0026-895X/87/040549-08$02.00/0

MOLECULAR PHARMACOLOGY, 32:549-556

Mechanism of *S*-(1,2-Dichlorovinyl)-L-Cysteine- and *S*-(1,2-Dichlorovinyl)-L-Homocysteine-Induced Renal Mitochondrial Toxicity

LAWRENCE H. LASH and M. W. ANDERS

Department of Pharmacology, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642

Received February 3, 1987; Accepted July 22, 1987

SUMMARY

The mechanism by which the nephrotoxic *S*-conjugates *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC) and *S*-(1,2-dichlorovinyl)-L-homocysteine (DCVHC) produce toxicity in rat kidney mitochondria was studied by examining their effects on mitochondrial function, structural integrity, and metabolism. Both *S*-conjugates inhibited succinate-linked state 3 respiration and impaired the ability of mitochondria to retain Ca^{2+} and to generate a membrane potential; 30–60 min were required for maximal expression of these functional changes. Mitochondrial structure was damaged, as indicated by enhanced polyethylene glycol-induced shrinkage of matrix volume and by leakage of protein and malic dehydrogenase from the matrix; 60–120 min were required for maximal expression of these structural changes. Much shorter incubation times (15–30 min) were required for DCVC and DCVHC to decrease ATP concentrations, to alter the concentrations of several citric acid cycle intermediates, and to inhibit succinate:cytochrome *c* oxidoreductase and isocitrate dehydrogenase activities. Lipid peroxidation and oxidation of glutathione to glutathione disulfide also occurred. The relative time courses of these pathological changes indicate that the initial effects of DCVC and DCVHC in renal mitochondria are the inhibition of energy metabolism and the oxidation of glutathione. These changes then lead to alterations in mitochondrial function and ultimately to irreversible damage to mitochondrial structure.

The nephrotoxicity of DCVC is dependent on bioactivation by cysteine-conjugate β-lyase (EC 4.4.1.13), a pyridoxal phosphate-dependent enzyme found in the cytosolic and mitochondrial fractions of kidney cortex, to produce 1,2-dichlorovinylthiol, the putative toxic metabolite (1–6). *In vitro* studies indicate that mitochondria are the primary targets of toxic cysteine *S*-conjugates: DCVC inhibits state 3 respiration in isolated renal (5, 7, 8) and hepatic (7, 8) mitochondria and inhibits oxygen consumption and mitochondrial Ca^{2+} sequestration and decreases ATP concentrations in isolated rat kidney cells (6).

DCVHC, the homocysteine analogue of DCVC, is also a potent nephrotoxin in rats and is cytotoxic in isolated rat kidney cells (9, 10). Although the enzymes involved in DCVHC bioactivation have not been identified, pyridoxal phosphate-dependent metabolism may occur in rat kidney and may also generate 1,2-dichlorovinylthiol (9). Studies in isolated rat kidney cells, undertaken to compare the mechanisms of DCVHC- and DCVC-induced cytotoxicity, revealed that DCVHC also inhibits cellular respiration and mitochondrial Ca^{2+} sequestration and decreases cellular ATP concentrations by similar amounts compared with DCVC, indicating that mitochondria are intracellular targets for DCVHC as well (10).

Inhibition of DCVC metabolism with aminooxyacetic acid prevented DCVC-induced toxicity and the nonmetabolizable α-methyl analogue was not toxic (4–6), further supporting a role for cysteine conjugate β-lyase in DCVC bioactivation. Similarly, inhibition of DCVHC metabolism with aminooxyacetic acid or DL-propargylglycine diminished DCVHC-induced toxicity, and the nonmetabolizable α-methyl analogue was not toxic (9, 10). Thus, for both DCVC and its homocysteine analogue DCVHC, cellular and mitochondrial toxicity are associated with pyridoxal phosphate-dependent bioactivation.

The objective of the current studies was to gain a better understanding of the mechanism of nephrotoxic *S*-conjugate-induced mitochondrial dysfunction. Hence, the effects of DCVC and DCVHC on mitochondrial function, structural integrity, and metabolism in isolated rat kidney mitochondria were examined. By comparing the courses and magnitudes of the observed changes, primary effects could be distinguished from

This research was supported by National Institute of Environmental Health Sciences Grant ES03127 (M. W. A.) and National Research Service Award ES05357 (L. H. L.).

ABBREVIATIONS: DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DCVHC, *S*-(1,2-dichlorovinyl)-L-homocysteine; β-lyase, cysteine conjugate β-lyase; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; arsenazo III, 2,2′-(1,8-dihydroxy-3,6-disulfo-2,7-naphthalene-bisazo)-dibisbenzenearsonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GSH, glutathione; GSSG, glutathione disulfide; PEG, polyethylene glycol; safranine O, 3,7-diamino-2,8-dimethyl-5-phenylphenazium chloride; TBA, thiobarbituric acid.

secondary effects, thereby allowing elucidation of the sequence of events in *S*-conjugate-induced mitochondrial dysfunction. The results show that the initial effects of DCVC and DCVHC in renal mitochondria involve inhibition of flux through the citric acid cycle and oxidation of GSH to GSSG; these changes are followed by impairment of mitochondrial function and, ultimately, damage to mitochondrial structural integrity. A preliminary report of this work has been presented (11).

Experimental Procedures

Materials. TMPD, 1-fluoro-2,4-dinitrobenzene, ferricytochrome *c* (type VI), arsenazo III [purified as described by Kendrick (12)], Lubrol PX, rotenone, coenzyme A (trilithium salt), valinomycin, PEG (M_r = 200, 300, 600, and 1000), L-lactate dehydrogenase (EC 1.1.1.27), malic dehydrogenase (EC 1.1.1.37), citrate (*pro*-3*S*)-lyase (EC 4.1.3.6), isocitrate dehydrogenase (NADP$^+$) (EC 1.1.1.42), glutamate dehydrogenase (NAD(P)$^+$) (EC 1.4.1.3), adenylate kinase (EC 2.7.4.3), pyruvate kinase (EC 2.7.1.40), hexokinase *(EC 2.7.1.1)*, and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were purchased from Sigma Chemical (St. Louis, MO). FCCP was purchased from Chemical Dynamics (South Plainfield, NJ). Oligomycin and succinate CoA ligase (EC 6.2.1.5) were purchased from Boehringer Mannheim (Indianapolis, IN). Safranine O was purchased from Aldrich (Milwaukee, WI). DCVC and DCVHC were synthesized as previously described (4, 13). All other chemicals were reagent grade.

Preparation of mitochondria. Male Fischer 344 rats (200–300 g, Charles River Laboratories, Wilmington, MA) were anesthetized with ether and killed by puncturing the diaphragm. The kidneys were immediately removed and placed in ice-cold 20 mM triethanolamine/HCl buffer (pH 7.4), containing 225 mM sucrose, 10 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, and 20 mM KCl; this buffer was used except where indicated. The mitochondrial fraction of renal cortical homogenates was isolated by the method of Johnson and Lardy (14). Marker enzyme activities for the various subcellular fractions were measured to assess the purity of the mitochondria, as previously described (5). Because mitochondrial function deteriorates and respiration rates are too rapid to be accurately measured at higher temperatures, all incubations were performed at 28°.

