0·1 mM (Staal & Veeger, 1969). The copper concentrations used in the experiments of Deiss *et al.* (1970) are so high that the effect of the GSSG concentration used in their experiments (5·3 mM) is offset.

About the mode of action of copper on the holoenzyme one can only speculate. Direct GSSG binding by copper is unlikely, because copper ions chelate with two peptide molecules at most (Tommel, 1969). From our experiments it can be seen that the GSSG-copper ratio at which the enzyme is inhibited is much higher than two. SH-groups of the enzyme are probably involved in the inhibition by Cu^{2+}. Studies on this subject are in progress.

Our recombination experiments indicate that copper is a competitive inhibitor of FAD-apoenzyme recombination at high physiological erythrocyte copper concentrations. This observation might be of value in the treatment of haemolytic anaemia caused by high erythrocyte copper concentrations. It is well-known that administration of riboflavin gives a higher content of FAD in the erythrocyte (Beutler, 1969). In glutathione reductase deficiency, administration of riboflavin results in an increase of the glutathione reductase activity. Therefore, it seems reasonable to administer riboflavin to patients with chronic haematological disorders in which case it is often found that the copper content has increased.

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