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MECHANISMS OF DIETHYLDITHIOCARBAMATE-INDUCED LOSS OF CYTOCHROME P-450 FROM RAT LIVER

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Abstract—A single, intraperitoneal injection of diethyldithiocarbamate (DDTC) to adult, male Sprague–Dawley rats decreased hepatic cytochrome P-450 (P-450) concentrations. This effect was dose-dependent over a range of 250 to 750 mg/kg and most prominent 24–36 hr after dosing. Depletion of hepatic glutathione (GSH) by diethylmaleate (DEM) administration significantly decreased P-450 8 hr after concurrent treatment with DDTC at a dose which given alone had little effect on P-450 concentrations. When hepatic microsomes were incubated with DDTC in the presence of NADPH, P-450 was converted to cytochrome P-420 (P-420). Similar incubations employing [^{35}S]DDTC demonstrated strict NADPH-dependent binding of labeled sulfur to microsomal membranes, suggesting that diminished P-450 concentrations are related to the metabolic activation of DDTC. Addition of reduced GSH to incubation mixtures blocked the binding of ^{35}S to microsomal membranes, as well as conversion of P-450 to P-420. DDTC inhibited NADPH-ADP^{3+} mediated peroxidation of microsomal lipids *in vitro*, suggesting that the effect of DDTC on P-450 does not result from stimulation of lipid peroxidation, but may be influenced by the levels of hepatic GSH. DDTC treatment 1 hr after P-450 was pulse labeled by an intravenous injection of [^{3}H]δ-aminolevulinic acid resulted in a 2-fold increase in the rate of loss of radioactivity associated with membrane-bound P-450 heme during the next 20 hr. Within this time interval, hepatic heme oxygenase (HO) activity increased and at 8 hr after dosing was 7-fold greater than control values in the livers, but was unchanged in the kidneys and spleens of DDTC-treated animals. An elevation of hepatic δ-aminolevulinic acid synthetase (δ-ALAS) activity occurred at 8 and 24 hr after DDTC treatment. Since this enzyme is rate limiting in the biosynthesis of heme, its increased activity may represent a compensatory response to offset the DDTC-mediated loss of P-450 heme.

Various thiono-sulfur compounds, including disulfiram (DS), a drug occasionally used in alcohol avoidance therapy [1, 2], and diethyldithiocarbamate (DDTC), a major DS metabolite [3–5], inhibit the activity of P-450 mixed-function oxygenase of rats and mice *in vivo* and *in vitro* [6–14]. Similarly, DS impairs drug biotransformation in humans [15–17]. These effects, along with the hepatic necrosis produced by related compounds such as carbon disulfide and thioacetamide, apparently depend on the covalent modification of cellular macromolecules by reactive metabolites that arise during monooxygenase-catalyzed biotransformation of the thiono-sulfur group. Losses of P-450 and mixed-function oxygenase activity produced by such compounds may be related to the covalent binding of atomic sulfur to microsomal components, particularly P-450, whereas production of hepatic necrosis is related to covalent binding by electrophilic metabolites such as S-oxides, and atomic sulfur to cellular sites other than P-450 [18]. Although covalent binding of reactive metabolites to cellular macromolecules is associated frequently with changes in cell morphology and function, identification of such covalently bound material by itself fails to indicate the precise chemical events that ultimately cause these changes. Therefore, the present studies were undertaken to define the mechanism by which DDTC reduces hepatic P-450 concentrations and to investigate the role of metabolic activation in this process.

MATERIALS AND METHODS

Chemicals. The chemicals utilized in these studies were purchased from the following companies: J. T. Baker Chemical Co. (Phillipsburg, NJ), Eastman Kodak Co. (Rochester, NY), Fisher Scientific Co. (Fair Lawn, NJ), Mallinckrodt Chemical Works (St. Louis, MO), and Sigma Chemical Co. (St. Louis, MO). All chemicals were reagent grade and used without further purification.

Pre-mixed scintillators, tissue solubilizers, and the sodium salt of [^{35}S]diethyldithiocarbamic acid (5 mCi/mmole) were obtained from Amersham/Searle (Arlington Heights, IL). The radiochemical purity of [^{35}S]DDTC exceeded 95% as determined by the manufacturer using silica gel thin-layer

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chromatography and solvent systems of methanol–pyridine–water (20:1:10) and chloroform–methanol (16:3). Tritiated δ-aminolevulinic[3,5-^{3}H (N)] acid hydrochloride (δ-ALA[^{3}H]), with a specific activity of 5.050 Ci/mmole, was purchased from the New England Nuclear Corp. (Boston, MA). The radiochemical purity of this compound as determined by paper chromatography with solvent systems composed of *n*-butanol–acetic acid–water (25:4:10) and *t*-butanol–methylethylketone–formic acid–water (8:6:3:3) was claimed by the manufacturer to exceed 96%. Both radiolabeled compounds were used without further purification.

Enzymes. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), prepared from Bakers Yeast, and steapsin (triacylglycerol acyl-hydrolase EC 3.1.1.3), prepared from hog pancreas, were purchased from the Sigma Chemical Co.

Animals and animal treatments. Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 175–250 g were used for all experiments. They had free access to Purina lab chow and tap water unless otherwise specified.

All animals were decapitated after 24 hr of starvation. Hemoglobin contamination of microsomes was minimized by perfusing livers *in situ* through the vena cava with ice-cold 0.1 M sodium phosphate buffer (pH 7.4, 1.15% KCl). This buffer was also employed to isolate microsomes as previously described [6].

DDTC was dissolved in deionized water and administered in a 0.5 to 1.0 ml volume by intraperitoneal injection.

Diethylmaleate (DEM), in sesame oil (1:1, v:v), was given at a dose of 644 mg/kg by intraperitoneal injection [19].

δ-ALA[^{3}H] in 0.9% saline was administered by tail vein injection at a dose of 100 μCi/kg.

