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# OXYGEN REQUIREMENT FOR CUPRIC ION INDUCED HEMOLYSIS

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Summary: The Cu(II) induced lysis of rabbit erythrocytes occurs in two phases. First there is a lag period of 60 min where few cells lyse, followed by a period of rapid lysis where most of the remaining cells undergo hemolysis. Lysis is effectively inhibited if the incubation is conducted in nitrogen or if the erythrocytes are pre-saturated with carbon monoxide. These results suggest that oxygenated hemoglobin is necessary for lysis. It has been reported that copper binds to oxyhemoglobin and releases superoxide ion. This additional oxidative stress can initiate lipid peroxidation which leads to cell hemolysis.

The mechanism of copper toxicity to the red cell needs clarification. Early studies focused on the inhibition of erythrocyte enzymes by Cu(II), particularly those enzymes involved in the glycolytic pathway (1, 2). Recently, using isolated systems, new mechanisms involving the oxidative reactions of Cu(II) have been proposed.

Kumar (3) and Hochstein (4) have shown that Cu(II) reacts with membrane sulfhydryls and $O_2$. This reaction generates $O_2^{\cdot-}$ and $H_2O_2$, which can initiate lipid peroxidation. Salhany (5) has shown that Cu(II) can directly oxidize membrane sulfhydryls, with or without the presence of $O_2$. He suggests that this leads to disulfide links, which could lead to cell lysis. Rifkind, (6) and Winterborn and Carrell (7) have shown that Cu(II) can catalyse the oxidation of $HbO_2$ to $O_2^{\cdot-}$ and metHb. It has been suggested that this internal superoxide or possibly other oxygen intermediates are responsible for the initiation of cell lysis.

To test the various mechanisms, intact rabbit erythrocytes were incubated under air and nitrogen atmospheres. Additional experiments were

Abbreviations: $HbO_2$, oxyhemoglobin; HbCO, carbonmonoxyhemoglobin; GSH, reduced glutathione; NEM, N-ethyl maleimide; EDTA, ethylenediaminotetraacetic acid disodium salt; SOD, superoxide dismutase.

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conducted, where the cells were first exposed to CO to convert the hemoglobin to HbCO. These cells were then incubated in air equilibrated buffer. SOD and catalase were added to detect the presence of $O_2^-$ and $H_2O_2$, respectively, in each case.

Materials and Methods: All chemicals used were AR quality. Superoxide dismutase (EC 1.15.1.1) catalase (EC 1.11.1.6) and bovine serum albumin were purchased from the Sigma Chemical Co. Erythrocytes from rabbit blood were washed five times with cold phosphate buffered saline (155 mM NaCl in 20 mM phosphate buffer, pH 7.4). Reactions were carried out in the same buffer with a total volume of 3 ml in 25 ml Erlenmeyer flasks in a shaking waterbath at 37°C. The concentrations of cells was a 1.5% hematocrit. When the reactions were complete the samples were transferred to test tubes and spun in a table top centrifuge for 10 min at 1000 xg. Percent hemolysis was determined according to Kellogg (8). In the anaerobic experiments, $N_2$ was bubbled through the cell suspension for 15 min before the Cu(II) was added. The flask containing the cells was sealed for the entire experiment. Erythrocytes containing HbCO were prepared by flushing the cells with CO for fifteen min before the addition of Cu(II). The cells were then added to air incubated buffer, because of the great affinity of hemoglobin for CO these flasks were not sealed.

Results: Fig. 1 shows that the Cu(II) induced lysis of rabbit erythrocytes in the presence of $O_2$ occurs in two phases. First there is a slow phase where less than 15% of the cells are lysed. This is followed by a rapid phase where 80% of the remaining cells hemolyse.

The presence of Cu(II) is not required for the rapid phase of lysis to occur. If EDTA is added to the incubation media at one hour there is no change in the lysis pattern. Likewise if the cells are washed at one hour and resuspended in fresh incubation buffer, lysis is unaffected. This indi-

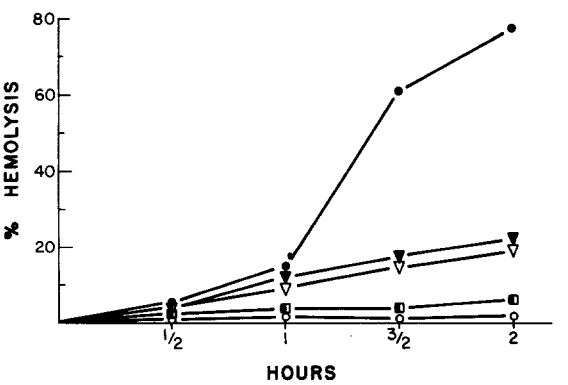


Figure 1: The effect on Cu(II) induced lysis on erythrocytes by the presence of oxyhemoglobin, (filled circles), deoxyhemoglobin, filled squares, and carbonmonoxyhemoglobin, filled triangles, the open symbols are blanks and do not contain Cu(II).

cates that after Cu(II) initiates the process of lysis it is no longer needed. (Results not shown.)

To determine if Cu(II) induced lysis is $O_2$ dependent, cell suspensions were flushed with $N_2$ for 15 min prior to the addition of Cu(II). Fig. 1 shows there is no Cu(II) induced lysis. Cells which were presaturated with CO, and contain largely HbCO, had a greatly diminished rate of lysis.

