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THE 1,2-DICHLOROETHYLENES: THEIR METABOLISM BY HEPATIC CYTOCHROME P-450 *IN VITRO**

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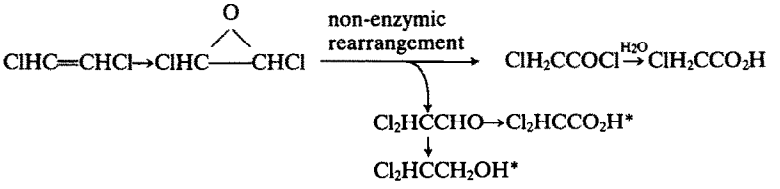
Abstract—*Cis*- and *trans*-1,1-dichloroethylene bound to the active site of hepatic microsomal cytochrome P-450 with the production of a Type I difference spectrum and stimulated CO-inhibitable hepatic microsomal NADPH oxidation. Incubation of *cis*- and *trans*-1,2-dichloroethylene plus hepatic microsomes, NADPH-generating system−EDTA resulted in the production of measurable levels of 2,2-dichloroethanol and dichloroacetaldehyde but not of 2-chloroethanol, chloroacetaldehyde or chloroacetic acid and, also, resulted in decreased levels of hepatic microsomal cytochrome P-450 and heme. In addition, dichloroacetic acid was produced from *trans*-dichloroethylene under these experimental conditions. The omission of any component of the incubation mixture eliminated the above effects, while the inclusion of SKF-525A, metyrapone or $CO:O_2$ (80, v/v) diminished these effects. The effects of β-naphthoflavone and phenobarbital pretreatment on the values of K_s, ΔA_{max}, K_m and V_{max} for the binding and metabolism of the 1,2-dichloroethylenes are reported. The binding and metabolism of the 1,2-dichloroethylenes and the 1,2-dichloroethylene-mediated inactivation of cytochrome P-450 were enhanced per mg of microsomal protein, but generally not per nmole of cytochrome P-450 by prior induction with β-naphthoflavone or phenobarbital. It is concluded that multiple forms of hepatic microsomal cytochrome P-450 bind and metabolize the 1,2-dichloroethylenes. The role of cytochrome P-450 in the metabolic activation of the dichloroethylenes is considered.

The *cis*- and *trans*-1,2-dichloroethylenes are widely used as industrial solvents for fats, oils, waxes and, particularly, rubber. The deleterious effects of the 1,2-dichloroethylenes have not been investigated extensively, but these compounds are reportedly hepatotoxic to laboratory rodents [1]. However, the 1,2-dichloroethylenes are not mutagenic in the presence or absence of liver activating enzymes and are not known to be carcinogenic in laboratory animals [2].

To date, the metabolism of the 1,2-dichloroethylenes has not been studied extensively *in vivo* or *in vitro*, but the following pathways for their metabolism *in vivo* have been proposed [3, 4]:



where an asterisk indicates metabolites of the 1,2-dichloroethylenes which have been identified from perfused isolated rat liver [4].

It would appear that the hepatic microsomal cytochrome P-450 enzyme system may be involved in the metabolism of the 1,2-dichloroethylenes. The 1,2-dichloroethylenes have been proposed to inhibit the demethylation of aminopyrine *in vitro* and the metabolism of hexobarbital *in vivo* by competing for binding to the active site of cytochrome P-450 [5, 6]. Furthermore, the 1,2-dichloroethylenes have been reported to be metabolized by hepatic post-mitochondrial supernatant fraction plus NADPH to an unknown metabolite, which was not monochloroacetate [3].

Since direct evidence for the interaction of the 1,2-dichloroethylenes with cytochrome P-450 appeared to be lacking, we have investigated the binding and metabolism of these compounds by hepatic microsomal cytochrome P-450. The role of different forms of cytochrome P-450 in these processes was investigated with the use of specific inducing agents. These studies represent one avenue in our investigations of the effect of the chlorine substitution on the metabolism of the chlorinated ethylenes by hepatic cytochrome P-450.

EXPERIMENTAL

Materials. The pure *cis*- and *trans*-1,2-dichloroethylenes were from Ferak, Berlin, FRG, and Fluka AG, Buchs, Switzerland, respectively. The mixture

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of the *cis*- and *trans*-1,2-dichloroethylenes (30:70) was from Merck Chemicals, Darmstadt, FRG. SKF-525A (β-diethylaminoethyl-2,2-diphenylvalerate) and metyrapone [2-methyl-1,2-bis-(3-pyridyl)-1-propane] were gifts from Smith Kline & French Ltd., Isando, Transvaal, R.S.A., and from Ciba-Geigy Ltd., Basle, Switzerland.

Dichloroacetaldehyde was prepared from freshly distilled chloral according to the method of Swietoslawski and Silowieki [7]. Stock solutions of dichloroacetaldehyde in water were prepared immediately after distillation of the freshly prepared dichloroacetaldehyde in order to prevent polymerization [8]. The acetaldehyde content of the stock solutions was confirmed by the method of Malhotra and Anand [8].

Treatment of animals. Male Long–Evans rats (180–200 g) were used for all experiments. Animals were permitted free access to Epol laboratory chow (protein min. 20%; fat 2.5%; fibre max. 6%; calcium 1.4%; phosphorus 0.7%; obtained from Epol Ltd., Goodwood, C.P., R.S.A.) and water. β-Naphthoflavone was given as a single i.p. injection (80 mg/kg in corn oil) 36 hr prior to sacrifice [9]. Phenobarbital was administered i.p. for 3 days at 80 mg per kg per day in 0.9% saline [10]. Animals were starved for 18 hr prior to being killed. Hepatic microsomes were isolated by differential centrifugation [11].

Introduction of the dichloroethylenes. The 1,2-dichloroethylenes were added to hepatic microsomes as 33% (v/v) solutions in absolute ethanol and were dispersed by vortex mixing for 30 sec. In no case was more than a single addition of dichloroethylene made per sample of microsomal suspension.