Analytical procedures. Freshly prepared microsomes were used for all assays. Cytochromes P-450, P-420, and b_5 were determined by difference spectroscopy as described by Omura and Sato [20, 21], with extinction coefficients of 91 $mM^{-1}\,cm^{-1}$, 111 $mM^{-1}\,cm^{-1}$, and 185 $mM^{-1}\,cm^{-1}$ respectively. The total heme content of microsomes was determined by difference spectroscopy as described by Falk [22] with an extinction coefficient of 32.4 $mM^{-1}\,cm^{-1}$.

The biuret procedure was employed to measure microsomal protein relative to bovine serum albumin standards [23]. Hepatic GSH concentrations were estimated by determination of nonprotein sulfhydryl groups in 9000 *g* supernatant fractions of liver homogenates according to the method of Beutler *et al.* [24].

In vitro *incubations of microsomes with DDTC.* Pooled microsomes isolated from perfused livers of starved, untreated rats were used for these studies. The final concentrations of incubation components, in 0.1 M phosphate buffer, pH 7.4, were as indicated: microsomal protein, 5 mg/ml; DDTC, 1 mM; glucose-6-phosphate, 10 mM; NADP, 0.43 mM; $MgCl_2$, 5 mM; and glucose-6-phosphate dehydrogenase, 1 enzyme unit/ml. For a given experimental incubation the composition of the corresponding control incubation was identical except for omission of DDTC. Similarly, the compositions of corresponding incubations with and without the NADPH-generating system were identical except for the omission of NADP from the latter. All incubations were done in triplicate at 37° with air as the gaseous phase in a shaking Dubnoff metabolic incubator. After 30 min the incubation mixtures were rapidly cooled to 0°, and microsomes were isolated from the mixtures by ultra-centrifugation (105,000 g_{av}, 15 min). Microsomal pellets were resuspended in fresh phosphate buffer and then analyzed for P-450, P-420, b_5, and total heme.

When microsomes were incubated with [^{35}S]DDTC, the protein concentration was reduced to 2.5 mg/ml, and 2.5 μCi of radioactivity was added to each incubation flask along with cold DDTC so that the final DDTC concentration remained 1 mM. These incubations were also terminated by rapidly cooling the incubation mixtures to 0°, followed by ultra-centrifugation. The incubation supernatants fractions were decanted, and the microsomal pellets were washed repeatedly with hot (50°) methanol to remove non-bound radioactivity. When radioactivity in the methanol washes was reduced to background levels, the microsomal protein was hydrolyzed by incubation in 3 N NaOH for 1 hr at 50°. Aliquots of the hydrolysate were mixed with Aquasol (New England Nuclear) and analyzed for radioactivity with a Beckman liquid scintillation counter (model LS-100).

Labeling of hemoproteins in endoplasmic reticulum. The heme moiety of hepatic cytochrome P-450 was labeled by i.v. administration of δ-ALA[^{3}H] as described by Levin and Kuntzman [25]. One hour after isotope administration, DDTC (750 mg/kg) or water was given by intraperitoneal injection. The rates at which radioactivity was lost from the labeled pools of membrane-bound P-450 heme in DDTC-treated and control rats were then followed by isolating microsomes at various times after treatment, and analyzing for membrane radioactivity after b_5 was solubilized by anerobic digestion of microsomes with steapsin [21]. The digestion medium was buffered at pH 7.4 with 0.1 M phosphate buffer and contained microsomal protein and steapsin at concentrations of 2 mg/ml and 0.2% (w:v) respectively. After 1 hr at 37°, the microsomal digests were rapidly cooled to 0°; the microsomes were pelleted by ultra-centrifugation (105,000 g_{av}, 15 min) then washed, and resuspended in fresh phosphate buffer. Aliquots of this suspension were solubilized with NCS tissue solubilizer (Amersham Corp.) and then analyzed for radioactivity using a toluene, PPO–POPOP* scintillation mixture.

Enzymes assays. Hepatic δ-aminolevulinic acid synthetase (δ-ALAS) activity was determined in whole liver homogenates as described by Marver *et al.* [26]. Livers were homogenized in 3 vol. of Tris–HCl buffer (75 mM, pH 7.2, 0.9% NaCl); then 0.5-ml portions of homogenate were added to incubation flasks containing 1.5 ml of Tris buffer, 200 mM with respect to glycine, and 13 mM with respect to

* Abbreviations: PPO, 2,5-diphenyloxazole; and POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

EDTA. The reaction was carried out at 37° for 45 min and then stopped by the addition of 0.5 ml of 25% trichloroacetic acid (TCA). The formation of δ-ALA was determined spectrophotometrically using modified Ehrlich reagent after δ-ALA was converted to its pyrrole by condensation with acetylacetone [27].

The assay for hepatic heme oxygenase (HO) activity was that of Tenhunen *et al.* [28] as modified by Maines and Kappas [29]. Final concentrations of assay components in 0.1 M phosphate buffer, pH 7.4, were as follows: microsomal protein, 2.5 mg/ml for liver and kidney, and 1.25 mg/ml for spleen; cytosolic protein, 2.0 mg/ml; glucose-6-phosphate, 0.85 mM; NADP, 0.8 mM; $MgCl_2$, 2 mM; glucose-6-phosphate dehydrogenase, 0.5 enzyme units/ml; and hemin, 17 μM. The reaction was monitored within the cell block of an Aminco–Chance dual wavelength spectrophotometer (model DW-2) maintained at 37°, and the conversion of hemin to bilirubin was quantitated by the increase in optical density difference between 464 and 530 nm using an extinction coefficient of 40 $mM^{-1}\,cm^{-1}$.

