Biochemical Pharmacology, Vol. 41, No. 6/7, pp. 985–993, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

HEMOLYTIC AND MICROBICIDAL ACTIONS OF DIETHYLDITHIOCARBAMIC ACID

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(*Received* 2 *June* 1990; *accepted* 18 *September* 1990)

Abstract—Micromolar concentrations of diethyldithiocarbamic acid (DDC) kill fungi, bacteria and malaria. DDC forms chelates with copper and the microbicidal effectiveness of this drug is enhanced greatly by small amounts of copper. DDC, in the presence of at least 1 molar equivalent of copper, also causes lysis of human erythrocytes. To explore the cytocidal actions of DDC and copper, we have used human erythrocytes and *Escherichia coli* as models. We found that: (1) the combination of DDC and copper also lysed *E. coli* spheroplasts, suggesting a possible common mechanism of hemolytic and microbicidal action; (2) higher ratios of drug:metal (>4:1) diminished hemolytic and, as observed earlier, microbicidal effects; (3) cobalt, known to suppress the microbicidal effects of DDC:Cu, also prevented red cell lysis; (4) despite the necessary involvement of copper in DDC-mediated hemolysis, there was no evidence of oxidative damage to erythrocytes, and both lysis of erythrocytes and killing of *E. coli* were undiminished in the absence of oxygen; (5) the DDC:Cu chelate preferentially located in organic solvents and in membranes of erythrocytes. The chelate was quite soluble in chloroform but much less so in a C-16 hydrocarbon (hexadecane) which resembled erythrocyte membrane lipid. In hexadecane and at $>10^{-4}$M DDC and 5×10^{-5}M copper, an amphipathic drug:metal complex accumulated at the organic:aqueous interface; and (6) this amphipathic complex may permeabilize the lipid bilayer, causing leakage of ions and cell water and eventuating in colloid osmotic lysis. Red cells and *E. coli* exposed to the chelate showed early loss of intracellular rubidium ($^{86}Rb^+$). Furthermore, lysis of erythrocytes and *E. coli* spheroplasts was suppressed by the inclusion of either dextran or sucrose. Thus, it appears that DDC:Cu chelates are cytocidal by virtue of concentrating in the lipid bilayer and, perhaps, forming amphipathic complexes which disrupt membrane integrity. Drugs with similar behavior hold promise for therapy of malaria because metals capable of forming such complexes may accumulate within parasitized red cells.

The microbicidal effects of diethyldithiocarbamic acid (DDC§) and the closely related compound, dimethyldithiocarbamic acid, have been known for several decades [reviewed in Ref. 1]. The antimicrobial actions of both drugs are synergistically magnified by copper. More recently, DDC has been found to be effective against *Plasmodium falciparum* in culture [2], and antimalarial action is also greatly enhanced by small amounts of copper [3]. These dithiocarbamates bind copper and certain other metals such as cobalt. It is thought that copper chelates may form in the ratios of 1:1–2:1 (drug:metal) and that the chelates represent the active form of the drugs [1]. The chelation of limited amounts of trace metals by DDC and dimethyldithiocarbamate may underlie the unusual phenomenon of "concentration quenching." For example, in the presence of a low concentration of copper, small amounts of dimethyldithiocarbamate readily kill *Aspergillus niger*, but increasing drug concentrations actually spare the fungi [1, 4]. This may be due to the fact that a drug:metal chelate of low ratio is most toxic whereas the addition of increasing concentrations of drug leads to the formation of less toxic chelates having a higher drug:metal ratio.

At present, the mechanisms responsible for the antimalarial and microbicidal effects of these chelators, especially when present as copper chelates, are unknown. We have found recently that micromolar amounts of Cu^{2+} and DDC cause lysis of human red cells, whereas neither agent alone exerts any hemolytic effect [3]. This observation suggested that the hemolytic, antimalarial and microbicidal effects of DDC:Cu may be exerted through a single mechanism. Therefore, we have carried out a series of experiments, using the human erythrocyte as the primary model, in an attempt to elucidate the basis of the cytocidal actions of DDC:Cu.

MATERIALS AND METHODS

Erythrocytes. Heparinized venous blood was obtained from normal adults following informed consent. The plasma was removed and the erythrocytes were washed three times in 172 mM (isotonic) Tris (tris hydroxymethyl aminomethane) buffer, pH 7.4, at 25°.

Erythrocyte lysis. Stock solutions of DDC, copper

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§ Abbreviations: DDC, diethyldithiocarbamic acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; GSH, reduced glutathione; and DTNB, 5,5′-dithio-bis-nitrobenzoic acid.

(as $CuSO_4$), and cobalt (as $CoCl_2$)—either 3 or 100 mM—were prepared in isotonic Tris buffer daily. Tris was employed because this buffer has a weak binding affinity for metals and may help prevent the incidental precipitation of copper on cell surfaces and as insoluble copper salts. Washed erythrocytes were diluted to an hematocrit of 10 vol. % in isotonic Tris buffer. Small volumes of stock DDC were added to the red cells, followed by small volumes of copper or cobalt. This order of addition was followed invariably. In most cases, lysis of the erythrocytes was assessed spectrophotometrically by measuring at 542 nm the concentration of hemoglobin in appropriate dilutions of the centrifugal supernatant (2000 *g* for 5 min) of the cell suspension. Percentage lysis was calculated with reference to the measured concentration of hemoglobin in the total incubation mixture. Lysis of dilute suspensions of red cells (hematocrit 0.01 to 0.1 vol.%) was determined by following spectrophotometrically the loss of scattering at 600 nm. To determine whether lysis of the cells involved oxygen-driven reactions, limited experiments were carried out in which the target cells, suspended in isotonic Tris buffer as above, were first deoxygenated through equilibration with 100% nitrogen in an IL 237 blood gas tonometer. Deoxygenated solutions of DDC and copper were then added anaerobically to these cell suspensions.