Spectral assays. Difference spectra with hepatic microsomal cytochrome P-450 were measured at 25° as described earlier [12]. The rates of NADPH oxidation by hepatic microsomes (2 mg protein/ml) were measured spectrally at 30° in the presence of 7.2 mM dichloroethylene and 0.18 mM NADPH in 0.02 M Tris–HCl, pH 7.4, as described earlier [12]. Reported rates of NADPH oxidation are corrected for the rates of non-cytochrome P-450 dependent NADPH oxidation using $CO:O_2$ according to the method of Stripp *et al.* [13]. For determining the effects of the 1,2-dichloroethylenes on the levels of hepatic microsomal cytochrome P-450 and heme, hepatic microsomes (2 mg protein/ml) were incubated at 30° for 15 min with shaking at 60 cycles/min in the presence or absence of 7.2 mM dichloroethylene, 0.2 mM EDTA and an NADPH-generating system [14] in 0.02 M Tris–HCl, pH 7.4.

The concentration of cytochrome P-450 was determined from the difference spectrum of CO-ferrocytochrome P-450 versus ferrocytochrome P-450 according to the method of Omura and Sato [15], using an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbance between 450 nm and 490 nm. Microsomal heme was determined spectrally as the reduced pyridine hemochrome ($\varepsilon_{557-575}$ 32.4 mM^{-1} cm^{-1}) according to Omura and Sato [15]. H_2O_2 was assayed by the method of Hildebrandt *et al.* [16].

Identification of metabolites of the dichloroethylenes. To assess the metabolism of the 1,2-dichloroethylenes, incubations were carried out as described above, except that the concentration of the hepatic microsomes was 4 or 6 mg protein/ml and incubations were for 20 or 30 min. To determine the chlorinated carboxylic acids and chlorinated acetaldehydes, 6 ml of hepatic microsomes (4 mg protein/ml) was incubated with dichloroethylene as described above. The reaction was terminated by precipitation of the protein with 200 μl of 2 N H_2SO_4 and 200 μl of 10% Na_2WO_4 [17]. The precipitate was removed by centrifugation at 1000 *g* for 10 min. The supernatant fraction was extracted with 4 ml of cold diethyl ether, and the ether layer was removed and dried with anhydrous Na_2SO_4. The methyl esters of the chlorinated carboxylic acids were prepared by bubbbling with diazomethane under a stream of nitrogen. The resulting ether extract was analyzed by gas–liquid chromatography using a 1 m × 6 mm glass column of Chromosorb 101 [18]. Column, injector, and detector temperatures were 200°, 200° and 250° respectively. Retention times were as follows: methyl monochloroacetate, 348 sec; methyl dichloroacetate, 550 sec; acetyl 2,2-dichloroethanol, 491 sec; chloracetaldehyde, 145 sec; and dichloroacetaldehyde, 210 sec. For monitoring the production of the chlorinated ethanols, incubation mixtures contained 15 ml of hepatic microsomes (6 mg protein/ml). After precipitation and removal of the protein as described above, the supernatant fraction was extracted with 5 ml of ethyl acetate. The organic phase was removed, dried with anhydrous Na_2SO_4, and analyzed by gas–liquid chromatography using a 3 m × 6 mm glass column of 10% Carbowax 20 M on Chromosorb W (80/100 mesh) [19]. Column, injector and detector temperatures were 170°, 200° and 230° respectively. Retention times were as follows: 2-chloroethanol, 137 sec; 2,2-dichloroethanol, 270 sec; and methyl dichloroacetate, 656 sec. Standards for gas–liquid chromatography were prepared from analytical grade reagents or freshly prepared chemicals which were added to hepatic microsomes and treated exactly as were the incubation mixtures. Gas–liquid chromatographic analyses were performed on a Packard model 428 gas–liquid chromatograph, using a ^{63}Ni electron capture detector. Peak areas were calculated by a Pye Unicam DP 88 computing integrator. A Varian Aerograph 2700 gas–liquid chromatograph linked to a Varian MAT 311A mass spectrometer was utilized for obtaining mass spectra of authentic standards and of metabolites extracted from reaction mixtures.

Calculations and statistical analysis. Reported values are means ± standard deviations for assays in triplicate or quadruplicate on two or more preparations of hepatic microsomes. Student's *t*-test for unpaired data was utilized to calculate significant differences between means. A significant difference was taken as $P < 0.01$. Binding (K_s) and Michaelis (K_m) constants, maximum extents of binding (ΔA_{max}) and maximum rates of metabolism (V_{max}) were calculated from Hanes plots.

RESULTS

Binding of the 1,2-dichloroethylenes to hepatic microsomal cytochrome P-450. The *cis*- and *trans*-

1,2-dichloroethylenes bound to hepatic microsomal cytochrome P-450 with the production of a Type I difference spectrum (λ_{max} = 386 nm; λ_{min} = 416 nm), which is indicative of binding to the active site of cytochrome P-450 [20]. Hanes plots of the binding of the 1,2-dichloroethylenes to hepatic microsomal cytochrome P-450 were biphasic (see, for example, Fig. 1), and therefore two binding constants (K_s) and two corresponding values for the maximum extents of binding (ΔA_{max}) were calculated for the binding of each of the 1,2-dichloroethylenes to hepatic cytochrome P-450 in microsomes from untreated and pretreated rats. The effects of induction of different forms of hepatic microsomal cytochrome P-450 on the values of K_s and ΔA_{max} for the binding of the 1,2-dichloroethylenes to hepatic microsomal cytochrome P-450 are shown in Table 1.

The K_s values for the binding of a mixture of *cis*- and *trans*-1,2-dichloroethylene as well as *trans*-1,2-dichloroethylene to hepatic microsomal cytochrome P-450 were not altered following β-naphthoflavone induction. Following phenobarbital treatment, there was no effect on K_s for the high-affinity site but there was a significant decrease in the K_s for the low-affinity site for the mixture of isomers and for the pure *trans*-isomer.

