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Cysteine Conjugate Toxicity, Metabolism, and Binding to Macromolecules in Isolated Rat Kidney Mitochondria

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SUMMARY

The ^{14}C-labeled, ^{35}S-labeled, and unlabeled nephrotoxic cysteine conjugates *S*-(1,2-dichlorovinyl)-L-cysteine, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine, *S*-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine (PCBC), and *S*-(1,1,2,3,3,3-hexafluoropropyl)-L-cysteine were synthesized and their toxicities were compared in isolated rat renal mitochondria. Inhibition of respiration, covalent binding to macromolecules, metabolism by mitochondria, metabolism by a purified cysteine conjugate β-lyase (β-lyase), and octanol/water partition coefficients were studied. All of the conjugates inhibited mitochondrial state 3 respiration. Only PCBC was found to uncouple oxidative phosphorylation. (Aminooxy)acetic acid, a β-lyase inhibitor, blocked the effects of the conjugates on state 3 respiration except for the uncoupling effect of PCBC, which was not blocked. Binding of ^{35}S label to macromolecules was observed after treatment with each of the ^{35}S-labeled conjugates, and (aminooxy)acetic acid blocked the binding. The relative amounts of metabolism of the conjugates did not correlate well with their relative binding and toxicities, indicating some differential reactivity of metabolites and/or selectivity for binding targets. Some of the binding from ^{35}S-labeled conjugates was removed by treatment with the disulfide-reducing agent dithiothreitol, suggesting that some of the binding was via mixed disulfides. The amount of dithiothreitol-sensitive binding differed among the conjugates. The metabolism of PCBC by permeabilized mitochondria, but not by a purified β-lyase, was consistent with its relative toxicity and covalent binding, suggesting the involvement of other β-lyase enzymes in the activation of PCBC to toxic species in mitochondria.

In the more than 30 years since McKinney *et al.* (1) first discovered DCVC as the toxic factor in trichloroethylene-extracted soybean meal, the potential for the toxicity of cysteine conjugates derived from halogenated ethylene compounds has been widely recognized. A number of investigations have led to the development of a general model for the activation of these compounds to toxic species by the cysteine conjugate β-lyase pathway. Briefly, *in vivo* conjugation of the halogenated vinyl compound with glutathione occurs in the liver through the action of glutathione *S*-transferases (EC 2.5.1.18). Elimination of a reactive thiol-containing fragment from a subsequently formed cysteine conjugate then occurs in the kidney during a shunt from the mercapturic acid pathway. This β-elimination reaction is catalyzed by a renal enzyme known as cysteine conjugate β-lyase (EC 4.4.1.13). Covalent binding of the fragment to cellular macromolecules is presumed to be the cause of the observed nephrotoxicity. Several recent reviews that discuss this and other metabolic transformations of glutathione and cysteine conjugates are available (2–5).

McKinney *et al.* (1) demonstrated the alkylating ability of DCVC in their original report. Shultze and co-workers then demonstrated the binding of radiolabel from ^{35}S-labeled DCVC to protein (6, 7) and DNA (8) and proposed the involvement of a β-elimination reaction catalyzed by PLP (7). Stevens *et al.* (9) found that a major cysteine conjugate β-lyase of rat kidney cytosol and mitochondria is identical to the PLP-dependent enzyme GTK (EC 2.6.1.64). Lock and Ishmael (10) and Vamvakas *et al.* (11) have also demonstrated the binding to protein of ^{14}C label from the cysteine conjugate of ^{14}C-labeled HCBD, a nephrotoxin and nephrocarcinogen. Vamvakas *et al.* (11), Dekant and co-workers (12–14), and Veltman *et al.* (15) have recently elucidated the chemical mechanisms involved in the transformation of α-halothiolate elimination fragments from several toxic cysteine conjugates to thioacylating species.

A considerable amount of evidence implicates mitochondria

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ABBREVIATIONS: DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; PCBC, *S*-(1,2,3,4,4-pentachlorobutadienyl)-L-cysteine; CTFC, *S*-(2-chloro-1,1,2-trifluoroethyl)-L-cysteine; TFEC, *S*-(1,1,2,2-tetrafluorethyl)-L-cysteine; HFPC, *S*-(1,1,2,3,3,3-hexafluoropropyl)-L-cysteine; AOAA, (aminooxy)acetic acid; DTT, dithiothreitol; PLP, pyridoxal phosphate; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TCA, trichloroacetic acid; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; GTK, glutamine transaminase K; HCBD, hexachlorobutadiene; MTB, α-keto-γ-methiolbutyrate; TFA, trifluoroacetic acid; ANOVA, analysis of variance; FAB, fast atom bombardment; MS, mass spectrometry.

as a primary cellular target for cysteine conjugate toxicity. Stonard and Parker (16) first reported the metabolism of DCVC in the soluble fraction of mitochondria along with the inhibition of mitochondrial respiration. After further studies (17), they proposed the 2-oxoacid dehydrogenases as the molecular targets of DCVC toxicity. Wallin and co-workers (18) reported the collapse of the membrane potential and release of sequestered calcium from isolated mitochondria treated with PCBC. Lash and Anders (19) showed similar effects after treatment with DCVC.

Despite the evidence for mitochondria as primary cellular targets and the implied involvement of covalent binding in this proposed model of cysteine conjugate toxicity, binding of cysteine conjugate metabolites to mitochondrial macromolecules has not been investigated. Therefore, to further explore the hypothesis that mitochondria are a primary target for cysteine conjugate toxicity and to investigate the relationship to toxicity of structural features of cysteine conjugates, we investigated the structure-activity relationship for cysteine conjugate metabolism, binding of metabolites, and toxicity in isolated renal mitochondria. To that end, ^{35}S-labeled, ^{14}C-labeled, and unlabeled cysteine conjugates of several toxic chlorinated and/or fluorinated vinyl compounds were synthesized. Structures of the conjugates are shown in Fig. 1. Inhibition of mitochondrial respiration, binding of elimination fragments to mitochondrial macromolecules, metabolism of conjugates by mitochondria, metabolism of conjugates by a purified β-lyase (i.e. GTK), and octanol/water partition coefficients were all examined.

The data demonstrate that each of the conjugates tested was metabolized by mitochondrial enzymes to generate metabolites that bound to macromolecules and inhibited respiration. Qualitative as well as quantitative differences in the binding of metabolites were detected. Additionally, the results indicate that a β-lyase other than GTK may be present in rat kidney mitochondria. These studies suggest that binding of reactive β-elimination fragments to mitochondrial targets may be a common mechanism for the nephrotoxicity of cysteine conjugates activated via the β-lyase pathway.