Prelytic changes in erythrocyte membrane permeability. RBC membrane permeability was estimated by measuring the rate of release of rubidium ($^{86}Rb^+$) (a potassium analogue) from erythrocytes exposed to sublytic concentrations of DDC and copper as described above. Briefly, 2 mL of washed red cells were preincubated in a shaking water bath for 1 hr at 37° in an equal volume of a buffer consisting of Tris (20 mM), NaCl (150 mM) and glucose (10 mM), pH 7.4, at 37°, containing 2 μCi/mL $^{86}Rb^+$. The cells were then quickly washed thrice in ice-cold isotonic Tris buffer, suspended in the same buffer to hematocrit 10 vol.%, and exposed for various periods to DDC (30 μM) and copper (30 μM). At the indicated times, 1-mL samples were removed and rapidly centrifuged (12,000 *g* for 60 sec). One hundred microliters of the supernatant was added to 900 μL of 10% trichloroacetic acid (TCA) and a deproteinized extract was prepared by centrifugation (2000 *g* for 10 min). The extent of release of $^{86}Rb^+$ was determined by measurement of the radioactivity in 500 μL of the supernatant. Results were adjusted for $^{86}Rb^+$ released through trace amounts of lysis which occurred during these brief incubations (not more than 1.3% of the total red cells) and for the small amount of $^{86}Rb^+$ spontaneously released before the start of the experiment (<1% of total radioactivity). Because considerable lysis ensued later in such experiments, only short-term measurements of $^{86}Rb^+$ release could be made.

Oxidation of erythrocyte membrane lipids. The extent of membrane oxidation was estimated by the accumulation of thiobarbituric acid reactive substances (TBARS). These are actually a mixture of aldehydic by-products of the peroxidation of unsaturated fatty acids. Following incubations of erythrocytes with DDC:Cu, protein-free extracts were prepared by the addition of 1 vol. of 15% trichloroacetic acid containing 0.375% (w/v) thiobarbituric acid and 0.25N HCl as described earlier [5]. Following immediate centrifugal removal of precipitate (15 min at 10,000 *g*) the supernatants were heated to 100° for 15 min and the concentration of TBARS was estimated spectrophotometrically.

Erythrocyte reduced glutathione. Concentrations of reduced glutathione (GSH) were measured by the method of Prins and Loos [6]. Briefly, erythrocyte proteins were precipitated by the sequential addition of 4 vol. of sulfuric acid (0.08 N) followed by 1 vol. of 0.3 M sodium tungstate. Precipitated proteins were sedimented by centrifugation (2000 *g* for 10 min), and the concentrations of GSH in the clear supernatant were determined by reaction with 5,5′-dithio-bis-nitrobenzoic acid (DTNB).

Methemoglobin levels. Spectrophotometric determinations on dilutions of stroma-free erythrocyte lysates prepared in 20 mM phosphate buffer, pH 6.0, were performed as previously reported [7]. Absorbances of dilute lysates were measured at four wavelengths (500, 510, 542 and 577 nm), and the concentration of methemoglobin was calculated from these absorbances.

Erythrocyte membrane partitioning of the DDC:Cu chelate. Red cell membranes prepared from washed erythrocytes were preincubated at 37° and hematocrit = 10 vol.% with 30 μM Cu^{2+} and various concentrations of DDC. Following 1 hr of incubation, the remaining intact cells and cell membranes were sedimented by centrifugation at 12,000 *g* for 20 min and percentage lysis was determined by measurement of the hemoglobin concentration in the supernatant. The residual cells and membranes were then washed (12,000 *g* for 20 min) twice in 9 vol. of isotonic Tris buffer, pH 7.4, containing 0.1% bovine serum albumin (to remove any DDC:Cu complex not associated with cell membranes). The remaining intact cells and cell membranes were then lysed and washed (12,000 *g* for 20 min) three times in 9 vol. of 20 mM Tris buffer, pH 7.8. The copper content of the membranes and initial supernatant fractions was determined by addition, to 500 μL of packed membranes (*ca.* 1 mg total protein) or supernatant, of 400 μL isotonic Tris, pH 7.4, 100 μL of 100 mM DDC and 1.0 mL of chloroform, followed by violent agitation. The concentration of DDC:Cu chelate in the organic phase was determined by measurement of optical density at 434 nm. Control incubations (containing only isotonic Tris buffer, 30 μM Cu^{2+} and equivalent concentrations of DDC but without added erythrocyte membranes) were carried out to ensure that incidental precipitation of the insoluble DDC:Cu chelates was not interfering with these determinations. Copper concentration was calculated from the measured millimolar absorption coefficient of chloroform solutions of DDC:Cu (>3:1 ratio of drug:metal) at 434 nm (10.8 $mM^{-1}cm^{-1}$).

