Role for an Episulfonium Ion in *S*-(2-Chloroethyl)-DL-cysteine-Induced Cytotoxicity and Its Reaction with Glutathione†

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ABSTRACT: The cysteine S conjugate of 1,2-dichloroethane, *S*-(2-chloroethyl)-DL-cysteine (CEC), is hepatotoxic, nephrotoxic, and mutagenic. To determine the cellular and chemical mechanisms involved in CEC-induced toxicity and to assess the role of an episulfonium ion, the effect of CEC on the viability of isolated rat hepatocytes was studied. CEC addition resulted in both a time- and concentration-dependent loss of cell viability. Depletion of intracellular glutathione concentrations (>70%) and inhibition of microsomal Ca^{2+} transport and Ca^{2+}-ATPase activity preceded the loss of cell viability, and initiation of lipid peroxidation paralleled the loss of viability. The depletion of glutathione concentrations was partially attributable to a reaction between glutathione and CEC to form *S*-[2-(DL-cysteinyl)ethyl]glutathione, which was identified by NMR and mass spectrometry. *N*-Acetyl-L-cysteine, vitamin E, and *N*,*N*′-diphenyl-*p*-phenylenediamine protected against the loss of cell viability. *N*,*N*′-Diphenyl-*p*-phenylenediamine inhibited CEC-initiated lipid peroxidation but did not protect against cell death at 4 h, indicating that lipid peroxidation was not the cause of cell death. The analogues *S*-ethyl-L-cysteine, *S*-(3-chloropropyl)-DL-cysteine, and *S*-(2-hydroxyethyl)-L-cysteine, which cannot form an episulfonium ion, were not cytotoxic, thus demonstrating a role for an episulfonium ion in the cytotoxicity associated with exposure to CEC and, possibly, 1,2-dichloroethane. These results show that an alteration in Ca^{2+} homeostasis and the generation of an electrophilic intermediate may be involved in the mechanism of cell death.

Although glutathione *S*-transferase dependent metabolism of xenobiotics has long been associated with detoxication reactions, glutathione *S*-transferase dependent bioactivation reactions have only recently been reported: Chloro- and fluoroalkene-induced nephrotoxicity is associated with glutathione S-conjugate formation followed by renal bioactivation (Anders et al., 1986; Elfarra & Anders, 1984), and glutathione *S*-transferase catalyzed sulfur mustard (β-haloethyl sulfide) formation has been implicated in the bioactivation of vicinal diahaloethanes (Inskeep et al., 1986; Koga et al., 1986; Livesey et al., 1982; Working et al., 1986).

1,2-Dichloroethane (EDC)[1] is used as a lead scavenger in gasoline, as an industrial solvent, and as a grain fumigant and is both hepatotoxic and nephrotoxic and a suspected animal carcinogen. Both cytosolic glutathione *S*-transferases and microsomal cytochrome P-450 dependent mixed-fraction oxidases metabolize EDC to reactive intermediates that become covalently bound to proteins and nucleic acids (Guenergich et al., 1980; McCann et al., 1975; Shih & Hill, 1981), but recent studies indicate that glutathione *S*-transferase dependent activation, rather than mixed-function oxidase-dependent activation, is responsible for the toxicity associated with EDC exposure (Inskeep et al., 1986; Koga et al., 1986; Ozawa & Guengerich, 1983; Working et al., 1986). *S*-(2-Chloroethyl)glutathione, the expected metabolite formed by reaction of glutathione with EDC, did not react with plasmid pBR322 DNA, but the corresponding cysteine conjugate *S*-(2-chloroethyl)-DL-cysteine (CEC) did induce DNA strand breaks (Vadi et al., 1985). CEC, which is a putative metabolite of EDC formed by the further metabolism of *S*-(2-chloroethyl)glutathione, has been implicated as the reactive intermediate involved in EDC-induced nephrotoxicity (Elfarra et al., 1985) and mutagenicity (Rannug et al., 1978; van Bladeren et al., 1979).

Because CEC is a putative metabolite of EDC and is readily accessible synthetically, it was chosen for study as a model sulfur mustard. The objective of the investigation was to study the cytotoxicity of CEC in isolated rat hepatocytes and to examine the cellular and chemical mechanisms by which CEC exerts its cytotoxic effects.

Materials and Methods

S-(2-Chloroethyl)-DL-cysteine hydrochloride, *S*-(3-chloropropyl)-DL-cysteine hydrochloride, and *S*-(2-hydroxyethyl)-DL-cysteine were prepared as previously described (Elfarra et al., 1985). *S*-Ethyl-L-cysteine, α-tocopherol acetate, glutathione, glutathione disulfide, and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Acetyl-L-cysteine and *N*,*N*′-diphenylphenylenediamine (DPPD) were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Eastman Kodak Co. (Rochester, NY), respectively. $^{45}Ca^{2+}$, as aqueous calcium chloride, was obtained from New England Nuclear Co. (Boston, MA). All other chemicals were of reagent grade and were obtained from commercial sources. A23187, the calcium ionophore, was a generous gift of Dr. W. Fields (Eli Lilly and Co., Indianapolis, IN).

