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# Hemolysis of Human Red Blood Cells Induced by the Combination of Diethyldithiocarbamate (DDC) and Divalent Metals: Modulation by Anaerobiosis, Certain Antioxidants and Oxidants

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The objective of the present communication is to describe the role played by combinations between diethydithiocarbamate (DDC) and divalent metals in hemolysis of human RBC. RBC which had been treated with DDC (10–50 μM) were moderately hemolyzed (about 50%) upon the addition of subtoxic amounts of $Cu^{2+}$ (50 μM). However, a much stronger and a faster hemolysis occurred either if mixtures of RBC–DDC were immediately treated either by $Co^{2+}$ (50 μM) or by a premixture of $Cu^{2+}$ and $Co^{2+}$ (Cu : Co) (50 μM).

While $Fe^{2+}$ and $Ni^{2+}$, at 50 μM, initiated 30–50% hemolysis when combined with DDC (50 μM), on a molar basis, $Cd^{2+}$ was at least 50 fold more efficient than any of the other metals in the initiation of hemolysis by DDC. On the other hand, neither $Mn^{2+}$ nor $Zn^{2+}$, had any hemolysis-initiating effects. $Co^{2+}$ was the only metal which totally blocked hemolysis if added to DDC prior to the addition of the other metals.

Hemolysis by mixtures of DDC + (Cu : Co) was strongly inhibited by anaerobiosis (flushing with nitrogen gas), by the reducing agents glutathione, N-acetyl cysteine, mercaptosuccinate, ascorbate, TEMPO, and α-tocopherol, by the $PLA_2$ inhibitor bromophenacylbromide (BrPACBr), by tetracycline as well as by phosphatidyl choline, cholesterol and by trypan blue. However, TEMPO, BrPACBr and PC were the only agents which inhibited hemolysis induced by DDC : $Cd^{2+}$ complexes.

On the other hand, none of the classical scavengers of reactive oxygen species (ROS) employed e.g dimethylthiourea, catalase, histidine, mannitol, sodium benzoate, nor the metal chelators desferal and phenanthroline, had any appreciable inhibitory effects on hemolysis induced by DDC + (Cu : Co).

DDC oxidized by $H_2O_2$ lost its capacity to act in concert either with $Cu^{2+}$ or with $Cd^{2+}$ to hemolyze RBC.

While either heating RBC to temperatures greater than 37°C or exposure of the cells to glucose-oxidase-generated peroxide diminished their susceptibility to hemolysis, exposure to the peroxyl radical from AAPH, enhanced hemolysis by DDC + (Cu : Co).

The cyclovoltammetry patterns of DDC were drastically changed either by $Cu^{2+}$, $Co^{2+}$ or by $Cd^{2+}$ suggesting a strong interaction of the metals with DDC. Also, while the absorbance spectrum of DDC at 280 nm was decreased by 50% either by $Co^{2+}$, $Cd^{2+}$ or by $H_2O_2$, a 90% reduction in absorbance occurred if DDC + $H_2O_2$ mixtures were treated either by $Cu^{2+}$ or by $Co^{2+}$, but not by $Cd^{2+}$.

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Taken together, it is suggested that DDC–metal chelates can induce hemolysis by affecting the stability and the integrity of the RBC membrane, and possibly also of the cytoskeleton and the role played by reducing agents as inhibitors might be related to their ability to deplete oxygen which is also supported by the inhibitory effects of anaeobiosis.

*Keywords:* Hemolysis, DDC, divalent metals, antioxidants, anaerobiosis



## INTRODUCTION

The interest in diethyldithiocarbamate (DDC) emerged from the discovery that this compound is a potent inhibitor of superoxide dismutase (SOD)[1] as well as a chelator of copper and an antimalarial agent.[2] DDC has a dual effect on cells. On the one hand, it acts as an antioxidant agent[3–7] but on the other hand, it might also act as a potent pro-oxidant because of its capacity to deplete glutathione (GSH) and other thiol compounds.[5] It was also demonstrated[8] that DDC combined with $Cu^{2+}$ hemolyzed human red blood cells (RBC) and that small amounts of $Co^{2+}$ tended to suppress DDC–copper-induced hemolysis. Since hemolysis by DDC : $Cu^{2+}$ was not inhibited under anaerobic conditions, the role of oxidative damage in hemolysis was excluded.[8] Instead, it was proposed that damage to RBC was due to the concentration of DDC : $Cu^{2+}$ in the lipid bilayer and perhaps due to the formation of amphipathic complexes which disrupted membrane integrity. Therefore, this drug behavior might hold promise for treatment of malaria because metals capable of forming such complexes might accumulate within parasitized RBC.[2]

Recent studies from our laboratories[9] have shown that subtoxic concentrations of DDC very markedly potentiated the killing of endothelial cells and of monkey kidney epithelial cells in culture and also the release of arachidonate from cell membranes when employed together with membrane-perforating agents and oxidants (a distinct synergistic effect). Because DDC acted in these *in vitro* models as a distinct pro-inflammatory agent it was of interest to further investigate the modulating effects of divalent metals, a variety of scavengers of reactive oxygen species (ROS) and of certain oxidants on the hemolysis of RBC initiated by DDC.

## MATERIAL AND METHODS

### Human Erythrocytes: (RBC)

Human blood in heparin was obtained from normal human donors with their consent. The blood was washed once with normal saline and twice with Hanks balanced salt solution (HBSS). We employed RBC suspensions which, following lysis by triton X-100, gave an absorbance reading of 1.8–2.0 at 550 nm. The RBC suspensions were kept on ice throughout the studies.

### Hemolytic Assays

One ml aliquots of RBC suspensions were treated with increasing concentrations of DDC and with various amounts of a variety of metal salts ($CuCl_2$, $CuO$, $CoCl_2 \cdot 6H_2O$, $MnCl_2$, $FeCl_2 \cdot 4H_2O$, $FeCl_3$, $ZnCl_2$, $NiCl_2$, $VCl_2$, $CdSO_4$). In some experiments, certain mixtures of the various metal salts as well as the sequences of their addition to RBC suspensions were also tested for their capacity to modulate RBC hemolysis in the presence of DDC. In some experiments, RBC suspensions were flushed for 10 min with $N_2$ using a glass flask with a long and narrow neck to deplete oxygen. Following incubation of the RBC with the various reactants for 15 min at 37°C, the tubes were centrifuged at 2000 rpm in a Clay Adams clinical centrifuge and the degree of hemolysis was read at 550 nm in a double-beam Unicam spectrophotometer.