Effect of DDTC on lipid peroxidation and fatty acid composition. Microsomal lipid peroxidation was determined by measuring the formation of malondialdehyde (MA), a breakdown product of lipid peroxides formed when microsomes are incubated with NADPH [30]. *In vitro* peroxidation of microsomal lipids was studied in the following mixtures: (1) microsomes alone, (2) microsomes plus NADP generator, and (3) microsomes plus NADPH generator plus ADP-Fe^{3+}. All incubations were performed at 37° in a Dubnoff metabolic incubator and contained 3 mg/ml microsomal protein, 56 mM Tris–HCl, pH 7.4, and 50 mM KCl in a total volume of 3 ml. Final concentrations of cofactors in incubations with NADPH generator were: 10 mM glucose-6-phosphate, 5 mM $MgCl_2$, 0.43 mM NADP, 12.5 mM nicotinamide, and 0.5 enzyme units/ml of glucose-6-phosphate dehydrogenase. Incubations with ADP-Fe^{3+} contained ADP and $FeCl_3$ at final concentrations of 4 mM and 20 μM respectively. The incubations were stopped after 45 min by adding 0.1 ml of 6.12 M TCA to incubation flasks which had been placed on ice. Samples (2 ml) of the acidified reaction mixtures were pipetted into centrifuge tubes containing 2.0 ml of 1.84 M TCA and 0.2 ml of 5 M HCl. After mixing, 2.0 ml of 52 mM 2-thiobarbituric acid (TBA) was added, and the tubes were placed in a boiling water bath for 15 min. These suspensions were centrifuged at 1500 *g* for 15 min in an International model EDX centrifuge, and the absorbance of clear supernatant fluid was measured at 535 nm. An extinction coefficient of 156 $mM^{-1}\,cm^{-1}$ was used to calculate MA concentration [30].

The fatty acid composition of the total lipid fraction of incubated microsomes was determined by gas–liquid chromatographic analysis of samples which had been derivatized to their corresponding methyl esters. The 3.0 ml microsomal incubation mixture was first extracted with 15 ml of chloroform–methanol (2:1, v:v) [31], then water (7.5 ml) was added, and the resulting mixture was centrifuged at 425 *g* for 15 min. After centrifugation, the aqueous layer was aspirated, and the protein layer was punctured to remove an aliquot of the organic layer, which was then evaporated to dryness under a stream of N_2. The dried material was dissolved in 4.0 ml of 1 N KOH in methanol and then 0.5 ml of concentrated HCl was added. The fatty acid methyl esters thus formed were extracted into 2.0 ml of hexane [32]. One milliliter of the hexane extract and 1.0 μl of internal standard (0.35 mM methyl heptadecanonate in hexane) were sealed in 1-ml sample vials (Hewlett–Packard 5080-8712). One microliter of this mixture was then injected onto a glass column (2.0 mm i.d. × 6 ft) packed with 10% SD-222-PS (Supelco Inc.) previously conditioned at 220°.

A Hewlett–Packard 5700A gas chromatograph equipped with a model 7671 automatic sampler and interfaced with a model 3373B integrator and 9810A calculator was used to separate, identify, and quantitate the fatty acid methyl esters. The optimal operating conditions were: N_2 carrier flow, 20 ml/min; auxillary gas (N_2) flow, 15 ml/min; H_2 flow, 80 ml/min; air flow, 260 ml/min; column temperature, 200° and flame ionization detector temperature, 300°.

Statistical analyses. Student's *t*-test was used to evaluate the significance of differences between two group means. Multiple comparisons between several experimental groups and one control group were made using the Dunnett procedure subsequent to analysis of variance [33].

RESULTS

Dose- and time-response effects of DDTC on microsomal hemoproteins. As shown in Fig. 1, DDTC administration, in doses ranging from 250 to 750 mg/kg, appeared progressively to decrease P-450 concentrations. Increasing the dose of DDTC to 1 g/kg did not further decrease the P-450 concentration. The action of DDTC was hemoprotein specific in that b_5 concentrations were unaffected by DDTC at all doses examined. A trend to decreased concentrations of microsomal heme also appeared to be evident in DDTC-treated animals. However, only

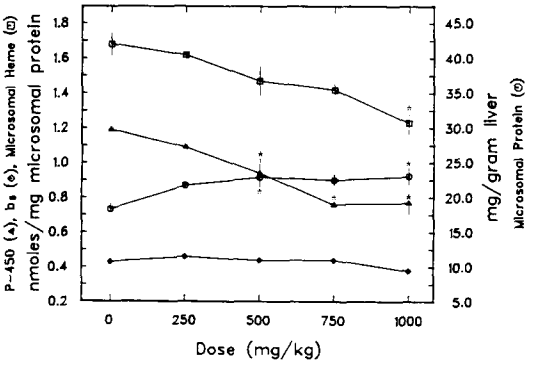

Fig. 1. Dose–response curves showing the affects of DDTC on microsomal cytochrome P-450, cytochrome b_5, total heme, and microsomal hemoprotein. Adult, male Sprague–Dawley rats were killed 24 hr after the i.p. administration of DDTC (or H_2O) at the indicated doses. All values are the means ± S.E. for each group of four animals. Key: (*) significantly different from control, $P < 0.05$.

