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# VINYLIDENE CHLORIDE: ITS METABOLISM BY HEPATIC MICROSOMAL CYTOCHROME P-450 *IN VITRO**

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**Abstract**—The effects of inducing agents on the binding and metabolism of vinylidene chloride by hepatic microsomal cytochrome P-450 are reported. Hanes plots for the Type I binding of vinylidene chloride to cytochrome P-450 were biphasic with hepatic microsomes from untreated and β-naphthoflavone- or phenobarbital-treated male rats. Neither pretreatment affected the value of the $K_s$ (*ca.* 0.22 mM) for the high-affinity binding site for vinylidene chloride, while phenobarbital induction, but not β-naphthoflavone treatment, decreased the value of the $K_s$ for the low-affinity site by 3-fold to *ca.* 1.6 mM. The maximum extents of binding ($\Delta A_{max}$ or $\Delta A_{max}$/nmole cytochrome P-450) of vinylidene chloride were decreased or not affected by β-naphthoflavone induction, while $\Delta A_{max}$ but not $\Delta A_{max}$/nmole cytochrome P-450 was elevated following phenobarbital induction. The rate of vinylidene chloride stimulated CO-inhibitable hepatic microsomal NADPH oxidation was not affected by β-naphthoflavone induction, but was increased significantly following phenobarbital induction. Vinylidene chloride was converted to monochloroacetate and to the previously unreported metabolite, dichloroacetaldehyde, by hepatic microsomes plus NADPH-generating system. Measurable levels of 2-mono- and 2,2-dichloroethanol, and of chloroacetaldehyde and dichloroacetic acid, were not produced from vinylidene chloride under these conditions. SKF-525A and $CO:O_2$ (80:20, v/v) inhibited the conversion of vinylidene chloride to monochloroacetate and dichloroacetaldehyde by approximately 60%. The rates of production of monochloroacetate and dichloroacetaldehyde in the presence of NADH were *ca.* 15% of the rates seen with NADPH-generating system. The rate of monochloroacetate production per mg microsomal protein was not affected by β-naphthoflavone induction but was increased slightly following phenobarbital induction. In contrast, the $V_{max}$ values per mg microsomal protein for the metabolism of vinylidene chloride to dichloroacetaldehyde were not elevated by either pretreatment. Incubation of vinylidene chloride, NADPH-generating system, EDTA and hepatic microsomes from untreated and β-naphthoflavone- or phenobarbital-treated rats did not result in any significant alterations in the levels of microsomal cytochrome P-450 and heme or in the covalent binding of the mono- or dichloroacetyl moieties to microsomal or buffer constituents, but it did result in significant production of $H_2O_2$. It is concluded that multiple forms of cytochrome P-450 bind and metabolize vinylidene chloride. However, the form of the enzyme elevated by phenobarbital plays, at most, a minor role in these processes, while the form induced by β-naphthoflavone is not involved in either process. The effect of metabolism of vinylidene chloride by cytochrome P-450 on the relationship between the metabolism and toxicity of vinylidene chloride *in vivo* and its mutagenicity *in vitro* is considered.

Vinylidene chlorine ($Cl_2C{=}CH_2$; 1,1-dichloroethylene) is widely used industrially in the manufacture of Saran-type plastics. Widespread interest in the toxic, mutagenic and carcinogenic potential of this compound has arisen as a consequence of its structural similarity to the carcinogen vinyl chloride ($ClHC{=}CH_2$).

Vinylidene chloride has been found to be toxic *in vivo*. The toxicity of vinylidene chloride *in vivo* is unaffected or decreased following the induction of hepatic cytochrome P-450, but it is exacerbated by the depletion of hepatic GSH [1–3]. In addition, vinylidene chloride has been found to be mutagenic in the presence of liver-activating enzymes [4–8] and carcinogenic in rats and mice, although the sex, species and strain of the experimental animal affect its carcinogenic potential [9–12]. The mutagenic and carcinogenic effects of vinylidene chloride are thought to be produced by reactive metabolites of the parent compound such as dichloroethylene oxide or chloroacetyl chloride (see, for example, Refs. 6 and 8). Neither of these compounds, however, has been identified as a metabolite of vinylidene chloride *in vivo* or *in vitro*. The only metabolites of vinylidene chloride that have been identified are monochloroacetate, conjugates, thiodiglycollic acid and related products [13–15]. All other intermediates in the proposed metabolic pathways for vinylidene chloride have not been identified (see Fig. 1).

