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COPPER-INDUCED GENERATION OF SUPEROXIDE IN HUMAN RED CELL MEMBRANE

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Summary: The addition of $CuSO_4$ to erythrocyte membrane preparations causes the generation of superoxide radicals as measured by the superoxide dismutase-sensitive oxidation of epinephrine. The formation of O_2^- is accompanied by the reduction of copper to the cuprous form. These events are inhibited by pCMB suggesting the involvement of membrane -SH groups in the reduction of copper, and a subsequent autoxidation of Cu^+ to generate O_2^- and Cu^{++}. It is proposed that these mechanisms may be the basis for the cytotoxicity of copper in individuals exposed to copper either through a genetic deficiency of copper binding proteins or as a result of the acute ingestion of copper salts.

The mechanisms of copper-induced hemolysis in individuals with Wilson's Disease (1) and as a consequence of acute copper intoxication (2) are not well understood. Many investigators have focused attention on the effects of the addition of copper salts, in vitro, on the glycolytic enzymes of both hemolysates and cells. Inhibitions of hexokinase (3), pyruvate kinase (4), glucose-6-phosphate dehydrogenase and glutathione reductase (5) have been described as possible causes of hemolysis. However, Metz and Sagone (6) have suggested that the inhibition of erythrocyte enzymes alone may not account for the toxicity of this metal. Since it seems likely that some "oxidant" action of copper forms the basis for its cytotoxicity, it is of interest that the accumulation of lipofuscin-like pigments has been described in the livers of patients with Wilson's Disease (7). Such pigments, whose formation may result from the peroxidation of endogenous phospholipids, have also been found in the livers of experimental animals subsequent to the administration of copper (8). It has also been recently noted that superoxide dismutase has a protective effect on ox-brain phospholipid liposomes undergoing copper-catalyzed lipid peroxidation (9). The forgoing observations suggest that the toxicity of copper may be mediated, in part, through the generation of oxygen radicals

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with the capacity for initiating peroxidation reactions in membrane phospholipids. The experiments described in this paper demonstrate the formation of superoxide anions during the interaction of cupric ions with erythrocyte membrane preparations.

Materials and Methods: Blood from healthy male volunteers was drawn into heparinized syringes and subsequently centrifuged at 500 x g for 5 minutes. The plasma and buffy coat were removed and the cells were washed thrice with 10-15 volumes of isotonic saline solution. The packed cells were then lysed in 10 mM Tris-HCl, pH 7.5, and held in an ice bath for at least 5 minutes to insure complete lysis. The resulting hemolysate was centrifuged at 30,000 x g for 20 minutes and the supernatant removed. After discarding the hard button at the bottom of the tubes, the remaining fluffy membranes were washed at least three times until they were free of visible hemoglobin. The hemoglobin-free ghosts were finally resuspended in the Tris buffer to a protein concentration of 2.5 to 3.0 mg/ml.

Superoxide was determined by measuring the formation of adrenochrome from epinephrine (10). To 2.9 ml of 10 mM borate buffer, pH 10.1, 0.1 ml of the membrane suspension and $CuSO_4$ to a final concentration of 25 μM were added. The mixture was held in a cuvette at 37° C for exactly 5 minutes. At the end of this preincubation period, 0.02 ml of 10 mM epinephrine was added and the increase in absorption at 480 nm was recorded. Control cuvettes contained no membranes. In experiments with either pCMB or superoxide dismutase these agents were added to the cuvettes during the preincubation period and before the addition of $CuSO_4$. For the detection of cuprous copper, the red cell ghosts (3.0 mg protein) were resuspended in 0.9 ml Tris-NaCl, pH 7.0, (prepared by mixing equal volumes of 0.14 M Tris-HCl and 0.9% NaCl) 0.05 ml of 5 mM bathocuproin was then added to the suspension of ghosts and the reactions initiated by the addition of $CuSO_4$ solutions to achieve a final concentration of 0.1 mM. The increase in absorbance at 483 nm was recorded.

Results: Figure 1 (curve 1) shows the oxidation of epinephrine, to form adrenochrome, caused by the addition of cupric ions to the membrane preparations. In the absence of copper (curve 4) there was a small but measurable increase in absorption over six minutes of observation. In the absence of membranes there was a stimulation of epinephrine oxidation (curve 3) which was not inhibited by superoxide dismutase and which is apparently unrelated to the generation of superoxide. However, cupric-stimulated epinephrine oxidation, in the presence of membranes, was almost completely blocked when superoxide dismutase was included in the reaction system (curve 2). This suggests that the increment in adrenochrome formation observed in the presence of membranes is associated with superoxide generation.

The generation of superoxide described in the preceding experiments requires the presence of membrane sulfhydryl groups. Thus, as illustrated in Figure 2

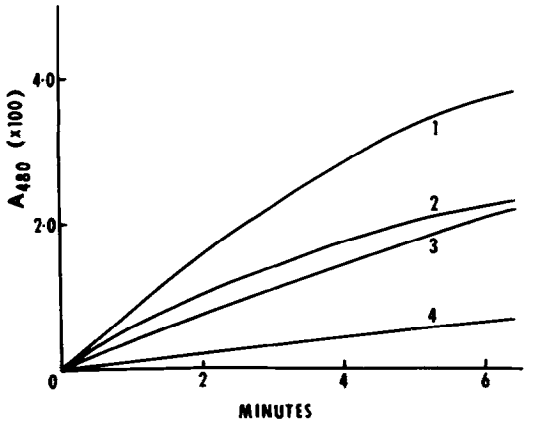

Figure 1: The effect of $CuSO_4$ on adrenochrome formation in the presence of erythrocyte membranes. Curve 1, the complete system included $CuSO_4$ (25 μM), epinephrine (100 μM) and membranes (.25 mg protein); Curve 2, complete system plus superoxide dismutase (290 units); Curve 3, complete system minus membranes; Curve 4, complete system minus $CuSO_4$.