### The Effect of Inhibitors on Hemolysis

The following agents were employed to modulate hemolysis: (1) the ROS scavengers catalase, histidine, mannitol, DMSO, dimethylthiourea (DMTU), benzoate, selenite, lipoic acid, melatonin, ascorbate, trolox, vitamin A acetate; (2) the

thiol compounds, glutathione, N-acetylcysteine, mercapto-succinate, penicillamine; (3) 2,2,6,6-tetramethyl-1-piperidine-n-oxyl (TFMPO); (4) the catalase inhibitors, sodium azide (AZ) and 3-amino, 2,4 triazole (ATAZ); (5) the metal chelators-1-10 phenanthroline (dissolved in ethanol) and deferoxamine mesylate; (6) the non-specific $PLA_2$ inhibitors, bromophenacylbromide (BrPACBr) (dissolved in ethanol) and the non-penetrating inhibitor CME (carboxymethyl cellulose bound to phosphtatidylethanolamine;[10,11] (7) phosphatidyl choline (PC) (dissolved in ethanol); (8) cholesterol (dissolved in hot ethanol); (9) the azo dye trypan blue. In most of the experiments, one ml aliquots of RBC suspensions were first pre-incubated for 10 min at 37°C with various amounts of the inhibitors at (50–1000 μM). This was followed by the addition of DDC (10–50 μM) followed immediately either by a mixture of $Cu^{2+}$ (50 μM) and $Co^{2+}$ (50 μM) (written as $Cu^{2+}:Co^{2+}$) or by $Cd^{2+}$ (see below). To study the possible mechanisms by which the inhibitors affected DDC–metal-induced hemolysis, these compounds were also first pre-incubated for 10 min either with DDC or with $Cu^{2+}:Co^{2+}$ prior to the addition of the other reactants. The reaction mixtures were then further incubated for 20 min at 37°C and the extent of hemolysis was determined after centrifugation.

### The Effect of Temperature and of Oxidants on the Susceptibility of RBC to Hemolysis

RBC suspensions were kept for 3 h on ice, or at 22°C, or at 37°C, or heated to 50°C. The cells were then treated with increasing concentrations of DDC (5–50 μM) combined with $Cu^{2+}:Co^{2+}$ both at (50 μM). In other experiments, RBC suspensions were treated for 30 min at 37°C either with glucose oxidase (0.001 Units/ml), which generated approximately 1 mM of $H_2O_2$ per hour, with azobis-diamidinopropane dihydrochloride (AAPH) (100 mM), a donor of peroxyl radical, or with HOCl (100–500 μM). These concentrations of oxidants were non-hemolytic. The oxidant-treated RBC's were washed twice with HBSS and their susceptibility to hemolysis by increasing amounts of DDC (5–50 μM) combined with $Cu^{2+}:Co^{2+}$ (50 μM) was determined.

### Spectrophotometric Analysis of DDC–Metal–Oxidant Mixtures

DDC (1.5 mM) in 1 ml HBSS was mixed either with 100 μM either of $Cu^{2+}$, $Co^{2+}$, or of $Cd^{2+}$ (at 100 μM), with $H_2O_2$ (1 mM), with HOCl (1–5 mM) or with combinations of the oxidants and metals and the absorbance spectrum at 280 nm was determined. In some experiments, changes in spectra after various sequences of addition of the various components were also tested.

### Cyclovoltammetry (CV)

CV measurements were performed to determine the total reductive capacities of DDC–metal-chelates.[12] This was performed in a BAS model CV-58 cyclic voltammeter (West Lafayette, IN, USA) equipped with a glassy carbon working electrode, a Pt wire auxiliary electrode and a Ag/AgCl reference electrode, as described. The measurements were carried out between −2.0 V and +2.0 V. DDC (300 μM) in 3 ml phosphate saline buffer pH 7.4 was flushed for 15 min with $N_2$. This was followed by the addition either of $Cu^{2+}$ (100 μM), $Co^{2+}$ (100 μM) or $Cd^{2+}$ (100 μM), $H_2O_2$ (1 mM) or of combinations between peroxide and metals. CV measurements were carried out mostly at a scan rate of 100 mV/s.

### Lipid Hydroperoxide and Thiobarbituric Acid Reactive Species (TBARS) Assays

Either RBC ghosts pretreated by hemolyzing RBC in 0.01 M phosphate buffer pH 7.4 containing 0.01 M EDTA or intralipid (100 μM of a 20% commercial emulsion rich in soybean lipids and egg phospholipids – Kabi Pharmacia, Sweden) were exposed for 10 h at 37°C either to DDC

(100 μM), $Cu^{2+}$ (50 μM), $Co^{2+}$ (50 μM), $Cd^{2+}$ (20 μM) or to combinations of DDC and metals. The TBARS and lipid hydroperoxides using the KI method were determined spectrophotometrically at 400 nm.[13] In some experiments, the antioxidants DMTU, glutathione, N-acetylcysteine and TEMPO at 1 mM were included in the reaction mixtures.

## RESULTS

### The Effect of DDC–Metal Complexes on Hemolysis

It had been reported[8] that human RBC suspended in TRIS-HCl (a non-physiological buffer) which had been treated with DDC (67 μM) were moderately hemolyzed (about 50%) within 30 min, following the addition of $Cu^{2+}$. We fully corroborated these results but chose throughout this study to use HBSS as a representing physiological medium. Figure 1 shows that while about 50% hemolysis occurred when RBC had been mixed with increasing concentrations of DDC in the presence of $Cu^{2+}$ (50 μM), equimolar concentrations of $Co^{2+}$ failed to induce any traces of hemolysis. On the other hand, a near-full hemolysis was induced, either if treatment of RBC with DDC was followed immediately by the addition of $Cu^{2+}$ and $Co^{2+}$ or even to a larger extent and much faster, by the addition of premixture of the two metals (see below). Surprisingly, however, no hemolysis occurred if RBC–DDC mixtures had first been treated with $Co^{2+}$, which was followed immediately by the addition of $Cu^{2+}$. This suggested that $Co^{2+}$ blocked some binding sites for $Cu^{2+}$ in the DDC molecule. It was also found that RBC, which had been first treated either by DDC or by the metals and then washed, failed to undergo hemolysis upon the addition of the corresponding additional agents. This suggested that all reagents had to be present simultaneously to induce hemolysis.