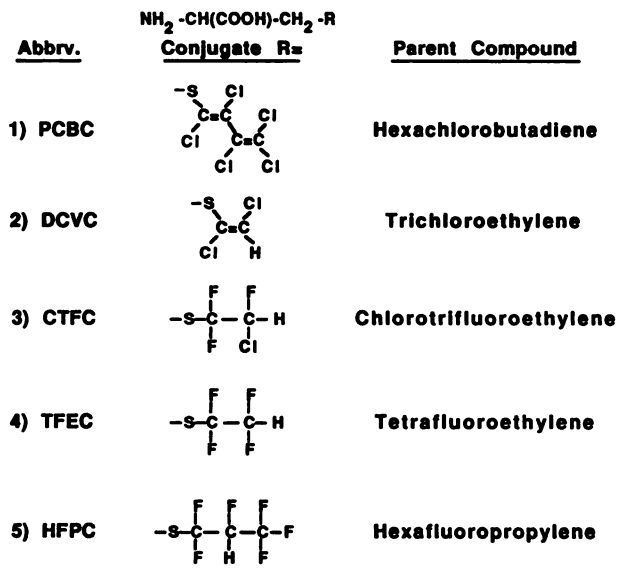

Fig. 1. Structures of synthetic cysteine conjugates.

Materials and Methods

Chlorotrifluorethylene, tetrafluoroethylene, and hexafluoropropylene were purchased from Matheson Gas Products (East Rutherford, NJ) L-[^{35}S]cystine and L-[^{14}C]cystine were purchased from New England Nuclear (Boston, MA). All other chemicals purchased from commerical sources were of at least reagent grade and were used without further purification. Male Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY).

Isolation of mitochondria and respiration experiments. Mitochondria were isolated from rat kidney cortex in buffer containing 70 mM sucrose, 220 mM mannitol, 2 mM HEPES (pH 7.4), 10 mM EGTA, and 0.5 mg/ml bovine serum albumin, following the procedure of Schnaitman and Greenawalt (20).

Mitochondria (approximately 2 mg) were incubated with various cysteine conjugates, but without respiratory substrates, for 1 hr at 25°. The same buffer used for mitochondrial preparations was used for the respiration experiments. Cysteine conjugates were added from a 2.0 mM stock solution made up in respiration buffer. The total volume of the incubation mixture was 1.0 ml. After 1 hr, the incubation mixtures were spun in an Eppendorf microcentrifuge at 16,000 × g for 5 min. The buffer containing the conjugate was then removed and the mitochondrial pellet was resuspended in fresh buffer minus conjugate. Respiration rates were determined by measuring oxygen consumption at 30° in a water-jacketed chamber equipped with a Clark-type oxygen electrode connected to an oxymeter (Yellow Springs Instrument Company, Yellow Springs, OH). To find the state 3 rate, potassium phosphate (10 mM), sodium succinate (5 mM), and rotenone (0.2 mM) (site II) or α-ketoglutarate (5 mM) plus malate (5 mM) (site I) were included in the buffer. ADP (250 nmol) was then added. The state 4 rate was measured after all the ADP had been consumed. Respiratory control values were calculated from the ratio of state 3/state 4 respiration rates.

^{35}S-binding experiments. Isolated mitochondria were incubated with 100 μM ^{35}S-labeled conjugates under conditions identical to the respiration experiments. The incubation was stopped by placing the incubation tubes in an ice-water bath. Macromolecules were precipitated by addition of 2.0 ml of ice-cold 10% TCA. After 10 min at 0–4°, precipitated macromolecules were collected by vacuum filtration on Whatman GF/C filters and rinsed twice with 5 ml of ice-cold 5% TCA. ^{35}S label associated with precipitated macromolecules was considered to be covalently bound. Because no modified adducts were actually isolated and identified, this is an operational definition only. The amount of binding was quantitated by scintillation counting of the radioactivity trapped on the filters. Boiled protein controls were included to determine any nonspecific binding. AOAA (100 μM) was included in some incubations to determine β-lyase specific binding.

In some experiments, the amount of ^{35}S label that could be removed by DTT was determined. Incubations of mitochondria with ^{35}S-labeled conjugates were terminated as described above, followed by addition of 250 μl of 0.1 N KOH and 5 mM DTT. After incubation for 30 min at 25°, macromolecules were precipitated and binding was determined as described above. Control samples treated with base only showed no loss of bound ^{35}S label.

Octanol/buffer partition experiments. Partition coefficients were determined by dissolving ^{14}C-labeled conjugates in 1.0 ml of isolation buffer to a concentration of 100 μM (specific activity, 2.5 × 10^5 dpm/μmol), followed by addition of 1.0 ml of octanol and vigorous mixing. The phases were separated by centrifugation. The amount of ^{14}C label in 100 μl of each phase was determined by liquid scintillation counting. The π values were calculated from the log of the ratio (P) of ^{14}C label found in the octanol phase to that in the aqueous phase.

Purification of cysteine conjugate β-lyase. Cysteine conjugate

β-lyase (GTK) was purified from the kidney cortex cytosol of 50 male Sprague-Dawley rats by the procedure of Stevens *et al.* (9), to a specific activity of 3.66 μmol/10 min·mg, using 1.0 mM DCVC and 5 mM MTB as substrates.

Synthesis of cysteine conjugates. General methods for the synthesis of radiolabeled cysteine conjugates have been reported previously (21). DCVC was synthesized according to the method of McKinney *et al.* (1). PCBC, CTFC, TFEC, and HFPC were synthesized by a modification of the procedure described by van Bladeren *et al.* (22) for the synthesis of mercapturic acids. Elemental analysis was performed by Galbraith Laboratories (Knoxville, TN). ^{1}H NMR spectra (400 MHz) were collected with a JEOL GX-400 spectrometer. FAB mass spectra were run on a Kratos MS-50 with an accelerating voltage of 8 kV. TLC was used to follow the reactions and was done on 0.25-mm silica gel F-254 layers obtained from EM Science (Cherry Hill, NJ). The solvent system used for TLC development was propanol/water/acetic acid (70:30:1). Ninhydrin spray reagent was used to detect amino acid products on TLC plates.

DCVC. Analysis, calculated for $C_5H_7NO_2SCl_2$: C, 27.79; H, 3.27; N, 6.48, 0, 14.81; found: C, 27.97; H, 3.22; N, 6.57; 0, 15.14. ^{1}H NMR (D_2O, 400 MHz) δ:3.39 (d of d, J_β = 15.51 Hz, J_α = 7.42 Hz, 1 H), 3.56 (d of d, J_β = 15.51 Hz, J_α = 4.05 Hz, 1 H), 3.91 (d of d, J_{β_1} = 4.05 Hz, J_{β_2} = 7.42 Hz, 1 H), and 6.75 (s, 1 H). FAB MS: 217 ([MH]$^+$). An isotopic distribution characteristic for two chlorine atoms was observed.