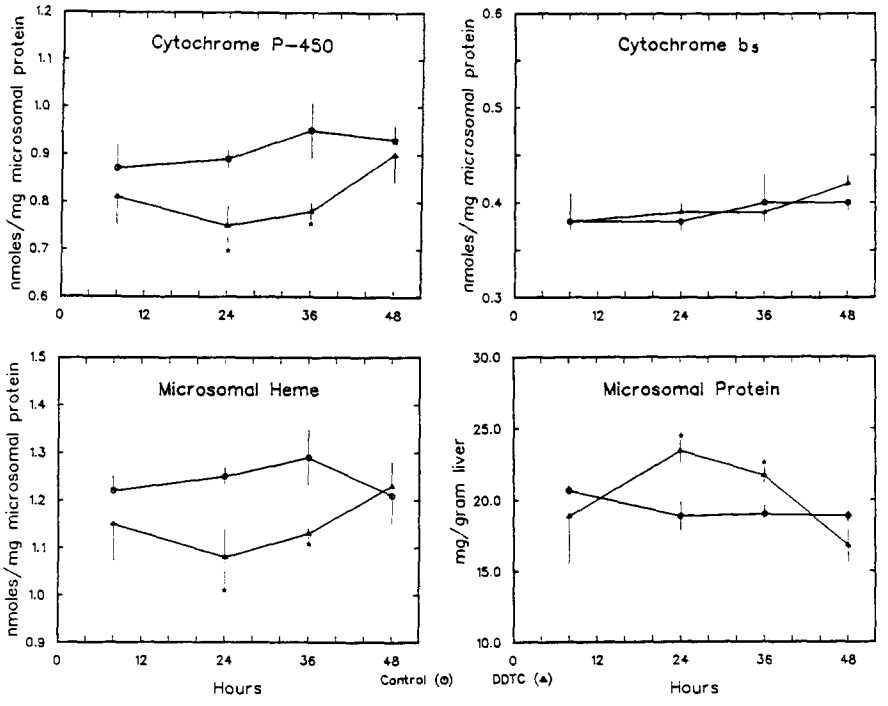

Fig. 2. Time–response curves of the affects of DDTC on microsomal cytochrome P-405, cytochrome b_5, total heme, and microsomal protein. DDTC (750 mg/kg) was administered i.p. to rats at 8, 24, 36 and 48 hr prior to a common time of sacrifice. All values represent the mean ± S.E. for each group of four animals. Key: (*) significantly different from control, $P < 0.05$.

for the highest dosage group was this change statistically significant. On the basis of these results, a 750 mg/kg dose of DDTC was chosen for most subsequent studies. The time–course of the action of DDTC on microsomal hemoproteins is shown in Fig. 2. At the time points examined, maximum depression of P-450 concentrations occurred 24–36 hr after DDTC administration. By 48 hr, P-450 concentrations had returned to control values.

Effect of DDTC on heme loss from microsomal P-450. To determine whether the DDTC-mediated losses of P-450 were due to accelerated P-450 degradation, experiments were performed to evaluate the effect of DDTC on the rate at which radioactivity associated with pulse-labeled P-450 heme was lost from the endoplasmic reticulum *in vivo*. As shown in Fig. 3, membrane radioactivity, indicative of P-450 heme, declined in a biphasic manner with time. Heme half-life values, calculated using a biexponential description of the data and the feathering technique [34], were decreased from 6 to 3 hr in DDTC-treated animals during the fast phase of heme loss. During the slow phase, half-life values increased from 27 to 33 hr. Thus, within hours of its administration, DDTC doubled the rate at which radioactivity associated with P-450 heme was lost from endoplasmic reticulum.

Effects of DDTC on HO and δ-ALAS. Since DDTC altered the homeostasis of P-450 heme in endoplasmic reticulum, we wished to determine whether this observation could be extended to include effects on the activities of HO, the rate-limiting enzyme for heme degradation, and δ-ALAS, the rate-limiting enzyme for heme synthesis. As illustrated in Table 1, hepatic δ-ALAS activity was elevated significantly 8 hr after DDTC administration and remained at higher levels until at least 24 hr after dosing. The effect on hepatic HO activity was more dramatic. Two hours after DDTC was administered, HO activity had increased 276%, and by 8 hr was 705% greater than control values. No changes in HO activity were detected in kidney or spleen.

In vitro *effects of DDTC on microsomal membranes.* To evaluate the degradative potential of DDTC on P-450 *in vitro*, microsomes were incubated with the compound under conditions where the P-450 monooxygenase system was either active (NADPH present) or inactive (NADPH absent). The results obtained in these experiments (Fig. 4) indicate that DDTC alone had little effect on the hemoprotein content of microsomal membranes. However, substantially diminished P-450 concentrations occurred when microsomes were incubated with DDTC in the presence of NADPH. This loss of P-450 was accompanied by an increased membrane content of P-420, but b_5 concentrations were not altered significantly and total microsomal heme remained unchanged. When microsomes were incubated with NADPH alone, their content of P-450, P-420 and total heme was decreased slightly. These changes, shown in the right panel of Fig. 4, probably reflect heme destruction mediated by formation of lipid peroxides [35, 36], an event which can be prevented by DDTC (Table 2).

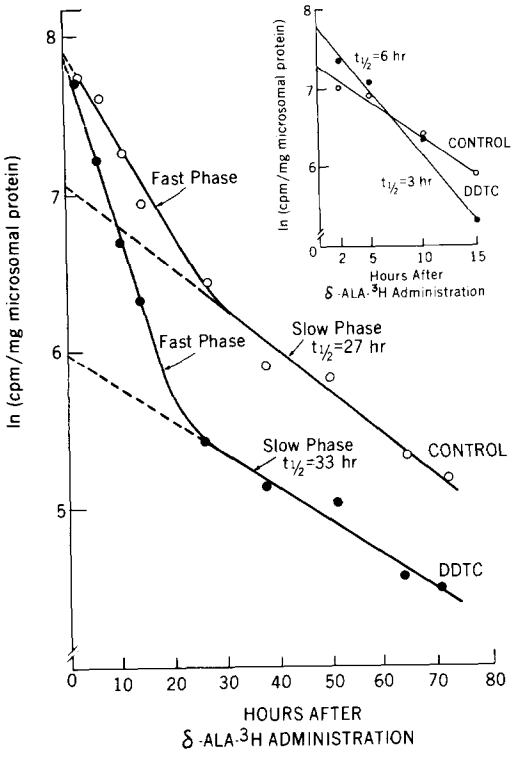

Fig. 3. Effect of DDTC on *in vivo* loss of [^{3}H]P-450 heme from endoplasmic reticulum. Microsomal hemoproteins were labeled by i.v. administration of δ-ALA[^{3}H]; 1 hr later, the animals were divided into two groups and given either water or DDTC by i.p. injection. After various time intervals, microsomes were isolated from control and treated animals and then analyzed for radioactivity after b_5 was solubilized from the membrane. Each time point represents the specific activity of pooled microsomes isolated from two rats. The inset presents fast phase data points which have been replotted after transformation by the feathering technique [34]. Additional details are given in the text.