The metabolism of vinylidene chloride has been proposed to involve hepatic microsomal cytochrome P-450 on the basis of the observation that the conversion of vinylidene chloride to monochloroacetate and an unknown metabolite is catalyzed by hepatic post-mitochondrial supernatant fraction plus NADPH-generating system [15].

We have investigated the interaction of vinylidene chloride with different forms of hepatic microsomal cytochrome P-450 *in vitro*, using hepatic microsomes isolated from rats which were pretreated, or not, with inducing agents to specifically elevate the levels

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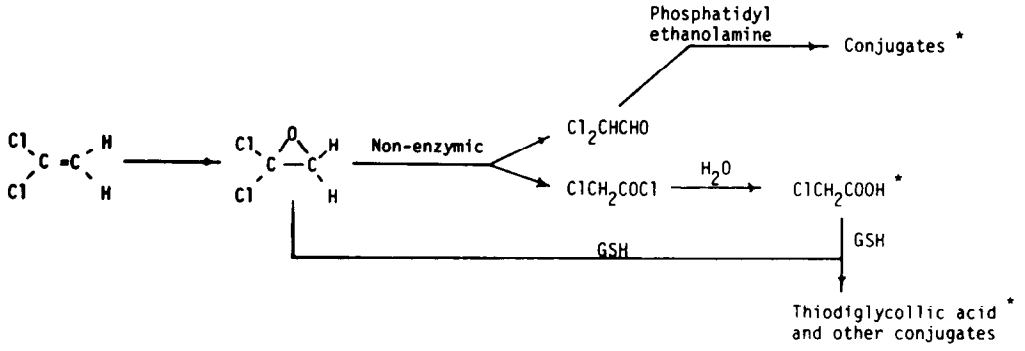


Fig. 1. Proposed pathways for the metabolism of vinylidene chloride *in vivo* [8, 13, 16]. An asterisk (*) indicates identified metabolites.

of different forms of cytochrome P-450. This study represents one avenue in our investigations into the metabolism and toxicity of the chlorinated ethylenes.

## EXPERIMENTAL

*Materials.* Vinylidene chloride, 2-chloroethanol, 2,2-dichloroethanol, and the mono- and dichloroacetic acids were reagent grade chemicals from Merck Chemicals, Darmstadt, West Germany. Chloroacetaldehyde was purchased from Fluka AG, Buchs, Switzerland, and redistilled prior to use. 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. Sodium phenobarbital and β-naphthoflavone were obtained from Maybaker, Port Elizabeth, R.S.A., and the Aldrich Chemical Co., Milwaukee, WI, U.S.A., respectively. NADPH and NADH were purchased from Miles Laboratories, Cape Town, R.S.A. All other chemicals were analytical grade reagents. Water was distilled and deionized. Dichloroacetaldehyde was prepared from freshly distilled chloral according to the method of Swietoslawski and Silowieki [17]. Stock solutions of dichloroacetaldehyde in water were prepared immediately after distillation of the freshly prepared dichloroacetaldehyde in order to prevent polymerization [18]. The acetaldehyde content of the stock solutions was confirmed by the method of Malhotra and Anand [18].

*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%; phosphorous 0.7%; obtained from Epol Ltd., Goodwood, C.P., R.S.A.) and water. β-Naphthoflavone was given as a single injection (80 mg/kg in corn oil) 36 hr prior to killing the animals [19]. Phenobarbital was administered i.p. for 3 days at 80 mg/kg in 0.9% saline [20]. Animals were starved for 18 hr prior to being killed.