CTFC. Chlorotrifluorethylene gas was bubbled through a methanol solution containing L-cysteine·HCl and 3 molar equivalents of 1.0 N aqueous NaOH. Progress of the reaction was monitored by TLC. When the reaction was judged to be complete, the solvent was removed *in vacuo*. The residue was dissolved in water and CTFC was precipitated from the solution by the addition of acetic acid to pH 5. The product was recrystallized from ethanol/water or by isoelectric precipitation. Analysis, calculated for $C_5H_7NO_2F_3SCl$: C, 25.27; H, 2.98: N, 5.90; F, 23.99; found: C, 24.93; H, 3.11; N, 5.70; F, 23.76. ^{1}H NMR (D_2O, 400 MHz) δ:3.35 (d of d, J_β = 14.84 Hz, J_α = 7.42 Hz, 1 H), 3.60 (d of d, J_β = 14.84 Hz, J_α = 4.01 Hz, 1 H), 4.06 (d of d, J_{β_1} = 4.01 Hz, J_{β_2} = 7.42 Hz, 1 H), and 6.70 (d of t, J_{HF} = 47.8 Hz, J_{HF} = 6.74 Hz, 1 H). The presence of additional unresolved splitting within the major signals was consistent with the presence of diasteriomers, as shown for the glutathione conjugate of chlorotrifluoroethylene (23); however, a contribution by long range HF coupling cannot be excluded. Mass spectral analysis (FAB MS) revealed a molecular ion at *m/z* 238 ([MH]$^+$) and an associated ion at *m/z* 240 ([MH]$^+$), as expected for the isotopic distribution of the single chlorine atom.

TFEC. TFEC was synthesized from tetrafluoroethylene gas by a procedure analogous to that described above for CTFC. Analysis, calculated for $C_5H_7NO_2F_4S$: C, 27.15; H, 3.20; N, 6.33; F, 34.36; found: C, 27.13; H, 3.33; N, 5.97; F, 33.84. ^{1}H NMR (D_2O, 400 MHz) δ: 3.35 (d of d, J_β = 14.84 Hz, J_α = 7.42 Hz, 1 H), 3.55 (d of d, J_{β_1} = 14.84 Hz, J_{β_2} = 4.05 Hz, 1 H), 4.00 (d of d, J_β = 4.05 Hz, J_α = 7.41 Hz, 1 H), and 6.20 (t, J_{HF} = 53.28, 1 H). FAB MS: *m/z* 222 ([MH]$^+$).

HFPC. HFPC was synthesized from hexafluoropropylene gas by a procedure analogous to that described for CTFC and TFEC. Analysis, calculated for $C_5H_7NO_2F_6S$: C, 26.57; H, 2.61; N, 5.17; F, 42.04; found: C, 26.74; H, 2.62; N, 5.20; F, 41.55. NMR analysis of HFPC was performed as for the other cysteine conjugates; however, we could not estimate *J* values with accuracy since the NMR data were obtained in the moderate coupling regime, where $J/\Delta\ \delta$ is insufficient to resolve assymetric multiplets at 3.64, 4.05, and especially 3.41 ppm. The complexity of the NMR spectrum is most likely due to the presence of diastereomers, as will be discussed in Results. ^{1}H NMR (D_2O, 400 MHz) δ: 3.41 [d of d (overlapping) J_β = 18–20 Hz, 1 H], 3.67 (apparent d of t, J_β = 14.84, 1 H; due to geminal coupling and unresolved vicinal coupling in an overlapping d of d for two possible diastereomers), 4.05 (d of d, J_β = 4.07–7.0 Hz, 1 H), and 5.61 (d of m, J_{HF} = 42.5 Hz, 1 H). FAB MS: *m/z* 272 ([MH]$^+$).

PCBC. PCBC was synthesized by addition of 2 equivalents of HCBD to a methanol solution containing L-cysteine·HCl and 3 equivalents of 1.0 N aqueous NaOH. The reaction was stirred under a nitrogen atmosphere for 3 days. Typically, only about 50% product formation could be obtained, as judged by TLC and HPLC. The solvent was removed *in vacuo* to obtain a yellow oil, which solidified upon standing. This material was dissolved in water and extracted 3 times with ethyl acetate to remove residual HCBD. The aqueous phase was reduced to ¼ the original volume, whereupon the solution became cloudy and precipitation occurred. After refrigeration overnight, the precipitated material was collected, dissolved in hot 50% ethanol, adjusted to pH 4.0 with 10% HCl, treated with activated charcoal, and filtered while hot. The resulting solution was reduced *in vacuo* until precipitation occurred. The precipitated product was collected by vacuum filtration. Analysis, calculated for $C_7H_6NO_2SCl_5$: C, 24.34; H, 1.75; N, 4.06; 0, 9.26; Cl, 51.31; found: C, 22.74; H, 1.90; N, 3.72; O, 10.90; Cl, 53.05. ^{1}H NMR (D_2O, 400 MHz) δ:3.46 (d of d, J_β = 15.52 Hz, J_α = 7.42 Hz, 1 H), 3.71 (d of d, J_β = 15.52 Hz, J_α = 5.62 Hz, 1 H), and 3.93 (d of d, J_β = 5.62 Hz, J_α = 7.42 Hz, 1 H). FAB MS: *m/z* 346 ([MH]$^+$). An isotopic distribution in the molecular ion characteristic of multiple chlorine atoms was observed.

Radiolabeled cysteine conjugates. ^{14}C- and ^{35}S-labeled compounds were synthesized from either L-[^{14}C]cystine or L-[^{35}S]cystine by the method of Hayden *et al.* (21). Products were characterized using a Waters μBondapak C18 analytical (3.9 mm i.d. × 30 cm) column and purified by semipreparative HPLC, using a Waters μBondapak C18 (7.8 mm i.d. × 30 cm) column. All compounds were >97% radiochemically pure, as determined by HPLC. Solvent A was aqueous 0.1% TFA; solvent B was acetonitrile/0.1% TFA. Under isocratic conditions, DCVC, CTFC, and TFEC were eluted with 15% B. HFPC and PCBC were eluted with 20 and 30% solvent B, respectively.

Other assays. Metabolism of cysteine conjugates was determined by measuring either the appearance of α-ketoacid products, using a previously reported extraction assay (24), or the disappearance of substrate, determined by HPLC. HPLC was done with a Rainin Rabbit HP dual pump system equipped with a Gilson 116 UV detector. Waters μBondapak C18 columns (Milford, MA) were used for separations. Solvent A was aqueous 0.1% TFA; solvent B was 0.1% TFA in acetonitrile. The solvent composition varied between 0 and 30% solvent B, depending on the conjugate. Peaks were quantitated by UV absorption at 210 nm and/or post-column collection and scintillation counting with ^{14}C-labeled conjugates. Protein was assayed by the Bio-Rad method, using bovine IgG as standard.

Statistical analysis. When means of two groups were compared, significant differences were detected using Student's *t* test. For multiple comparisons, significant differences were derived from ANOVA and multiple comparisons based on least significant differences. In either case, the level of significance was set at $p < 0.05$.