Assays. Oxygen consumption was measured polarographically with a Clark-type electrode at 28°. The electrode was calibrated with air-saturated buffer at 28°, and zero oxygen concentration was obtained by the addition of sodium sulfite. Respiration rates were measured according to the procedure of Estabrook (15): State 3 rates were measured by addition of either 4 mM glutamate + 2 mM malate or 3.3 mM succinate or 1 mM ascorbate + 0.2 mM TMPD and 0.3 mM ADP to 1.5–3.0 mg mitochondrial protein; state 4 rates were measured as the rate of oxygen consumption after exhaustion of ADP.

S-Conjugate-induced Ca^{2+} release from mitochondria was measured with arsenazo III by dual-wavelength spectroscopy at 654–685 nm with an SLM-Aminco DW-2C spectrophotometer (16). For these experiments, mitochondria were suspended in 240 mM sucrose, 10 mM potassium phosphate (pH 7.4), 20 mM triethanolamine/HCl (pH 7.4), and 20 mM KCl. Before addition of *S*-conjugates, mitochondria (1 mg protein/ml) were incubated with 50 μM $CaCl_2$ for 5 min. At the indicated times, arsenazo III (final concentration 40 μM) was added to mitochondrial fractions, and Ca^{2+} release was determined.

The ability of mitochondria to generate a membrane potential was evaluated with safranine O (17). After incubation for the indicated times with DCVC or DCVHC, 1-ml samples of mitochondria (1 mg protein/ml) were added to a cuvette containing 5 μg/ml rotenone, 5 μg/ml oligomycin, 10 μM safranine O, and 5 mM succinate to energize the mitochondria, and the absorbance at 511–533 nm was recorded with an SLM-Aminco DW-2C spectrophotometer. The membrane potential was abolished by adding 1 μM FCCP and 20 mM KCl. The safranine O signal was calibrated by inducing a K^+ diffusion potential with 50 ng/ml valinomycin and extramitochondrial K^+ concentrations of 0.12–20 mM. The potential was calculated according to the Nernst equation; the matrix K^+ concentration was assumed to be 120 mM (18). The magnitude of the signal was the difference in absorption after addition of valinomycin compared with that after further addition of 1 μM FCCP and 20 mM KCl. The calibration curve was fitted to a straight line by linear regression (r = 0.993, y = 0.0004X–0.0085).

The permeability of the inner membrane of *S*-conjugate-treated mitochondria was assessed by the ability of isomolar concentrations of PEG of various molecular weights to produce a decreased matrix volume, as measured by an increase in the absorbance at 540 nm (19, 20). Shrinkage was induced by addition of 0.3 ml of 60% PEG solutions of M_r = 200, 300, 600, or 1000 to 1 ml mitochondria (1 mg protein) incubated for various times with DCVC or DCVHC. Sucrose (0.3 ml, 0.25 M) was added to the reference cuvette.

S-Conjugate-induced lipid peroxidation was determined by measurement of malondialdehyde formation by the thiobarbituric acid assay (21).

Malic dehydrogenase activity was measured by the method of Ochoa (22). NAD$^+$-isocitrate dehydrogenase activity was measured by the method of Plaut and Sung (23). Succinate:cytochrome *c* oxidoreductase (EC 1.3.99.1) activity was measured by the method of Fleischer and Fleischer (24). All mitochondrial enzymes were assayed in samples previously treated with 0.05% (vol/vol) Lubrol PX.

The concentrations of these metabolites were measured in neutralized trichloroacetic acid extracts (final concentration 5%, wt/vol) of mitochondria (1.5–3.0 mg protein) by spectrophotometric pyridine nucleotide-linked enzyme assays at 340 nm, as described by Bergmeyer (25). Citrate was measured with citrate lyase and malic dehydrogenase; isocitrate was measured with isocitrate dehydrogenase; α-ketoglutarate was measured with glutamate dehydrogenase; succinate was measured with succinate thiokinase, pyruvate kinase, and lactate dehydrogenase; malate was measured with malic dehydrogenase and the oxaloacetate formed was trapped as the hydrazone; ATP was measured with hexokinase and glucose 6-phosphate dehydrogenase; ADP and AMP were measured with adenylate kinase, pyruvate kinase, and lactate dehydrogenase. Mitochondrial GSH, GSSG, glutamate, and aspartate concentrations were determined by ion-exchange high-pressure liquid chromatography on a 5μ Econosphere NH_2 column (4.6 × 150 mm; Alltech Applied Science, Deerfield, IL) with a methanol-acetate solvent system according to the method of Reed *et al.* (26). Neutralized trichloroacetic acid extracts (final concentration 5%, wt/vol) of mitochondria were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene, and the derivatives were detected and quantified as previously described (6). Protein concentrations were measured by the method of Read and Northcote (27) with bovine serum albumin as the standard.

Statistical significance was assessed by Student's t test, with differences at the $p < 0.05$ level considered significant.

Results

In previous studies with isolated rat kidney cells (6, 10), cellular respiration, adenine nucleotide status, and FCCP-releasable Ca^{2+} were employed as indexes of mitochondrial function within the intact cell, and we found that DCVC and DCVHC were potent inhibitors of these processes. Having thus established that mitochondria are intracellular targets for these compounds, the mechanism by which DCVC or DCVHC induce mitochondrial dysfunction was next studied in isolated renal mitochondria.

Mitochondrial function. As a first step toward examining how DCVC and DCVHC cause mitochondrial toxicity, their effects on three processes central to mitochondrial function were studied. First, concentration-dependent inhibition of succinate-linked state 3 respiration by DCVC and DCVHC was observed (Fig. 1). Mitochondria were equally susceptible to the effects of the two *S*-conjugates; 50% inhibition occurred at a

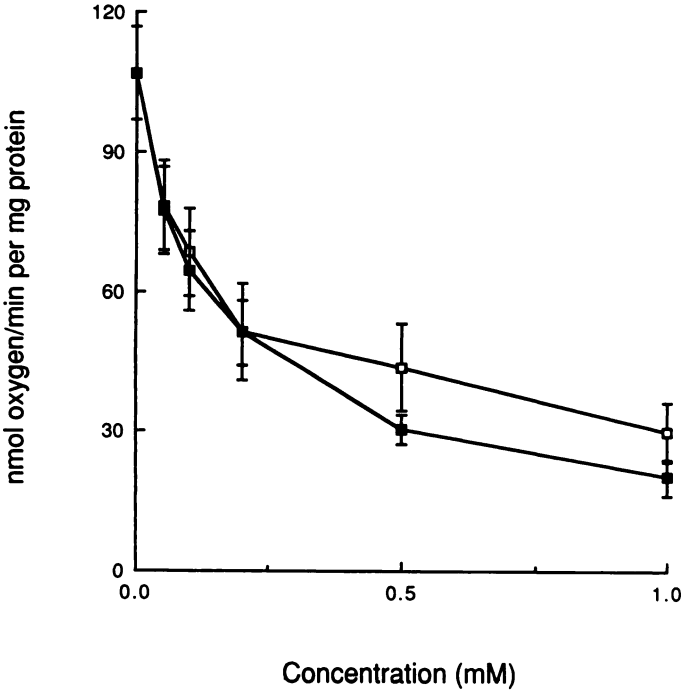

Fig. 1. Inhibition of mitochondrial state 3 respiration by DCVC and DCVHC. Oxygen consumption was measured as described in Experimental Procedures in mitochondria incubated with the indicated concentrations of DCVC (□) or DCVHC (■) for 120 min. State 3 respiration was measured in the presence of 3.3 mM succinate and 0.3 mM ADP. Results are means ± SE of mitochondrial preparations from three animals.