Organic:Aqueous partitioning of DDC:Cu chelates. The extent of partitioning was determined by mixing 2.0 mL of an aqueous 172 mM Tris buffer solution containing the indicated amounts of DDC and copper with 1 vol. of either chloroform or hexadecane. The concentrations of copper in the aqueous and organic phases were estimated by the

addition of 1.0 mL of 172 mM Tris buffer (to 1.0 mL of the organic phase) or 1.0 mL of chloroform (to 1.0 mL of the aqueous phase). In both cases, 100 μL of 100 mM DDC was added and, following extensive mixing, the copper (now present as a DDC:Cu chelate in the organic phase) was measured spectrophotometrically at 434 nm.

In the case of hexadecane-containing phase mixtures (consisting of 2 mL hexadecane and 8 mL of 172 mM Tris buffer containing either 180 μM:60 μM or 360 μM:120 μM DDC:Cu), the concentrations of interfacial (amphipathic) DDC:Cu chelate were estimated by centrifuging the mixture in a horizontal rotor at 10,000 *g* for 15 min. Following centrifugation, precisely 1.5 mL of supernatant hexadecane and 7.5 mL of subnatant aqueous phase were removed carefully. The copper concentrations in organic, aqueous and interfacial fractions were then measured as described above.

Redox activity of DDC:Cu chelates. The catalytic redox activity was determined by measuring the rate of copper-enhanced oxidation of ascorbic acid. DDC and Cu^{2+} were added to a solution of 60 μM ascorbic acid in 50 mM Tris buffer, pH 7.4. The rate of ascorbate oxidation was monitored spectrophotometrically at 265 nm. Loss of optical density at this wavelength reflects the copper-catalyzed oxidation of ascorbic acid [8].

Microbicidal actions of DDC:Cu chelates. Escherichia coli (LE 392(+) strain) [9] were grown overnight in static cultures in brain heart infusion, washed in isotonic NaCl containing 20 mM Tris, pH 7.4, and resuspended to a final concentration of approximately 2×10^6 organisms/mL. *E. coli* were then equilibrated with either air or 100% nitrogen in an IL 237 blood gas tonometer. Following complete equilibration with the gas, small volumes of previously deoxygenated DDC and Cu^{2+} solutions were added under continuing gas flow. Numbers of surviving organisms were estimated by plating appropriate dilutions of the bacterial suspension on nutrient agar (GIBCO). Colonies were enumerated with an Artek Auto Count plate counter. Spheroplasts (*E. coli* lacking external membrane and cell wall) were prepared from *E. coli*, grown as above, according to the technique of Osborn *et al.* [10]. Spheroplasts were suspended to a final concentration of 2.5×10^8 organisms/mL in 150 mM NaCl, 10 mM Tris, pH 7.4. Lysis of spheroplasts was assessed by monitoring spectrophotometrically the change in turbidity of cell suspensions at 600 nm.

Bacterial membrane permeability. Alterations in permeability were assessed by preincubation of 10 mL of *E. coli* (10^9 organisms/mL) for 15 min at 25° in phosphate-buffered saline with glucose (10 mM), pH 7.4, at 37°, containing 10 μCi $^{86}Rb^+$. Following rapid washing in ice-cold phosphate-buffered saline, the organisms were resuspended to 10^8/mL and the extent of $^{86}Rb^+$ release was estimated as described for erythrocytes (see above).

RESULTS

Mechanisms of hemolytic effect. The addition of Cu^{2+} and DDC to washed human erythrocytes caused substantial lysis of the cells (Fig. 1). The lysis began almost immediately and progressed in linear fashion for at least 1 hr under these conditions. This lysis requires that both copper and DDC be present; neither agent alone has any lytic effect. Just as in microbicidal systems [1, 4], the ratio of DDC:Cu is crucial; maximal hemolytic potency was achieved at a drug:metal ratio of between 2:1 and 3:1. In fact, the addition of excessive amounts of DDC (yielding drug:metal ratios >4:1) actually decreased the extent of hemolysis of erythrocytes (Fig. 1). Interestingly, hemolysis of erythrocytes was blocked by the addition of cobalt (Fig. 2), a maneuver which also diminishes the microbicidal actions of the closely related drug, dimethyldithiocarbamate [1].

The lytic effect of DDC:Cu evidently depends upon drug-mediated entry of the chelate into the cell membrane. Investigations of the organic:aqueous partitioning of the drug:metal complexes indicated that, at increasing concentrations of DDC, copper

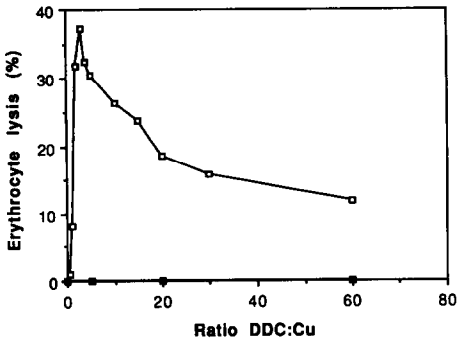

Fig. 1. Lysis of human erythrocytes exposed to 30 μM Cu^{2+} (as $CuSO_4$) or DDC (90 μM) alone (■) or 30 μM Cu^{2+} and increasing concentrations of DDC, from 0 to 1800 μM (□). Washed erythrocytes, hematocrit = 10 vol.%, were suspended in isotonic Tris buffer and incubated with DDC:Cu for 60 min (see Materials and Methods for additional details).