Hepatic microsomes were isolated by differential centrifugation [21]. Experiments were conducted with microsomal suspensions of 2 mg protein/ml in 0.02 M Tris–HCl, pH 7.4, unless otherwise indicated. Microsomal protein was determined by the method of Lowry *et al.* [22] as modified by Chaykin [23].

*Introduction of vinylidene chloride.* Vinylidene chloride was added to hepatic microsomes as a 33% (v/v) solution in absolute ethanol and was dispersed by vortex mixing for 30 sec. In no case was more than a single addition of vinylidene chloride made per sample of microsomal suspension. Experiments were routinely conducted in glass vials with serum stoppers or in cuvettes with Teflon stoppers. Identical results were obtained when experiments were conducted using sealed hypodermic vials.

*Spectral assays.* Difference spectra with hepatic microsomal cytochrome P-450 were measured at 25° with hepatic microsomes at a concentration of 2 mg protein/ml as described earlier [24]. The magnitude of the resulting difference spectrum was measured as the difference in absorbance ($\Delta A$) between the peak at 386 nm and the trough at 418 nm.

The rates of NADPH oxidation by hepatic microsomes were measured spectrally at 30° in the presence of 6.9 mM vinylidene chloride and 0.18 mM NADPH as described earlier [24]. 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.* [25]. $H_2O_2$ was assayed by the method of Hildebrandt *et al.* [26].

For determining the effects of vinylidene chloride 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 6.9 mM vinylidene chloride, 0.2 mM EDTA and an NADPH-generating system [27] 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 [28], using an extinction coefficient of 91 $cm^{-1}$ $mM^{-1}$ 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 [28].

*Identification of possible metabolites of vinylidene chloride.* To assess the metabolism of vinylidene chloride, 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 vinylidene chloride as described above. The reaction was terminated by precipitation of the protein with 200 μl of 2N $H_2SO_4$ and 200 μl of 10% $Na_2WO_4$ [29]. 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 bubbling 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 [30]. Column, injector, and detector temperatures were 200°, 200° and 250° respectively. Retention times were as follows: methyl monochloroacetate, 348 sec; methyl dichloroacetate, 550 sec; monochloroacetaldehyde, 145 sec; and dichloroacetaldehyde, 210 sec. When 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) [31]. Column, injector and detector temperatures were 170°, 200° and 230° respectively. Retention times were as follows: 2-chloroethanol, 137 sec; and 2,2-dichloroethanol, 270 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 ($\Delta A_{max}$), and maximum rates of metabolism ($V_{max}$) were calculated from Hanes plots.

## RESULTS

*Binding to hepatic microsomal cytochrome P-450.* Vinylidene chloride bound to hepatic cytochrome P-450 in microsomes from uninduced and induced rats, resulting in the production of a Type I difference spectrum ($\lambda_{max}$ = 386 nm; $\lambda_{min}$ = 418 nm). Hanes plots of the binding of vinylidene chloride to hepatic microsomal cytochrome P-450 were in all cases biphasic and were characterized by two $K_s$ values and two corresponding $\Delta A_{max}$ values (see, for example, Fig. 2). The effects of inducing agents for different forms of cytochrome P-450 on the binding constants ($K_s$) and the maximum extents of binding ($\Delta A_{max}$) for the binding of vinylidene chloride to hepatic microsomal cytochrome P-450 are shown in Table 1. The $K_s$ values for the binding of vinylidene chloride to hepatic microsomal cytochrome P-450 were not altered significantly by induction with β-naphthoflavone, and the values of $\Delta A_{max}$ were either not changed or were decreased by this inducing agent. Following phenobarbital induction, only the $K_s$ value for the low-affinity site for the binding of vinylidene chloride to hepatic microsomal cyto-