Summarized in Table 2 are the results of experiments designed to investigate whether the *in vitro* effects of DDTC on P-450 occurred secondarily to peroxidation of microsomal lipids. When added to incubations containing only microsomes, DDTC, at concentrations ranging from 10^{-5} M to 10^{-3} M, decreased the formation of MA. A similar effect was observed when DDTC was added to incubations containing microsomes plus NADPH. However, under these conditions MA formation was inhibited by DDTC concentrations as low as 5×10^{-7} M. Even when the rate of MA formation was greatly stimulated, as in microsomal incubations containing NADPH plus ADP-Fe^{3+}, DDTC remained an effective inhibitor of MA formation, but only if present at concentrations exceeding 10^{-5} M.

To ensure that MA formation reflected peroxidative changes in microsomal lipids, samples of microsomes were taken from the incubations presented in Table 2 and analyzed for fatty acid content. As indicated in Table 2, MA formation increased if the microsomal incubation medium contained either NADPH or NADPH plus ADP-Fe^{3+}. However, Table 3 shows that significant changes in microsomal fatty acid composition were observed only when MA was found in substantial quantities, as in those incubations containing NADPH plus ADP-Fe^{3+}. The most noteworthy alterations in microsomal fatty acid composition included decreased concentrations of total fatty acids, and complete depletion of 20:4 and 22:6 polyunsaturated fatty acids from the microsomes. These results are consistent with other observations concerning fatty acid destruction during lipid peroxidation [37]. DDTC not only inhibited MA formation in microsomal incubations containing NADPH plus ADP-Fe^{3+}, but it also prevented destruction of polyunsaturated fatty acids as seen in incubation D of Table 3. Similar results occurred when DS, instead of DDTC, was added to incubation mixtures [38].

In other experiments, [^{35}S]DDTC was employed to evaluate the role of metabolism of DDTC in the covalent binding of sulfur to microsomal membranes. As shown in Fig. 5, some covalent binding of radioactive material was detected in microsomes incubated with [^{35}S]DDTC in the absence of NADPH. However, the extent of this binding was greatly stimulated when NADPH was present in the medium. These results suggest that metabolic activation of DDTC and subsequent covalent binding

Table 1. Effect of DDTC on δ-aminolevulinic acid synthetase and heme oxygenase activities*

Time of treatment†	δ-Aminolevulinic acid synthetase‡	Heme oxygenase§		
	Liver	Liver	Kidney	Spleen
Control	28.18 ± 2.44	3.15 ± 0.32	1.27 ± 0.14	31.63 ± 0.98
2	25.83 ± 1.02	8.68 ± 1.24‖	1.17 ± 0.08	25.16 ± 1.98
8	43.67 ± 1.86‖	22.21 ± 2.17‖	1.80 ± 0.10	31.56 ± 2.18
24	40.39 ± 4.11‖	5.69 ± 1.30	1.32 ± 0.16	34.09 ± 2.95
48	34.99 ± 1.69	5.20 ± 0.24	1.28 ± 0.19	28.06 ± 3.47

* DDTC (750 mg/kg) was administered i.p. to rats at 2, 8, 24, and 48 hr prior to a common time of sacrifice. Controls were injected with H_2O, 2 hr before sacrifice. All values refer to the mean ± S.E. for each group of three animals.

† Hours prior to sacrifice.

‡ Expressed in nmoles δ-ALA formed per g liver per hr.

§ Expressed in nmoles bilirubin formed per mg protein per hr.

‖ Significantly different from control, $P < 0.05$.

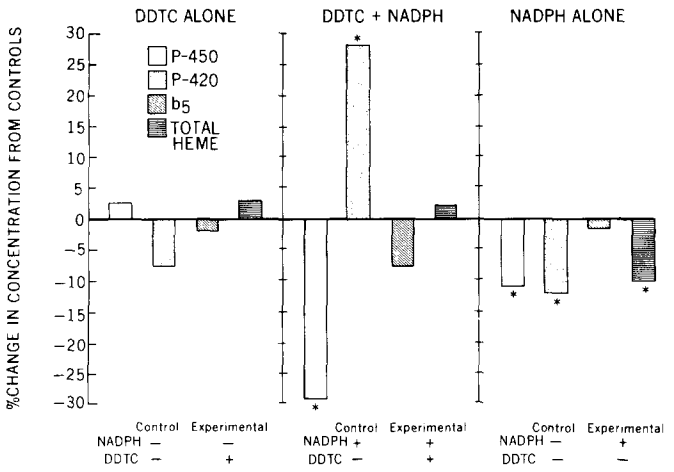

Fig. 4. NADPH-dependent changes in hemoprotein content of microsomes incubated with DDTC. DDTC alone, or DDTC plus an NADPH-generating system were incubated for 30 min at 37° with microsomes as indicated. The incubation mixtures were rapidly cooled to 0°; then microsomes were isolated by centrifugation and analyzed for hemoproteins and heme. All incubations were performed in triplicate, using pooled microsomes isolated from perfused livers of untreated rats. One hundred percent values, shown as the mean ± S.E. of three determinations and expressed as nmoles/mg protein, were: P-450, 1.17 ± 0.03; P-420, 0.43 ± 0.01; b_5, 0.56 ± 0.01; and total heme, 2.12 ± 0.06. Key: (*) significantly different from control, $P < 0.05$.

Table 2. Inhibition of *in vitro* lipid peroxidation by DDTC*

	Lipid peroxidation†		
Incubation additions	Microsomes	Microsomes plus NADPH	Microsomes plus NADPH and ADP-Fe^{3+}
None	0.22	1.59	25.79
DDTC			
1×10^{-3} M	0.01	0.85	0.94
1×10^{-4} M	0.04	0.76	1.07
1×10^{-5} M	0.09	0.69	1.12
1×10^{-6} M	0.21	0.75	25.46
5×10^{-7} M	0.30	0.80	24.70

* Pooled hepatic microsomes from male rats were incubated for 45 min at 37°, either alone, or with NADPH, or with NADPH plus ADP-Fe^{3+} as indicated. DDTC was added prior to the other additions. Malondialdehyde formation, taken as an indicator of lipid peroxidation, was determined as described in Materials and Methods.