The values of ΔA_{max} and ΔA_{max}/nmole cytochrome P-450 for the mixture if isomers and for *trans*-1,2-dichloroethylene were generally decreased following β-naphthoflavone treatment. In contrast, ΔA_{max} for the mixture of isomers and for pure *trans*-1,2-dichloroethylene was generally elevated by phenobarbital induction, while ΔA_{max}/nmole cytochrome P-450 was not. For each type of induction, the values of K_s and ΔA_{max} were within a factor of 2 for the mixture of isomers and for the pure *cis*- and *trans*-1,2-dichloroethylenes, except for the K_s for the low-affinity site for the *cis*-isomer which, following phenobarbital induction, was 3-fold greater than the corresponding values for the mixture of isomers or pure *trans*-1,2-dichloroethylene (Table 1).

NADPH oxidation. The rate of hepatic microsomal CO-inhibitable NADPH oxidation was stimu-

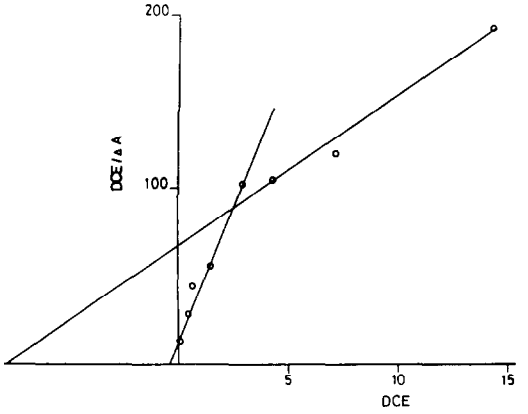

Fig. 1. Hanes plot of the binding of *cis*-1,2-dichloroethylene to cytochrome P-450 in hepatic microsomes (2 mg protein/ml) from phenobarbital-treated rats at room temperature. *cis*-1,2-dichloroethylene (DCE), mM; ΔA, $A_{386\,nm} - A_{418\,nm}$.

Table 1. Effect of inducing agents on the binding of the 1,2-dichlorinated ethylenes to hepatic microsomal cytochrome P-450*

Inducing agent	*Cis*- and *trans*-1,2-dichloroethylene (30:70)		*Trans*-1,2-dichloroethylene		*Cis*-1,2-dichloroethylene	
	K_s (mM)	$10^2\Delta A_{max}$	K_s (mM)	$10^2\Delta A_{max}$	K_s (mM)	$10^2\Delta A_{max}$
None	0.2 ± 0.1	4.1 ± 0.3	2.0 ± 0.5 (2.2 ± 0.6)	4.0 ± 0.8 (4.4 ± 0.9)	0.4 ± 0.1	8.2 ± 3.9
βNF	0.3 ± 0.1	5.2 ± 0.8	1.0 ± 0.3† (0.7 ± 0.2‡)	3.3 ± 0.3 (2.2 ± 0.2‡)	0.6 ± 0.2	8.6 ± 0.8
PB	0.3 ± 0.1	2.2 ± 0.3‡	3.6 ± 1.2‡ (1.7 ± 0.6)	6.8 ± 0.2‡ (3.2 ± 0.1)	0.4 ± 0.2	2.5 ± 1.3‡

Inducing agent	*Trans*-1,2-dichloroethylene		*Cis*-1,2-dichloroethylene	
	K_s (mM)	$10^2\Delta A_{max}$	K_s (mM)	$10^2\Delta A_{max}$
None	1.3 ± 0.1 (1.6 ± 0.1)	4.9 ± 1.0 (5.6 ± 1.1)	ND	ND
βNF	1.6 ± 0.1	6.5 ± 0.7† (5.2 ± 0.6)	ND	ND
PB	3.9 ± 0.8‡ (1.8 ± 0.4)	8.9 ± 3.2‡ (4.0 ± 1.5)	0.4 ± 0.1	7.7 ± 0.2

Inducing agent	*Cis*-1,2-dichloroethylene	
	K_s (mM)	$10^2\Delta A_{max}$
None	ND	ND
βNF	ND	ND
PB	2.8 ± 0.4 (1.5 ± 0.2)	9.0 ± 0.6 (5.2 ± 0.4)

* Values are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes except for results reported for pure *cis*-1,2-dichloroethylene where results were in triplicate from a single preparation of hepatic microsomes. Experimental details are described in the Experimental section. Values in parentheses are per nmole cytochrome P-450. Abbreviations: ND, not determined; βNF, β-naphthoflavone; and PB, phenobarbital.

† Probably differs from corresponding value for microsomes from untreated rats, $P < 0.05$.

‡ Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

lated by the mixture of isomers and by pure *cis*- and pure *trans*-1,2-dichloroethylene. Following β-naphthoflavone induction, there was no significant effect on the rate of NADPH oxidation per mg microsomal protein or per nmole cytochrome P-450 for either the mixture of isomers or for *trans*-1,2-dichloroethylene, while following phenobarbital induction, the rate of CO-inhibitable NADPH oxidation was increased per mg of microsomal protein but not per nmole of cytochrome P-450. The rates of CO-inhibitable NADPH oxidation in the presence of the mixture of isomers or of pure *cis*- or *trans*-1,2-dichloroethylene were, for each type of induction, identical (Table 2).