concentration of approximately 0.2 mM. In contrast to the inhibition of state 3 respiration, neither *S*-conjugate affected state 4 respiration (data not shown). The progressive decrease in respiratory control ratios from approximately 4 to 1 as *S*-conjugate concentrations were increased from 0 to 1 mM is attributable to decreases in rates of state 3 respiration. A concentration of 1 mM of each *S*-conjugate was used in all subsequent incubations. As observed in intact kidney cells (6, 10), the use of different respiratory substrates in isolated mitochondria results in differing degrees of inhibition of oxygen consumption by DCVC or DCVHC (Table 1). State 3 respiration was inhibited only when succinate was used as the respiratory substrate. No effect on state 4 respiration was observed, indicating that the uncoupling was due to the decreases in state 3 respiration rather than to increases in state 4 respiration. Oxygen consumption with all respiratory substrates was inhibited (>95%) by 1 mM KCN, indicating the involvement of the electron transport chain.

Second, because previous studies with isolated kidney cells showed that both DCVC and DCVHC inhibited mitochondrial Ca^{2+} sequestration (6, 10), this mitochondrial function was examined in isolated mitochondria (Fig. 2). Mitochondria were incubated with 50 μM Ca^{2+}, and the effect of DCVC and DCVHC on Ca^{2+} retention was examined at various times. Both *S*-conjugates caused a progressive release of Ca^{2+} from mitochondria. The time of this effect was similar to that previously observed for the inhibition of respiration (5).

Third, addition of DCVC or DCVHC impaired the ability of mitochondria to generate a membrane potential when energized with succinate (Fig. 3). Untreated mitochondria generated a membrane potential of greater than 140 mV (negative inside) throughout the time course of the incubation. *S*-conjugate-

TABLE 1

Effect of respiratory substrate on *S*-conjugate-induced changes in mitochondrial respiration

Mitochondria (1.5–3.0 mg protein/ml) were incubated for 120 min with the indicated additions. State 3 respiration was measured by addition of the indicated respiratory substrates and 0.3 mM ADP to mitochondria in an oxygen electrode at 28°C. State 4 respiration was measured as the rate of oxygen consumption after exhaustion of ADP. Respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 rates of oxygen consumption. Results are means ± SE of mitochondrial preparations from three animals.

	State 3	State 4	RCR
	nmol O_2/min/mg protein		
4 mM Glutamate + 2 mM malate			
Control	21.5 ± 2.0	5.8 ± 1.1	3.7 ± 0.2
1 mM DCVC	18.4 ± 1.8	5.9 ± 1.1	3.1 ± 0.3
1 mM DCVHC	17.2 ± 1.6	5.6 ± 1.3	3.1 ± 0.3
3.3 mM Succinate			
Control	78.7 ± 2.0	20.4 ± 1.4	3.9 ± 0.4
1 mM DCVC	31.7 ± 2.9*	19.7 ± 1.1	1.6 ± 0.3*
1 mM DCVHC	23.2 ± 3.1*	17.9 ± 0.3	1.3 ± 0.2*
1 mM Ascorbate + 0.2 mM TMPD			
Control	113 ± 9	24.2 ± 5.0	5.1 ± 1.1
1 mM DCVC	108 ± 10	24.4 ± 5.1	5.0 ± 1.2
1 mM DCVHC	106 ± 9	24.7 ± 5.3	4.7 ± 1.0

* Statistically significant difference ($p < 0.05$) from control.

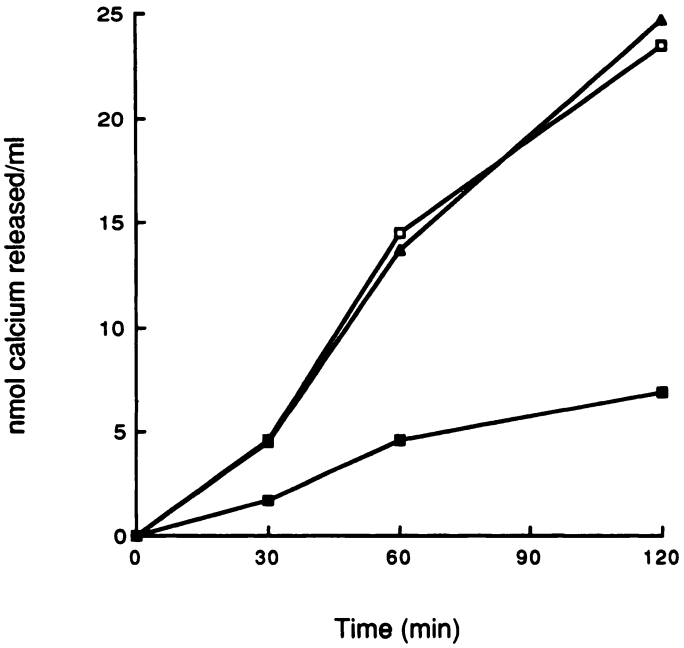

Fig. 2. *S*-conjugate-induced Ca^{2+} release. Mitochondria (1 mg protein/ml) were incubated with 50 μM $CaCl_2$ for 5 min before addition of buffer (■), 1 mM DCVC (□), or 1 mM DCVHC (▲). At the indicated times, Ca^{2+} release was determined by the arsenazo III method, as described in Experimental Procedures. Results are from one mitochondrial preparation, which is representative of preparations from three animals.

treated mitochondria gradually showed a decreased ability to generate a membrane potential; by 2 hr of incubation the membrane potential was <100 mV.