Hepatocytes were prepared from male Long–Evans rats (Charles River Breeding Laboratories, Kingston, NY)

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[1] Abbreviations: CEC, *S*-(2-chloroethyl)-DL-cysteine; CPC, *S*-(3-chloropropyl)-DL-cysteine; DPPD, *N*,*N*′-diphenyl-*p*-phenylenediamine; EDC, 1,2-dichloroethane; SCEG, *S*-[2-(DL-cysteinyl)ethyl]glutathione; LDH, lactate dehydrogenase; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

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weighing 225–300 g according to a previously described procedure (Moldéus et al., 1978). Viability of hepatocytes was determined by trypan blue exclusion, by release of lactate dehydrogenase (LDH) into the medium, and by succinate-stimulated oxygen consumption (Bauer et al., 1975). Only preparations exhibiting ≥90% trypan blue exclusion and ≤10% stimulation in the rate of oxygen consumption after succinate (5.0 mM) addition were used. All incubations were performed with a hepatocyte concentration of 2.0×10^6 cells/mL under a 95% O_2/5% CO_2 atmosphere at 37 °C. Microsomal fractions were obtained after the isolated hepatocytes were washed in cold (4 °C) homogenization buffer [80 mM KCl, 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) dipotassium salt, 5.0 mM NaN_3, and 1.0 mM $MgCl_2$, pH 6.8] by disrupting the cells with six strokes of a zero-clearance, Potter–Elvehjem homogenizer (Kontes, Vineland, NJ) or by three 8-s bursts of a Brinkmann polytron (Westbury, NY). Cell homogenates were centrifuged at 9000*g* for 20 min, and the supernatant was centrifuged at 100000*g* for 35 min. The microsomal pellet was resuspended to 1.5–6.0 mg of protein/mL with homogenization buffer Protein concentrations were measured according to the method of Lowry et al. (1951) with bovine serum albumin as the standard.

LDH activity was measured spectrophotometrically by determining the increase in NADH formation with L-lactate and NAD^+ as the substrates, according to Sigma Lactate Dehydrogenase Technical Bulletin 226-UV (Sigma Chemical Co., St. Louis, MO). Intracellular glutathione concentrations were determined according to a previous method (Hissin & Hilf, 1976). In some experiments, the hepatocyte concentrations of thiols, disulfides, mixed disulfides, and protein mixed disulfides were measured by HPLC according to the methods of Reed et al. (1980) and Meredith (1983). Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reacting substances, according to the method of Stacey et al. (1980). Microsomal Ca^{2+} transport was measured as previously described (Webb & Anders, 1985). Ca^{2+}-ATPase activities were measured under identical conditions as the Ca^{2+} transport studies, according to the procedures of Dawson and Fulton (1983) and Lanzetta et al. (1979).

The adduct formed between CEC and glutathione was synthesized by adding an aqueous solution of glutathione (50 mM) over 15 min to an aqueous solution of CEC (50 mM); the mixture was heated in a water bath at 37 °C with shaking for an additional 15 min. The reaction mixture was derivatized with 1-fluoro-2,4-dinitrobenzene and analyzed by HPLC according to the methods of Reed et al. (1980) and Meredith (1983). The peaks corresponding to the adduct were collected, and the eluent was lyophilized.

^1H NMR spectra were obtained with an IBM WP-270-SY instrument with a 5-mm tube at a probe temperature 23.5 °C. The 2,4-dinitrophenyl derivative of SCEG was dissolved in [$^2H_4$]methanol (1 mg/mL) containing a small amount of tetramethylsilane as an internal standard. Chemical shifts are reported in ppm from tetramethylsilane. Spectra were acquired with a 4000-Hz spectral width, a 3-μs pulse width, and a 2-s repetition time. The residual $^1H^2HO$ signal at 4.7–4.9 ppm was attenuated by continuous irradiation with the decoupler.

The negative-ion LSIMS analysis was performed with a Kratos (Manchester, U.K.) MS-5OS mass spectrometer equipped with a 23-kG magnet, a Cs^+ primary beam gun, and a postacceleration detector (Fallick et al., 1986). The temperature was ambient. The 2,4-dinitrophenyl derivative of SCEG was mixed with glycerol prior to placing the sample on the target. The spectrum was recorded over the range of m/z 200–900.

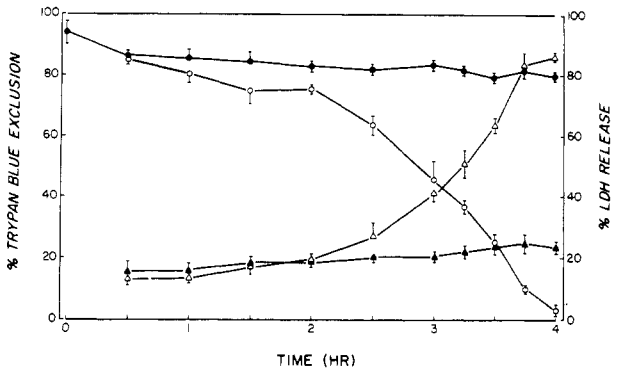


FIGURE 1: Time course of *S*-(2-chloroethyl)-DL-cysteine (CEC)-induced cytotoxicity in isolated rat hepatocyte suspensions. CEC (7.5 mM) was incubated with hepatocytes (2×10^6 cells/mL). At various times, samples were removed, and the percent trypan blue exclusion and percent lactate dehydrogenase release (LDH) were determined according to Materials and Methods. Trypan blue exclusion of control (●) and CEC-treated (○) hepatocytes; lactate dehydrogenase release from control (▲) and CEC-treated (△) hepatocytes. Values represent the mean ± SE of three experiments.