Identification of metabolites of the 1,2-dichloroethylenes. The 2-chloro- and 2,2-dichloro-derivatives of ethanol, acetaldehyde and acetic acid were investigated as possible metabolites of the pure *cis*- and *trans*-1,2-dichloroethylenes. Incubation mixtures contained the dichloroethylene (7.2 mM), hepatic microsomes from phenobarbital-treated rats, EDTA, and an NADPH-generating system. 2-Chloroethanol, chloroacetaldehyde and chloroacetic acid were not produced in significant amounts in these incubation mixtures, the limits of detection being 1.0, 0.06, and 0.15 nmoles·(mg microsomal protein)$^{-1}$·(30 min)$^{-1}$ respectively. 2,2-Dichloroethanol and dichloroacetaldehyde were produced in measurable amounts in incubation mixtures containing either *cis*- or *trans*-1,2-dichloroethylene. The rates of production of 2,2-dichloroethanol were 1.2 ± 0.1 and 0.32 ± 0.04 nmoles·(mg microsomal protein)$^{-1}$·(20 min)$^{-1}$ from the *cis*- and *trans*-isomers respectively. The identification of 2,2-dichloroethanol was based on its retention time for gas–liquid chromatography plus the conversion of the peak, following acetyl chloride treatment [21] of extracts of reaction mixtures, to one that chromatographed identically to an authentic sample of acetyl 2,2-dichloroethanol. The rates of production of 2,2-dichloroethanol were not diminished if freshly redistilled samples of the *cis*- and *trans*-1,2-dichloroethylenes were utilized in incubation mixtures. It was not possible to obtain metabolic parameters for the production of 2,2-dichloroethanol due to the low amounts produced under the conditions of our experiments and due to the relative insensitivity of the electron capture detector to this product.

Dichloroacetaldehyde was identified in extracts of reaction mixtures by its retention time for gas–liquid chromatography and by the application of the Fehling's test for aldehydes. This test was positive for extracts of reaction mixtures, and its application to extracts eliminated the peak attributed to dichloroacetaldehyde from gas–liquid chromatograms. Furthermore, the metabolite was oxidized by the following method for the oxidation of aldehydes to carboxylic acids. The reaction mixture (15 ml) was treated with 0.2 ml NaOH (10%, w/v) plus 10 ml of potassium permanganate (0.1 M), acidified with H_2SO_4 (2 N), and then an aqueous solution of sodium bisulfite (5%, w/v) was added until all colour was lost [22]. Protein was removed by centrifugation, and the supernatant fraction was extracted with 10 ml diethyl ether. This treatment eliminated the chromatographic peak with the retention time of dichloroacetaldehyde (210 sec) and, following methylation with diazomethane, resulted in the appearance of a new peak at 550 sec, which corresponded to the retention time of methyl dichloroacetate.

Dichloroacetic acid [0.88 nmole·(mg protein)$^{-1}$·(20 min)$^{-1}$] was found to be a metabolite of *trans*-1,2-dichloroethylene on the basis of its retention time, following methylation, being identical to that of methyl dichloroacetate on two gas–liquid chromatography columns (see Experimental section) and the peak being eliminated by the neutralization of reaction mixtures prior to methylation or by the omission of the methylation reaction.

Following incubation of *cis*-1,2-dichloroethylene with hepatic microsomes in the presence of the NADPH-generating system and EDTA, and subsequent treatment overnight with 20% H_2SO_4 at 100° [4], significant levels of dichloroacetic acid (identified as above) were produced [viz. 5.4 nmoles·(mg protein)$^{-1}$·(20 min)$^{-1}$], where only traces of dichloroacetic acid [<0.1 nmole·(mg protein)$^{-1}$·(20 min)$^{-1}$] were found in incubation mixtures before hydrolysis or in hydrolyzed, but not incubated, reaction mixtures. CO, metyrapone and SKF-525A had no effect on the production of the covalently

Table 2. Effect of inducing agents on the stimulation of hepatic microsomal CO-inhibitable NADPH oxidation by the 1,2-dichloroethylenes*

	NADPH oxidation [nmoles·min^{-1}·(mg microsomal protein)$^{-1}$]		
Inducing agent	*cis*- and *trans*-DCE (30:70)	*trans*-DCE	*cis*-DCE
None	1.1 ± 0.3	1.0 ± 0.3	ND
	(1.2 ± 0.3)	(1.1 ± 0.3)	
β-Naphthoflavone	1.0 ± 0.2	1.3 ± 0.1	ND
	(0.9 ± 0.2)	(1.2 ± 0.1)	
Phenobarbital	3.4 ± 0.8†	3.1 ± 1.1†	3.0 ± 0.3
	(1.6 ± 0.4)	(1.5 ± 0.5)	(1.4 ± 0.1)

* Values are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are as described in the Experimental section. Values in parentheses are in nmoles·min^{-1}·nmole cytochrome P-450^{-1}. Abbreviations: DCE, 1,2-dichloroethylene; and ND, not determined.

† Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

bound dichloroacetyl moiety, and it was subsequently found that the omission of the NADPH-generating system from incubation mixtures did not diminish the rate of formation of covalently bound dichloroacetate. These results indicate that the covalently bound dichloroacetate was produced as a function of time, but that its production was not the result of a cytochrome P-450-dependent reaction.

Kinetics of dichloroacetaldehyde production. The conversion of *cis*- or *trans*-1,2-dichloroethylene to dichloroacetaldehyde was linear for 10 min with microsomes from untreated or β-naphthoflavone-induced rats and for 3 min with hepatic microsomes from phenobarbital-induced rats (Fig. 2). The rates of dichloroacetaldehyde production per mg microsomal protein were identical whether incubation mixtures of hepatic microsomes at concentrations of 2 or 4 mg protein/ml were utilized (data not shown).

K_m and V_{max} for dichloroacetaldehyde production. The metabolism of *cis*-1,2-dichloroethylene to dichloroacetaldehyde gave rise to biphasic Hanes plots regardless of the pretreatment of the rats from which the hepatic microsomes were isolated (see, for example, Fig. 3). However, for the metabolism of *trans*-1,2-dichloroethylene to dichloroacetaldehyde, biphasic Hanes plots only arose following phenobarbital induction. In microsomes from untreated and β-naphthoflavone-induced rats, the conversion of *trans*-1,2-dichloroethylene to dichloroacetaldehyde gave rise to monophasic Hanes plots (see, for example, Fig. 4). For the *cis*- and *trans*-1,2-dichloroethylenes, β-naphthoflavone induction did not significantly affect the values of K_m or of V_{max} per nmole cytochrome P-450 except for the low-affinity K_m for the *cis*-isomer, but generally resulted in an increase in V_{max} per mg microsomal protein. Phenobarbital induction generally resulted in increases in the values of K_m and V_{max} per nmole cytochrome P-450, but did not significantly affect V_{max} per mg microsomal protein. For each type of pretreatment, V_{max} (either per mg microsomal protein or per nmole cytochrome P-450) was significantly greater for *cis*- than for *trans*-1,2-dichloroethylene ($P < 0.01$) (Table 3).