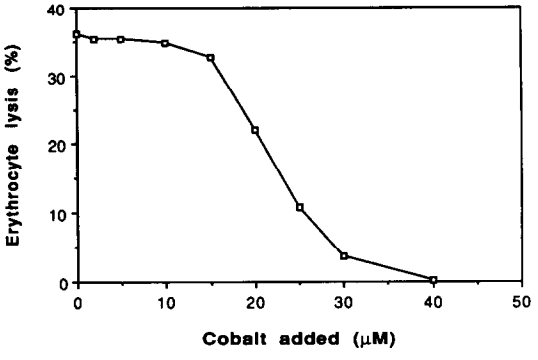

Fig. 2. Suppression of DDC:Cu-dependent erythrocyte lysis by cobalt. Red cells, hematocrit = 10 vol.%, were incubated with 30 μM Cu^{2+} (as $CuSO_4$), 60 μM DDC and the indicated concentrations of added cobalt (as $CoCl_2$) for 60 min at 37°.

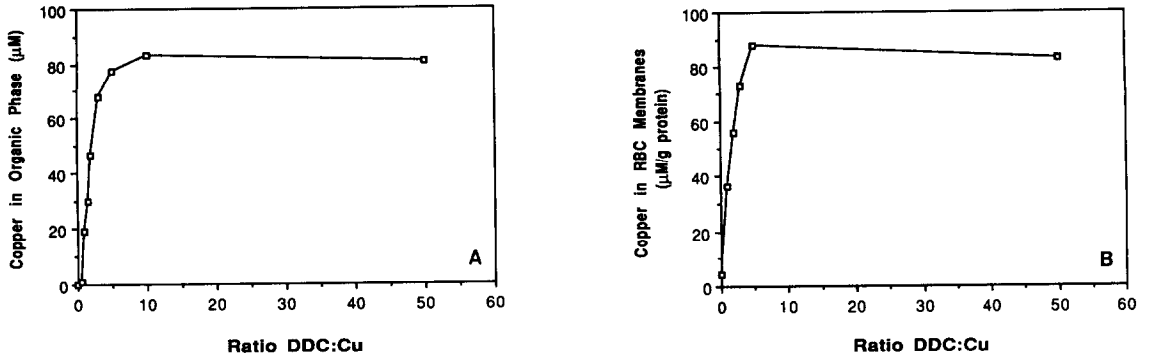

Fig. 3. DDC-mediated solvation of copper in an organic solvent (chloroform) and erythrocyte membranes. In (A), 1 mL of 172 mM Tris buffer containing 90 μM Cu^{2+} (as $CuSO_4$) and increasing amounts of DDC was vigorously shaken with 1 mL of chloroform. Concentrations of copper in the organic phase were determined as described under Materials and Methods. In (B), hemoglobin-free erythrocyte membranes (2 mg/mL total protein) were prepared from intact erythrocytes previously incubated with 30 μM Cu^{2+} and increasing amounts of DDC. Amounts of membrane-associated Cu^{2+} were determined as described under Materials and Methods.

Table 1. Indices of erythrocyte oxidation following incubation of cells with 30 μM DDC and 30 μM Cu^{2+} for 1 hr at 37°

Sample	TBARS* (nmol/mg protein)	GSH (μmol/g Hb)	MetHb (% of total)
Control	5.0/6.6	5.7/6.1	<1%
+Cu^{2+}	5.8/6.7	5.1/5.9	<1%
+DDC	5.6/5.0	6.1/6.4	<1%
+Cu^{2+} + DDC	5.0/4.5	4.9/5.4	<1%

Values are means of duplicate determinations derived from two experiments employing different red cell donors.
* Thiobarbituric acid reactive substances.

was preferentially transferred into organic solvents such as chloroform (Fig. 3A). There was a similar DDC-dependent solvation of copper in erythrocyte membranes (Fig. 3B). This enhanced association of copper with the erythrocyte membrane probably represents a drug-mediated transfer of the metal (probably as 2:1 DDC:Cu chelates) to the lipid bilayer.

The above results indicate that the principle of hemolytic action of DDC:Cu likely involves the translation of the metal chelate into the lipid bilayer of the erythrocyte membrane. Because transition metals such as copper will greatly accelerate the oxidation of unsaturated fatty acids, thiols and hemoglobin, we suspected that oxidative events might underly the hemolytic effects of DDC:Cu. However, we were unable to detect signs of oxidative effects of the chelate: (1) Following 60 min of incubation with 30 μM DDC:30 μM Cu (1:1) (a concentration which, as shown in Fig. 1, caused modest hemolysis), there was no detectable generation of thiobarbituric acid reactive substances (Table 1). (2) Similarly, erythrocytes incubated up to 60 min with 30 μM DDC:Cu also showed no significant change in GSH concentrations (Table 1). (3) After a 60-min incubation of erythrocytes with 30 μM DDC:Cu, no detectable oxidation of hemoglobin occurred (i.e. methemoglobin content of the cells remained less than 1% of total hemoglobin) (Table 1). (4) Likewise, similar incubations caused no change in the activity of red cell membrane calcium ATPase (Ca^{2+}-ATPase), an enzyme known to be readily inhibited through oxidation of one or more sensitive sulfhydryl groups (results not shown). (5) Measurements of copper-catalyzed oxidation of ascorbic acid indicated that DDC:Cu chelates in ratios above 2:1 to 3:1 were not oxidatively active. Thus, as shown in Fig. 4, although copper alone is an effective catalyst of ascorbate oxidation, the addition of DDC in ratios exceeding 1:1 (DDC:Cu) progressively lessened copper-driven ascorbate oxidation, suggesting that, at least in an aqueous milieu, the DDC-bound copper is catalytically inactive. (6) Perhaps the strongest evidence against the involvement of oxidative processes in the hemolytic effects of DDC:Cu derived from experiments in which erythrocytes were exposed to the chelate while deoxygenated. Complete exclusion of oxygen from the system (through equilibration of the cell suspension with 100% nitrogen in a blood gas tonometer) had absolutely no effect upon the