To determine the specificity of $Cu^{2+}$ as an inducer of DDC-hemolysis, additional divalent metals were tested. Figure 2 shows that on a molar basis, $Cd^{2+}$ was by far the most potent and the fastest inducer of DDC-hemolysis and as little as 7.5 μM of the metal initiated full hemolysis within 1–3 min in the presence of 50 μM of DD. $Cd^{2+}$ in its efficiency as an hemolysis-promoting metal was followed, in a descending order, by $Cu^{2+}$, $Ni^{2+}$ and $Fe^{2+}$. On the other hand, at equimolar concentrations, neither $Mn^{2+}$, $Zn^{2+}$, nor $V^{2+}$ (not shown) had any appreciable hemolysis-inducing effects.

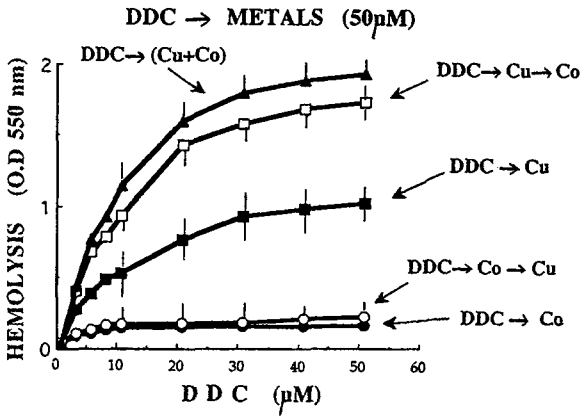


FIGURE 1 Hemolysis induced by DDC followed by the addition either of $Cu^{2+}$, $Co^{2+}$, or of mixtures of $Cu^{2+}$ and $Co^{2+}$ Note the blocking effect of $Co^{2+}$ on hemolyis. The data are averages ± S.D from 5 different experiments.

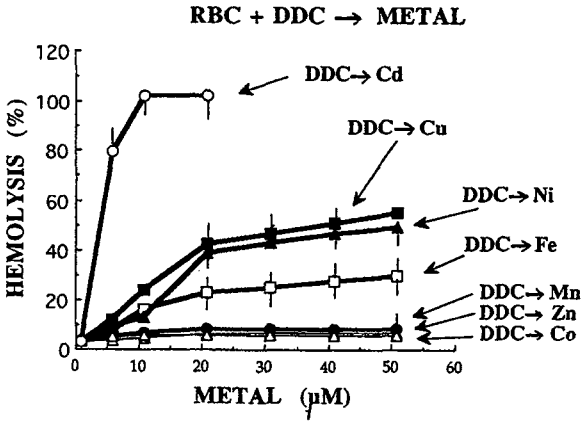


FIGURE 2 Hemolysis induced by treatment with DDC (100 μM) followed by increasing amounts of different divalent Note: that on a molar basis when combined with DDC, $Cd^{2+}$ is by far the most potent hemolysis-inducing metal. The data are averages ± S.D from 4 different experiments.

Under similar conditions, neither CuO (predissolved in ethanol) nor $Fe^{3+}$ had any appreciable hemolysis-inducing capacities when tested with DDC. We also examined the hemolytic potencies of combinations among several divalent metals when added to RBC–DDC mixtures. It was found that while combinations of DDC (100 μM) with (Cu + Co) (50-μM) yielded maximal hemolysis (100%), the complexes, $Cu^{2+}:Fe^{2+}$, $Cu^{2+}:Mn^{2+}$, $Cu^{2+}:Zn^{2+}$, $Cu^{2+}:Ni^{2+}$ and $Cu^{2+}:V^{2+}$ induced only 30% hemolysis. However, the inclusion of $Fe^{2+}$ with all these combinations markedly depressed hemolysis compared with the effects exerted by $Cu^{2+}:Co^{2+}$. The results suggest that the dominating metals causing DDC-induced hemolysis were $Cd^{2+}$ and $Cu^{2+}:Co^{2+}$. Since $Cu^{2+}:Co^{2+}$, at 50 μM, yielded maximal hemolysis when added to mixtures of RBC and DDC (10–50 μM), it was also of interest to establish whether pre-incubation of RBC–DDC mixtures with the various metals might alter or even block hemolysis upon the addition of $Cu^{2+}:Co^{2+}$. It was found that while $Cu^{2+}$ and $Co^{2+}$, at 30 μM, strongly inhibited hemolysis if added to RBC–DDC mixtures prior to the addition of $Cu^{2+}:Co^{2+}$, neither $Fe^{2+}$, $Mn^{2+}$, $Zn^{2+}$ nor $V^{2+}$ (not shown) had any inhibitory effects.

Since $Cd^{2+}$, combined with DDC, had the highest hemolysis-promoting activity (Figure 2), we also examined the possible blocking effects of several divalent metals on $Cd^{2+}$-induced hemolysis. It was found (Figure 3) that while pre-incubation of RBC–DDC mixtures either with $V^{2+}$ or with $Mn^{2+}$ did not alter the effect of $Cd^{2+}$, a 40–50% inhibition of hemolysis occurred when RBC–DDC mixtures were pre-incubated, either with $Zn^{2+}$, $Fe^{2+}$, $Ni^{2}$ or $Cu^{2+}$ prior to the addition of $Cd^{2+}$. On the other hand, pre-incubation with $Co^{2+}$ caused a near-total inhibition of hemolysis. The findings with $Co^{2+}$ are essentially similar to those shown in Figure 1, where this metal was shown to totally block the effect of $Cu^{2+}$ on DDC-induced hemolysis.