Results

^{1}H NMR spectra. The proton NMR spectra of TFEC (Fig. 2A) and HFPC (Fig. 2B) are shown. The complex splitting pattern in the HFPC spectrum is due to the presence of diasteriomers resulting from the additional chiral center at C_2 of the propyl group, in addition to the vicinal HF coupling. The data were collected in the moderate coupling regime, resulting in incomplete resolution of coupling constants for α and β protons (see Materials and Methods). Heating the sample increased resolution some (data not shown), but peak broadening was observed in all spectra, perhaps suggesting that HFPC assumes some tertiary structure in solution. Such behavior is not unusual for amphipathic molecules. Diasteriomers have also been observed in the chemical synthesis of *S*-(2-chloro-1,1,2-trifluoroethyl)glutathione (23). The doublet of multiplets splitting pattern at δ 5.61 ppm indicates the interaction of the C_2 proton with one geminal fluorine. The structure assigned to HFPC is thus *S*-(1,1,2,3,3,3-hexafluoropropyl)-L-

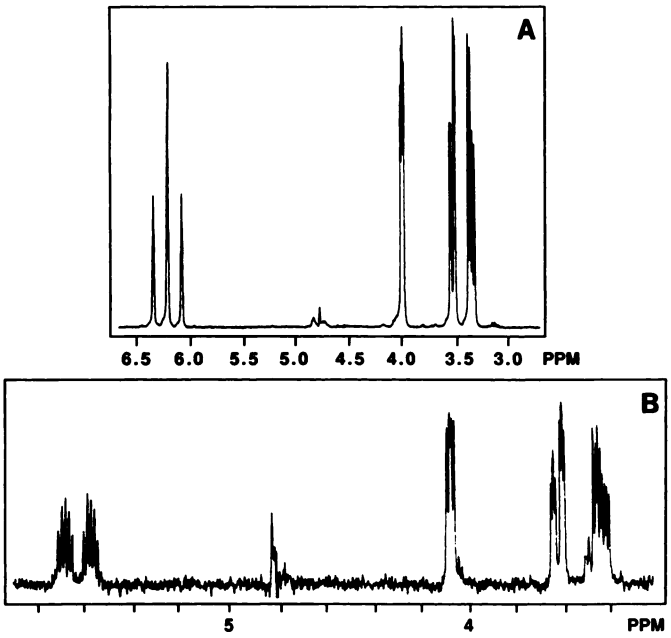

Fig. 2. ^{1}H NMR of TFEC (A) and HFPC (B). Chemical shifts are in ppm relative to trimethylsilane. Spectra at 400 MHz were acquired on a JEOL GX-400 spectrometer.

cysteine. By comparison, the spectrum of TFEC, which does not contain an additional chiral center, shows a simple pattern consistent with the structural assignment. Green and Odum (25) reported the synthesis of a cysteine conjugate of hexafluoropropylene to which they assign the structure *S*-(1,1,2,2,3,3-hexafluoropropyl)-L-cysteine. The difference between the structure reported here for HFPC and that of Green and Odum (25) may be due to the different conditions of the synthesis. This raises the question as to which conjugate is formed *in vivo*.

Inhibition of mitochondrial respiration by cysteine conjugates. To determine the relative potencies of the cysteine conjugates for producing mitochondrial toxicity, the dose-response relationship for inhibition of mitochondrial oxygen consumption was determined for each conjugate (Fig. 3). α-Ketoglutarate plus malate (site I) and succinate (site II) were used as substrates. Both resting (state 4) and ADP-stimulated (state 3) oxygen consumption were monitored. With the exception of PCBC, the major effect of the cysteine conjugates on oxygen consumption was an inhibition of ADP-stimulated (state 3) respiration. PCBC caused both an increase in state 4, indicating an uncoupling effect for this compound, and an inhibition of state 3 oxygen consumption (Fig. 3; and see Table 2).

To quantitate the relative degree of toxicity of the cysteine conjugates, the concentrations required to inhibit state 3 respiration by 50% (IC_{50} values) were calculated from the dose-response data in Fig. 3. The results are tabulated in Table 1. The order of potency for inhibition of respiration was PCBC = DCVC > TFEC = CTFC > HFPC at site I, but for site II the relative order was PCBC > DCVC = TFEC = CTFC > HFPC. With the exception of DCVC, which is more toxic at site I than at site II, no significant difference in sensitivity was seen between sites I and II with any of the conjugates.

To confirm that cysteine conjugate metabolism by a PLP-dependent β-lyase is responsible for the observed effects on oxygen consumption, the PLP enzyme inhibitor AOAA was included in some incubations along with the cysteine conjugate (Fig. 4). With the exception of PCBC, against which it had little effect, AOAA effectively prevented the loss of respiratory control ratios in conjugate-treated mitochondria. AOAA alone had no effect on mitochondrial respiration (data not shown).

Except for PCBC, the effect of the conjugates was almost entirely on state 3 respiration (Fig. 3). However, in the case of PCBC, the loss of respiratory control could be caused by either the decrease in state 3 or the increase in state 4 respiration. A comparison of the effect of AOAA on both state 3 and state 4 respiration in PCBC-treated mitochondria (Table 2) indicates that the increase of state 4 respiration (uncoupling) is the AOAA-insensitive component.

Binding of β-elimination fragments in mitochondria. It is generally accepted that the β-lyase pathway yields reactive thiol-containing metabolites that covalently bind to macromolecules. It has been shown that several chemical species (thiols, thionoacyl halides, and thioketenes) that should be capable of covalently binding to macromolecules can be generated by β-elimination of various cysteine conjugates (12–14). These different binding species may have various consequences for toxicity. Therefore, we assayed the ability of each conjugate to bind to mitochondrial macromolecules and determined whether the extent of binding correlated with the observed order of mitochondrial toxicity. In addition, the disulfide-reducing agent DTT was used to investigate how much of the observed binding might be attributed to the formation of mixed disulfides (Table 3).

PCBC produced by far the most binding, followed by DCVC, CTFC, TFEC, and HFPC in order of decreasing binding. For each conjugate, binding to protein was effectively blocked by AOAA, indicating that metabolism by a β-lyase is necessary for binding. The data also indicate that a large proportion of the binding from CTFC and HFPC may be due to disulfide formation. TFEC, PCBC and DCVC, on the other hand, show much less evidence for disulfide formation.

Metabolism of cysteine conjugates by mitochondria. The conjugate that produces the most binding need not be metabolized to the greatest extent. Differences in reactivity of the elimination fragment could lead to differences in binding, thus leading to differential toxicity of conjugates that are metabolized equally well. To explore these possibilities, the relative rates of metabolism of cysteine conjugates in whole mitochondria were determined (Table 4). These results show that the relative rates of metabolism in mitochondria are consistent with the trends observed for DTT-insensitive ^{35}S binding and toxicity (i.e., the most toxic conjugate also shows the most metabolism and binding, whereas the least toxic conjugate shows the lowest amount of metabolism and binding). Interestingly, AOAA was not completely effective in preventing metabolism of PCBC, even though it was very effective in inhibiting binding.