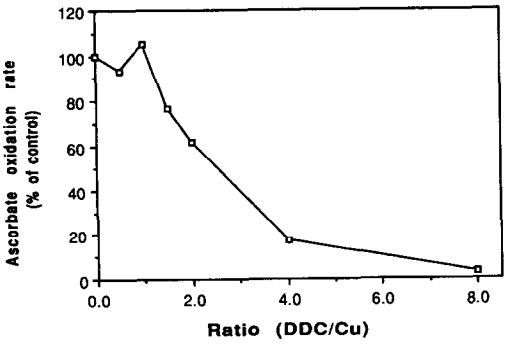

Fig. 4. Rate of oxidation of ascorbic acid catalyzed by 1 μM Cu^{2+} in the presence of increasing amounts of added DDC. Results are expressed as a percentage of maximal rate of ascorbate oxidation in the presence of 1 μM Cu^{2+} (under these conditions, 3.3 μM ascorbate oxidized/min). Control rate of ascorbate oxidation in the absence of added Cu^{2+} was <0.06 μM/min, equivalent to the results obtained with the highest ratio of added DDC.

Table 2. Lysis of erythrocytes incubated with Cu^{2+} (30 μM) and DDC (90 μM) for 30 min under air or nitrogen

Experiment	Conditions	Lysis (%)
1	Aerobic	15/16
	Anaerobic	20/21
2	Aerobic	17/18
	Anaerobic	16/21

Results are duplicate determinations from two experiments employing red cells from different donors. Lysis in the absence of DDC:Cu was <1%.

extent of hemolysis caused by DDC (90 μM final concentration) and copper (30 μM final concentration) (Table 2). Note that under these conditions (in the "cup" of a tonometer), the extent of lysis at these DDC:Cu concentrations was somewhat less than that observed in similar incubations in test tubes (see, for example, Fig. 1).

The results of additional experiments suggest that the hemolytic action of DDC:Cu may arise from non-oxidative disruption of membrane barrier function. As shown in Fig. 5A, the addition of 20 mM dextran (molecular weight ~7900 daltons) to red cells simultaneously incubated with hemolytic concentrations of DDC:Cu suppressed lysis of the cells. It should be noted that the inclusion of dextran did not diminish the amounts of copper accumulated by the red cell membranes in these experiments (not shown). Sucrose (300 mM) similarly blocked DDC:Cu-induced hemolysis (Fig. 5A), suggesting that the chelate does not greatly increase membrane permeability to solutes of molecular weight >300 daltons.

Direct evidence for a pre-lytic increase in erythrocyte membrane permeability upon incubation with DDC and Cu^{2+} was provided by experiments with erythrocytes pre-loaded with $^{86}Rb^+$. Upon subsequent short-term incubation in buffer alone, these red cells extruded only a small fraction of intracellular $^{86}Rb^+$. However, cells incubated with DDC:Cu showed an accelerated early loss of $^{86}Rb^+$ which occurred in advance of most hemolysis (Fig. 6).

Mechanism of microbicidal action. In *E. coli*, the presence of a cell wall precludes simple lytic experiments such as were carried out with human red cells (*vide supra*). However, enzymatic removal of the cell wall with lysozyme yielded still viable, intact bacterial spheroplasts capable of osmotic lysis. When *E. coli* spheroplasts were exposed to DDC:Cu (15 μM:15 μM), lysis rapidly ensued just as it does in the case of human red cells. This lysis was also blocked by the addition of either dextran (20 mM) or sucrose (150 mM) (Fig. 5B).

The bactericidal effects of DDC were also found to be independent of oxygen. As shown in Table 3, the addition of DDC:Cu (30 μM:30 μM) to *E. coli* resulted in killing of approximately 70% of the organisms during a 30-min incubation at 37°. Complete deoxygenation of the bacterial suspension prior to addition of DDC:Cu caused no diminution in the bactericidal effects of the chelate (Table 3). Note that, in these experiments, some loss of viable bacteria occurred during incubation due to the lack of nutrients in the suspending medium (isotonic NaCl/Tris).

To determine whether the bactericidal action of DDC:Cu may involve the same increase in membrane ion permeability as was observed with erythrocytes, *E. coli* were preloaded with $^{86}Rb^+$. Loss of intracellular $^{86}Rb^+$ was then assessed following exposure to DDC:Cu. As shown in Fig. 7, there was enhanced efflux of intracellular $^{86}Rb^+$ from those organisms exposed to DDC:Cu. However, this loss was not quite as rapid as that from DDC:Cu-exposed erythrocytes (Fig. 6), and $^{86}Rb^+$ loss from untreated controls was substantial.