Mitochondrial structural integrity. The functional alterations described above may lead to or be due to structural damage to the mitochondria. This possibility was investigated with two methods. In the first method, the effect of DCVC and DCVHC on the ability of isosmolar solutions of PEG of various

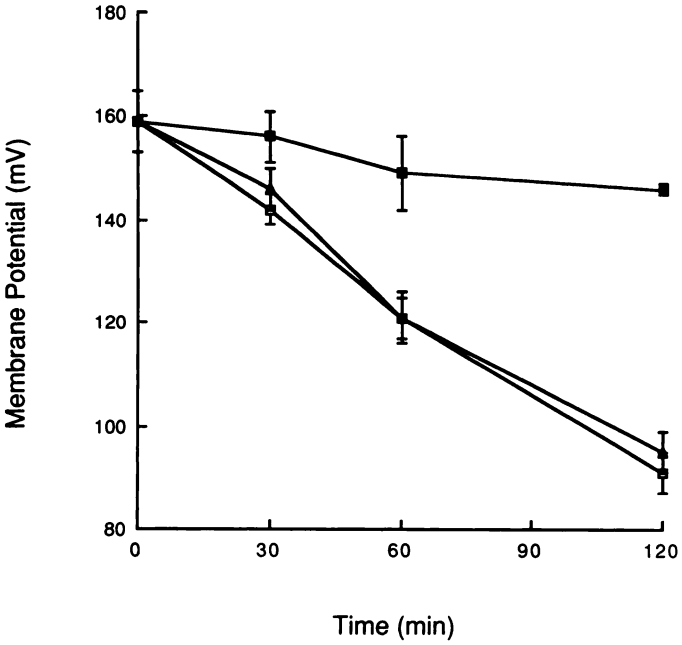

Fig. 3. Effect of DCVC and DCVHC on ability of mitochondria to generate a membrane potential. The ability of mitochondria, incubated for the indicated times with buffer (■), 1 mM DCVC (□), or 1 mM DCVHC (▲) to generate a membrane potential was investigated with safranine O by measuring changes in the absorbance at 511–533 nm. Samples of mitochondria (1 ml, 1 mg protein/ml) were added to cuvettes containing 5 μg/ml rotenone, 5 μg/ml oligomycin, 10 μM safranine O, and 5 mM succinate, and the absorbance was recorded. The membrane potential was abolished by addition of 1 μM FCCP and 20 mM KCl. The safranine O signal was calibrated with valinomycin-induced K^+-diffusion potentials as described under Experimental Procedures. Results are means ± SE of mitochondrial preparations from three animals. Significantly different ($p < 0.05$) from control: DCVC, all times; DCVHC, 30, 60, 120 min.

molecular weights to shrink swollen mitochondria was examined. Mitochondria exposed to 1 mM DCVC or 1 mM DCVHC exhibited a time-dependent increase in their permeability to PEG of $M_r = 1000$, as indicated by large increases in absorbance at 540 nm. Suspensions of mitochondria incubated with sucrose had A_{540} values of 0.04, 0.05, 0.06, and 0.08 at 0, 30, 60, and 120 min, respectively. In contrast, suspensions of mitochondria incubated with DCVC had A_{540} values of 0.08, 0.15, and 0.30 at 30, 60, and 120 min, respectively, and those incubated with DCVHC had A_{540} values of 0.06, 0.15, and 0.32 at 30, 60, and 120 min, respectively. Mitochondria are normally impermeable to PEG molecules of $M_r \geq 200$ and therefore will not show volume changes in such solutions (20). Incubation of mitochondria with DCVC or DCVHC for 2 hr resulted in progressively larger increases in A_{540} as the PEG molecular weight was increased from 200 to 1000 (data not shown).

In the second method, further evidence of *S*-conjugate-induced permeability changes was obtained by measuring leakage of protein and of the matrix enzyme malic dehydrogenase from mitochondria. After incubation for various times, mitochondria were isolated by centrifugation for 4 min at 10,000 × *g*, and protein concentrations and malic dehydrogenase activities were measured in the mitochondrial pellets and in the supernatants. Initially, control incubations contained 4.9% of the total protein in the supernatant; the amount of protein found in the supernatant was 7.0% of the total after 120 min incubation with buffer and was 9.0 and 9.5% of the total after 120 min incubation with 1 mM DCVC or 1 mM DCVHC, respectively. Larger differences were seen when malic dehydrogenase leakage was assessed: the percentage of total activity in the supernatant fraction from control mitochondria increased from an initial value of 6% to 8, 11, and 12% after 30, 60, and 120 min, respectively; mitochondria incubated with 1 mM DCVC had 9, 13, and 22% of the total activity in the supernatant after 30, 60, and 120 min, respectively, and mitochondria incubated with 1 mM DCVHC had 9, 15, and 22% of the total activity in the supernatant after 30, 60, and 120 min, respectively. Significant leakage of enzyme activity, indicating irreversible damage to the mitochondrial inner membrane, was not observed until 120 min of incubation with the *S*-conjugates. *S*-Conjugates did not inhibit malic dehydrogenase activity (data not shown).

Mitochondrial metabolism. The effects of DCVC and DCVHC on mitochondrial function and structural integrity indicated that more fundamental changes, such as those involving the activity of key enzymes or the concentrations of important metabolites, may also occur. Indeed, marked and rapid effects of DCVC and DCVHC on the concentrations of several intermediates in the citric acid cycle were observed (Fig. 4). In all cases, the two *S*-conjugates had nearly identical effects on metabolite concentrations. Citrate concentrations were not increased by incubation with DCVC or DCVHC (Fig. 4A), whereas isocitrate concentrations were increased by DCVHC within 15 min of incubation (Fig. 4B). In contrast, α-ketoglutarate concentrations were decreased after 30 min of incubation with DCVC (Fig. 4C). The largest effects were seen with succinate concentrations, which doubled within 15 min of exposure to the *S*-conjugates (Fig. 4D); malate concentrations were only slightly increased (30%) after 60 min of *S*-conjugate exposure (Fig. 4B). Effects on the concentrations of glutamate (Fig. 4F) and aspartate (Fig. 4G) were also examined, because these two amino acids interact with the citric acid cycle via transamination reactions. Glutamate concentrations decreased 20 and 40% after 15 and 30 min, respectively, of incubation with DCVC or DCVHC. Aspartate concentrations, however, nearly doubled after 15 min. Although there was some variability in the pattern and time course of the *S*-conjugate-induced changes in mitochondrial metabolite concentrations, DCVC and DCVHC produced alterations in substrate utilization that generally preceded *S*-conjugate-induced inhibition of mitochondrial structure and function.

The changes in citric acid cycle metabolites produced by DCVC and DCVHC were accompanied by pronounced inhibitory effects on isocitrate dehydrogenase and succinate:cytochrome *c* oxidoreductase activities (Fig. 5). Both enzyme activities were inhibited by more than 60% by both *S*-conjugates. As noted above, malic dehydrogenase activity was not inhibited by DCVC or DCVHC, indicating that the observed inhibition was not due to a general decrease in mitochondrial metabolism.

Rapid, *S*-conjugate-induced changes were also observed in the adenine nucleotide status of the mitochondria (Fig. 6). Incubation of mitochondria with DCVC or DCVHC decreased the ATP concentrations from 4.5 nmol/mg protein to approximately 2 nmol/mg protein within 30 min; concomitantly, ADP and AMP concentrations were increased in the presence of DCVC but were unaltered by DCVHC (data not shown).