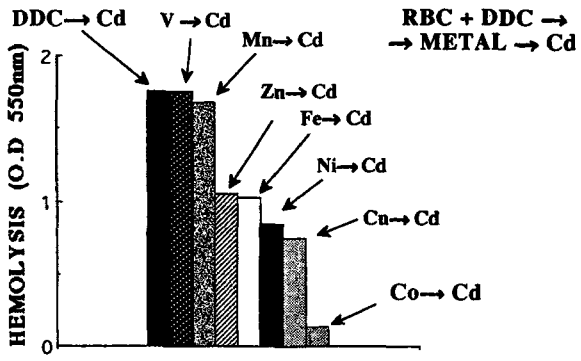


FIGURE 3 The blocking effects of divalent metals (at 50 μM) on hemolysis induced by mixtures of DDC (100 μM) and $Cd^{2+}$ (10 μM). The data are averages from 4 different experiments.

## The Effect of Anaerobiosis on Hemolysis

Hemolysis by DDC : $Cu^{2+}$ was reported to proceed, unabated, even after the apparent exclusion of oxygen (in a gas tonometer), suggesting that membrane damage resulted from a non-oxidative process.[8] Since under normal physiological conditions RBC generate a low flux of ROS[13] and since transition metals might participate in a Fenton reaction yielding toxic oxygen radicals, it was of interest to reinvestigate the possible role of ROS in DDC–metal-induced hemolysis. Figure 4 shows that a very significant inhibition of

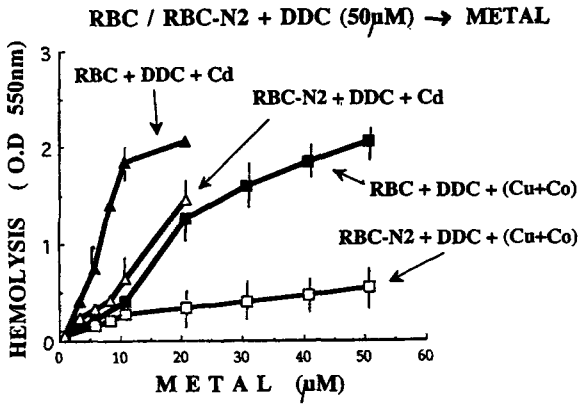


FIGURE 4 The effect of anaerobiosis on hemolysis. RBC were flushed with nitrogen gas for 10 min. Hemolysis was then induced by DDC (100 μM) followed by the addition of (Cu + Co) (50 μM) Note: that unlike control RBC cells, pretreated by nitrogen gas ($N_2$) became highly resistant to hemolysis. The data are averages ± S.D from 5 different experiments.

hemolysis, induced either by DDC : $Cu^{2+}$ : $Co^{2+}$ or by DDC : $Cd^{2+}$, occurred when RBC suspensions were first exposed for 10 min to 100% nitrogen gas. However, the $N_2$ effect was reversed by flushing the RBC suspensions with air (not shown). These findings suggested that oxygen-dependent processes catalyzed by copper might participate in DDC–metal-induced hemolysis (see below).

### The Effect of Inhibitors on Hemolysis

To further investigate the possible dependency on oxygen of hemolysis induced by DDC : $Cu^{2+}$ : $Co^{2+}$, RBC suspensions were pre-treated with a series of agents which included antioxidants, metal chelators, $PLA_2$ inhibitors and phospholipids and then were exposed to DDC (50 μM), followed immediately by the addition either of $Cu^{2+}$ : $Co^{2+}$ (50 μM) or of $Cd^{2+}$ (10 μM). These combinations were shown to induce maximal hemolysis (Figures 1 and 2). Table I shows that when tested at 100 μM, a larger than 90% inhibition of hemolysis induced by DDC : $Cu^{2+}$ : $Co^{2+}$ was induced by the thiols, glutathione N-acetylcysteine, penicillamine, mercaptosuccinate, by ascorbate, by the adduct TEMPO, by alpha tocopherol, by the non-specific $PLA_2$ inhibitor BrPACBr, by the non-penetrating $PLA_2$ inhibitor, CME (not shown), as well as by PC, cholesterol and by the azo dye trypan blue (the latter three agents are potent inhibitors of streptococcal hemolysins). A lesser degree of inhibition (75%) was induced by tetracycline. On the other hand, none of the "classical" antioxidants tested (catalase DMTU, histidine, benzoate, selenite, mannitol and DMSO (the latter 3 agents not shown)), had any inhibitory effects. It was, however, very surprising that even high concentrations (1–5 mM) either of phenathroline or of desferal, known chelators of divalent metals, failed to significantly inhibit hemolysis by DDC : $Cu^{2+}$ : $Co^{2+}$. On the other hand, hemolysis by

TABLE I The effect of inhibitors on hemolysis

| Inhibitor* | Inhibition of hemolysis (%) for sequence of addition | |
|---|---|---|
| | RBC Inhibitor DDC (50 μM) Cu : Co(50 μM) | RBC Inhibitor DDC (50 μM) $Cd^{2+}$ (10 μM) |
| N-Acetyl cysteine (100 μM) | 97 | 0 |
| Penicillamine (100 μM) | 96 | 0 |
| Glutathion E (100 μM) | 96 | 0 |
| Mercaptosuccinate (100 μM) | 97 | 0 |
| Ascorbate (20 μM) | 100 | 0 |
| Tempo (250 μM) | 95 | 90 |
| Vitamin A Acetate (100 μM) | 98 | ND |
| BrPACBr (100 μM) | 90 | 80 |
| PC (50–100 μM) | 100 | 100 |
| Cholesterol (100 μM) | 100 | 0 |
| Trypan Blue (50 μM) | 100 | 0 |
| Tetracycline (100 μM) | 75 | 0 |
| Catalase (1000 Units) | 19 | 0 |
| DMTU (1–2 mM) | 6 | 0 |
| Histidine (1 mM) | 33 | 0 |
| Sodium selenite (1 mM) | 0 | 0 |
| Sodium benzoate (1 mM) | 0 | 0 |
| Phenanthroline (1–5 mM) | 35 | 0 |
| Desferal (1–5 mM) | 17 | 0 |

*The data are averages of 5 experiments performed with RBC obtained from 5 different donors.

DDC : $Cd^{2+}$ was significantly inhibited only by TEMPO, BrPACBr and by PC.