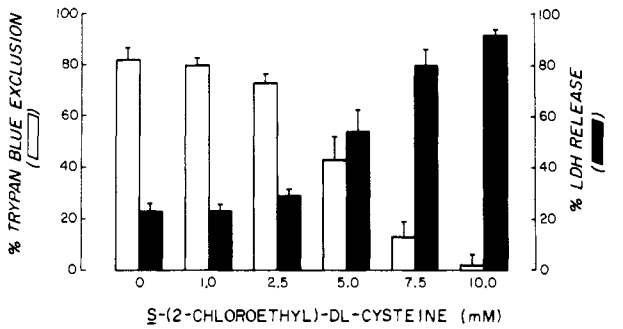


FIGURE 2: Concentration dependency of *S*-(2-chloroethyl)-DL-cysteine (CEC)-induced cytotoxicity. Hepatocytes (2×10^6 cells/mL) were incubated with various concentration of CEC. After 3 h of incubation, samples were removed, and trypan blue exclusion and lactate dehydrogenase release were determined according to Materials and Methods. Values represent the mean ± SE of three experiments.

RESULTS

S-(2-Chloroethyl)-DL-cysteine was cytotoxic to isolated rat hepatocytes, as determined by trypan blue exclusion and LDH release (Figure 1) ; nearly complete cell death was apparent at 3.5–4.0 h. CEC-induced cell death was also concentration dependent (Figure 2). CEC concentrations greater than 2.5 mM resulted in enhanced LDH release and a decrease in the number of cells able to exclude trypan blue at 3 h.

The effect of several agents on CEC-induced cytotoxicity was studied to explore the possible mechanisms involved (Table I). Vitamin E and DPPD, both inhibitors of lipid peroxidation, protected against CEC-induced cytotoxicity. Cysteine, a poor precursor of glutathione (Estrela et al., 1983), did not prevent CEC-induced cytotoxicity, and *N*-acetyl-L-cysteine, which is an effective glutathione precursor (Estrela et al., 1983), partially prevented CEC-induced cytotoxicity. Probenecid, an organic acid transport system inhibitor (Weiner et al., 1960), protected against CEC-induced cytotoxicity, indicating that *S*-(2-chloroethyl)-DL-cysteine may enter the cell via an organic ion transport system.

Incubation of hepatocytes with CEC decreased the intracellular glutathione content to 25% of the control values within 1 h (Figure 3). The loss of glutathione was not accompanied by a leakage of glutathione into the medium, the formation

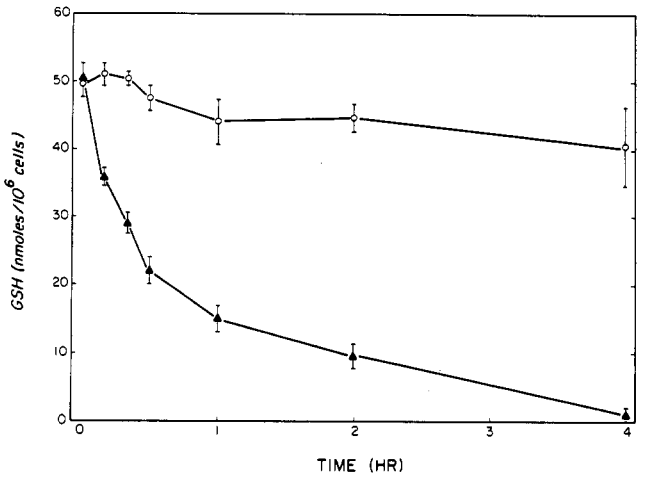


FIGURE 3: Effect of *S*-(2-chloroethyl)-DL-cysteine (CEC) on intracellular glutathione concentrations. Hepatocytes (2×10^6 cells/mL) were incubated with buffer or CEC (7.5 mM). Samples of control (○) and CEC-treated (▲) hepatocytes were removed at various times, and the intracellular glutathione concentration was determined, as described under Materials and Methods.