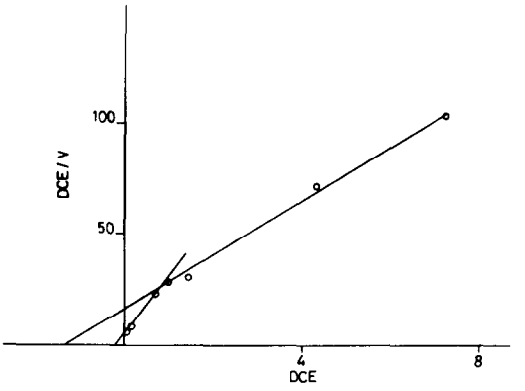

Fig. 3. Hanes plot of the production of dichloroacetaldehyde from *cis*-1,2-dichloroethylene by hepatic microsomes from β-naphthoflavone-treated rats. *Cis*-1,2-dichloroethylene (DCE), mM; *V*, nmoles dichloroacetaldehyde·(mg microsomal protein)·(10 min)$^{-1}$. Experimental details are given in the legend of Fig. 2.

Effects of inhibitors of cytochrome P-450 on the metabolism of the cis- *and* trans-1,2-*dichloroethylenes.* CO, metyrapone and/or SKF-525A effectively inhibited the metabolism of the *cis*- and *trans*-1,2-dichloroethylenes to 2,2-dichloroethanol and dichloroacetaldehyde (Table 4). Metyrapone was the least effective inhibitor of the three for the conversion of the *cis*-isomer to dichloroacetaldehyde but was the most effective inhibitor of the conversion of the *trans*-isomer to this metabolite. For each inhibitor investigated, the extent of inhibition of 2,2-dichloroethanol production was equivalent to the extent of inhibition of dichloroacetaldehyde production.

Effect of the 1,2-*dichloroethylenes on hepatic microsomal H_2O_2 production.* Incubation of *cis*- or *trans*-1,2-dichloroethylene (7.2 mM), hepatic microsomes (2 mg protein/ml) from phenobarbital-treated rats, NADPH-generating system, EDTA (0.1 mM) and sodium azide (0.2 mM) for 5 min at 30° with shaking at 60 cycles/min resulted in 0.7 nmole

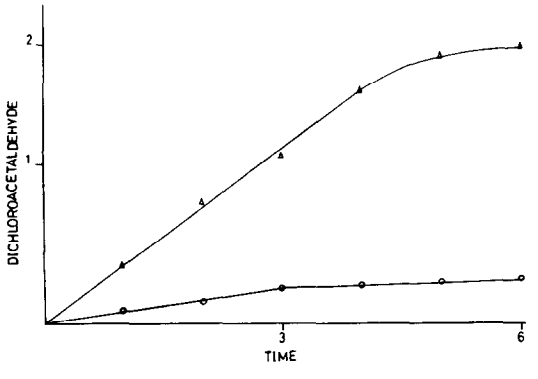

Fig. 2. Production of dichloroacetaldehyde from *cis*- and *trans*-1,2-dichloroethylene as a function of time. Dichloroacetaldehyde, nmoles/mg microsomal protein; time, min. Reaction mixtures containing hepatic microsomes (4 mg microsomal protein/ml) from phenobarbital-treated rats, NADPH-generating system [13], EDTA (0.2 mM) and *cis*- (△) or *trans*- (○) 1,2-dichloroethylene (7.2 mM) were incubated at 30° with shaking at 60 cycles/min.

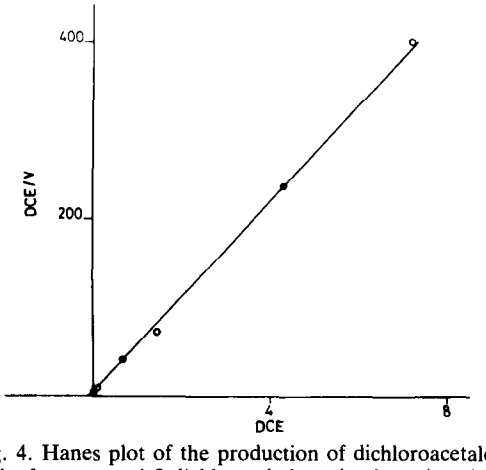

Fig. 4. Hanes plot of the production of dichloroacetaldehyde from *trans*-1,2-dichloroethylene by hepatic microsomes from β-naphthoflavone-treated rats. *Trans*-1,2-dichloroethylene (DCE), mM; *V*, nmoles dichloroacetaldehyde·(mg microsomal protein)$^{-1}$·(10 min)$^{-1}$. Experimental details are given in the legend of Fig. 2.

Table 3. Effect of inducing agents on the hepatic microsomal conversion of the *cis*- and *trans*-1,2-dichloroethylenes to dichloroacetaldehyde*

Inducing agent	*Trans*-1,2-dichloroethylene				*Cis*-1,2-dichloroethylene			
	K_m (mM)		V_{max} [nmoles·min^{-1}·(mg protein)$^{-1}$]		K_m (mM)		V_{max} [nmoles·min^{-1}·(mg protein)$^{-1}$]	
None	0.06 ± 0.01		0.006 ± 0.001 (0.007 ± 0.001)		0.04 ± 0.01	1.3 ± 0.2	0.03 ± 0.01 (0.03 ± 0.01)	0.07 ± 0.02 (0.08 ± 0.02)
β-Naphthoflavone	0.08 ± 0.01		0.016 ± 0.003† (0.009 ± 0.002)		0.14 ± 0.05†	2.2 ± 0.9	0.05 ± 0.01 (0.03 ± 0.01)	0.14 ± 0.06† (0.08 ± 0.04)
Phenobarbital	0.15 ± 0.06†	1.66 ± 0.32†	0.023 ± 0.003† (0.007 ± 0.001)	0.067 ± 0.006† (0.020 ± 0.002†)	0.26 ± 0.14†	2.1 ± 1.2	0.11 ± 0.03† (0.03 ± 0.01)	0.30 ± 0.11† (0.09 ± 0.03)

* Values are means ± S.D. for experiments performed in triplicate with two or three different preparations of hepatic microsomes. Experimental conditions are as described in the Experimental section.