To further examine whether the various inhibitors which affected hemolysis by DDC : $Cu^{2+}Co^{2+}$ acted either by binding to DDC or to the metals, these inhibitors were either pre-incubated with DDC or with $Cu^{2+}$ : $Co^{2+}$ prior to the addition of the other agents. Under these conditions, similar patterns of inhibition were obtained.

To determine whether the various inhibitory agents acted by binding to RBC to block a further interaction either with DDC or to the metals, the following experiment was performed: two sets of tubes containing 1 ml of RBC were first pre-incubated for 10 min at 37°C with a variety of agents, at 1 mM. One set of tubes was then centrifuged and the RBC were washed with HBSS to remove unbound agent, while the second set remained unwashed. The two sets were then treated for 1 min at 37°C with DDC (50 μM), followed by the addition of 50 μM of $Cu^{2+}$ : $Co^{2+}$. It was found that only BrPACBr and to a lesser extent, PC, markedly inhibited hemolysis even in the washed system. This suggested that these agents had the capacity to bind to the cells and to interfere with the lytic process, presumably by inhibiting $PLA_2$ activity (see below).

## Effect of Oxidants on Hemolysis by Mixtures of DDC and Metals

Since DDC (a thiol compound) could be oxidized either to the dimer, disulfiram or to 4 possible additional forms,[3] it was of interest to determine whether oxidized DDC might still retain its hemolysis-promoting capacity. In parallel, it was also of interest to test the possible modulating effects of oxidants either on RBC, DDC or on $Cu^{2+}$ : $Co^{2+}$. Table II shows that treatment of RBC with $H_2O_2$, did not alter their susceptibility to hemolysis upon addition of DDC and $Cu^{2+}$ : $Co^{2+}$. On the other hand, DDC which had been pretreated with $H_2O_2$ (200 μM–1 mM) almost totally lost its hemolysis-inducing properties. The effect of peroxide was however totally abolished by catalase, suggesting that oxidized DDC no longer promoted hemolysis.

Since the failure of $H_2O_2$ to affect hemolysis might have been due to the presence, in RBC, of large amounts of catalase capable of breaking down peroxide, RBC were first exposed either to AZ (1 mM) or to ATAZ (1 mM), both potent inhibitors of catalase, washed in HBSS and then exposed to $H_2O_2$. This was followed by the addition of DDC and $Cu^{2+}$ : $Co^{2+}$. Table II shows that while RBC treated by 1 mM of $H_2O_2$ were still

TABLE II The effect of $H_2O_2$, catalase, azide and aminotriazole on hemolysis induced by mixtures of DDC and (Cu : Co)

| Reaction mixture | Followed by | Followed by | Followed by | Hemolysis (%) |
|---|---|---|---|---|
| RBC | | DDC | (Cu : Co)* | 100 |
| (RBC + $H_2O_2$)** | | DDC | (Cu : Co) | 100 |
| (RBC + $H_2O_2$) | CAT*** | DDC | (Cu : Co) | 100 |
| RBC + CAT | $H_2O_2$ | DDC | (Cu : Co) | 100 |
| DDC*** | | RBC | (Cu : Co) | 100 |
| DDC + $H_2O_2$ | | RBC | (Cu : Co) | 6 |
| DDC + CAT | $H_2O_2$ | RBC | (Cu : Co) | 95 |
| DDC + $H_2O_2$ | CAT | RBC | (Cu : Co) | 7 |
| RBC + AZIDE**** | $H_2O_2$ | DDC | (Cu : Co) | 5 |
| RBC + ATZ**** | $H_2O_2$ | DDC | (Cu : Co) | 5 |

*(Cu : Co) 50 μM.
**$H_2O_2$ – 1 mM.
***CAT – Catalase (1000 Units/ml).
****AZIDE, ATZ (Aminotriazole) (1 mM). Results are averages of 3 experiments performed in duplicates.

fully hemolyzed upon the addition of DDC and $Cu^{2+}:Co^{2+}$, RBC pre-treated either with AZ or with ATAZ and then exposed to DDC and $Cu^{2+}:Co^{2+}$ became highly refractory to hemolysis. These findings suggested that inhibition of catalase allowed the residual $H_2O_2$ to oxidize DDC and thus to abolish its capacity to act in concert with $Cu^{2+}:Co^{2+}$ to hemolyze the RBC.

### Effect of Oxidants on DDC

To test the specificity of $H_2O_2$ in DDC inactivation, we also examined the effect of additional oxidizing agents on hemolysis. In these experiments, DDC was pre-incubated either with $H_2O_2$, HOCl, AAPH (a generator of peroxyl radical) or of menadione (vitamin $K_3$), a known generator of ROS. Either catalase or taurine (scavengers of peroxide and HOCl, respectively) were then added, followed by RBC and (Cu + Co). Figure 5 shows that whereas pre-treatment with very small amounts of $H_2O_2$ inactivated DDC, even 4 mM of HOCl failed to do the same. Under similar conditions (not shown), neither AAPH (100 mM) nor menadione (at 1 mM) had the ability to alter hemolysis initiated by $DDC:Cu^{2+}:Co^{2+}$.

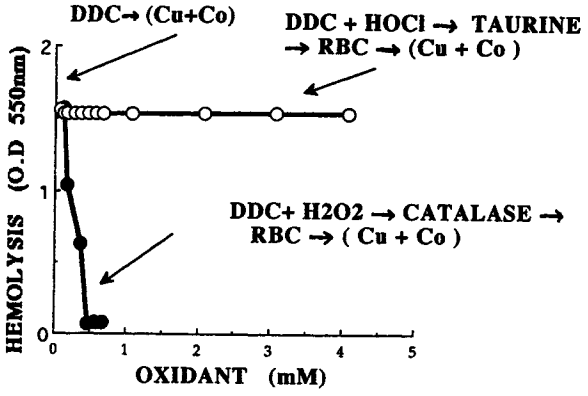


FIGURE 5 The effect of oxidants of hemolysis. DDC (100 μM) was treated for 5 min by increasing amounts either of $H_2O_2$ or of reagent HOCl. This was followed by the addition either of catalase (1000 Units/ml) or of taurine (1 mM) respectively, to scavenge the oxidants. This was followed by the addition of RBC and (Cu + Co) (50 μM). Note: that while very small mounts of peroxide were needed to oxidize DDC, even very large amounts of HOC failed to oxidize DDC. Neither catalse nor taurine had any modulating effects on hemolysis. Data are from a typical experiment.