GSH concentrations rapidly declined in *S*-conjugate-treated mitochondria, decreasing by approximately 70% during the 2 hr incubation (Fig. 7A). The GSH lost was nearly quantitatively

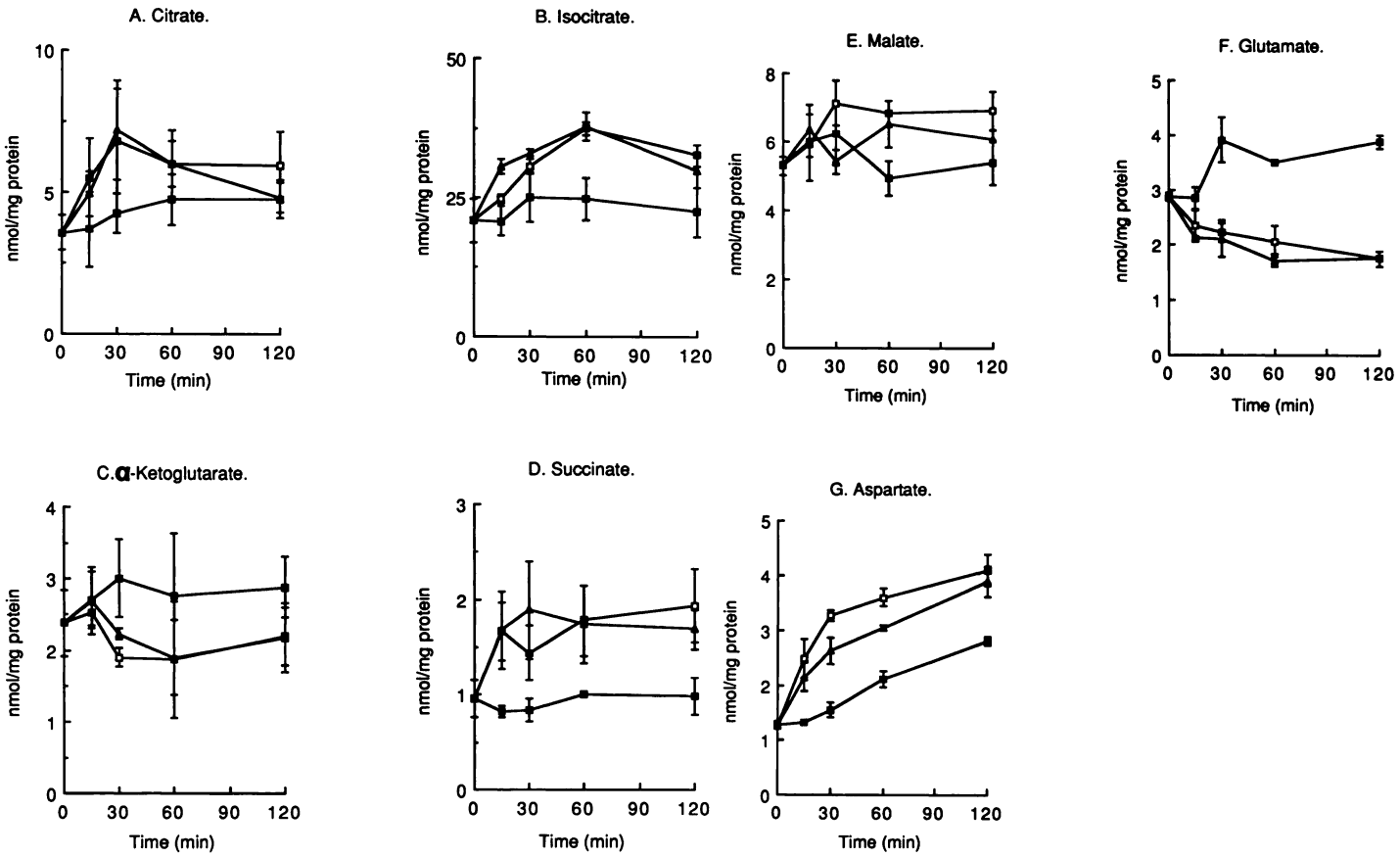

Fig. 4. Effects of DCVC and DCVHC on concentrations of mitochondrial metabolites. Mitochondria were incubated with buffer (■), 1 mM DCVC (□), or 1 mM DCVHC (▲). At the indicated times, neutralized trichloroacetic acid extracts were assayed for various metabolites. Results are means ± SE of mitochondrial preparations from three animals. Significant difference ($p < 0.05$) from control: A, DCVC none; DCVHC none; B, DCVC 60, 120 min; DCVHC 15, 30, 60 min; C, DCVC 30 min; DCVHC none; D, DCVC all; DCVHC 15, 30, 120 min; E, DCVC 60, 120 min; DCVHC 60 min; F, DCVC 30, 60, 120 min; DCVHC all; G, DCVC all; DCVHC all.

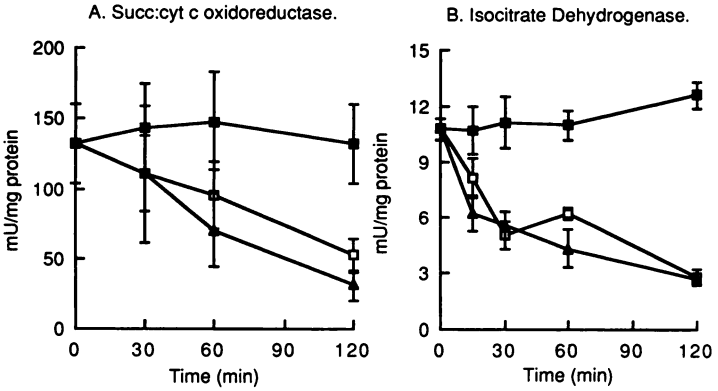

Fig. 5. Inhibition of isocitrate dehydrogenase and succinate:cytochrome *c* oxidoreductase activities by DCVC and DCVHC. Mitochondria were incubated with buffer (■), 1 mM DCVC (□), or 1 mM DCVHC (▲). At the indicated times, samples were treated with 0.05% (vol/vol) Lubrol PX, and isocitrate dehydrogenase (A) and succinate:cytochrome *c* oxidoreductase (B) activities were measured. Results are means ± SE of mitochondrial preparations from four animals. Significant difference ($p < 0.05$) from control: A) DCVC 120 min; DCVHC 120 min; B, DCVC 30, 60, 120 min; DCVHC all.