† Expressed in nmoles malondialdehyde formed per mg protein per 45 min. Each value is the average of duplicate incubations; this experiment was repeated with similar results.

Table 3. Effect of DDTC on destruction of polyunsaturated fatty acids during microsomal lipid peroxidation*

	Incubation additions			Fatty acid content†						
Incubation	Cofactor	ADP-Fe^{3+}	DDTC‡	Total	16:0	18:0	18:1	18:2	20:4	22:6
A	−	−	−	0.412	0.076	0.107	0.033	0.068	0.105	0.024
B	+	−	−	0.439	0.078	0.111	0.034	0.070	0.110	0.036
C	+	+	−	0.216	0.066	0.086	0.026	0.038	0.000	0.000
D	+	+	1×10^{-5} M	0.409	0.077	0.107	0.028	0.066	0.106	0.026

* Pooled hepatic microsomes from male rats were incubated as described in Table 2. Microsomal fatty acids were measured as described in Materials and Methods.

† Expressed in μmoles fatty acid/mg protein. Each value is the average of duplicate analyses; this experiment was repeated with similar results.

‡ Final concentration.

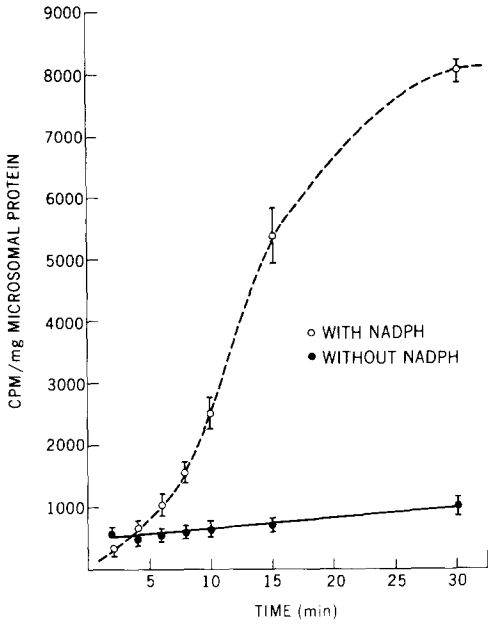

Fig. 5. Binding of ^{35}S to microsomes after incubation with [^{35}S]DDTC. Microsomes were incubated at 37° with [^{35}S]DDTC in the presence or absence of an NADPH-generating system. After various time intervals, the incubation mixtures were rapidly cooled to 0°; the microsomes were isolated by centrifugation, extensively washed with hot (50°) methanol, and then analyzed for covalently bound radioactivity. Each time point represents the mean ± S.E. of four separate incubations of pooled microsomes isolated from perfused livers of untreated rats.

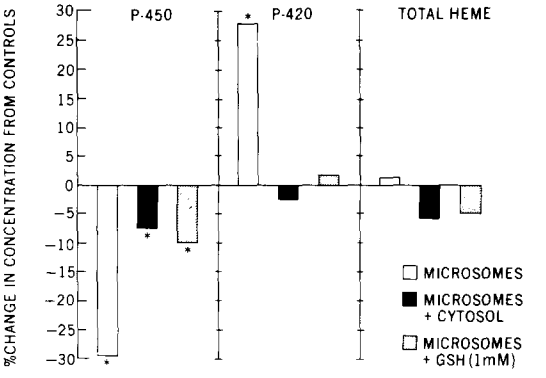

Fig. 6. Effects of liver cytosol and glutathione on NADPH-dependent conversion of P-450 to P-420 in microsomes incubated with DDTC. DDTC plus an NADPH-generating system were incubated with microsomes, microsomes plus liver cytosol, or microsomes plus exogenous GSH under the same conditions as described in the legend of Fig. 4. One hundred percent values, shown as the means ± S.E. of three determinations and expressed as nmoles/mg protein, were: P-450, 1.01 ± 0.03; P-420, 0.41 ± 0.02; and total heme, 2.02 ± 0.05. Key: (*) significantly different from control, $P < 0.05$.

of sulfur may contribute to the effects of DDTC on microsomal membranes.

Modification of the effects of DDTC on microsomal membranes. Table 4 shows results of an experiment designed to determine whether the effects of DDTC on P-450 concentrations *in vivo* could be modified by depletion of hepatic GSH. DEM, administered alone or concurrently with DDTC, produced a marked but transient depletion of hepatic GSH. Treatment with DDTC alone had no effect on GSH concentrations and, under the conditions chosen for this experiment, did not alter significantly P-450 concentrations. Only when DEM was administered concurrently with DDTC were P-450 concentrations appreciably decreased.

That GSH can modify the effects of DDTC on microsomal membranes is further illustrated in Fig. 6. In these *in vitro* studies, liver cytosol and exogenous GSH were each evaluated for their ability to influence DDTC-mediated conversion of P-450 to P-420. As noted previously, an NADPH-dependent

Table 4. Effect of concurrent administration of DDTC and diethylmaleate (DEM) on hepatic glutathione, microsomal hemoproteins, and microsomal heme*

	Reduced glutathione†		Cytochrome P-450‡	Cytochrome b_5‡	Total heme‡
Treatment	1 hr	8 hr	8 hr		
Control (deionized H_2O + sesame oil)	4.60 ± 0.64	5.13 ± 0.43	1.03 ± 0.04	0.40 ± 0.01	1.37 ± 0.04
DEM (644 mg/kg)	1.20 ± 0.44§	4.78 ± 0.39	1.04 ± 0.06	0.41 ± 0.01	1.36 ± 0.06
DDTC (500 mg/kg)	4.85 ± 0.24	5.18 ± 0.36	0.95 ± 0.06	0.39 ± 0.02	1.31 ± 0.07
DEM + DDTC	1.33 ± 0.12§	4.84 ± 0.51	0.82 ± 0.04§	0.38 ± 0.03	1.22 ± 0.02

*Rats were administered DDTC (500 mg/kg) and/or DEM (644 mg/kg) by i.p. injection and then randomly killed in groups of three after 1 hr, or in groups of four after 8 hr. All values represent the mean ± S.E. for each treatment group.