DDC:Cu may form a lytic amphipathic complex. In an attempt to define the nature of the lytic effects of DDC:Cu, we studied the behavior of the chelate in hexadecane, an organic phase thought to closely resemble that of normal lipid bilayer (in terms of solubility properties, apolarity and transverse diffusion rates [11]). In contrast to chloroform, the solubility of the DDC:Cu chelate in hexadecane was quite limited. Whereas very high concentrations of DDC:Cu can be solvated in chloroform (>10^{-2} M), the limit of solubility of the chelate in hexadecane would appear to be <10^{-4} M (Fig. 8). When concentrations of DDC:Cu well above the limit of solubility were mixed with an organic phase of hexadecane (C-16), almost all of the complex was withdrawn from the aqueous subnatant. However, the majority of the DDC:Cu complex laid, as a turbid, insoluble gel, at the organic:aqueous interface. Figure 9 shows the distribution of copper in fractions obtained by sampling the contents of tubes containing phase-separated mixtures of isotonic Tris buffer, hexadecane, and two concentrations of DDC:Cu (180 μM:60 μM and 360 μM:120 μM respectively). The aqueous fractions contained almost no detectable copper. The hexadecane supernatant contained roughly 80–90 μM at both

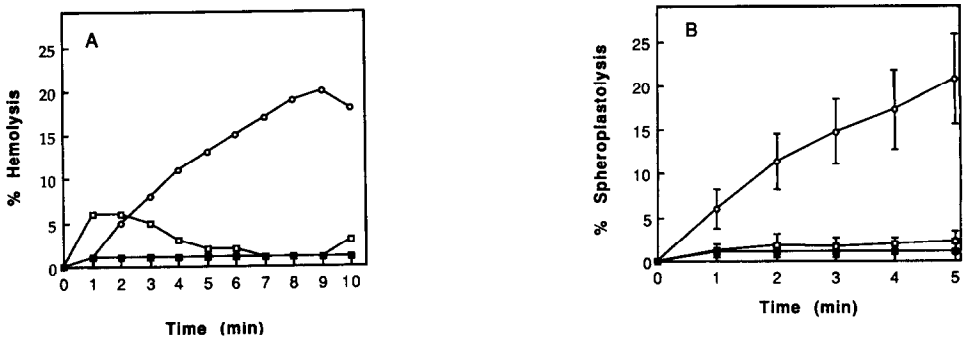

Fig. 5. (A) Lysis of erythrocytes incubated with 60 μM Cu^{2+} and 180 μM DDC (○) and with added 300 mM sucrose (□), or 20 mM dextran (■) (nominal molecular weight = 7900 daltons). (B) Lysis of *E. coli* spheroplasts incubated with 15 μM DDC and 15 μM Cu^{2+} (○) and with added 150 mM sucrose (□), or 20 mM dextran (■).

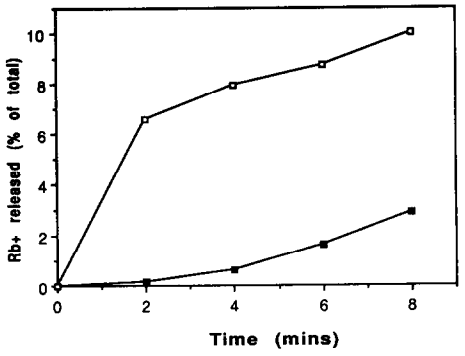

Fig. 6. Release of $^{86}Rb^+$ from erythrocytes pre-equilibrated with the isotope and then incubated in 172 mM Tris buffer with (□) and without (■) 30 μM Cu^{2+} and 30 μM DDC. Values are the results of a single experiment. Note that only minimal lysis occurred in the sample incubated with DDC:Cu (1.2% vs 0.4% in the control) and that the expected release of Rb^+ secondary to this lysis was subtracted from the results shown. Total initial intracellular $^{86}Rb^+ = 1.4 \times 10^6$ cpm/mL.

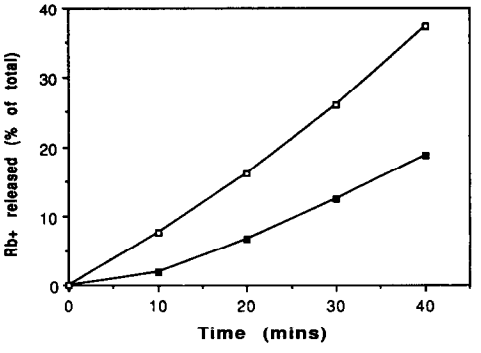

Fig. 7. Efflux of $^{86}Rb^+$ from *E. coli* during a 40-min incubation in the presence (□) and absence (■) of 30 μM DDC and 30 μM Cu^{2+}. Total initial intracellular $^{86}Rb^+ = 8.6 \times 10^5$ cpm/10^9 bacteria.

Table 3. Killing of *E. coli* incubated for 30 min with DDC:Cu (30 μM:30 μM) under aerobic and anaerobic conditions

Sample	Atmosphere	Viable *E. coli*/mm^3
Initial suspension		2458 ± 34
No addition	Aerobic	1902 ± 46*
	Anaerobic	2047 ± 61*
+DDC:Cu	Aerobic	563 ± 90†
	Anaerobic	535 ± 4†

Results shown are the means (±1 SD) of triplicate determinations.