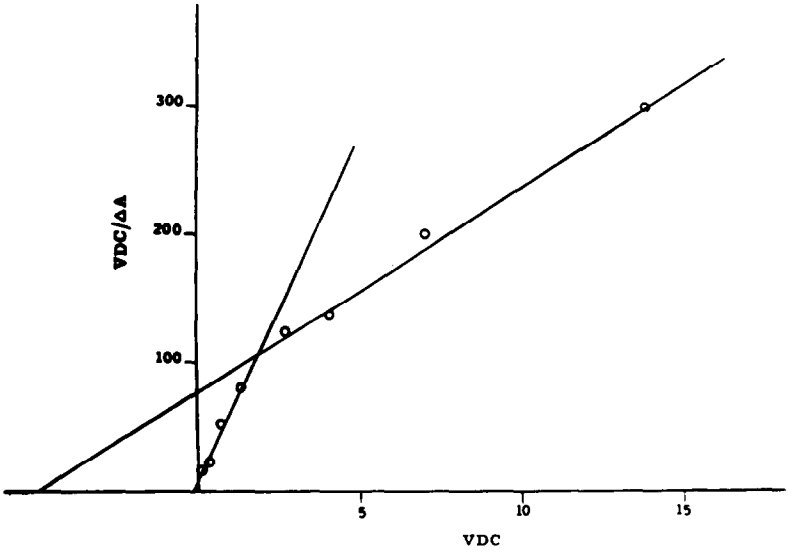


Fig. 2. Hanes plot for the binding of vinylidene chloride to cytochrome P-450 in hepatic microsomes (2 mg protein/ml) from β-naphthoflavone-treated rats. Concentration of vinylidene chloride (VDC), mM; $\Delta A$, $A_{386\,nm}$–$A_{418\,nm}$. Room temperature.

Table 1. Binding by hepatic microsomal cytochrome P-450 and stimulation of hepatic microsomal NADPH oxidation by vinylidene chloride*

| Inducing agent | Cytochrome P-450 (nmoles/mg microsomal protein) | $K_s$ (mM) | | $10^2\ \Delta A_{max}$ ($A_{386\,nm}-A_{418\,nm}$) | | NADPH oxidation [nmoles · min$^{-1}$ · (mg microsomal protein)$^{-1}$] | |
|---|---|---|---|---|---|---|---|
| None | 0.9 ± 0.2 | 0.22 ± 0.06 | 5.8 ± 1.3 | 1.3 ± 0.5 | 7.2 ± 1.7 | 1.6 ± 0.6 | |
| | | | | (1.4 ± 0.6) | (8.0 ± 1.9) | (1.8 ± 0.7) | |
| β-Naphthoflavone | 1.2 ± 0.1† | 0.19 ± 0.06 | 4.8 ± 0.6 | 1.1 ± 0.6 | 4.5 ± 1.4 | 1.1 ± 0.6 | |
| | | | | (0.9 ± 0.5) | (3.8 ± 1.2†) | (0.9 ± 0.5) | |
| Phenobarbital | 2.1 ± 0.1† | 0.26 ± 0.01 | 1.6 ± 0.1† | 4.0 ± 0.4† | 11.4 ± 0.1† | 3.1 ± 0.8† | |
| | | | | (1.9 ± 0.2) | (5.4 ± 0.1†) | (1.5 ± 0.4) | |

* 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 text. Values in parentheses are per nmole of cytochrome P-450.
† Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

chrome P-450 was decreased significantly, while both $\Delta A_{max}$ values were increased significantly.

*Hepatic microsomal NADPH oxidation.* The rate of hepatic microsomal CO-inhibitable NADPH oxidation was increased by vinylidene chloride *in vitro* (Table 1). The rate of vinylidene chloride stimulated CO-inhibitable NADPH oxidation was not altered significantly following β-naphthoflavone induction but was elevated significantly following phenobarbital induction (Table 1).