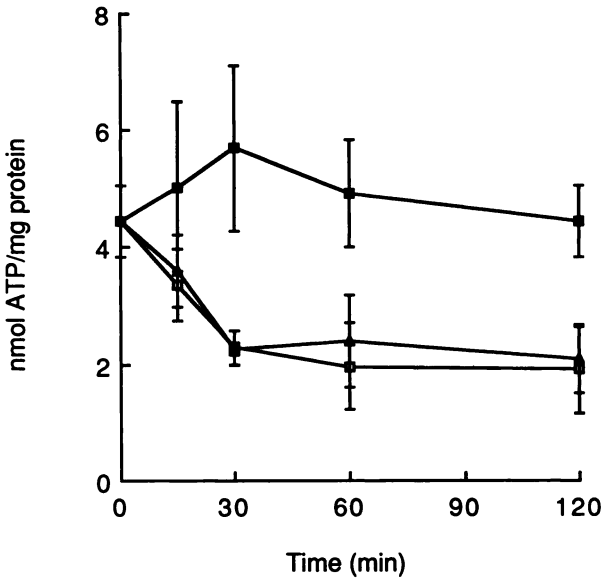

Fig. 6. Effects of DCVC and DCVHC on mitochondrial ATP concentrations. Mitochondria were incubated with buffer (■), 1 mM DCVC (□), or 1 mM DCVHC (▲). At the indicated times, neutralized trichloroacetic acid extracts were assayed for ATP concentrations. Results are means ± SE of mitochondrial preparations from three animals. Significant difference ($p < 0.05$) from control: DCVC 30, 60, 120 min; DCVHC 30, 60, 120 min.

recovered as GSSG (Fig. 7B). During the course of the incubations, GSSG concentrations increased from less than 10% of the total acid-soluble glutathione pool (GSH + 2 GSSG) to nearly 80% of the total pool size. TBA reactants, a measure of

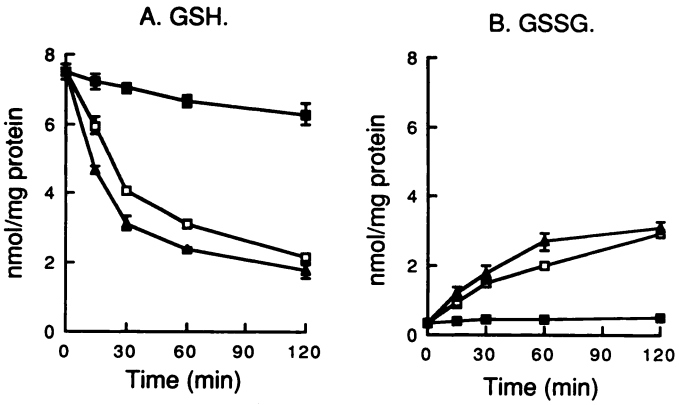

Fig. 7. Alteration of mitochondrial glutathione status by DCVC and DCVHC. Mitochondria were incubated with buffer (■), 1 mM DCVC (□), or 1 mM DCVHC (▲). At the indicated times, neutralized trichloroacetic acid extracts were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene and were analyzed by high-pressure liquid chromatography for GSH (A) and GSSG (B) concentrations as described under Experimental Procedures. Results are means ± SE of mitochondrial preparations from three animals. Measurements in the presence of either DCVC or DCVHC were significantly different ($p < 0.05$) from paired controls at all times.

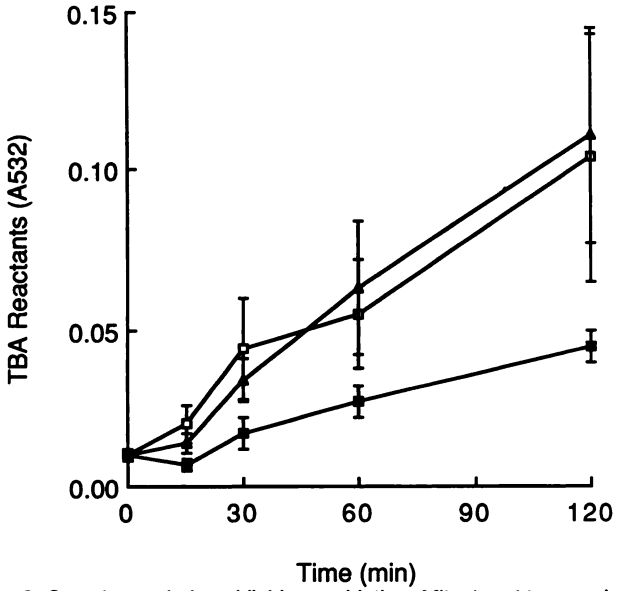

Fig. 8. S-conjugate-induced lipid peroxidation. Mitochondria were incubated with buffer (■), 1 mM DCVC (□), or 1 mM DCVHC (▲) for the indicated times. Lipid peroxidation was measured as malondialdehyde formation and is expressed as TBA reactants in absorbance units. Results are means ± SE of mitochondrial preparations from four animals. Measurements in the presence of either DCVC or DCVHC were significantly different ($p < 0.05$) from paired controls at all times.

lipid peroxidation, were increased by incubation of mitochondria with DCVC or DCVHC (Fig. 8). Comparison of this time course with the more rapid oxidation of GSH to GSSG indicates that GSH oxidation precedes the onset of lipid peroxidation.

Discussion

Renal proximal tubular cells are highly dependent on mitochondria to perform the various energy-dependent activities necessary for normal cellular function (28). The toxic effects of both DCVC and the homocysteine analogue DCVHC in isolated kidney cells and isolated kidney mitochondria are prevented by inhibition of the bioactivating enzymes, indicating that metabolites of DCVC and DCVHC and not the *S*-conjugates themselves are responsible for the toxicity (5, 6, 9–11). Although previous studies (5–8, 10) established that mitochondrial function is inhibited by DCVC and DCVHC, the current work uses isolated mitochondria to examine mitochondrial function, structural integrity, and metabolism in greater detail to gain a better understanding of how these *S*-conjugates produce mitochondrial toxicity and ultimately cell death.

Although significant differences exist in the mechanism of DCVC- and DCVHC-induced cytotoxicity in isolated kidney cells (6, 9, 10), no significant differences were observed in the present study in the mechanism of DCVC- and DCVHC-induced toxicity in isolated kidney mitochondria. This indicates that the factors responsible for the greater potency and different mode of action of DCVHC compared with DCVC in the intact cell are extramitochondrial in origin. This conclusion suggests further that chemically induced cytotoxicity may be produced by mechanisms that do not involve interference with mitochondrial function.

Both *S*-conjugates inhibited three important mitochondrial functions: state 3 respiration, Ca^{2+} sequestration, and the ability to generate a membrane potential. Only state 3 respiration with succinate as the electron donor was inhibited. The lack of inhibition with ascorbate + TMPD as respiratory substrates indicates that DCVC and DCVHC do not act at complex IV (cytochrome oxidase). The small amount of inhibition (<20%) with glutamate + malate as respiratory substrates indicates that complex I (NADH dehydrogenase) is also not the site of action of the *S*-conjugates. The potent inhibition of state 3 respiration with succinate as respiratory substrate and the inhibition of succinate:cytochrome *c* oxidoreductase activity indicate that complex II (succinate dehydrogenase) may be a primary site of action of DCVC and DCVHC. The results may, however, also be explained by inhibition of electron transfer between ubiquinone and complex III (cytochrome *b*-cytochrome c_1 complex). Because the rate of electron transfer from glutamate + malate is much slower than that from succinate, inhibition of flux through complex III with the former respiratory substrates as electron donors may not be detected by measurement of oxygen consumption.

Stonard and Parker (29) found that DCVC caused inhibition of state 3 and uncoupled respiration in liver mitochondria with substrates that donate electrons to NADH dehydrogenase. The difference between this and our results (Table 1) may be attributable to differences between liver and kidney mitochondria or to differences in incubation conditions. Although the explanation is unclear at present, we have previously observed the same pattern of inhibition with different respiratory substrates in isolated kidney cells (6, 10).