Metabolism of cysteine conjugates by purified GTK. A major cysteine conjugate β-lyase in rat kidney has been shown to be GTK (9). This enzyme has both cytosolic and mitochondrial forms, which are very similar. It was of interest to see whether the substrate specificity of this enzyme for cysteine conjugates is consistent with the observed order of metabolism, binding, and toxicity of these compounds in mitochondria. Therefore, the β-lyase activity of purified GTK was measured with various cysteine conjugate substrates, in the presence and

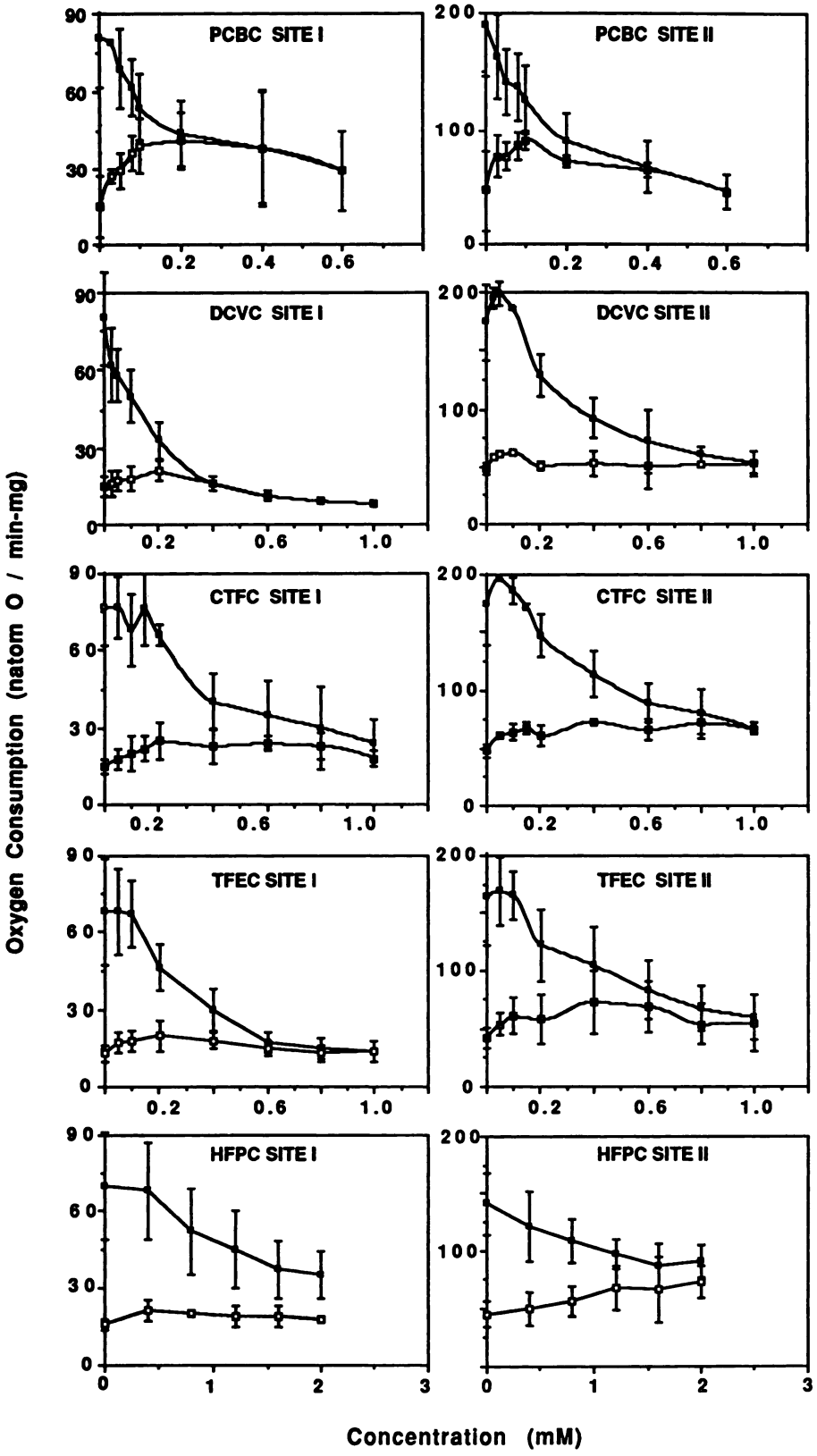

Fig. 3. Dose-response curves for cysteine conjugate inhibition of mitochondrial respiration. Approximately 2 mg of mitochondrial protein were incubated for 1 hr with various concentrations of conjugate after which they were pelleted by centrifugation and resuspended in fresh buffer. Oxygen consumption was then measured as described in Materials and Methods. ■, State 3 respiration; □, State 4 respiration. Values are the mean ± standard deviation of three experiments.

absence of MTB (Table 5). In contrast to metabolism results obtained with mitochondria, CTFC and TFEC were the best substrates, whereas PCBC was a poor substrate for the purified β-lyase. Furthermore, MTB only slightly stimulated metabolism of PCBC by the purified enzyme.

Hydrophobicity and access of cysteine conjugates to mitochondrial β-lyase. Log P (π) values of octanol/water partition coefficients (P) are commonly used as an indicator of hydrophobic character. This property may have some bearing on the toxicity of cysteine conjugates in relation to their ability to traverse biological membranes or their ability to serve as substrates for the enzymes involved in activation (26). There-

TABLE 1

Summary of the inhibition of ADP-stimulated respiration by cysteine conjugates

The values are the mean ± standard deviation for three separate experiments in which the ability of the conjugate to inhibit ADP-stimulated respiration was measured. The data were calculated from the percentage of control ADP-stimulated (State 3) oxygen consumption. The concentration required to inhibit ADP-stimulated respiration by 50% was derived by converting the percentage of control to probit values and plotting the log of the concentration (μM) versus the probit data by linear regression analysis.

Conjugate	IC_{50}	
	Site I	Site II
	μM	
PCBC	65 ± 11[a]	45 ± 8[a]
DCVC	76 ± 26[a, b, d]	236 ± 40[b]
TFEC	191 ± 80[a, b]	216 ± 81[b]
CTFC	260 ± 67[b]	236 ± 72[b]
HFPC	979 ± 203[c]	779 ± 142[c]

[a, b, c] Significantly different from site I ($p < 0.05$). Values with the same letter designation are not different, whereas those with different letters were significantly different ($p < 0.05$) by ANOVA analysis, as described in Materials and Methods.