*Metabolism by hepatic microsomal cytochrome P-450.* The 2-chloro- and 2,2-dichloro-derivatives of ethanol, acetaldehyde and acetic acid were investigated as possible metabolites of vinylidene chloride by hepatic microsomal cytochrome P-450. Incubation mixtures contained vinylidene chloride (6.9 mM), hepatic microsomes (4 or 6 mg protein/ml) from phenobarbital-induced rats, EDTA (0.2 mM) and NADPH-generating system. The amounts of 2-chloroethanol, chloroacetaldehyde and dichloroacetic acid produced from these incubation mixtures were below their limits of detection, viz. 2-chloroethanol, <16.2 nmoles · (mg protein)$^{-1}$ · (30 min)$^{-1}$; chloroacetaldehyde, <0.06 nmole · (mg protein)$^{-1}$ · (30 min)$^{-1}$; and dichloroacetic acid, <0.08 nmole · (mg protein)$^{-1}$ · (30 min)$^{-1}$.

Low levels of 2,2-dichloroethanol [0.22 nmole · (mg protein)$^{-1}$ · (20 min)$^{-1}$] were initially found in incubation mixtures. However, following redistillation of the reagent grade vinylidene chloride, no 2,2-dichloroethanol [limit of detection, 0.15 nmol · (mg protein)$^{-1}$ · (20 min)$^{-1}$] was produced, suggesting that its appearance was due to metabolism of a contaminant of the commercially available vinylidene chloride, possibly such as of 1,1-dichloroethane [16].

Following the extraction of acidified reaction mixtures and methylation with diazomethane, one of the metabolites of vinylidene chloride chromatographed identically to methyl chloroacetate. Neutralization of the reaction mixture prior to methylation, or the omission of the methylation reaction from the isolation procedure, eliminated the observed chromatographic peak. The mass spectrum of the methylated reaction product was identical to that of an authentic sample of methyl chloroacetate: significant peaks at 108, 77, 59 and 49 corresponded to the $ClCH_2COOCH_3^+$, $ClCH_2CO^+$, $COOCH_3^+$ and $ClCH_2^+$ segments respectively.

A chromatographic peak with a retention time equal to dichloroacetaldehyde was also produced following the incubation of vinylidene chloride with hepatic microsomes, NADPH-generating system and EDTA. The Fehling's test for aldehydes was positive for extracts of reaction mixtures containing this peak, and the application of this test to reaction mixtures eliminated this peak from gas–liquid chromatograms. Because of its volatility, this metabolite was lost during attempts to concentrate ether extracts of reaction mixtures for assay by GC–MS. Therefore, the following treatment for the oxidation of aldehydes to carboxylic acids was applied. 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 M), and then an aqueous solution of sodium bisulfite (5%, w/v) was added