Table I: Effect of Various Additions on *S*-(2-Chloroethyl)-DL-cysteine-Induced Cytotoxicity[a]

addition	% inhibition of cytotoxicity	
	trypan blue exclusion	LDH release
vitamin E (0.2 mM)	85 ± 1	79 ± 4
L-cysteine (1.0 mM)	5 ± 8	5 ± 7
N-acetyl-L-cysteine (1.0 mM)	54 ± 17	53 ± 21
DPPD (10 μM)	88 ± 6	98 ± 2
probenecid (0.5 mM)	65 ± 7	70 ± 6

[a] Hepatocyte (≃2.0×10^6 cells/mL) suspensions were incubated with the various additions for 10 min before the addition of *S*-(2-chloroethyl)-DL-cysteine (7.5 mM). Hepatocyte viability was assessed after 3.0 h. Trypan blue exclusion of control cells was 81 ± 3%, and 14 ± 11% of the CEC-exposed cells excluded trypan blue. The percent LDH release was 24.0 ± 6% and 81 ± 8% for the control and CEC-treated cells, respectively. Values represent the mean ± SD of three experiments.

of intracellular or extracellular glutathione disulfide, or the formation of protein mixed disulfides (Table II). The failure to observe an increased glutathione concentration in the medium or the appearance of glutathione disulfide within the cell or in the medium and the failure to detect protein mixed disulfides indicated that CEC may deplete glutathione concentrations by forming glutathione adduct. Indeed, HPLC analysis of incubation mixtures revealed a new peak on the HPLC chromatogram (Figure 4D, peak I). This peak had a retention time identical with the chemically synthesized *S*-[2-(DL-cysteinyl)ethyl]glutathione (SCEG) and was found in the medium when *S*-(2-chloroethyl)-DL-cysteine was incubated with hepatocytes. No adduct was detected when *S*-(3-chloropropyl)-DL-cysteine was incubated with hepatocytes. The peak in the chromatogram (Figure 4A) labeled "Glu" may

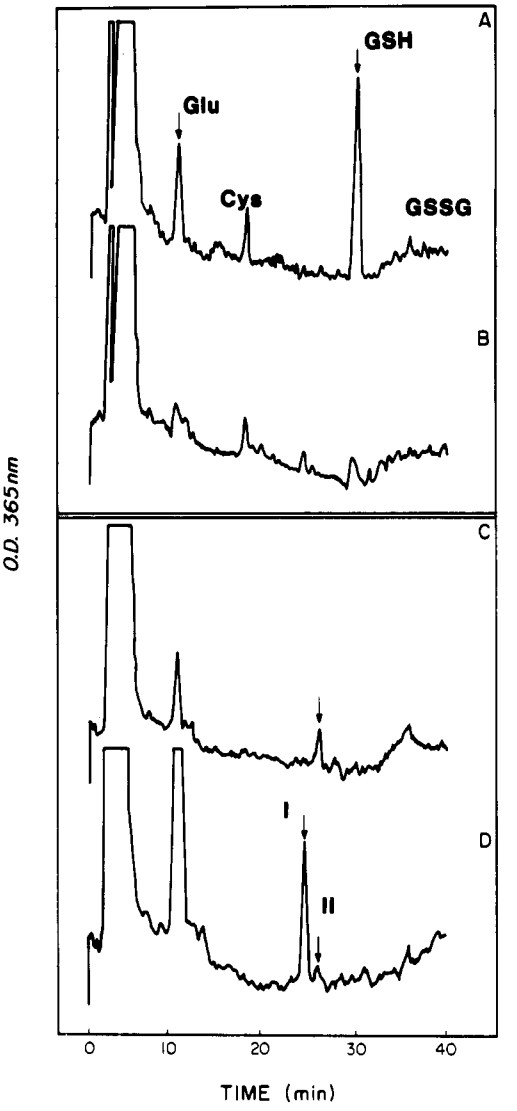


FIGURE 4: HPLC chromatograms of glutathione, glutathione disulfide, and mixed disulfides from control and *S*-(2-chloroethyl)-DL-cysteine (CEC)-treated hepatocytes and their respective incubation media. Cells were incubated with CEC (7.5 mM) for 1 h, and GSH, GSSG, and S conjugates were derivatized and chromatographed, as outlined under Materials and Methods. (Panel A) HPLC chromatogram of control cells; (panel B) HPLC chromatogram of CEC-treated cells; (panel C) HPLC chromatogram of control cell medium; (panel D) HPLC chromatogram of CEC-treated cell medium. I = *S*-[2-(DL-cysteinyl)ethyl]glutathione; II = *S*-cysteinylglutathione. Glu = glutamate; Cys = cysteine; GSH = glutathione; GSSG = glutathione disulfide.

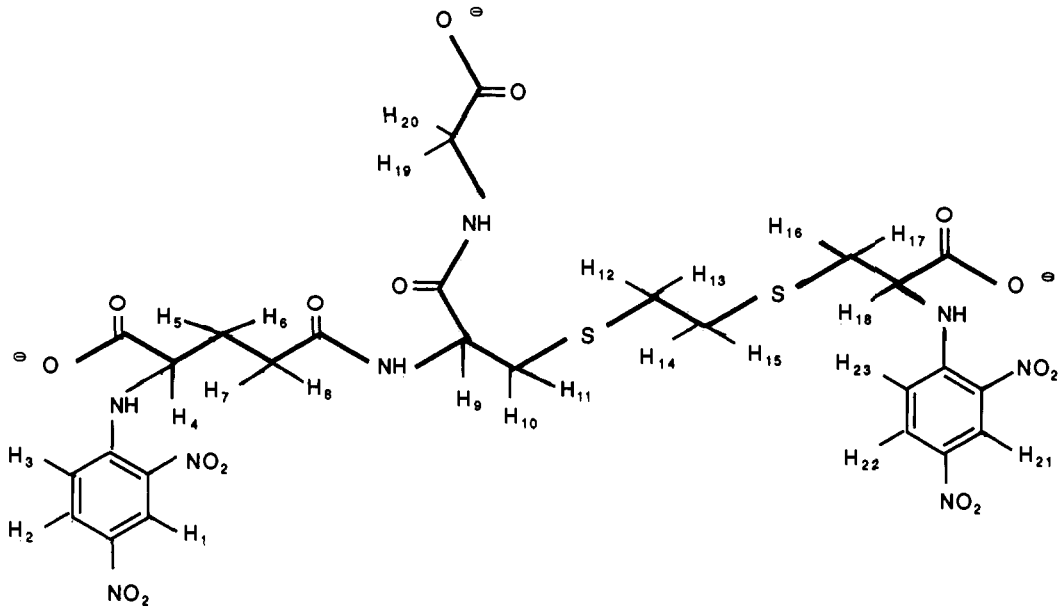
represent glutamate or *S*-(2-hydroxyethyl)-DL-cysteine, the solvolysis product of CEC; both compounds have the same retention time.