† Expressed in μmoles/g liver.

‡ Expressed in nmoles/mg microsomal protein.

§ Significantly different from control, $P < 0.05$.

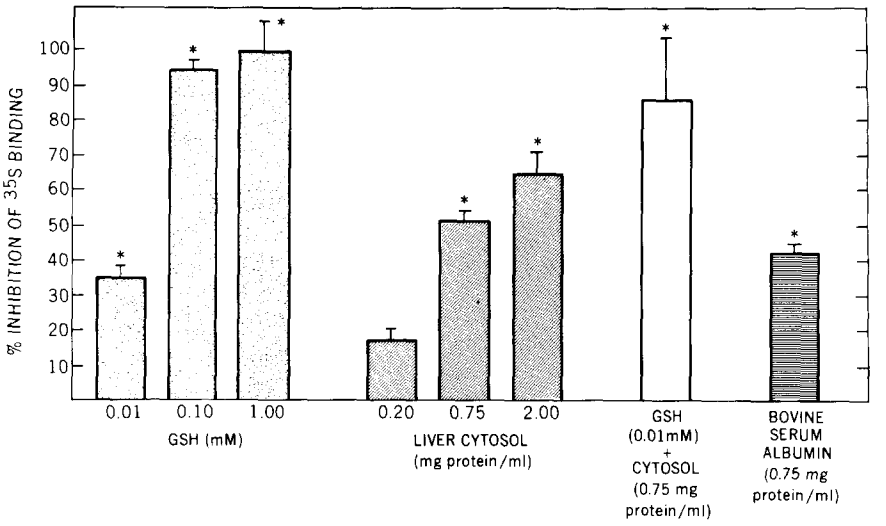

Fig. 7. Inhibitory effects of glutathione and soluble protein on NADPH-dependent binding of ^{35}S to microsomes during incubations with [^{35}S]DDTC. To incubation mixtures containing microsomes, [^{35}S]DDTC, and an NADPH-generating system, each of the following was added at the concentrations indicated: GSH, dialyzed liver cytosol, or bovine serum albumin (BSA). After incubation at 37° for 10 min, microsomes were analyzed for bound radioactivity as described in the legend of Fig. 5. Each incubation was performed in triplicate, and the percent inhibition of ^{35}S-binding ± S.E. was expressed relative to ^{35}S-binding in control incubations that did not contain GSH, liver cytosol, or BSA (see Fig. 5). The control (100%) value for binding of ^{35}S was 2335 ± 237 cpm/mg microsomal protein. Key: (*) significantly different from control, $P < 0.05$.

decrease in P-450 concentrations, accompanied by increased concentrations of P-420, was observed in microsomes incubated with DDTC. When liver cytosol or exogenous GSH was added to the incubation medium, the DDTC-induced conversion of P-450 to P-420 was no longer demonstrable.

In a similar experiment, exogenous GSH, cytosol dialyzed to remove endogenous GSH, and dialyzed cytosol in combination with exogenous GSH were each evaluated for their ability to prevent covalent modification of microsomal membranes during incubations with [^{35}S]DDTC. Also included in this experiment was an incubation containing bovine serum albumin (BSA) to determine if inhibition of ^{35}S-binding might be a general property of proteins capable of forming mixed disulfides. As shown in Fig. 7, almost total inhibition of ^{35}S-binding to microsomes was observed when GSH was present in the incubation medium at concentrations of 1.0 or 0.1 mM. Dialyzed cytosol also diminished ^{35}S-binding. The extent of this inhibition ranged from 16 to 62% for cytosolic protein concentrations of 0.2 and 2.0 mg/ml respectively. BSA was somewhat less effective than cytosolic protein in preventing covalent binding of ^{35}S to microsomes. At a concentration of 0.75 mg/ml, cytosolic protein produced a 49% inhibition of ^{35}S-binding. An equivalent concentration of BSA inhibited ^{35}S-binding by 38%. When a combination of exogenous GSH and cytosolic protein was added to the incubation medium, additive inhibition of ^{35}S-binding to microsomes was observed.

DISCUSSION

Our studies demonstrate that metabolic activation of DDTC impairs P-450-dependent monooxygenase activity. They also suggest that additional factors within the hepatocyte play a role in determining the degree of this impairment. These additional factors include the availability of GSH and activity of δ-ALAS and HO, the key enzymes in the synthesis and degradation, respectively, of P-450 heme.

DDTC treatment, 1 hr after i.v. administration of δ-ALA[3], accelerated the loss of ^{3}H-labeled P-450 heme from endoplasmic reticulum, suggesting that preexisting P-450 molecules were subject to an increased rate of degradation (Fig. 3). This effect, apparent within a few hours of DDTC treatment, was sustained for approximately 20 hr. Therefore, stimulation of P-450 degradation by DDTC was maintained over much of the time interval required for P-450 concentrations to become maximally depressed (Fig. 2).

Heme is degraded *in vivo* to bilirubin in a two-step reaction in which HO-catalyzed formation of biliverdin is rate limiting [28, 39]. The best substrate for HO appears to be protohemin IX. However, HO is also active toward a variety of hemoprotein substrates, providing the heme–protein complex is easily dissociable [28]. If metabolic activation of DDTC, with subsequent binding of metabolites to P-450, destabilizes the P-450 apoprotein–heme complex, diminished P-450 concentrations might be attributed to increased vulnerability of P-450 heme to catabolism by HO. Other studies have shown that the heme moiety of P-420, but not that of P-450, is dissociable from its apoprotein and can be transferred from the microsomal membrane to albumin during *in vitro* incubations [40, 41]. Thus, DDTC-mediated conversion of P-450 to P-420 as demon-

strated in our *in vitro* studies (see below) might provide HO with a better pool of substrate.