[d] Although the values for DCVC and TFEC were not different within the ANOVA analysis, a direct comparison of these two groups showed that TFEC is significantly larger than DCVC (one-tailed Student's t test; $p < 0.05$).

fore, the π values for the conjugates were determined (Table 6). PCBC was the only compound that was found to have a position π value, indicating its distinct preference for the hydrophobic phase. The other conjugates clearly prefer the aqueous phase.

In view of the hydrophobic character of PCBC, compared with the other conjugates, it seems plausible that its extensive metabolism in mitochondria might be attributed to an ability to cross the inner mitochondrial membrane. This preferential access to the matrix might explain the relatively large amount of PCBC metabolism in spite of its poor activity as a substrate for the purified β-lyase, i.e., GTK. However, when metabolism of cysteine conjugates was studied in mitochondria that had been permeabilized with digitonin, thereby eliminating access as a factor in metabolism (Table 7), PCBC was still the most extensively metabolized conjugate. This result indicates that preferential access of PCBC to the matrix compartment cannot account for its extensive metabolism, compared with other conjugates. Considering the poor activity of PCBC as a substrate for GTK, compared with the other conjugates, these results suggest that a β-lyase other than GTK is responsible for the majority of PCBC metabolism in mitochondria.

Discussion

The primary goal of these studies was to compare the metabolism, binding of ^{35}S-metabolites to macromolecules, and toxicity of several related cysteine conjugates. Additionally, we wanted to examine the influence of structure on the above parameters. In this regard, several points are worthy of discussion. PCBC, the completely chlorinated, unsaturated, four-carbon conjugate, was the most potent inhibitor of respiration at both sites I and II. DCVC, which is also unsaturated, had comparable toxicity to CTFC and TFEC at site II but was equipotent with PCBC at site I. HFPC, the fluorinated three-carbon conjugate, was the least toxic at both sites. Although only a limited number of compounds were studied, the data suggest that structural features such as halogen substitution and unsaturation may play a role in the mechanism of mitochondrial toxicity. It would be interesting to study a larger group of conjugates to determine whether structure and the type of halogen substituent plays a significant role in mitochondrial toxicity.

Commandeur *et al.* (27) have recently reported that, among several two-carbon-substituted mercapturate conjugates tested (saturated mercapturic acids of tetrafluoroethylene, 1,1-dichloro-1,2-difluoroethylene, and 1,1-dibromo-2,2-difluoroethylene), the tetrafluoroethyl conjugate was the most potent for nephrotoxicity in the rat. Their data (27) suggest that, within a saturated series, the fluorinated compounds are more toxic than those with chloro- or bromo- substituents. By this reasoning, the unsaturated nature of PCBC and DCVC, rather than halogen substituent, may play a more important role in their potent effects on site I respiration.

Metabolism in mitochondria and binding of ^{35}S-metabolites to mitochondrial macromolecules was demonstrated for each conjugate. However, the relative extent of metabolism and binding of the conjugates did not correlate well with their calculated IC_{50} values. For example, PCBC was metabolized approximately 4-fold better than HFPC but produced 10-fold more binding and was 15-fold more toxic than HFPC. Likewise, PCBC was metabolized about 2-fold more than DCVC, CTFC, and TFEC, yet its binding and toxicity were 10-fold and 5-fold greater at site II. At site I, DCVC was comparable in toxicity to PCBC. These differences suggest differential reactivity of the metabolites, possibly leading to differences in binding targets.

Differential reactivity of cysteine conjugate metabolites was previously suggested by Green and Odum (25) to account for differences in metabolism, toxicity, and mutagenicity. The fluorinated conjugates were suggested to produce stable thiols, which are less reactive than the chlorinated conjugates and are not capable of alkylating DNA. Thiol metabolites of chlorinated conjugates on the other hand, by virtue of the better leaving

TABLE 2

Effect of AOAA on state 3 versus state 4 respiration of mitochondria treated with PCBC

Experiments were performed by the same procedure as for the dose-response curves, using 0.2 mM PCBC and 0.1 mM AOAA. Values are the mean ± standard deviation of three experiments.

	Respiration			
	Site I		Site II	
	State 3	State 4	State 3	State 4
	natom of oxygen/mg · min			
Control	61.7 ± 5.6	11.3 ± 0.9	149.3 ± 17.6	39.1 ± 5.3
+ PCBC	34.1 ± 9.7[a]	32.4 ± 9.2[a]	79.4 ± 13.3[a]	70.2 ± 8.8[a]
+PCBC and AOAA	55.9 ± 9.8	21.1 ± 3.9[a]	141.9 ± 9.2	72.1 ± 9.8[a]

[a] Significantly different from control ($p < 0.05$) by Student's t test.

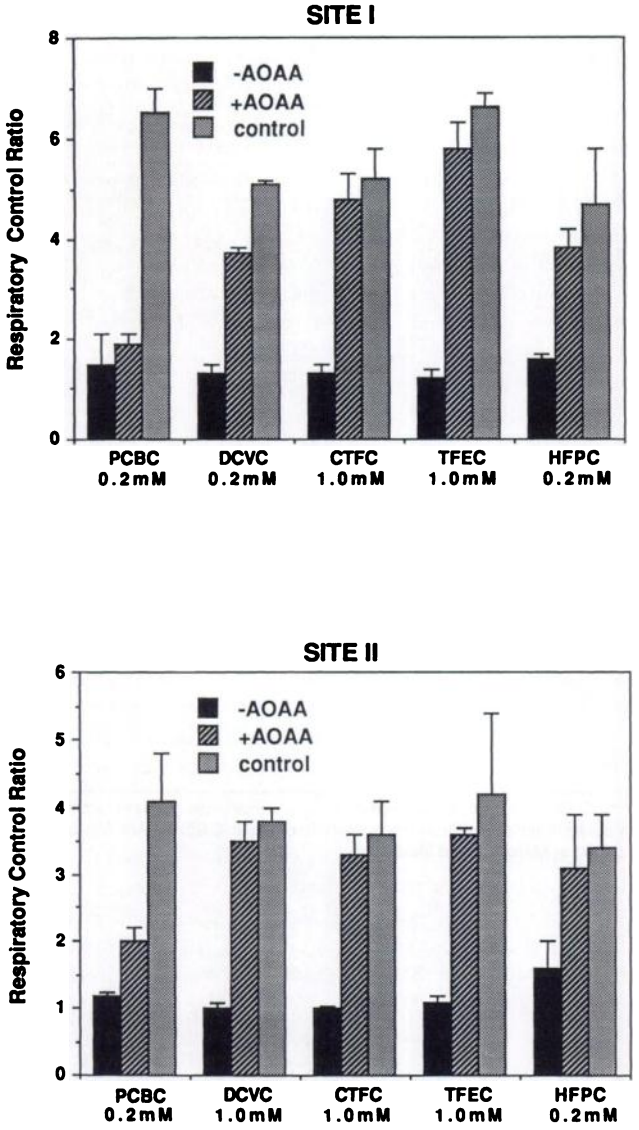

Fig. 4. Protection of mitochondrial respiratory control ratios by AOAA. Experiments were performed by the same procedure as for the dose-response curves, except that 100 μM AOAA was added along with the conjugate at the concentrations indicated. Values are the mean ± standard deviation of three experiments.

group ability of chloride versus fluoride, were proposed to undergo further elimination of chloride and rearrangement to an electrophilic species capable of reacting with nucleophilic sites on proteins and DNA.