The characterization of SCEG was performed after formation of the 2,4-dinitrophenyl derivative and isolation by HPLC. The ^{1}H NMR spectrum indicates that the adduct is

Table II: Effect of *S*-(2-Chloroethyl)-DL-cysteine (CEC) on Glutathione, Glutathione Disulfide, and Protein Mixed-Disulfide Concentrations in Isolated Rat Hepatocytes[a]

	intracellular				extracellular				protein mixed disulfides	
	glutathione		glutathione disulfide		glutathione		glutathione disulfide			
	60 min	120 min	60 min	120 min	60 min	120 min	60 min	120 min	60 min	120 min
control	57.8 ± 14.4	40.2 ± 1.2	6.2 ± 2.1	5.3 ± 1.4	<LD	<LD	8.6 ± 4.0	11.1 ± 6.0	1.1 ± 0.6	0.9 ± 0.5
CEC	11.8 ± 5.9[b]	5.7 ± 2.6[b]	<LD[c]	<LD	<LD	<LD	6.9 ± 3.2	6.5 ± 2.4	1.1 ± 0.9	0.8 ± 0.3

[a] Isolated rat hepatocytes (2×10^6 cells/mL) were incubated with *S*-(2-chloroethyl)-DL-cysteine (7.5 mM) for the times indicated. Values are expressed as nanomoles of glutathione or glutathione disulfide for 10^6 cells and represent the mean ± SD for three different hepatocyte preparations. [b] Significantly different from control, $p < 0.05$. [c] <LD = below limit of detection (<4.2 nmol/10^6 cells).



Proton	Chemical Shift[a]	Multiplicity[b]	Coupling Constants (Hz)
$H_{1,21}$	9.07, 9.06, 9.05	3-s	
$H_{2,22}$	8.31, 8.30, 8.29	3-d	$H_2 - H_3 = 9.5$
$H_{3,23}$	7.17, 7.15	2-d	$H_{22} - H_{23} = 9.5$
H_4	4.64	m	
$H_{5,6}$	2.35	m	$H_4 - H_{5,6} = 5.7$
$H_{7,8}$	2.5	t	$H_{5,6} - H_{7,8} = 6.8$
H_9	4.55	d of d	
$H_{10,16}$	3.0	m	$H_9 - H_{10} = 5.9$
$H_{11,12,13,14,15,17}$	2.8	m	$H_9 - H_{11} - 8.6$
H_{18}	Under DOH peak		
$H_{19,20}$	3.92	s	

FIGURE 5: Structure and ^1H NMR parameters of the 2,4-dinitrophenyl derivative of *S*-[2-(DL-cysteinyl)ethyl]glutathione. The sample was dissolved in [$^2H_4$]methanol. Chemical shifts are downfield from internal tetramethylsilane, which was assigned a chemical shift of 0.0 ppm. The spectral resolution was 0.488 Hz/point. For multiplicity, s = singlet, d = doublet, t = triplet, and m = multiplet.

the 2,4-dinitrophenyl derivative of SCEG. The spectrum shows the presence of the cysteine and glutathione residues as well as the ethylene bridge. The assignments of chemical shifts and coupling constants (Figure 5) are based on ^1H–^1H decoupling experiments and on published spectra of other glutathione and cysteine conjugates (Fennell et al., 1984; Rosevear et al., 1984). CEC is the racemic modification; thus, reaction of CEC with glutathione results in a mixture of diasteroisomers. The presence of a diastereoisomeric mixture is reflected in the ^1H NMR by double peaks for H_{21} and H_{22} (Figure 5).

The negative-ion LSIMS of the isolated, derivatized adduct yielded a pseudomolecular ion $(M-H)^-$ of m/z 785, which is consistent with the expected molecular weight of 786 for the 2,4-dinitrophenyl derivative of SCEG. The spectrum contained major ions derived from the fragmentation of the 2,4-dinitrophenyl derivative of SCEG (Figure 6). Fragments of the 2,4-dinitrophenyl derivative of SCEG appear at m/z 300 and 486 and correspond to cleavage of the ethylene bridge. The glutathione portion undergoes further fragmentation by successive loss of the glycine moiety and decarboxylation to yield fragments of m/z 385 and 341. There was also a significant peak at m/z 791 and smaller peaks at m/z 769 and 807; these were assigned to $[M - H_2O + Na^+]^-$, $[M - OH]^-$, and $[M - H + Na^+]^-$, respectively.