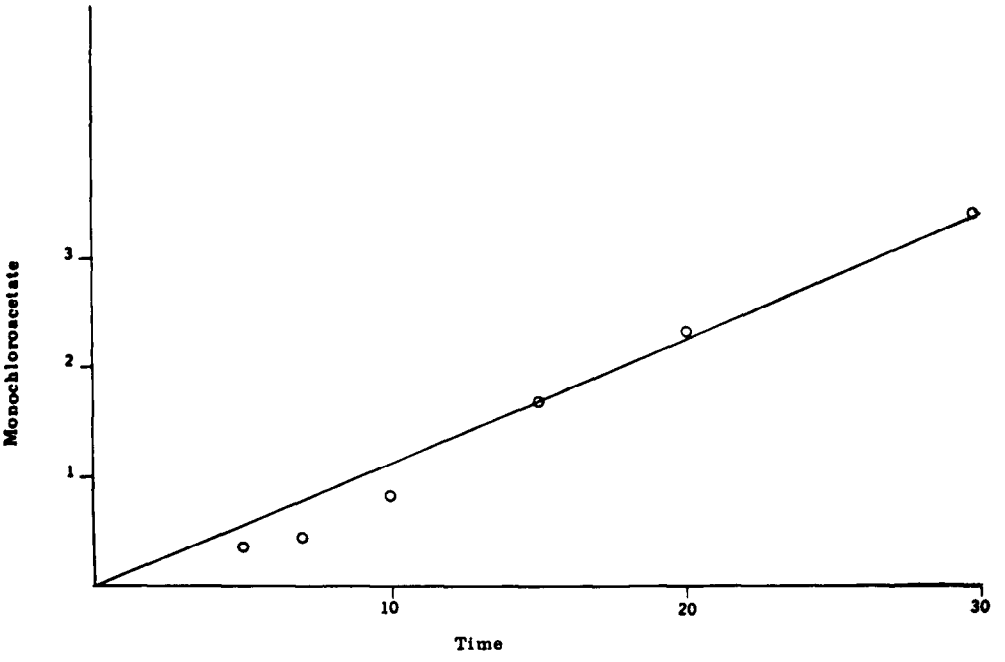


Fig. 3. Production of monochloroacetate as a function of time. Monochloroacetate, nmoles/mg microsomal protein; time, min. Reaction mixtures contained hepatic microsomes (4 mg protein/ml) from phenobarbital-treated rats and NADPH-generating system [27], EDTA (0.2 mM) and vinylidene chloride (6.9 mM) were incubated at 30° with shaking.

until all colour was lost [32]. 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). Following methylation with diazomethane, a new peak appeared at 550 sec, which corresponded to the retention time of methyl dichloroacetate.

In no case were any chlorinated metabolites measurable in extracts of incubation mixtures of

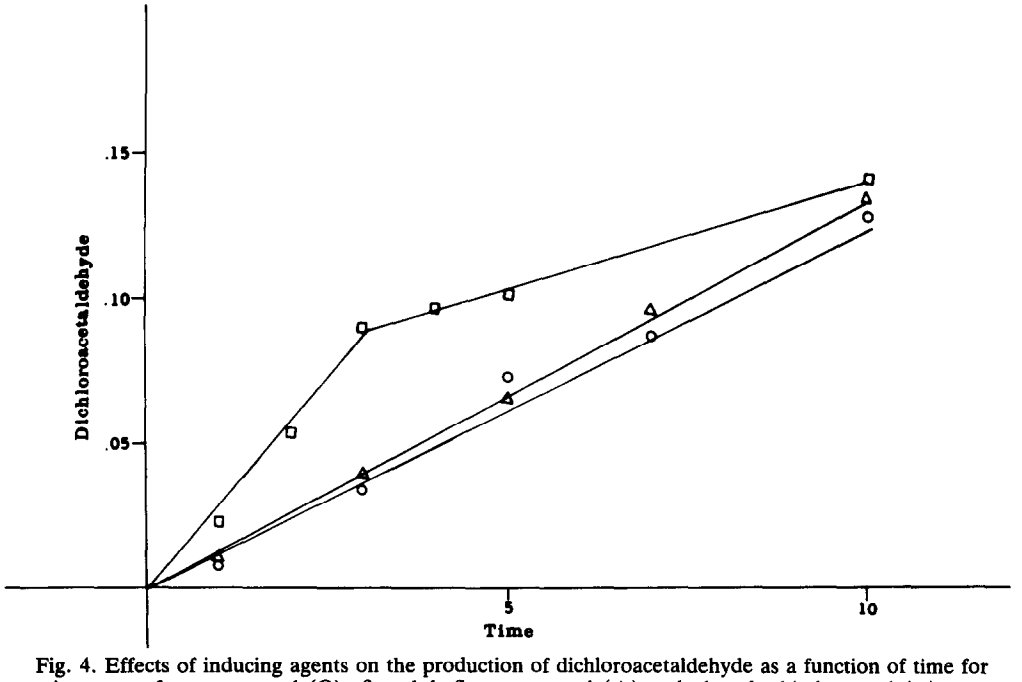


Fig. 4. Effects of inducing agents on the production of dichloroacetaldehyde as a function of time for microsomes from untreated (○), β-naphthoflavone-treated (△) and phenobarbital-treated (□) rats. Dichloroacetaldehyde, nmoles per min per mg microsomal protein; time, min. Experimental conditions were as described in the legend of Fig. 3.