Dekant and co-workers (12–14), however, reported the formation of thionoacyl halide or thioketene metabolites from PCBC, DCVC, and CTFC, demonstrating that fluoride (in the case of CTFC) can be a leaving group in elimination reactions of thiol metabolites. However, that CTFC has less tendency than completely chlorinated conjugates to eliminate halide is indicated by the fact that its thiol metabolite could also be trapped as the sulfide by methyl iodide and benzyl bromide (12). Sulfides were not obtainable from PCBC or DCVC, indicating the extreme reactivity of the thiols generated from these conjugates (13, 14). Further evidence for fluoride elimination comes from the work of Commandeur et al. (27), who have recently reported the *in vitro* and *in vivo* metabolism of TFEC to difluoroacetic acid, presumably through hydrolysis of a thionoacyl fluoride intermediate. However, other toxic conjugates used in that study (27) did not show evidence for thionoacyl fluoride formation.

Mixed disulfides between protein thiols and thiol cleavage fragments, hydrogen sulfide, or thiosulfate (27, 28) might account for some of the ^{35}S binding. However, that all of the conjugates tested in this study can form species capable of acylating macromolecules is suggested by the DTT-resistant binding. In agreement with the results of Dekant and co-workers discussed above, CTFC metabolites showed evidence for both the disulfide formation expected from a stable thiol and the DTT-resistant binding expected from a thionoacylating species. HFPC also showed evidence for significant disulfide formation. If the disulfide binding of stable thiols is not as damaging as thionoacylation, this might account for some of the discrepancy between IC_{50} values and binding. Indeed, when the binding is adjusted to represent only the DTT-resistant portion, some better correlations between binding and toxicity are obtained. Thus, PCBC is about 15-fold more toxic than HFPC and shows about 15-fold more binding.

Still, adjusting the binding to represent only the DTT-resistant portion cannot resolve all the discrepancies between binding and toxicity. Specificity in the binding targets may be the key to these differences. The significantly greater sensitivity of site I than site II to inhibition of respiration by DCVC also suggests some selectivity in binding targets. In addition to differences in reactivity, possible reasons for different binding targets for cysteine conjugate metabolites might include differences in the site of activation or in hydrophobicity of metabolites.

Differences in the site of activation imply that more than one enzyme may be responsible for the metabolism of cysteine conjugates. This possibility has been suggested previously by Stevens *et al.* (29). Further evidence in support of more than one β-lyase comes from the data obtained for cysteine conjugate metabolism by purified β-lyase and isolated mitochondria. The specificity of the conjugates for the purified β-lyase was found to be inconsistent with their metabolism by mitochondria (Tables 4, 5, and 7). In particular, TFEC was found to be about 5-fold better as a substrate for GTK than was PCBC. Yet TFEC and PCBC were metabolized to about the same extent by permeabilized mitochondrial. This suggests that an enzyme or enzymes other than GTK is responsible for approximately 80% of the PCBC metabolism in mitochondria. The blockage of the majority of PCBC metabolism by AOAA indicates that the non-GTK activity may be at least partially PLP-dependent.

PCBC was also found to have an uncoupling effect on oxidative phosphorylation. This is consistent with the previous reports of Schnellmann and co-workers (30), and Wallin *et al.* (18). Schnellmann and Lock (31) also reported that, in rabbit kidney mitochondria at 37°, AOAA effectively blocks the uncoupling effects of PCBC (5–100 μM) at short time points (2–5 min). However, our data with rat kidney mitochondria show that, at longer time points (1 hr), the uncoupling effect is not effectively blocked by AOAA. Inasmuch as AOAA blocked greater than 90% of the PCBC binding under identical conditions, some other mechanism for uncoupling may be involved. Therefore, the differences in the results may be due to different species, PCBC concentrations, and incubation conditions.

These studies show that all of the nephrotoxic cysteine conjugates tested are metabolized by renal mitochondrial β-

TABLE 3

Binding of ^{35}S-labeled cysteine conjugate metabolites to mitochondrial macromolecules

Mitochondria (1 mg) were incubated with 100 μM ^{35}S-labeled conjugates for 1 hr at 25°. Protein was precipitated by addition of 10% TCA and collected by vacuum filtration on glass fiber filters. Incorporation of ^{35}S label into protein was quantitated by scintillation counting. To determine DTT-sensitive binding, metabolism was stopped and mitochondria were dissolved by addition of 250 μl of 0.1 N KOH. Incubation with 5 mM DTT was continued for 30 min at 25° before precipitation of protein and quantitation of ^{35}S incorporation, as described above and in Materials and Methods. Values are the mean ± standard deviation of three experiments.

Conjugate	^{35}S bound	Binding blocked by AOAA	Binding removed by DTT	DTT-insensitive binding
	nmol/60 min · mg	%	%	*nmol/60 min · mg*
[^{35}S]PCBC	10.2 ± 1.4^{a}	93.3 ± 5.2	11 ± 7^{a}	9.08 ± 1.4^{a}
[^{35}S]DCVC	1.4 ± 0.2^{b}	95.0 ± 3.2	11 ± 8^{a}	1.25 ± 0.2^{b}
[^{35}S]CTFC	1.3 ± 0.2^{b}	97.2 ± 1.5	43 ± 15^{b}	0.73 ± 0.2^{c}
[^{35}S]TFEC	1.1 ± 0.2^{b}	96.1 ± 1.8	5 ± 8^{a}	1.08 ± 0.2^{b,c}
[^{35}S]HFPC	0.9 ± 1.0^{b}	91.7 ± 3.5	36 ± 5^{b}	0.60 ± 0.1^{c}

^{abc} Values with the same letter designation are not different, whereas those with different letters were significantly different ($p < 0.05$) by ANOVA analysis as described in Materials and Methods.

TABLE 4

Metabolism of cysteine conjugates in mitochondria

^{14}C-labeled cysteine conjugates (100 μM) were incubated with mitochondria (1 mg) for 1 hr at 25° in mitochondrial isolation buffer. After 1 hr, protein was precipitated with perchloric acid. After neutralization with potassium hydroxide, metabolism was measured using HPLC and liquid scintillation counting to detect disappearance of the cysteine conjugate. Values are the mean ± standard deviation of three experiments.