Lipid peroxidation, as measured by MDA formation, was detected in hepatocytes exposed to CEC (Figure 7). The initiation of lipid peroxidation did not begin until 1.5 h after CEC addition, at which time intracellular glutathione concentrations were depleted. When DPPD was included in the

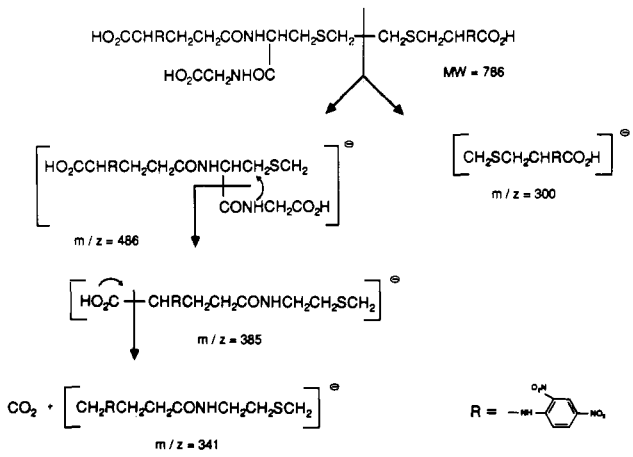


FIGURE 6: Negative ions generated in the LSIMS of the 2,4-dinitrophenyl derivative of *S*-[2-(DL-cysteinyl)ethyl]glutathione dissolved in glycerol.

incubation medium, no lipid peroxidation was detected in the CEC-treated cells, and the viability of cells was maintained for 2.5 h, when the cells began to die at a rate similar to cells treated with CEC alone. DPPD had no effect on the trypan blue exclusion or lipid peroxidation of control hepatocytes (Figure 7).

Alterations in intracellular Ca^{2+} homeostasis, as a result of inhibition of the microsomal Ca^{2+} transport and Ca^{2+}-ATPase activity, have been suggested as a mechanism by which hepatotoxic agents may exert their effects (Ascosta & Sorensen, 1983; Bellomo et al., 1982; Bridges et al., 1983; Trump & Berezesky, 1984). To determine if the microsomal Ca^{2+}-ATPase activity is inhibited by CEC and if this inhibition was associated with cytotoxicity, the Ca^{2+} transport and Ca^{2+}-ATPase activities of microsomal fractions obtained from CEC-treated hepatocytes were measured. CEC inhibited both activities in a time-dependent process (Figure 8); significant inhibition was not observed until ≥2 h.

To determine if an episulfonium ion is involved in the cytotoxicity of CEC, the analogues *S*-(3-chloropropyl)-, *S*-(2-hydroxyethyl)-, and *S*-ethyl-L-cysteine were studied. *S*-Ethyl-L-cysteine is unable to form an episulfonium ion because of the lack of a leaving group in the β position. The hydroxyl group of *S*-(2-hydroxyethyl)-DL-cysteine is a poor leaving group, and, therefore, episulfonium ion formation would be unlikely to occur. *S*-(3-Chloropropyl)-DL-cysteine is unlikely to form an episulfonium ion, because the formation of a four-membered ring is kinetically much less favored than the formation of a three-membered ring (Smit et al., 1979). *S*-(3-Chloropropyl)-, *S*-(2-hydroxyethyl)-, and *S*-ethyl-L-cysteine, at equimolar concentrations with CEC, were not cytotoxic (Table III). These results indicate that the electrophilic episulfonium ion is directly involved in CEC-induced cytotoxicity.

Table III: Effects of Various Cysteine S Conjugates on Hepatocyte Viability[a]

conjugate	trypan blue exclusion (%)	LDH release (% of control)
S-(2-chloro)-DL-cysteine	7.3	89.0
S-ethyl-L-cysteine	82.4	21.5
S-(2-hydroxyethyl)-DL-cysteine	85.3	20.8
S-(3-chloropropyl)-DL-cysteine	80.6	22.3

[a] Hepatocytes were incubated with the S conjugates (7.5 mM) for 3.5 h under a 95% O_2/5% CO_2 atmosphere. Trypan blue exclusion and LDH release from control cells were 81.8% and 20.4%, respectively.

DISCUSSION

CEC was cytotoxic to isolated rat hepatocytes as shown by a concentration- and time-dependent loss of viability. An initial event associated with CEC exposure is the rapid loss of intracellular glutathione; depletion to ≤25% of control values was observed within 1 h. Glutathione disulfide and *S*-cysteinylglutathione concentrations were not elevated after incubation with CEC, and there was no increased concentration of protein mixed disulfides. In addition, the rapid depletion of glutathione is not a result of lipid peroxidation, because lipid peroxidation is not detectable until 1.5 h after CEC addition. Hence, the formation of an adduct between CEC and glutathione may be responsible for the loss of glutathione; this hypothesis was validated by the identification of SCEG in the extracellular medium. The presence of SCEG, which is presumably formed intracellularly, in the medium may be explained by lysis of damaged cells and release of SCEG into the medium or by active transport of the conjugate from viable cells. Rat liver canalicular membrane vesicles (Inoue et al., 1984) and rat hepatocyte plasma membrane fractions (Nicotera et al., 1985) both contain transport systems that may function in the cellular extrusion of glutathione S conjugates.