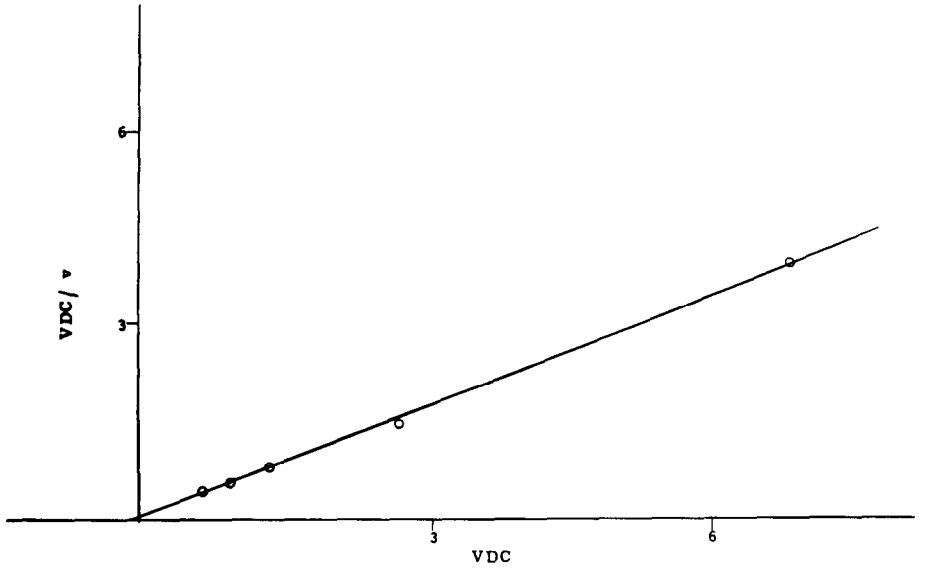


Fig. 5. Hanes plot for the production of monochloroacetate from vinylidene chloride by microsomes from phenobarbital-treated rats. Vinylidene chloride (VDC), mM; $v$, nmoles monochloroacetate per mg microsomal protein per 20 min. Experimental details, except for vinylidene chloride concentration, were as in Fig. 3.

hepatic microsomes plus NADPH-generating system and EDTA or of hepatic microsomes plus vinylidene chloride.

*Kinetics of metabolite production.* The conversion of vinylidene chloride to monochloroacetate was linear for 30 min for incubation mixtures containing hepatic microsomes (4 mg protein/ml) from phenobarbital-induced rats, vinylidene chloride (6.9 mM), EDTA (0.2 mM) and an NADPH-generating system (Fig. 3). The rates of monochloroacetate production per mg microsomal protein per 20 min were identical whether incubation mixtures of hepatic microsomes from phenobarbital-induced rats were utilized at concentrations of 2 or 4 mg protein/ml.

The production of dichloroacetaldehyde from vinylidene chloride in incubation mixtures prepared exactly as described above was linear for 3 min for microsomes from phenobarbital-induced rats and for 10 min for microsomes from untreated or β-naphthoflavone-induced rats (Fig. 4).