Furthermore, the increase in P-420 following DDTC treatment may partially account for the dramatic increase in hepatic HO activity observed in the present study, since increased availability of substrate is reported to induce HO activity [42, 43]. It is not clear why HO activity was not also increased in spleen or kidney by DDTC. Our data suggest that metabolic activation of DDTC is required to initiate the biochemical events leading to increased HO activity. If this is the case, DDTC activation by P-450 is probably too low in these tissues to initiate HO induction.

Since DDTC is a potent inhibitor of copper-containing enzymes [44, 45], P-450 could be decreased through inhibition of ferrochelatase, a copper-containing enzyme involved in heme synthesis [46]. In the present study, b_5 concentrations were unchanged by DDTC treatment, suggesting that heme synthesis is not impaired by the compound. Marselos *et al.* [47] in their studies with DS and DDTC reached a similar conclusion.

Our data demonstrate that hepatic heme synthesis may be increased by DDTC, as indicated by sustained elevation of δ-ALAS activity in treated animals. Since heme is believed to regulate δ-ALAS activity by negative feedback control [48–50], perhaps the increase in δ-ALAS activity that we observed was secondary to an increased degradation and eventual depletion of the regulatory heme pool. This would explain the maintenance of P-450 concentrations at or near control levels during the first 8 hr after DDTC treatment and implies that hepatocytes are capable of a limited adaptive response to the effect on P-450 degradation.

In vitro studies show that the interaction of DDTC with the microsomal membrane leads to conversion of P-450 to P-420 (Fig. 4). This effect was observed only in microsomal incubations containing NADPH, suggesting that mixed-function oxygenase activity is necessary for DDTC to initiate this conversion. Furthermore, *in vitro* metabolic activation of DDTC is not accompanied by destruction of P-450 heme, since the total heme content of microsomes remained unchanged after cytochrome conversion. Incubation of microsomes with [^{35}S]DDTC under the same conditions that converted P-450 to P-420 also resulted in covalent binding of sulfur-containing material to the microsomal membrane (Fig. 5). The chemical nature of this covalently-bound material was not established.

Endogenous substances normally present within hepatic cytosol appear to modify the impact of DDTC on endoplasmic reticulum. In rats depleted of hepatic GSH by DEM administration, P-450 concentration was reduced significantly by DDTC at a dose insufficient when given alone to alter P-450 concentration (Table 4). Similarly, GSH prevented the occurrence of two effects associated with the *in vitro* metabolic activation of DDTC: conversion of P-450 to P-420 (Fig. 6) and covalent binding of ^{35}S-containing material to microsomal membranes (Fig. 7). Dialyzed cytosol also inhibited covalent modification of microsomes, as did BSA (Fig. 7). Apparently, alternative binding sites provided by soluble protein [51, 52], as well as GSH [53–55], act to protect the microsomal membrane from DDTC and its metabolites either by interfering with the metabolic activation of DDTC, the binding of metabolites to microsomal components, or both.

In other experiments, peroxidation of microsomal lipids was monitered closely since this phenomenon is accompanied by destruction of P-450 heme [35, 36]. Since CS_2, which can be formed from DDTC *in vivo* [3, 5], has been reported to stimulate lipid peroxidation [54, 55], we considered the possibility that similar effects might occur *in vitro*, especially if degradation of DDTC produced significant quantities of CS_2. This apparently did not occur; DDTC actually inhibited lipid peroxidation since DDTC prevented both the destruction of microsomal polyunsaturated fatty acids and the formation of MA (Tables 2 and 3). Therefore, generalized membrane autooxidation suggested to occur after CS_2 exposure [56], is unlikely to account for the effect of DDTC on P-450 concentrations.

As noted previously, nonenzymatic degradation of DS and DDTC may give rise to CS_2 [3, 5]. Therefore, inhibition of P-450-dependent reactions by DS and DDTC may depend on CS_2 formation [57]. Our previous work [6, 8–10] demonstrated that DS, DDTC, and CS_2 share similar patterns of inhibitory effects on P-450-catalyzed reactions characterized by early inhibition of aniline hydroxylation, followed later by reduced *N*-demethylase activity, and diminished P-450 concentrations. However, the potency and time course of these effects very considerably for each compound.

DS and DDTC also appear to have biological activities not shared by CS_2 of potential significance to the regulation of microsomal enzymes. As indicated in the present study, δ-ALAS activity is increased by DDTC (Table 1), but CS_2 is reported to inhibit this enzyme only weakly [58]. Similarly, DS and DDTC inhibit both plasma and microsomal carboxyesterases, whereas CS_2 does not [8, 10]. Different effects on lipid peroxidation are also evident as discussed above. In light of these observations, it seems unlikely that CS_2 formation is responsible for most of the effects that DS and DDTC exert on P-450.

In summary, DDTC appeared to lower P-450 concentrations by altering the homeostasis of P-450 heme within the hepatocyte. Soon after its administration, DDTC accelerated loss of membrane radioactivity from prelabeled pools of P-450 heme. In addition, DDTC greatly stimulated the capacity for heme catabolism by increasing hepatic heme oxygenase activity. Both effects may be related to NADPH-dependent formation of sulfur-containing metabolites that covalently bind to microsomal components and initiate conversion of P-450 to P-420. Through this mechanism, the dissociation of the heme prosthetic group from its apoprotein may be facilitated, thereby increasing the vulnerability of P-450 heme to degradation by heme oxygenase. Induction of δ-ALAS several hours after DDTC administration indicates that the capacity of the hepatocyte to synthesize heme, and perhaps new P-450, increases in response to enhanced catabolism of P-450 heme mediated by active metabolites of

DDTC. The later effect, together with an apparent ability to block lipid peroxidation, may serve to limit the degree of hepatotoxicity following a single dose of DDTC. Taken together, the results from this study suggest that the onset, degree, and duration of impairment of cytochrome P-450-dependent monooxygenase activity by compounds such as DDTC depend not only on the formation of reactive metabolites, but on the ability of the cell to respond to the toxic insult.

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