The inability of mitochondria to retain Ca^{2+} has been associated with increased inner membrane permeability and with the failure of the mitochondria to generate a membrane potential (16, 20, 30). The *S*-conjugate-induced decrease in the ability to generate a membrane potential and the swelling of matrix volume and leakage of matrix protein and malic dehydrogenase observed in the present study are also associated with the observed perturbations in Ca^{2+} sequestration. Although membrane damage and alterations in mitochondrial Ca^{2+} homeostasis are also often associated with uncoupling of respiration due to increases in state 4 respiration, DCVC and DCVHC did not

stimulate state 4 respiration. Rather, respiration was effectively uncoupled due to decreases in the state 3 rate and the inhibition of substrate metabolism.

Effects on mitochondrial function were observed only after 30–60 min incubation with the *S*-conjugates (Ref. 5 and Figs. 2 and 3). In contrast, deterioration of mitochondrial structure occurred primarily between 60 and 120 min incubation, indicating that alterations in mitochondrial structural integrity, which indicate irreversible damage, are preceded by, and may be caused by, the *S*-conjugate-induced impairment of function. Likewise, the changes in mitochondrial function cannot be ascribed to general damage to the organelle, because functional changes were observed before structural alterations were detectable.

To identify underlying causes of *S*-conjugate-induced inhibition of mitochondrial function and damage to mitochondrial structure, the effects of DCVC and DCVHC on mitochondrial metabolism were examined; these studies focused on the citric acid cycle, adenine nucleotide status, and GSH status. Changes in the concentrations of metabolites and the activities of affected enzymes caused by incubation with DCVC and DCVHC were apparent within 15–30 min (Figs. 4–7). Comparison of the time courses of these metabolic effects with those of the functional and structural changes indicates that the primary effects of DCVC and DCVHC in renal mitochondria are the inhibition of energy metabolism and the disturbance of thiol-disulfide status.

Alterations of mitochondrial GSH status may be more important than alterations of cytosolic GSH status in the mechanism of toxicity of many compounds (31–33). The oxidation of mitochondrial protein sulfhydryl groups, which may be associated with the oxidation of GSH to GSSG (34, 35), may also play a role in cytotoxicity, and the thiol-disulfide status of mitochondrial proteins is critical in the control of inner membrane permeability and structure (36, 37). In the present study, the sulfhydryl-dependent enzymes succinate:cytochrome *c* oxidoreductase and isocitrate dehydrogenase were inhibited by DCVC and DCVHC (Fig. 5). Earlier studies with rat liver mitochondria showed that two other sulfhydryl-dependent enzymes, pyruvate and α-ketoglutarate dehydrogenases, are also inhibited by DCVC (29, 38). Although Parker (7) and Stonard and Parker(29, 38) observed inhibition of state 3 respiration with pyruvate as the substrate, indicating inhibition at site I, only modest inhibition was observed with glutamate + malate as respiratory substrates in the present study (Table 1). This difference suggests that whereas some sulfhydryl-dependent enzymes are inhibited by DCVC and DCVHC under our experimental conditions, other enzymes, such as the pyruvate and α-ketoglutarate dehydrogenases, may not be inhibited. One explanation for these observations is that each of these enzymes may be differentially sensitive to compounds that alkylate or oxidize critical sulfhydryl groups.

S-conjugate-induced oxidation of GSH may be associated with the inhibition of sulfhydryl-sensitive enzymes. Malis and Bonventre (39) found, however, that mitochondrial accumulation of Ca^{2+} and generation of oxygen free radicals leads to inhibition of electron transport at NADH coenzyme Q reductase (site I of respiratory chain). Because the *S*-conjugates did not affect electron transport at site I, but did produce alterations in Ca^{2+} homeostasis, oxidation of GSH, and lipid peroxidation, oxidative stress is probably not the mechanism by which these enzymes are inhibited. Rather, a direct effect on the enzymes in question may be involved. Further studies are warranted to determine the mechanism of inhibition.

In mitochondria, both DCVC and DCVHC caused GSH oxidation and lipid peroxidation (Figs. 7, 8). In the intact kidney cell, however, DCVC produced only a small decrease, whereas DCVHC produced a much larger decrease in cellular GSH concentrations (6, 10). As suggested previously (10), this difference in effects on cellular GSH status may be due to the generation of a potent Michael acceptor in addition to 1,2-dichlorovinylthiol during DCVHC bioactivation, which may react with GSH; pyruvate is formed during DCVC bioactivation and will not deplete GSH. In contrast to the findings in isolated mitochondria described above, neither compound increased GSSG concentrations or caused lipid peroxidation in intact cells. Most of the GSH lost in the intact cell may be due to mixed disulfide formation or to the formation of an adduct between GSH and an electrophile derived from DCVHC (10). These observations suggest that a localized oxidative stress is produced by DCVC and DCVHC and that extramitochondrial processes may prevent the initiation or propagation, or may promote termination, of lipid peroxidation in the intact renal cell. Apparently, isolated mitochondria lack these protective processes or are much more sensitive to oxidative stress.

In conclusion, the nephrotoxic *S*-conjugates DCVC and DCVHC inhibited energy metabolism and caused oxidation of GSH in renal mitochondria. These effects may lead to impairment of mitochondrial function and ultimately to irreversible structural damage. These findings describe a biochemical mechanism for the mitochondrial toxicity of halogenated *S*-conjugates. Future studies to determine the relationship between mitochondrial dysfunction and cytotoxicity will provide a better understanding of *S*-conjugate-induced cell and tissue damage.

Acknowledgment

The authors thank Lori J. Mittelstaedt for typing the manuscript.