† Differs significantly from the corresponding value for microsomes from untreated rats, $P < 0.01$.

Table 4. Effect of inhibitors on the metabolism of the *cis*- and *trans*-1,2-dichloroethylenes by hepatic microsomal cytochrome P-450*

Compound	Inhibitor	% Inhibition of	
		Dichloroacetaldehyde production	2,2-Dichloroethanol production
Cis-1,2-dichloroethylene	$CO:O_2$ (80:20, v/v)	47 ± 6	38 ± 3
	Metyrapone (2.3 mM)	37 ± 3	ND
	SKF-525A (200 mM)	53 ± 6	ND
Trans-1,2-dichloroethylene	$CO:O_2$ (80:20, v/v)	38 ± 4	29 ± 2
	Metyrapone (2.3 mM)	63 ± 3	62 ± 2
	SKF-525A (200 mM)	29 ± 4	ND

* Values are means ± S.D. for experiments performed in duplicate or triplicate with two different preparations of hepatic microsomes from phenobarbital-pretreated rats. Experimental details are given in the Experimental section. Abbreviation: ND, not determined.

$H_2O_2 \cdot min^{-1} \cdot (mg\ microsomal\ protein)^{-1}$ from the *cis*-isomer and none from the *trans*-isomer, relative to incubation mixtures in which the 1,2-dichloroethylene was absent.

Effect of the 1,2-dichloroethylenes on the levels of hepatic microsomal cytochrome P-450. The effects of a mixture of the *cis*- and *trans*-1,2-dichloroethylenes on the levels of hepatic microsomal cytochrome P-450 and heme are shown in Table 5. No appreciable degradation of hepatic microsomal cytochrome P-450 or heme was observed in the absence of the 1,2-dichloroethylenes or of the NADPH-generating system. Incubation for 15 min of the dichloroethylenes with hepatic microsomes and NADPH-generating system, however, resulted in significant losses of hepatic microsomal cytochrome P-450 and heme. In these incubation mixtures, the losses of cytochrome P-450 were approximately equivalent to the losses of heme for each type of induction. The total loss of microsomal cytochrome P-450 and heme was far more pronounced for microsomes from phenobarbital-induced rats than from untreated or β-naphthoflavone-treated rats (Table 5). Both CO and metyrapone inhibited the degradation of cytochrome P-450 by the 1,2-dichloroethylenes in hepatic microsomes from phenobarbital-treated rats. NADH (1 mM) did not measurably support the 1,2-dichloroethylene-mediated degradation of cytochrome P-450 in microsomes from phenobarbital-induced rats.

Following incubation of microsomes from phenobarbital-treated rats, NADPH-generating system, EDTA and pure *cis*- or *trans*-1,2-dichloroethylene, the losses of cytochrome P-450 were 1.27 ± 0.18 and 1.52 ± 0.30 nmoles cytochrome P-450/15 min. These losses are comparable to the losses seen with the mixture of isomers ($P > 0.1$) (Table 5).

Table 5. Effect of a mixture of *cis*- and *trans*-1,2-dichloroethylene (30:70) on the levels of hepatic microsomal cytochrome P-450*

Inducing agent	1,2-DCE	Other additions	Cytochrome P-450† (loss/initial level)	Heme† (loss/initial level)	Loss Heme†‡ / Loss cytochrome P-450
None	−	—	0.06/(0.82 ± 0.08)	0.04/(1.44 ± 0.04)	
	+	—	0.28/(0.72 ± 0.05)	0.28/(1.43 ± 0.11)	0.24/0.23
β-Naphthoflavone	−	—	0.10/(1.24 ± 0.06)	0.08/(1.84 ± 0.09)	
	+	—	0.51/(1.11 ± 0.09)	0.36/(1.76 ± 0.03)	0.28/0.41
Phenobarbital	−	—	0.16/(3.10 ± 0.28)	0.18/(4.19 ± 0.32)	
	+	—	1.58/(3.02 ± 0.28)	1.44/(4.10 ± 0.27)	1.26/1.42
	+	CO:O_2 (80:20)	1.17/(2.83 ± 0.35)		
	+	Metyrapone (2.3 mM)	0.54/(2.57 ± 0.35)		
	+	NADH (0.6 mM)§	0.39/(2.53 ± 0.26)		
	−	NADH (0.6 mM)§	0.21/(2.46 ± 0.12)		

* Values are means ± S.D. for experiments performed in triplicate with at least three different preparations of hepatic microsomes. Incubation mixtures (3 ml) contained hepatic microsomes (2 mg protein/ml), NADPH-generating system, EDTA (0.2 mM) and a mixture of *cis*- and *trans*-1,2-dichloroethylene (7.2 mM). Losses are reported for samples incubated for 15 min at 30° with shaking relative to zero-time samples of identical composition.
† In nmoles/mg microsomal protein.
‡ Corrected for background losses seen in incubation mixtures not containing 1,2-dichloroethylene.
§ NADPH-generating system omitted from incubation mixture.