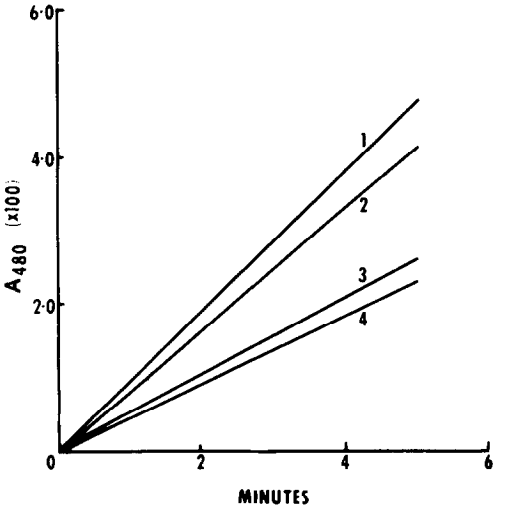

Figure 2: The effect of pCMB on adrenochrome formation catalyzed by $CuSO_4$ in the presence of erythrocyte membranes. Curve 1, the complete system included $CuSO_4$ (25 μM), epinephrine (100 μM) and membranes (0.3 mg protein); Curve 2, the complete system plus pCMB (0.3 mM); Curve 3, the complete system plus pCMB (1.5 mM); Curve 4, the complete system plus pCMB (3.0 mM).

pretreatment of the ghosts with pCMB (1.5 and 3.0 mM) results in an inhibition of epinephrine oxidation to superoxide dismutase-sensitive levels (curves 3 and

4 respectively). At a concentration of 0.3 mM pCMB an inhibition of about 25% was observed (curve 2). Similar inhibitory effects with either iodoacetamide or N-ethylmaleimide were not observed. However, their inability to do so and the requirement for high concentrations of pCMB may be a result of the high pH at which these assays were run.

The pCMB-sensitive generation of superoxide in the presence of copper suggests that membrane sulfhydryl groups may be involved in the reduction of the metal to Cu^{+}. Figure 3 shows that the accumulation of cuprous ions may indeed be measured after complex formation with bathocuproin (the curve marked with squares). In the absence of membranes there was little formation of the cuprous-bathocuproin complex (the curve marked with triangles). As was the case in experiments in which superoxide formation was measured, the reduction of copper by the membranes was markedly inhibited after treatment of the membranes with pCMB. In experiments also not presented in this paper, the content of membrane sulfhydryl groups was also shown to decline rapidly after the addition of cupric ions.

Discussion: The results described above demonstrate that cupric ions may react with plasma membrane sulfhydryl groups to cause the generation of superoxide anions and the reduction of the copper. These reactions are illustrated in equations 1 and 2.

1. $2Cu^{++} + 2\text{-}SH \longrightarrow 2Cu^{+} + S\text{-}S + 2H^{+}$

2. $Cu^{+} + O_2 \longrightarrow Cu^{++} + O_2^{-}$

The reduction of copper by sulfhydryl groups of proteins has ample precedent. For example, it is in agreement with the mechanism proposed by Takagi and Isemura (11) for the copper-induced activation of takaamylase. More recently, Jacobs et al., (12) have suggested such a mechanism in the cupric ion-mediated active transport of amino acids in membrane vesicles.

The reduction of copper and its subsequent autoxidation in erythrocyte membranes may be the initiating event in a cascade that leads to eventual

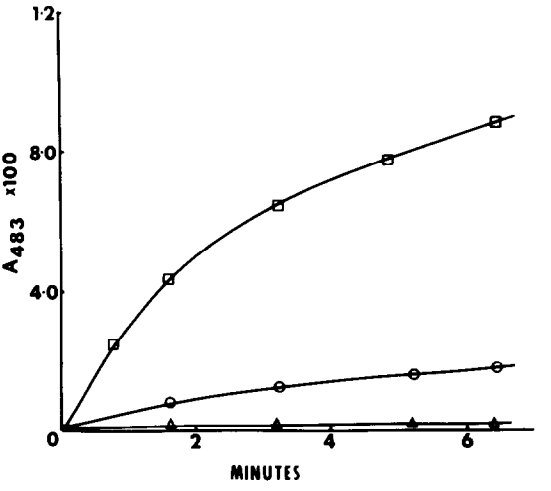

Figure 3: The reduction of Cu^{++} to Cu^{+} measured by complex formation with bathocuproin. The upper curve (squares), the complete system included $CuSO_4$ (100 μM) and bathocuproin (250 μM); the middle curve (circles), the complete system plus pCMB (1.5 mM); the lower curve (triangles), the complete system minus membranes.

hemolysis. In the current experiments with red cell membranes, mechanisms for regenerating membrane sulfhydryl groups were obviously not present. It is tempting to speculate that, in intact cells, membrane disulfides may undergo reduction via interaction with intracellular reducing equivalents. Such an event would make possible the catalytic generation of large amounts of superoxide, hydrogen peroxide, hydroxy radicals, etc., through the cyclic reduction and autoxidation of small amounts of copper. The toxic effects of these agents in the erythrocyte are by this time well established. The central role of the peroxidation of membrane phospholipids in copper-mediated cellular toxicity is substantiated by the findings that fluorescent chromolipids may be shown to accumulate in the membranes of copper-treated cells (13). Thus, it seems likely that the hemolysis associated with the release of unbound copper to the plasma in patients with Wilson's Disease, as well as that associated with acute copper intoxication, may result primarily from the interaction of copper with the erythrocyte membrane rather than from its inhibitory effects on intracellular enzymes.

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