### Generation of TBARS and Hydroperoxides by DDC–Metal Complexes

Since anaerobiosis (Figure 4) and a series of antioxidants (Table I) inhibited hemolysis induced by $DDC:Cu^{2+}:Co^{2+}$, we also tested the possibility that these agents might induce the generation of oxidation products either in RBC ghosts or in intralipid that can be measured as TBARS and as hydroperoxides. Figure 6 shows that while substantial amounts of hydroperoxides were generated either by copper alone or by copper + cobalt, about one third less peroxidation products were induced by combinations of DDC with $Cu^{2+}$ or with $Cu^{2+}:Co^{2+}$. On the other hand, neither DDC alone nor mixtures of DDC and $Cd^{2+}$ initiated any oxidation products. Generation of hydroperoxides induced either by copper or by mixtures of DDC and copper (not shown) was inhibited either by ascorbate or by glutathione. It was also found (not shown) that unlike thiols or ascorbate, DMTU failed to inhibit the peroxidation of intralipid induced by copper. This also

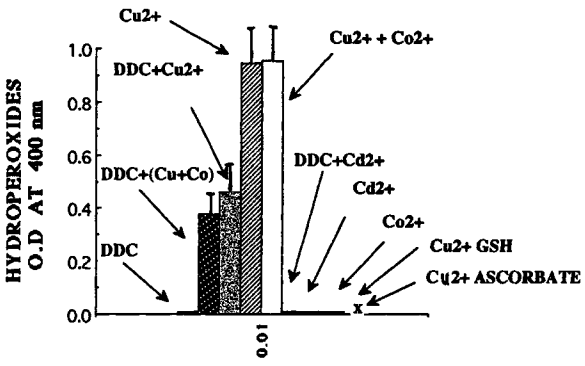


FIGURE 6 Generation of hydroperoxides in intralipid by DDC–metal complexes. 100 μl aliquots of intralipid were incubated for 18 h at 37°C with the various agents (see Materials and Methods). This was followed by the addition of 1 ml ethanol and 50 μl of a 50% solution of KI. The mixtures were kept in the dark for 30 min and the intensity of yellow color was determined O.D at 400 nm. The data are averages ± S.D from 3 separate experiments.

suggested that the hemolysis-initiating property of copper depended on its oxi/reductive state.

### The Effect of Oxidants on RBC

Since peroxidation of membrane components might affect metal-induced hemolysis, RBCs were pretreated for 30 min either with reagent $H_2O_2$ (1–5 mM), glucose-oxidase (generating approximately 1–2 mM peroxide/h) with non-hemolytic amount of AAPH (100 mM), a peroxyl radical generator or with HOCl (100 μM). This was followed by the addition of catalase and taurine to scavenge peroxide and hypochlorite, respectively. It was found (not shown) that whereas treatment with reagent $H_2O_2$ had no effect on hemolysis induced by DDC : $Cu^{2+}$ : $Co^{2+}$, RBC treated with GO-generated peroxide, which turned green presumably due to the formation of met hemoglobin, became 30–50% less susceptible to hemolysis. Treatment with HOCl yielded variable and irreproducible results. On the other hand, RBC treated with AAPH became 30–50% more susceptible to hemotysis induced by DDC : $Cu^{2+}$ : $Co^{2+}$.

### Effect of Temperature on Hemolysis

To determine whether exposure of RBC to different temperatures might affect their susceptibility to lysis by DDC : $Cu^{2+}$ : $Co^{2+}$, the cells were kept for 3 h at 4°C, 22°C, 37°C and at 50°C. It was found that while incubation at 37°C depressed hemolysis by 30%, incubation at 50°C caused about 50% inhibition of hemolysis induced by DDC : $Cu^{2+}$ : $Co^{2+}$.

### Absorbance Spectrum of DDC–Metal Complexes

Since DDC strongly absorbs light at 280 nm, it was of interest to follow the absorption patterns of DDC following treatment by peroxide, by copper and by cobalt, thiols and by peroxide. Table III shows that about a 40–50% decrease in absorbance occurred when DDC interacted either with $Cu^{2+}$, $Co^{2+}$ or with $Cd^{2+}$. Also, DDC treatment either with $H_2O_2$ or with HOCl also resulted in a significant drop in absorbance. However, a 90% drop in absorbance occurred if mixtures of DDC + $H_2O_2$ were further treated either by $Cu^{2+}$, $Co^{2+}$ or by $Cu^{2+}$ : $Co^{2+}$. On the other hand, the addition of $Cd^{2+}$ did not result in a decline in absorbance beyond that induced by DDC + $H_2O_2$. The presence of a thiol compound such as glutathione, which was shown to very strongly inhibit hemolysis induced by DDC : $Cu^{2+}$ : $Co^{2+}$ (see Table I), also resulted in a very drastic decrease in $A_{280}$, which was unaffected by the subsequent addition of peroxide. Oxidized DDC retained one third of its absorbance following the addition of GSH, while DDC pre-treated by copper, followed by the addition of GSH, retained two thirds of its light-absorbing properties. The results suggest that a partial oxidation of DDC by peroxide was further augmented either by $Cu^{2+}$

TABLE III Absorption spectrum of combinations among DDC, divalent metals, oxidants and glutathione

| Reaction mixture* | Followed by | $A_{280}$ |
|---|---|---|
| DDC (1.5 mM) | | 1.950 |
| DDC + $H_2O_2$ (10 mM) | | 1.135 |
| DDC + HOCl (10 mM) | | 0.885 |
| DDC + $Cu^{2+}$ (100 μM) | | 1.270 |
| DDC + $Cu^{2+}$ | $H_2O_2$ | 0.911 |
| DDC + $H_2O_2$ | $Cu^{2+}$ | 0.200 |
| DDC + $Co^{2+}$ (100 μM) | | 0.960 |
| DDC + $Co^{2+}$ | $H_2O_2$ | 0.720 |
| DDC + $H_2O_2$ | $Co^{2+}$ | 0.137 |
| DDC + $Cd^{2+}$ (100 μM) | | 1.190 |
| DDC + $Cd^{2+}$ | $H_2O_2$ | 1.216 |
| DDC + $H_2O_2$ | $Cd^{2+}$ | 0.922 |
| DDC + (Cu : Co) (100 μM) | | 0.870 |
| DDC + $H_2O_2$ | (Cu : Co) | 0.125 |
| DDC + GSH** (1 mM) | | 0.001 |
| DDC + GSH | $H_2O_2$ | 0.001 |
| DDC + $H_2O_2$ | GSH | 0.594 |
| DDC + $Cu^{2+}$ | GSH | 1.038 |