Compounds which react with sulfhydryl groups inhibit the Cu(II) induced lysis. Table 1 shows that NEM, glutathione, Hg(II), Zn(II), inhibit lysis. This inhibition was reversible. If the cells are preincubated with these compounds, washed, resuspended in fresh buffer, then incubated with Cu(II), lysis is unaffected.

Cu(II) can be chelated to an inactive form. Albumin and EDTA which bind Cu(II) strongly, were very effective in inhibiting lysis. Catalase and SOD have no effect on lysis. This is in agreement with Lovstad's (9) finding with rat erythrocytes. None of the compounds added in addition to Cu(II) had any effect on lysis by themselves.

Discussion: The evidence presented here suggests that $HbO_2$ is necessary for Cu(II) to induce cell lysis. Cells incubated under anaerobic conditions contain little $HbO_2$. These cells exhibited no Cu(II) induced lysis. If cells were first exposed to CO, to convert the $HbO_2$ to HbCO, then incu-

Table I. Effect of compounds on Cu(II) induced hemolysis.

| Compounds added | % Hemolysis | % Inhibition |
|---|---|---|
| Control ($Cu^{+2}$ 100$\mu$M) | 83 ± 5 | — |
| Catalase (30$\mu$g / ml) | 81 ± 4 | 2 |
| SOD (10$\mu$g / ml) | 84 ± 5 | 0 |
| Catalase + SOD | 81 ± 3 | 2 |
| $Zn^{+2}$ (1mM) | 5 ± 2 | 94 |
| $Hg^{+2}$ (1mM) | 2 ± 1 | 97 |
| NEM (1mM) | 2 ± 1 | 97 |
| GSH (1mM) | 2 ± 1 | 97 |
| Albumin (1%) | 3 ± 2 | 96 |
| EDTA (100$\mu$M) | 2 ± 1 | 97 |

Rabbit erythrocytes were incubated for 2 h at 37C after the addition of Cu(II). All compounds were added prior to the addition of Cu(II). None of the compounds induced lysis by themselves. Mean values of % hemolysis ±SEM are listed. % inhibition = 100 X (% hemolysis Cu(II) - % hemolysis (Cu(II) + compound added))/% hemolysis Cu(II).

bated with Cu(II), there is again almost no Cu(II) induced lysis, even though there is $O_2$ in the incubation buffer. So not only is $O_2$ required for lysis, but it must be complexed to hemoglobin. This is in agreement with the mechanism suggested by Rifkind (6) and later by Winterborn and Carrell (7).

Superoxide ion produced by the reaction of Cu(II) and $HbO_2$ can spontaneously self dismutate to form peroxide and singlet oxygen (10). It may also react with peroxide to form hydroxy radicals. It is not clear which of the oxygen species is responsible for damage to the cell, but it has been reported that "activated oxygen" (10) can initiate lipid peroxidation (8). Hochstein (4) has shown the presence of flouresent chromolipids in the membrane of cells incubated with Cu(II). These chromolipids are associated with lipid peroxidation. If antioxidants such as BHA (11), and ceruloplasmin (12) are included in the incubation medium, the appearance of the chromolipids is greatly reduced, and cell lysis is markedly inhibited.

In the early stages of the incubation the cell appears adequately protected against this oxidative threat by its own SOD, catalase or Glutathione peroxidase. As increasing amounts of Cu(II) enter the cell, Cu(II) reacts with $HbO_2$ and releases $O_2^{\cdot-}$. Since the cell does not have a mechanism for repairing much of the damage to its constituents, even small amounts of $O_2^{\cdot-}$ which are not destroyed constitute a real danger. Ribavore et al. (13) have shown that cells which are incubated with Cu(II) have diminished catalase activity, before the onset of lysis. The lag phase may be determined by how long the cell can adequately deal with the oxidative stress produced.

GSH may play a role in the cells' defenses against Cu(II). Sivertsen (14) has reported that the initial response to Cu(II) challenge is a reduction in the GSH concentration in the cell. This decrease always preceded lysis. We found that GSH protects erythrocytes from Cu(II) induced lysis. GSH could compete with $HbO_2$ as preferred substate for Cu(II) (15). Alternatively, its role as a necessary component of the glutathione peroxidase system could be involved.

Table 1 shows that Zn(II), Hg(II), and NEM inhibit Cu(II) lysis. This correlates closely with Rifkind's (16) results on isolated hemoglobin. He found that the Cu(II) catalyzed oxidation of hemoglobin was inhibited by these three compounds probably by interfering with the binding of Cu(II) and hemoglobin.

It is not surprising that added SOD or catalase show no inhibition of cell lysis, even though $O_2^{\cdot-}$ or $H_2O_2$ may be involved. The $O_2^{\cdot-}$ is produced on the inside of the cell, presumably inacessible to enzymes outside the cell.

The experiments shown here utilizing intact erythrocytes support the mechanism proposed by Rifkind (6) and Winterborn and Carrell (7), as responsible for Cu(II) induced lysis. Cu(II) enters the cell and reacts with $HbO_2$ to form $O_2^{\cdot-}$ and metHb. Superoxide ion $H_2O_2$ or possibly other oxygen species that can be formed, have the capacity to initiate lipid peroxidation which can lead to cell lysis.

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