References

1. Elfarra, A. A., and M. W. Anders. Renal processing of glutathione conjugates: role in nephrotoxicity. *Biochem. Pharmacol.* **33**:3729–3732 (1984).
2. Hassall, C. D., A. J. Gandolfi, R. C. Duhamel, and K. Brendel. The formation and biotransformation of cysteine conjugates of halogenated ethylenes by rabbit renal tubules. *Chem.-Biol. Interact.* **49**:283–297 (1984).
3. Stevens, J. L. Cysteine conjugate β-lyase activities in rat kidney cortex: subcellular localization and relationship to the hepatic enzyme. *Biochem. Biophys. Res. Commun.* **129**:499–504 (1985).
4. Elfarra, A. A., I. Jakobson, and M. W. Anders. Mechanism of *S*-(1,2-dichlorovinyl)glutathione-induced nephrotoxicity. *Biochem. Pharmacol.* **35**:283–288 (1986).
5. Lash, L. H., A. A. Elfarra, and M. W. Anders. Renal cysteine conjugate β-lyase: bioactivation of nephrotoxic cysteine *S*-conjugates in mitochondrial outer membrane. *J. Biol. Chem.* **261**:5930–5935 (1986).
6. Lash, L. H., and M. W. Anders. Cytotoxicity of *S*-(1,2-dichlorovinyl)-glutathione and *S*-(1,2-dichlorovinyl)-L-cysteine in isolated rat kidney cells. *J. Biol. Chem.* **261**:13076–13081 (1986).
7. Parker, V. H. A biochemical study of the toxicity of *S*-dichlorovinyl-L-cysteine. *Food Cosmet. Toxicol.* **3**:75–84 (1965).
8. Stonard, M. D., and V. H. Parker. The metabolism of *S*-(1,2-dichlorovinyl)-L-cysteine by rat liver mitochondria. *Biochem. Pharmacol.* **20**:2429–2437 (1971).
9. Elfarra, A. A., L. H. Lash, and M. W. Anders. Metabolic activation and detoxication of nephrotoxic cysteine and homocysteine *S*-conjugates. *Proc. Natl. Acad. Sci. USA* **83**:2667–2671 (1986).
10. Lash, L. H., A. A. Elfarra, and M. W. Anders. *S*-(1,2-Dichlorovinyl)-L-homocysteine-induced cytotoxicity in isolated rat kidney cells. *Arch. Biochem. Biophys.* **251**:432–439 (1986).
11. Lash, L. H., and M. W. Anders. *S*-Conjugate-induced rat kidney mitochondrial dysfunction. *Pharmacologist* **28**:198 (1986).
12. Kendrick, N. C. Purification of arsenazo III, a Ca^{2+}-sensitive dye. *Anal. Biochem.* **76**:487–501 (1976).
13. McKinney, L. L., J. C. Picken Jr., F. B. Weakley, A. C. Eldridge, R. E.

Campbell, J. C. Cowan, and H. E. Biester. Possible toxic factor of trichloroethylene-extracted soybean oil meal. *J. Am. Chem. Soc.* **81**:909–915 (1959).
14. Johnson, D., and H. Lardy. Isolation of liver and kidney mitochondria. *Methods Enzymol.* **10**:94–96 (1967).
15. Estabrook, R. W. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods Enzymol.* **10**:41–47 (1967).
16. Moore, G. A., S. A. Jewell, G. Bellomo, and S. Orrenius. On the relationship between Ca^{2+} efflux and membrane damage during t-butyl hydroperoxide metabolism by liver mitochondria. *FEBS Lett.* **153**:289–292 (1983).
17. Akerman, K. E. O., and M. K. F. Wikström. Safranine as a probe of the mitochondrial membrane potential. *FEBS Lett.* **68**:191–197 (1976).
18. Rossi, E., and G. F. Azzone. Ion transport in liver mitochondria. Energy barrier and stoichiometry of aerobic K^+ translocation. *Eur. J. Biochem.* **7**:418–426 (1969).
19. Haworth, R. A., and D. R. Hunter. The Ca^{2+}-induced membrane transition in mitochondria. II. Nature of the Ca^{2+} trigger site. *Arch. Biochem. Biophys.* **195**:460–467 (1979).
20. Mittnacht, S., Jr., and J. L. Farber. Reversal of ischemic mitochondrial dysfunction. *J. Biol. Chem.* **256**:3199–3206 (1981).
21. Stacey, N. H., L. R. Cantilena Jr., and C. D. Klaassen. Cadmium toxicity and lipid peroxidation in isolated rat hepatocytes. *Toxicol. Appl. Pharmacol.* **53**:470–480 (1980).
22. Ochoa, S. Malic dehydrogenase from pig heart. *Methods Enzymol.* **1**:735–739 (1955).
23. Plaut, G. W. E., and S.-C. Sung. Diphosphopyridine nucleotide isocitric dehydrogenase from animal tissues. *Methods Enzymol.* **1**:710–714 (1955).
24. Fleischer, S., and B. Fleischer. Removal and binding of polar lipids in mitochondria and other membrane systems. *Methods Enzymol.* **10**:406–433 (1967).
25. Bergmeyer, H. U. *Methods of Enzymatic Analysis*, 2nd Ed. Academic Press, New York (1974).
26. Reed, D. J., J. R. Babson, P. W. Beatty, A. E. Brodie, W. W. Ellis, and D. W. Potter. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal. Biochem.* **106**:55–62 (1980).
27. Read, S. M., and D. H. Northcote. Minimization of variation in the responses to different proteins of the Coomassie blue G dye-binding assay for protein. *Anal. Biochem.* **116**:53–64 (1981).
28. Balaban, R. S., L. J. Mandel, S. P. Soltoff, and J. M. Storey. Coupling of active ion transport and aerobic respiratory rate in isolated renal tubules. *Proc. Natl. Acad. Sci. USA* **77**:447–451 (1980).
29. Stonard, M. D., and V. H. Parker. 2-Oxoacid dehydrogenases of rat liver mitochondria as the site of action of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2-dichlorovinyl)-3-mercaptopropionic acid. *Biochem. Pharmacol.* **20**:2417–2427 (1971).
30. Beatrice, M. C., J. W. Palmer, and D. R. Pfeiffer. The relationship between mitochondrial membrane permeability, membrane potential, and the retention of Ca^{2+} by mitochondria. *J. Biol. Chem.* **255**:8663–8671 (1980).
31. Meredith, M. J., and D. J. Reed. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J. Biol. Chem.* **257**:3747–3753 (1982).
32. Meredith, M. J., and D. J. Reed. Depletion *in vitro* of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). *Biochem. Pharmacol.* **32**:1383–1388 (1983).
33. Romero, F. J., S. Soboll, and H. Sies. Mitochondrial and cytosolic glutathione after depletion by phorone in isolated hepatocytes. Experientia **40**:365–367 (1984).
34. Ku, R. H., and R. E. Billings. The role of mitochondrial glutathione and cellular protein sulfhydryls in formaldehyde toxicity in glutathione-depleted rat hepatocytes. *Arch. Biochem. Biophys.* **247**:183–189 (1986).
35. Di Monte, D., G. Bellomo, H. Thor, P. Nicotera, and S. Orrenius. Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular Ca^{2+} homeostasis. *Arch. Biochem. Biophys.* **235**:343–350 (1984).
36. Le-Quoc, K., and D. Le-Quoc. Control of the mitochondrial inner membrane permeability by sulfhydryl groups. *Arch. Biochem. Biophys.* **216**:639–651 (1982).
37. Le-Quoc, K., and D. Le-Quoc. Crucial role of sulfhydryl groups in the mitochondrial inner membrane structure. *J. Biol. Chem.* **260**:7422–7428 (1985).
38. Stonard, M. D. Further studies on the site and mechanism of action of *S*-(1,2-dichlorovinyl)-L-cysteine and *S*-(1,2-dichlorovinyl)-3-mercaptopropionic acid in rat liver. *Biochem. Pharmacol.* **22**:1329–1335 (1973).
39. Malis, C. D., and J. V. Bonventre. Mechanism of calcium potentiation of oxygen free radical injury to renal mitochondria: a model for post-ischemic and toxic mitochondrial damage. *J. Biol. Chem.* **261**:14201–14208 (1986).

Send reprint requests to: Dr. M. W. Anders, Department of Pharmacology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642.