* Differs from initial suspension, $P < 0.01$ (Student's *t*-test).

† Differs from corresponding incubations without DDC:Cu, $P < 0.001$ (Student's *t*-test).

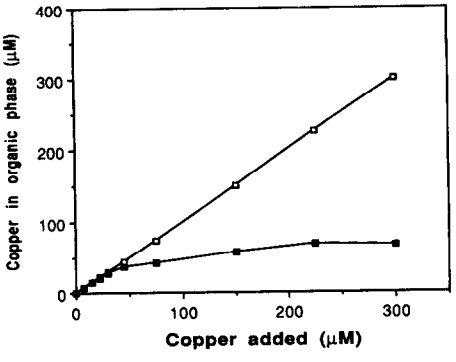

Fig. 8. Comparison of the solubility of DDC:Cu chelates in either chloroform (□) or hexadecane (■). The indicated concentrations of Cu^{2+} (as $CuSO_4$) were added in the presence of an excess (10 mM) of DDC. Following violent agitation and separation of the phases, the Cu^{2+} concentration in the organic phase was determined as described under Materials and Methods.

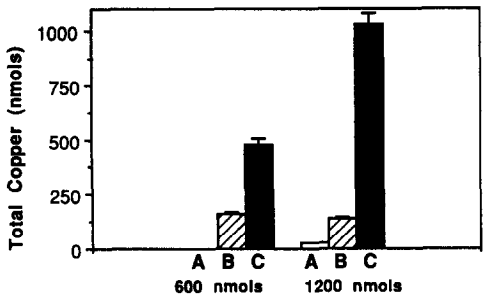

Fig. 9. Aqueous (A), organic (B) and interfacial (C) copper content in a mixed phase system. Copper concentrations within a tube containing an aqueous:organic mixture comprised of 8 mL of 172 mM Tris buffer, pH 7.4, and 2 mL hexadecane containing DDC:Cu (180 μM:60 μM and 360 μM:120 μM). Following thorough mixing, the tube was centrifuged at 2000 *g* and the Cu^{2+} content of each fraction was determined as described under Materials and Methods. Note that results are expressed as total Cu^{2+} in each phase. The vertical lines denote one standard deviation (N = 4).

lower and higher amounts of added DDC:Cu. However, the organic:aqueous interface, the expected site of deposition of amphipathic compounds, contained very large amounts of the DDC:Cu chelate.

DISCUSSION

The microbicidal effectiveness of DDC and several other metal-chelating compounds (including dimethyldithiocarbamate and 8-hydroxyquinoline) has been known for some time. Albert [1] has presented an excellent review of the characteristics of these compounds. Not only are these agents potent broad-spectrum microbicides, with both fungicidal and bactericidal actions, but they also have been shown to be antimalarial when tested against cultured *P. falciparum* [2, 3, 12].

It is thought that these drugs may exert toxic effects via the formation of lipophilic metal chelates (principally copper chelates in the case of DDC and dimethyldithiocarbamate, and iron chelates in the case of 8-hydroxyquinoline). One unique feature of these agents is so-called "concentration quenching," whereby the addition of supra-effective concentrations of the drug actually spares organisms which would be killed at lower doses. This has been explained on the basis of changes in the solubility of the drug:metal complexes such that >2:1 (drug:metal) chelates may have unfavorable solubility characteristics and be unable to cross membranes. Once the drug (or drug:metal chelate) enters the target, inhibition of enzymes of microbial glycolysis has been suggested as the lethal event in both fungi [13–15] and malaria [2]. However, direct proof of this proposition has not been forthcoming and the mechanisms of antimicrobial action have not been shown definitively.

We found that DDC:Cu chelates caused lysis of red cells and of *E. coli* spheroplasts. At least in the case of erythrocytes, lysis by DDC:Cu cannot be explained by inhibition of glycolysis, because human red cells will remain intact despite total metabolic depletion. Lysis of erythrocytes and *E. coli* required both copper and DDC; neither DDC nor copper, at low concentrations, had a measurable effect whereas DDC:Cu chelates (in ratios of apparently up to 3:1, drug:metal) were lytic. The hemolytic potency of DDC:Cu chelates is at least partially explicable on the basis of membrane solvation of the drug:copper complex. Thus, we found that the amount of copper associated with erythrocyte membranes was increased greatly when one or more molar equivalents of DDC were added to the system. In keeping with the phenomenon of "concentration quenching" mentioned above, the lysis of red cells and *E. coli* spheroplasts by DDC:Cu was actually lessened by the addition of more free drug. However, the simple organic solvation of a DDC:metal chelate cannot explain the lytic effects; although cobalt:DDC is non-lytic, the chelate (probably 2:1) was quite soluble in chloroform and hexadecane (not shown). Furthermore, the protection of red cells and *E. coli* against destruction by higher DDC concentrations cannot simply be explained by diminished organic solvation of the metal (see, for example, Fig. 1 vs Fig. 3). Rather, this paradoxical effect of high DDC:Cu ratios may be explained by more subtle changes in the long-range structure of the polymeric, amphipathic complexes formed by the chelate.