*DDC was pre-incubated for 5 min at room temperature with the various agents. This was followed by the addition of the other agents and a further incubation for 5 min was made before reading the absorbance at 280 nm.
**GSH – glutathione.

or by $Co^{2+}$ but to a lesser extent by $Cd^{2+}$. It is paradoxical however why $Cd^{2+}$, which, when combined with DDC, showed the highest and the fastest hemolysis-inducing activity, only partially altered the patterns of DDC absorption.

### Cyclovoltammetry of DDC–Metal Complexes

We also studied the reductive capacities of DDC–metal chelates using cyclovoltammetry.[12] DDC (1.5 mM) showed two distinct anodic waves at 280 mV and at 900 mV respectively. The addition of $Cu^{2+}$ (100 μM) caused a near-total disappearance of both anodic waves. As demonstrated above (Figure 1), DDC : $Cu^{2+}$ induced about 50% hemolysis. A similar disappearance of the anodic waves also took place when DDC was mixed either with $Co^{2+}$, which had no hemolysis-inducing activity (Figure 1) or with $Cd^{2+}$, which had the most potent hemolysis-promoting activity when combined with DDC (Figure 2). The CV patterns of DDC treated with $H_2O_2$ also changed. The major anodic wave at 900 mV disappeared and a small wave at 580 mV appeared. This wave might represent the oxidized form of DDC the dimer disulfiram. As expected, treatment of DDC with peroxide + $Cu^{2+}$ resulted in the complete disappearance of all waves.

## DISCUSSION

The purpose of the present communication is to describe the role played by combinations of DDC with certain divalent metals in hemolysis of human RBC and the possible role played by ROS in hemolysis. The main result presented show that:

(1) DDC at 10–1000 μM does not induce hemolysis.
(2) Strong hemolysis could however be induced by combinations either between micromolar amounts of DDC : $Cu^{2+}$ : $Co^{2+}$ or between DDC and $Cd^{2+}$(Figure 1).
(3) Hemolysis was blocked to a large extent if RBC had been pre-incubated with $Co^{2+}$ prior to the addition either of $Cu^{2+}$ : $Co^{2+}$ or of $Cd^{2+}$ (Figures 1 and 3).
(4) On a molar basis, $Cd^{2+}$ had the highest and the fastest hemolysis-promoting activities (Figure 2).
(5) Hemolysis depends on the presence of molecular oxygen (Figure 4), suggesting the involvement of reactive oxygen species in hemolysis. Also, both hydroperoxides (KI method) and TBARS were generated either by $Cu^{2+}$, by, $Cu^{2+}$ : $Co^{2+}$ and to a lesser extent by DDC : $Cu^{2+}$ : $Co^{2+}$.
(6) While hemolysis by DDC : $Cu^{2+}$ : $Co^{2+}$ was inhibited by the reducing agents thiols, ascorbate, TEMPO, vitamin E, by $PLA_2$ inhibitors, PC, cholesterol, tetracycline and by trypan blue, neither the classical antioxidants, catalase, DMTU, histidine, selenite, benzoate nor the metal chelators, desferal or phenanthroline had any inhibitory effects.
(7) Contrary to the results obtained with DDC : $Cu^{2+}$ : $Co^{2+}$, neither thiols, ascorbate, the classical antioxidants, nor the metal chelators inhibited hemolysis induced by DDC : $Cd^{2+}$, which could however be inhibited by TEMPO, by the non-specific $PLA_2$ inhibitor BrPACBr and by PC.
(8) Oxidation of DDC by $H_2O_2$ but not by HOCl resulted in loss of hemolysis in the presence of $Cu^{2+}$ and $Co^{2+}$ (Table III and Figure 5) and in a significant change in the spectrum of its absorption at 280 nm and in its CV patterns.

Although the mechanisms by which DDC–metal chelates cause hemolysis are still not fully understood, several explanations for this phenomenon can be offered.

(A) The findings that no hemolysis occurred in RBC–DDC mixtures had first been incubated with $Co^{2+}$ prior to the addition either of $Cu^{2+}$ or $Cd^{2+}$ (Figures 1 and 3) suggest that $Co^{2+}$ blocked the interaction of DDC with copper. The capacity of $Co^{2+}$ to block the interaction either of $Cu^{2+}$ or of

$Cd^{2+}$ with DDC (Figures 1 and 3) and to inhibit hemolysis is an important observation, suggesting that DDC–cobalt complexes might protect cells against the toxicity of cadmium (see below).

(B) The findings that hemolysis, either by DDC : $Cu^{2+}$ : $Co^{2+}$ or by mixtures of DDC with $Cd^{2+}$, was significantly inhibited under anaerobic conditions (flushing with $N_2$) (Figure 4), implicate the involvement of still undefined reactive oxygen species in hemolysis. These findings seem to contradict those published by Agar *et al.*,[8] who suggested that DDC : copper-induced hemolysis is not associated with oxidative processes. Instead, these authors, who induced anaerobiosis in RBC suspensions by using a tonometer, stated that "under these conditions (in the cup of the tonometer), the extent of lysis by DDC–copper mixtures was somewhat less than that observed in similar incubations in test tubes". It appears, therefore, that the level of anaerobiosis obtained under these conditions might have been insufficient. This did not justify the statement that oxidative mechanisms were not essential for hemolysis. Refuting the possibility that ROS might be involved in DDC : $Cu^{2+}$ hemolysis, Agar *et al.*[8] offered a different explanation for this phenomenon. They suggested that an amphipathic DDC : metal complex accumulated at the organic : aqueous interface and that this led to altered membrane permeability which resulted in colloid osmotic lysis. These apparently controversial results remain to be resolved (see below).