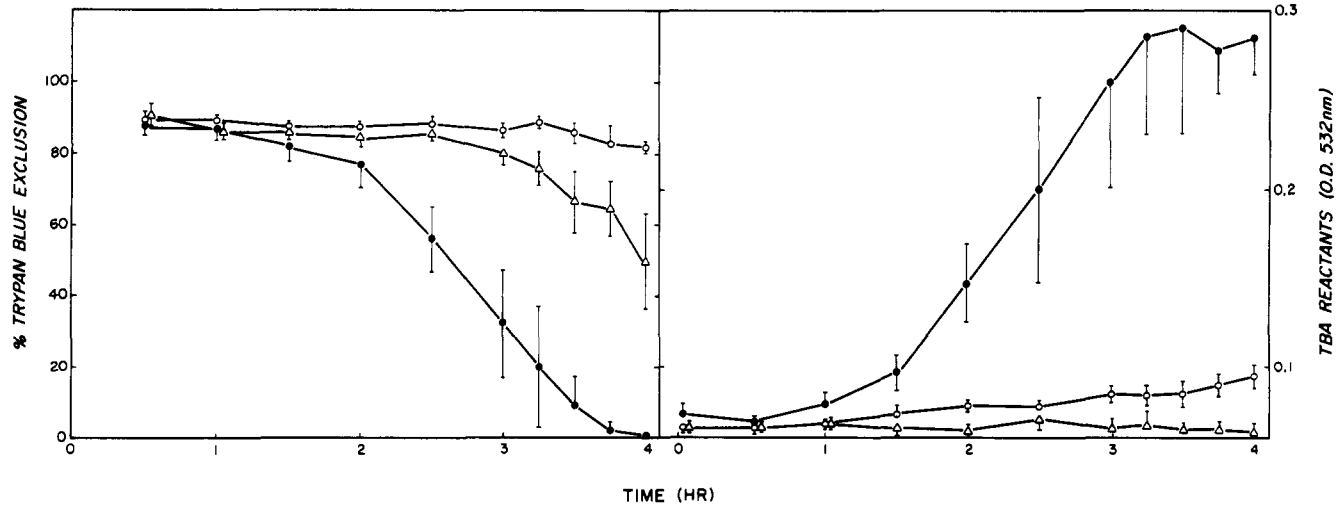


FIGURE 7: Effect of *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) on the time course of *S*-(2-chloroethyl)-DL-cysteine (CEC)-induced cytotoxicity and lipid peroxidation. Hepatocytes (2×10^6 cells/mL) were incubated with CEC (7.5 mM) in the presence or absence of DPPD (10 μM). Samples were removed at various times, and trypan blue exclusion (left panel) and lipid peroxidation (right panel) were measured, as described under Materials and Methods. Control (○), CEC (●), and CEC plus DPPD (△). Values represent the mean ± SD of three experiments.

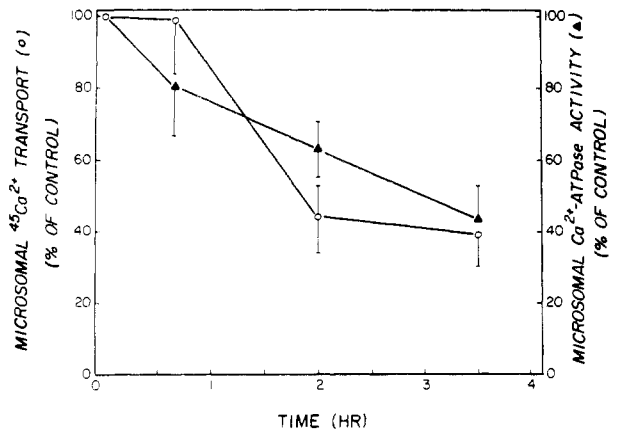


FIGURE 8: Effect of *S*-(2-chloroethyl)-DL-cysteine (CEC) on microsomal Ca^{2+} transport (○) and Ca^{2+}-ATPase (▲) activity. Hepatocytes (2×10^6 cells/mL) were incubated with buffer or CEC (7.5 mM), and microsomal fractions were prepared at various times. Values represent the mean ± SD of three experiments.

N-Acetyl-L-cysteine, a precursor of glutathione, protected against the cytotoxicity of CEC; L-cysteine, which is a poor glutathione precursor due to its rapid metabolism to thiopyruvate, did not protect against CEC-induced cytotoxicity.