DISCUSSION

The implicated involvement of hepatic cytochrome P-450 in the metabolism of the *cis*- and *trans*-1,2-dichloroethylenes *in vitro* (see Introduction) is confirmed by the results presented herein. Both the *cis*- and *trans*-1,2-dichloroethylenes appeared to bind to the active site of hepatic microsomal cytochrome P-450 since both produced a Type I difference spectrum and stimulated hepatic microsomal CO-inhibitable NADPH oxidation (Table 2). Furthermore, the conversion of both the *cis*- and *trans*-1,2-dichloroethylenes to 2,2-dichloroethanol and dichloroacetaldehyde required hepatic microsomes, the 1,2-dichloroethylene and an NADPH-generating system, with the omission of any component eliminating metabolite production (see Results). Finally, the inhibitors of cytochrome P-450—CO, metyrapone and/or SKF 525A [23]—inhibited the production of dichloroacetaldehyde and 2,2-dichloroethanol from the *cis*- and *trans*-1,2-dichloroethylenes (Table 4).

It would appear that the *cis*- and *trans*-1,2-dichloroethylenes had been bound and metabolized by several of the multiple forms of cytochrome P-450, including the forms induced by β-naphthoflavone and phenobarbital and those found in the liver of untreated rats but not elevated by either of the

above agents. Cytochrome P-448, which is induced by β-naphthoflavone, appeared to play a slight, but significant, role in the binding and metabolism of *cis*- and *trans*-1,2-dichloroethylene. Although following β-naphthoflavone treatment the values of ΔA_{max} and ΔA_{max}/nmole cytochrome P-450 were generally decreased for the mixture of isomers and for *trans*-1,2-dichloroethylene, the value of ΔA_{max} for the low-affinity site for the binding of *trans*-1,2-dichloroethylene was increased slightly (Table 1). Furthermore, although β-naphthoflavone treatment did not significantly affect the rates of hepatic microsomal CO-inhibitable NADPH oxidation in the presence of the mixture of isomers or of *trans*-1,2-dichloroethylene or affect V_{max} per nmole of cytochrome P-450 for the conversion of *cis*- or *trans*-1,2-dichloroethylene to dichloroacetaldehyde, it did significantly increase the maximum rates of dichloroacetaldehyde production per mg of microsomal protein from both isomers (Tables 2 and 3).

The form of cytochrome P-450 elevated by phenobarbital, appeared to play a significant role in the binding and metabolism of *cis*- and *trans*-1,2-dichloroethylene. Phenobarbital pretreatment significantly increased ΔA_{max} and the rate of NADPH oxidation per mg of microsomal protein for both the mixture of isomers and for *trans*-1,2-dichloroethylene, and the values of these parameters were comparable to those for *cis*-1,2-dichloroethylene (Tables 1 and 2). Furthermore, phenobarbital pretreatment increased the values of V_{max} per mg of microsomal protein for the conversion of the *cis*- and *trans*-1,2-dichloroethylenes to dichloroacetaldehyde (Table 3). Metyrapone, which reportedly selectively interacts only with the phenobarbital-inducible form of cytochrome P-450 [24], significantly inhibited the conversion of *cis*- and particularly *trans*-1,2-dichloroethylene to chlorinated metabolites (Table 4).

Finally, one or more of the multiple forms of cytochrome P-450 found in liver microsomes from untreated rats, but not elevated by β-naphthoflavone or phenobarbital, appeared to bind and metabolize the 1,2-dichloroethylenes since, per nmole of cytochrome P-450, the values of ΔA_{max} and V_{max} for the 1,2-dichloroethylenes were generally not elevated by either inducing agent relative to the values obtained with microsomes from untreated rats (Tables 1 and 3).

It would appear that the *cis*- and *trans*-1,2-dichloroethylenes may be converted by cytochrome P-450 to other metabolites besides 2,2-dichloroethanol and dichloroacetaldehyde (and dichloroacetate in the case of *trans*-1,2-dichloroethylene) or may be partial uncouplers of cytochrome P-450 [25] since the rates of dichloroethylene-stimulated CO-inhibitable NADPH oxidation exceeded the rates of metabolite production for both isomers (Tables 2 and 3). If metabolites other than 2,2-dichloroethanol and dichloroacetaldehyde were produced from the 1,2-dichloroethylenes by hepatic microsomal cytochrome P-450, they did not include 2-chloroethanol, chloroacetaldehyde or chloroacetic acid (see Results). However, it is possible that a portion of the dichloroacetaldehyde produced from the 1,2-dichloroethylenes was bound covalently to microsomal or buffer constitutents and thus was not measured by gas–liquid chromatography. This alternative is supported by the very short period (*ca.* 3 min) over which the production of dichloroacetaldehyde was linear with microsomes from phenobarbital-treated rats (Fig. 2). The alternative proposal, viz. that the 1,2-dichloroethylenes act as partial uncouplers of cytochrome P-450, is not consistent with the observation that the rate of H_2O_2 production in the presence of *trans*-1,2-dichloroethylene was negligible and that the rate of production of H_2O_2 in the presence of *cis*-1,2-dichloroethylene (see Results) was far below the rate of *cis*-1,2-dichloroethylene stimulated CO-inhibitable NADPH oxidation ($P < 0.01$) (Table 2). In contrast, the dichlorinated ethylene vinylidene chloride appears to partially uncouple hepatic cytochrome P-450 [26].

The results presented herein support the scheme proposed for the metabolism of the 1,2-dichloroethylenes (see Introduction). The production of 2,2-dichloroethanol from both isomers and of dichloroacetate from *trans*-1,2-dichloroethylene by hepatic microsomal cytochrome P-450 is fully consistent with the proposed metabolic pathway and with the report that the *cis*- and *trans*-1,2-dichloroethylenes are converted to 2,2-dichloroethanol and dichloroacetic acid by perfused liver [4]. Furthermore, the identification of dichloroacetaldehyde as a metabolite of both the *cis*- and *trans*-1,2-dichloroethylenes from hepatic microsomal cytochrome P-450 provides the first confirmation in any *in vitro* or *in vivo* test system that this proposed metabolite is produced from the 1,2-dichloroethylenes.*