	Metabolism	
	−AOAA	+AOAA
	nmol/60 min · mg	
PCBC	21.2 ± 6.7^{a}	6.8 ± 3.2^{a}
DCVC	9.2 ± 1.5^{b}	2.1 ± 2.1^{b}
CTFC	9.7 ± 5.3^{b}	1.7 ± 0.7^{b}
TFEC	10.5 ± 2.6^{b}	1.0 ± 0.9^{b}
HFPC	6.2 ± 1.1^{b}	2.0 ± 0.6^{b}

^{a,b} Values with the same letter designation are not different, whereas those with different letters were significantly different ($p < 0.05$) by ANOVA analysis, as described in Materials and Methods.

TABLE 5

Metabolism of cysteine conjugates by purified β-lyase

Metabolism of ^{14}C-conjugates by purified β-lyase was measured for 10-min incubations at 37° in 10 mM Tris, pH 8.0. Formation of ^{14}C-labeled α-keto acid products from ^{14}C-labeled DCVC, TFEC, CTFC, and HFPC were determined by the extraction procedure and scintillation counting as previously reported (9). Disappearance of [^{14}C]PCBC was determined by the HPLC assay (9) and scintillation counting as well as peak integration. Values reported are the mean ± standard deviation of three experiments.

Conjugate (1.0 mM)	Metabolism		
	+MTB	−MTB	+MTB/−MTB
	μmol/10 min · mg		
TFEC	5.9 ± 0.1^{a}	0.3 ± 0.1	22
CTFC	5.6 ± 1.0^{a}	0.2 ± 0.3	37
DCVC	3.6 ± 0.5^{b}	0.1 ± 0.2	30
HFPC^{d}	2.3 ± 0.2^{b,c}	0.1 ± 0.1	23
PCBC	1.1 ± 0.8^{c}	0.3 ± 1.0	3.7

^{a,b,c} Values with the same letter designation are not different, whereas those with different letters were significantly different ($p < 0.05$) by ANOVA analysis, as described in Materials and Methods.

^{d} Due to problems in obtaining ^{14}C-labeled HFPC of sufficient purity, the experiments with HFPC were done several months after the experiments with the other conjugates. The enzyme had lost a significant amount of activity during this time. Therefore, the data for HFPC reported here have been normalized by an internal comparison with DCVC metabolism to account for the loss in activity and allow direct comparison with the other conjugates.

lyases to reactive metabolites that bind to mitochondrial macromolecules and inhibit respiration. In addition, they support the notion that mitochondria are primary cellular targets for cysteine conjugate toxicity. It remains to be determined whether the same relationships among metabolism, binding, and toxicity exist in isolated renal cells.

TABLE 6

Octanol/buffer partition ratios for nephrotoxic cysteine conjugates

Octanol/buffer partition ratios were determined by adding ^{14}C-labeled conjugate to 1 ml of respiration buffer, followed by addition of 1 ml of octanol and vigorous mixing. The phases were separated by centrifugation and the amount of label in the two phases was determined by liquid scintillation counting. The ratio presented is the ratio of dpm recovered in the two phases.

Conjugate	Octanol/buffer	π
PCBC	4.83 ± 0.40	0.684^{a}
HFPC	0.21 ± 0.015	−0.684^{b}
DCVC	0.08 ± 0.003	−1.097^{c}
CTFC	0.08 ± 0.008	−1.097^{c}
TFEC	0.04 ± 0.002	−1.400^{d}

^{a,b,c,d} Values with the same letter designation are not different, whereas those with different letters were significantly different ($p < 0.05$) by ANOVA analysis, as described in Materials and Methods.

TABLE 7

Metabolism of cysteine conjugates by permeabilized mitochondria

Mitochondria were permeabilized by incubation in the usual buffer plus 0.25 mg of digitonin/mg of protein for 15 min before addition of conjugates. Schnaitmen and Greenawalt (20) have shown that, at concentrations higher than 0.15 mg of digitonin/mg of protein, malic dehydrogenase and cytochrome oxidase leak from the matrix, indicating complete permeabilization of the inner membrane. Metabolism was measured as before for whole mitochondria. The values are the mean ± standard deviation of three experiments. The values for metabolism in the absence of digitonin were generally higher than obtained in previous experiments (Table 3). Differences due to variations among rats may account for the discrepancies.

Conjugate (100 μM)	Metabolism	
	−Digitonin	+Digitonin
	nmol/60 min · mg	
PCBC	31.99 ± 6.57^{a}	35.30 ± 6.80^{a}
DCVC	19.69 ± 3.55^{a,b}	18.72 ± 2.91^{a,b}
TFEC	15.64 ± 9.80^{b,c}	22.39 ± 13.38^{a,b}
CTFC	14.07 ± 8.28^{b,c}	22.60 ± 1.73^{a,b}
HFPC	5.22 ± 0.02^{c}	8.45 ± 1.74^{b}

^{a,b,c} Values with the same letter designation are not different, whereas those with different letters were significantly different ($p < 0.05$) by ANOVA analysis, as described in Materials and Methods.

Foremost among the questions remaining to be answered before the toxic effects of cysteine conjugates can be understood are the critical molecular targets of interaction for their metabolites. One possible target for covalent binding that was not considered in this study is the soluble thiol glutathione. This low molecular weight compound possesses antioxidant properties and has been shown to be depleted in rat (18) and rabbit (32) kidney mitochondria after treatment with PCBC. Lash and Anders also reported depletion of glutathione as well as lipid peroxidation in isolated rat kidney mitochondria (33) but not in isolated rat kidney cells (34) after treatment with DCVC. Recently, Beuter *et al.* (35) reported malondialdehyde forma-

tion in renal cortical slices after *in vivo* treatment of mice with DCVC and suggested lipid peroxidation as a possible cause of toxicity. Although this study focused only on binding of ^{35}S-metabolites to macromolecules, the possibility that lipid peroxidation is a cause of cysteine conjugate toxicity is a topic of current interest in this laboratory.

Among the possible protein targets for cysteine conjugate metabolite interactions, Stonard and Parker (17) have proposed that the 2-oxoacid dehydrogenases are the primary targets for DCVC toxicity in isolated rat liver mitochondria. They showed that DCVC, at a concentration of 100 μM, effectively inhibits respiration with site I substrates (β-hydroxybutyrate or pyruvate/fumarate). At this concentration, they found that respiration with the site II substrate succinate was relatively unaffected. These results are consistent with the dose-response data reported here, which show that site II respiration is only affected at a dose higher than that required for inhibition of site I. The IC_{50} value of approximately 200 μM determined in this study for inhibition of site II respiration by DCVC in isolated renal mitochondria agrees with that reported by Lash and Anders (19). However, they found site I to be unaffected by DCVC treatment. Therefore, they have proposed succinate dehydrogenase as a primary target for DCVC. The reason for these discrepancies is not clear. They do, however, serve to underscore the formidable challenge that elucidation of the molecular targets continues to present. In addition to possibly resolving inconsistencies in structure-activity data, knowledge of specific molecular targets should yield important insights into fundamental mechanisms of cell death.

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