Although free copper is well known to exert oxidative effects in biological systems, we found no evidence that the lysis of red cells by DDC:Cu involves oxidative reactions. In particular, there was no detectable oxidation of cytoplasm (i.e. hemoglobin and GSH) or membrane (unsaturated fatty acid). Furthermore, although free copper catalyzes rapid oxidation of ascorbic acid, the addition of DDC actually suppressed this catalysis. In fact, the copper-catalyzed oxidation of cysteamine is similarly inhibited by DDC [16]. Perhaps most convincingly, the hemolytic effects of DDC:Cu proceeded unabated even in the complete absence of oxygen. We should note the apparent conflict between these observations and an earlier report [17] that DDC *per se* causes glutathione and hemoglobin oxidation. However, the concentrations of DDC employed in this earlier work (~10 mM) were far in excess of those which we have used.

Despite the absence of detectable oxidation, both red cells and *E. coli* exposed to sub-lytic concentrations of DDC:Cu did show accelerated loss of intracellular ions ($^{86}Rb^{+}$), perhaps reflecting chelate-induced increases in membrane permeability. Indeed, red cells suspended in high molecular weight dextran or even in sucrose-containing solutions were much less susceptible to lysis by DDC:Cu than were erythrocytes in isotonic sodium buffer. This blockade of DDC:Cu-mediated lysis by both dextran and sucrose is strong support for the proposition that the chelate causes enhanced ion and water permeation of the cell membrane, eventuating in colloid osmotic lysis of the red cells. Such impermeant solutes will oppose the secondary influx of water into the cells, which ordinarily leads to cell swelling and lysis.

There are several similarities between the hemolytic and bactericidal actions of DDC:Cu.

First, both occurred as readily under anaerobic as aerobic conditions. Second, the addition of cobalt blocked DDC:Cu-mediated hemolysis and has been shown previously to prevent the microbicidal effects of DDC:Cu [1]. Third, both the hemolytic and bactericidal actions of DDC:Cu showed evidence of "concentration quenching," wherein larger amounts of drug (in the presence of a constant amount of metal) actually spared the target. Fourth, shortly after exposure to DDC:Cu, both red cells and *E. coli* showed exaggerated loss of intracellular $^{86}Rb^{+}$. Although this loss differed kinetically between erythrocytes and *E. coli*, it does indicate that loss of membrane integrity is involved in both cases. Finally, DDC:Cu was lytic for *E. coli* spheroplasts as well as human erythrocytes.

The principle of lytic and, perhaps, microbicidal, action may involve the formation of high molecular weight amphipathic complexes of DDC and Cu^{2+} which cause irreparable disruption of membrane integrity. This is suggested by the observation that DDC:Cu had relatively limited solubility in hexadecane, a simple C-16 hydrocarbon with solubility and polarity properties resembling those of the lipid bilayer of red cells [10]. We found that when DDC:Cu was added to a two-phase aqueous:hexadecane system, most of the chelate localized at the phase interface, existing there primarily as a turbid mass, soluble in neither organic nor aqueous phases. A similar event would be expected when DDC:Cu is added to dilute suspensions of either bacteria or erythrocytes, especially because the actual volume of cell lipid is very much less than the total cell volume. The formation of such high molecular weight amphipathic complexes within the membrane of target cells would have obvious deleterious effects upon the barrier function of the cell membrane. These effects could arise, as has been suggested for the hemolytic agent, chlorpromazine, through the formation of a drug-rich zone at the center of the bilayer [17]. Alternatively, by localizing at the interface between the membrane and aqueous fractions, the insoluble DDC:Cu chelate may cause vertical disruption of the bilayer. In fact, this latter possibility is favored by calculations which indicate that, at hemolytic concentration of DDC:Cu, the mole fraction of the chelate is <10% of the red cell lipids. These calculations are based on the measurement of membrane-associated DDC:Cu and volume estimates previously used by Lieber *et al.* [18] for chlorpromazine intercalation into erythrocyte membranes.

Overall, our results support the following conclusions regarding the mechanism(s) of microbicidal, hemolytic and, perhaps, antimalarial action of DDC:Cu chelates. (1) The toxic effects of DDC upon target cells or microorganisms are likely mediated by the formation of drug:copper chelates. In the case of red cell lysis, the most effective chelate forms when 1:1 to 3:1 ratios of DDC:Cu are present, whereas ratios of up to 2:1 exert maximal bactericidal effect. (2) The DDC:Cu chelate is preferentially solvated not only in pure organic solvents such as chloroform but also concentrates in the membrane of target cells. (3) Once within the cell membrane, the limited solubility of the chelates in the lipid bilayer may lead to the formation of amphipathic complexes. (4) Finally, these insoluble complexes, concentrated within the cell membrane, disrupt the normal impermeability of the lipid bilayer.

It is possible that these principles of drug action may extend to other therapeutic agents which act through the mechanism of drug-induced insertion of metal chelates into biological membranes. Drugs of this sort may be particularly promising for therapy of malaria, because parasite-mediated degradation of host cell metalloproteins such as hemoglobin [19] and copper/zinc superoxide dismutase [20] may lead to the deposition of substantial amounts of metals available for the formation of such toxic chelates.

Acknowledgements—This work was supported in part by grants to J.W.E. from the National Institutes of Health (R01 AI-25625 and R01 ES-04748). The authors wish to thank Ms. Diane Konzen for help in manuscript preparation and Drs. Theodore Steck and Steven Meshnick for helpful discussions.

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