Since dichlorinated derivatives are the major products of the hepatic microsomal metabolism of the 1,2-dichloroethylenes, it is proposed that the nonenzymic rearrangement of the dichlorinated epoxide produced by the cytochrome P-450 enzyme system favors a chlorine shift (to yield dichloroacetaldehyde) rather than a hydride shift (to yield chloroacetyl chloride). The subsequent conversions of dichloroacetaldehyde to 2,2-dichloroethanol and dichloroacetate may be catalyzed by hepatic microsomal cytochrome P-450 or by alcohol dehydrogenase and aldehyde dehydrogenase contaminants of the hepatic microsomal preparations. The observation that dichloroacetaldehyde (0.4 mM) is converted to 2,2-dichloroethanol [at a rate of $0.26\ \text{nmole} \cdot (\text{mg protein})^{-1} \cdot (20\ \text{min})^{-1}$] following aerobic incubation in the presence of hepatic microsomes (6 mg protein/ml) and NADPH-generating system would suggest that an alcohol dehydrogenase contaminant of the hepatic microsomes (utilizing NADPH as electron donor) is responsible for the reduction of dichloroacetaldehyde to 2,2-dichloroethanol observed in hepatic microsomal incubation mixtures.†

* It is anticipated that dichloroacetaldehyde is the unidentified metabolite reported by Leibman and Ortiz [3] to be produced from *cis*- and *trans*-1,2-dichloroethylene by post-mitochondrial supernatant fraction plus NADPH.

† A similar conclusion has been drawn from the observation that chloroacetaldehyde is reduced to 2-chloroethanol in the presence of hepatic microsomes and NADPH [27].

It would appear that the cytochrome P-450 dependent metabolism of the *cis*- and *trans*-1,2-dichloroethylenes may be the rate-limiting step in their metabolism *in vivo*. Hepatic microsomal cytochrome P-450 metabolizes *cis*-1,2-dichloroethylene at a 4-fold greater rate than *trans*-1,2-dichloroethylene *in vitro* (Table 3), and the zero order rate of elimination of the *cis*-isomer *in vivo* is 4-fold greater than that of the *trans*-isomer [28]. In addition, *cis*-1,2-dichloroethylene is metabolized at a greater rate by isolated perfused liver than is *trans*-1,2-dichloroethylene [4].

The *cis*- and *trans*-1,2-dichloroethylenes appear to be converted by the hepatic microsomal cytochrome P-450 enzyme system to reactive metabolite(s) which can modify the heme moiety of hepatic microsomal cytochrome P-450. Incubation of either isomer of the 1,2-dichloroethylenes with hepatic microsomes and an NADPH-generating system resulted in the loss of microsomal cytochrome P-450 and heme, with the omission of any component of the incubation mixture eliminating the effect, and inhibitors of cytochrome P-450 decreasing the effect (Table 5). Furthermore, the losses of cytochrome P-450 were virtually eliminated if NADH, which is not an effective electron donor for cytochrome P-450, was substituted for the NADPH-generating system (Table 5). Since the loss of heme is equivalent to the loss of cytochrome P-450 for the mixture of the *cis*- and *trans*-1,2-dichloroethylenes, it would appear that the reactive species which are generated by the biotransformation of the *cis*- and *trans*-1,2-dichloroethylenes by hepatic cytochrome P-450 modify the heme moiety of this enzyme. The reactive metabolite(s) mediating the loss of cytochrome P-450 might, for example, be the Zwitterionic species of 1,2-dichloroethylene oxide (see Refs. 27 and 29).

It would appear that effects previously attributed to the inhibition of cytochrome P-450 by the *cis*- and *trans*-1,2-dichloroethylenes, viz. the abilities of the 1,2-dichloroethylenes to diminish the activity of aminopyrine demethylase *in vitro* and to increase hexobarbital sleeping time *in vivo* [5, 6], might, in part, reflect the abilities of metabolities of the 1,2-dichloroethylenes to modify the heme moiety at the active site of cytochrome P-450, rather than the reversible inhibition of this enzyme by the 1,2-dichlorinated ethylenes.

The abilities of the three isomeric dichloroethylenes [viz. the *cis*- and *trans*-1,2-dichloroethylenes and vinylidene chloride (1,1-dichloroethylene)] to modify the heme moiety of cytochrome P-450 do not correlate with their rates of metabolism by hepatic microsomal cytochrome P-450 *in vitro*. The *cis*- and *trans*-1,2-dichloroethylenes drastically decrease the levels of hepatic microsomal cytochrome P-450 *in vitro* (Table 5) while vinylidene chloride does not [26]. In contrast, the rate of metabolism of vinylidene chloride by hepatic microsomal cytochrome P-450 *in vitro* falls between the rates of metabolism of *cis*- and *trans*-1,2-dichloroethylene (Table 3) (see Results) [26].

The abilities of reactive metabolites of the dichlorinated ethylenes to modify the heme of cytochrome P-450 do, however, appear to correlate with the major rearrangement products of the epoxide intermediates. Epoxides that rearrange predominantly to aldehydes *in vitro* are from compounds that cause the modification of the heme moiety of cytochrome P-450 (viz. *cis*- and *trans*-1,2-dichloroethylene) (Table 5), while epoxides that rearrange mainly to acyl chlorides are from compounds that do not modify the heme moiety of cytochrome P-450 *in vitro* (viz. vinylidene chloride) [26].

The relative mutagenic and carcinogenic potentials of the dichloroethylenes do not correlate with their relative rates of metabolism by hepatic cytochrome P-450 *in vitro*. Vinylidene chloride is reportedly both mutagenic and carcinogenic while the isomeric 1,2-dichloroethylenes are neither [2, 30, 31], yet the rate of metabolism of vinylidene chloride by hepatic microsomal cytochrome P-450 *in vitro* is intermediate between the rates for *cis*- and *trans*-1,2-dichloroethylene (Table 3, see Results) [26]. It would, therefore, appear that the active metabolites of the 1,1- and 1,2-dichloroethylenes which give rise to their mutagenic effects differ from those that cause the modification of the heme moiety of cytochrome P-450.

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