(C) The probable involvement of ROS in hemolysis is also supported by the observation that, in addition to the creation of anaerobic conditions (Figure 4), hemolysis induced by DDC : $Cu^{2+}$ : $Co^{2+}$ was strongly inhibited by the reducing antioxidants, thiols, ascorbate, vitamin E and by TEMPO Table I. On the other hand, it is surprising why, except for TEMPO, all these antioxidants failed to inhibit hemolysis induced by DDC : $Cd^{2+}$. It is also intriguing why none of the classical antioxidants (catalase, DMTU, histidine, selenite, benzoate) had the capacity to inhibit hemolysis induced either by DDC : $Cu^{2+}$ : $Co^{2+}$ or by DDC : $Cd^{2+}$ (Table I). The inability of these antioxidants to affect hemolysis might not be related to their membrane permeating capacities. DMTU is known to freely diffuse through membranes and if OH was involved in hemolysis intracellularly, it was expected that DMTU would inhibit hemolysis.

(D) To explain the possible involvement of ROS in hemolysis induced by DDC : $Cu^{2+}$ : $Co^{2+}$ and DDC : $Cd^{2+}$, the following argument might also be valid: Since combinations of copper and reducing agents (ascorbate, GSH) can deplete molecular oxygen from the solution,[13] its removal can explain why hemolysis is inhibited. On the other hand, Figure 4 shows that anerobiosis significantly blocked hemolysis by DDC : $Cd^{2+}$ even when very low amounts of metal were used (less than 5 μM), suggesting that hemolysis by cadmium might proceed even in the absence of molecular oxygen. This indicates a different mechanism of action on RBC. Another way to explain the cytolytic effect of DDC–copper chelate is that copper interacts with certain amino acids in a site-specific manner which becomes susceptible to ROS.[14,15]

The assumption that DDC in its reduced state is necessary to promote hemolysis is based on the findings: (1) that treatment with hydrogen peroxide totally abolished its hemolysis-promoting effect (Table III and Figure 5); (2) that peroxide combined with copper changed the CV patterns, where the main anodic wave changed and totally disappeared; and (3) that a strong alteration of the absorbance of DDC at 280 nm occurred, especially following treatment with combinations of peroxide and copper Table III.

If ROS are involved in membrane damge leading to hemolysis, then it might have been expected that peroxidation products should be detected following treatment of RBC with DDC : $Cu^{2+}$ : $Co^{2+}$ complexes. While neither TBARS nor KI-reactive products (hydroperoxides) were detected either in RBC ghosts or in an intralipid preparation rich in phospholipids following

exposure to DDC alone, large amounts of hydropeoxides (Figure 6) and TBARS (not shown) were produced by copper alone but less so by mixtures of DDC with copper and cobalt. It is, however, intriguing why $Cd^{2+}$, which is the most potent initiator of DDC-induced hemolysis (Figure 2), failed to induce any peroxidation products either in ghosts or in intralipid (Figure 6). This further strengthens the assumption that hemolysis by DDC : $Cd^{2+}$ might proceed by a mechanism not requiring oxygen. Also, a recent publication[16] has suggested that DDC toxicity towards thymocytes involves a copper-catalyzed conversion to thiuram disulfide, which oxidized GSH in a redox cycle and did not release reactive oxygen species. This brings into question the absolute role of oxidants in RBC hemolysis in our systems, unless other components such as spectrin, an important cytoskeletal structure, were also oxidized (see Ref. [24]). This, however, could not be detected by the methods employed. This apparent paradox remains to be resolved.

It is intriguing, however, that while neither the metal chelators, desferal nor phenanthroline had significant inhibitory effects on DDC : $Cu^{2+}$ : $Co^{2+}$-induced hemolysis tetracycline (also a known metal chelator) significantly inhibited hemolysis (Table I). These findings might be due to the low affinity of both desferal and phenanthroline for the metals tested.

Since the role played by ROS in DDC–metals induced hemolysis is still unclear, can this process perhaps be explained by the activation by DDC–copper–cobalt complexes of membrane-associated $PLA_2$ which might permeabilize the membrane? Our findings that hemolysis induced by DDC–metal complexes was inhibited by BrPACBr (a non-specific $PLA_2$ inhibitor), by the non-penetrating $PLA_2$ inhibitor, CME[10] (not shown) and by the anti-hemolytic agents, PC, cholesterol and trypan blue[17,18](Table I), support this possibility. On the other hand, since there is a claim that unlike in sheep RBC, human RBC do not show a measurable $PLA_2$ activity, the involvement of this enzyme in hemolysis is also not clear.

The mechanisms by which treatment of RBC with AAPH enhanced hemolysis by DDC + metals are still not fully understood. AAPH was found to hemolyze RBC,[19] which was inhibited either by hypoxia or by 5-aminoalicylic acid but not to involve either a significant peroxidation of RBC lipids or of oxidation of proteins. It appears that the pretreatment of RBC membranes by subtoxic amounts of AAPH, as described by us, depleted GSH, resulting in the weakening of the membrane which was then permeabilized by oxidants generated by DDC : $Cu^{2+}$ : $Co^{2+}$.

Finally, the role of DDC as a metal chelator and as an antidote to cytotoxicity exerted by $Cd^{2+}$ should be considered.[20–26] It was proposed[24] that the hemolytic activity of $Cd^{2+}$ is related to the unique properties of the cytoskeleton of erythrocytes, which are largely determined by spectrin, the major component of the membrane. It was found that thiols protected spectrin in its tetrameric form from $Cd^{2+}$. Moreover, when RBC membranes were treated by $Cd^{2+}$, the spectrin tetramer was converted to a dimer, while $Zn^{2+}$ had no such an effect. A similar mechanism might also occur during hemolysis by DDC–copper chelates, which might involve oxyhemoglobin-catalyzed glutathione depletion and methemoglobin production.[27]

Taken together, our findings that on a molar basis, $Cd^{2+}$ was by far more effective than either $Cu^{2+}$, $Co^{2+}$, $Fe^{2+}$ or $Ni^{2+}$ in inducing DDC-hemolysis (Figure 2) and that DDC–cobalt complexes blocked the toxic effects of copper and cadmium (Figure 3), suggest that such complexes might be useful for treating metal intoxications.

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