Lipid peroxidation is not evident until 1.5 h after CEC addition, indicating that lipid peroxidation may be a consequence of glutathione depletion. Decreases in cellular glutathione concentrations have been associated with increases in lipid peroxidation (Anundi et al., 1979; Reiter & Wendel, 1982; Younes & Siegers, 1981, 1983, 1984; Younes et al., 1984). Despite the inhibition of lipid peroxidation by vitamin E and DPPD, accelerated cell death, compared to control cells, was observed in CEC-treated hepatocytes. Therefore, lipid peroxidation is not a causal event in CEC-induced cytotoxicity but may hasten the process leading to cell death. Similarly, Beales et al. (1985) found that CaEDTA blocked lipid peroxidation but did not protect cells from acetaminophen-induced cell damage; others have also concluded that lipid peroxidation, although seen, is not necessarily the key event in xenobiotic-induced cell death (Younes & Siegers, 1984; Smith et al., 1983).

The endoplasmic reticulum Ca^{2+} transport system is an important component in the maintenance of a low intracellular Ca^{2+} concentration (Becker et al., 1980; Murphy et al., 1980), and alterations in Ca^{2+} homeostasis may result from the inhibition of the Ca^{2+} pump by many hepatotoxins. Perturbations in intracellular Ca^{2+} concentrations are implicated in the pathogenesis of cell injury and cell death (Bellomo et al., 1982; Farber, 1981; Trump & Berezesky, 1984). CEC inhibited microsomal Ca^{2+} transport and Ca^{2+}-ATPase activity, and inhibition of Ca^{2+} transport and Ca^{2+}-ATPase activity preceded cytotoxicity, indicating that Ca^{2+} transport inhibition may be an important event in cell death. The mechanism of inhibition of the Ca^{2+} transport and Ca^{2+}-ATPase activity may involve protein alkylation by the proposed episulfonium ion intermediate or may result from the cellular depletion of glutathione concentrations, as described previously for other hepatotoxic agents (Bellomo et al., 1982; Jones et al., 1983; Moore, 1984; Prasad & Holtzman, 1985; Thor et al., 1982).

The role for an episulfonium ion intermediate in the cytotoxicity of CEC was supported by studies with the analogues *S*-(3-chloropropyl)-, *S*-ethyl-, and *S*-(2-hydroxyethyl)-DL-cysteine. CEC, the only compound studied that is capable of forming an episulfonium ion, was cytotoxic, whereas equimolar concentrations of non-episulfonium ion forming analogues were not cytotoxic. This observation strongly indicates a role for the episulfonium ion in CEC-induced cytotoxicity. Structural confirmation of the CEC–glutathione adduct as SCEG also provides strong evidence for an episulfonium ion intermediate. Although CPC does not readily form an episulfonium ion, nucleophilic displacement of the chlorine atom is possible. The failure to detect a glutathione adduct of CPC indicates that nucleophilic displacement (S_N2) of the chlorine atom by glutathione does not occur. Nucleophilic attack by glutathione on the thioether of *S*-(2-chloroethyl)glutathione followed by anti elimination would be expected to yield ethylene and glutathione disulfide (Livesey et al., 1982); the lack of increased *S*-cysteinylglutathione formation during incubation of glutathione with CEC indicates that this is a minor mechanism.

The present results are consistent with the hypothesis that alkylation of critical cellular targets by reactive intermediates may be an important event in xenobiotic-induced cell death. Further tests of this hypothesis, including investigations into the mechanisms involved in CEC-induced cytotoxicity and into the role of episulfonium ions in cytotoxicity, are warranted. Moreover, such compounds, which do not require bioactivation, may prove to be valuable tools for studying xenobiotic-induced cell death.

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Substrate Specificity of the Flavoprotein Trypanothione Disulfide Reductase from *Crithidia fasciculata*†

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ABSTRACT: The substrate specificity of the trypanosomatid enzyme trypanothione reductase has been studied by measuring the ability of the enzyme to reduce a series of chemically synthesized cyclic and acyclic derivatives of N^1,N^8-bis(glutathionyl)spermidine disulfide (trypanothione). Kinetic analysis of the enzymatic reduction of these synthetic substrates indicates that the mutually exclusive substrate specificity observed by the NADPH-dependent trypanothione disulfide reductase and the related flavoprotein glutathione disulfide reductase is due to the presence of a spermidine binding site in the substrate binding domain of trypanothione reductase. Trypanothione reductase will reduce the disulfide form of N^1-monoglutathionylspermidine and also the mixed disulfide of N^1-monoglutathionylspermidine and glutathione. The Michaelis constants for these reactions are 149 μM and 379 μM, respectively. Since the disulfide form of N^1-monoglutathionylspermidine and the mixed disulfide of N^1-monoglutathionylspermidine and glutathione could be formed in trypanosomatids, the binding constants and turnover numbers for the enzymatic reduction of these acyclic disulfides are consistent with these being potential alternative substrates for trypanothione reductase in vivo.

The tripeptide L-γ-glutamyl-L-cysteinylglycine (glutathione; GSH) is maintained in high concentration within the cells of eukaryotic and prokaryotic organisms and frequently constitutes the major thiol metabolite within these cells. Important general functions of this peptide are thought to include maintenance of the intracellular thiol redox balance (RSH/RSSR), the scavenging of free-radical species, and functioning as a cofactor in hydrogen peroxide metabolism (Meister,

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