*$K_m$ and $V_{max}$ for the metabolism of vinylidene chlor-*

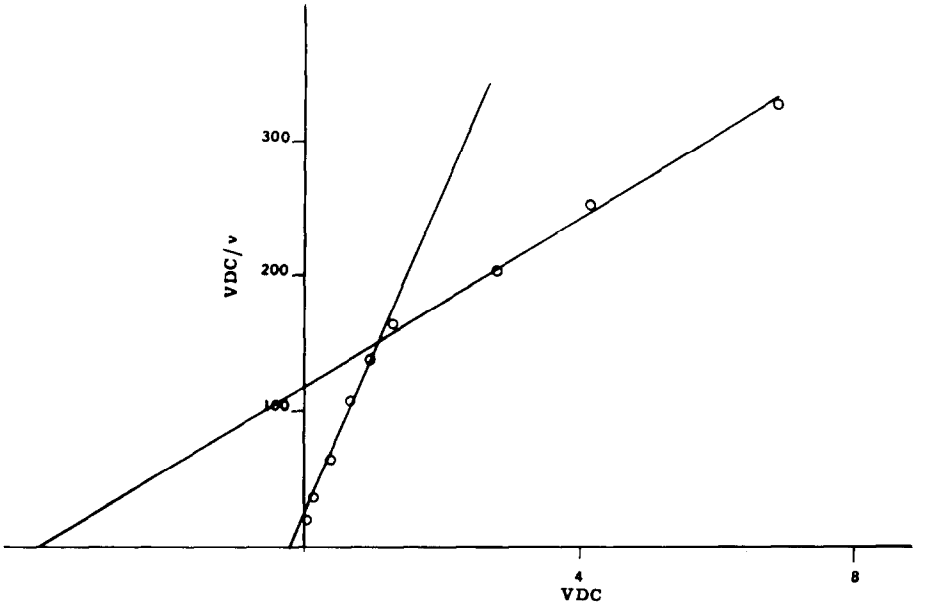


Fig. 6. Hanes plot for the production of dichloroacetaldehyde from vinylidene chloride by microsomes from phenobarbital-treated rats. Vinylidene chloride (VDC), mM; $v$, nmoles dichloroacetaldehyde per mg microsomal protein per min. Experimental details, except for vinylidene chloride concentration, were as in Fig. 3.

Table 2. Effect of induction on the conversion of vinylidene chloride to dichloroacetaldehyde by hepatic microsomal cytochrome P-450*

| Inducing agent | Cytochrome P-450 (nmoles/mg microsomal protein) | $K_m$ (mM) | $V_{max}$ [nmoles · (mg microsomal protein)$^{-1}$ · min$^{-1}$] |
|---|---|---|---|
| None | 1.1 ± 0.1 | 0.17 ± 0.05 | 0.025 ± 0.011 (0.023 ± 0.010) |
| | | 0.59 ± 0.05 | 0.052 ± 0.010 (0.048 ± 0.009) |
| β-Naphthoflavone | 1.4 ± 0.1† | 0.17 ± 0.04 | 0.034 ± 0.011 (0.024 ± 0.008) |
| | | 2.2 ± 0.2† | 0.070 ± 0.011 (0.057 ± 0.080) |
| Phenobarbital | 2.6 ± 0.0† | 0.21 ± 0.02 | 0.019 ± 0.002 (0.007 ± 0.001†) |
| | | 2.9 ± 1.0† | 0.054 ± 0.010 (0.021 ± 0.003†) |

* Values are means ± S.D. for experiments performed in triplicate with three or more different preparations of hepatic microsomes. Experimental conditions are described in the text. Values in parentheses are in nmoles · min$^{-1}$ · (nmole cytochrome P-450)$^{-1}$.
† Differs significantly from corresponding value for microsomes from untreated rats, $P < 0.01$.

*ide.* A Michaelis constant ($K_m$) of 0.16 ± 0.02 mM and a maximum rate of metabolism ($V_{max}$) of 1.85 ± 0.14 nmoles · (mg microsomal protein)$^{-1}$ · (20 min)$^{-1}$ [0.69 ± 0.05 nmole · (nmole cytochrome P-450)$^{-1}$ · (20 min)$^{-1}$] were calculated for monochloroacetate production from vinylidene chloride by hepatic microsomes from phenobarbital-pretreated rats (Fig. 5). The rates of monochloroacetate production were 1.32 ± 0.13 and 1.57 ± 0.18 nmoles · (mg microsomal protein)$^{-1}$ · (20 min)$^{-1}$ [1.59 ± 0.16 and 1.34 ± 0.15 nmoles · (nmole cytochrome P-450)$^{-1}$ · (20 min)$^{-1}$] for microsomes from untreated and β-naphthoflavone-treated rats respectively.

The metabolism of vinylidene chloride 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. 6). The $K_m$ for the high-affinity site for the production of dichloroacetaldehyde was unaffected by β-naphthoflavone and phenobarbital treatment, while the $K_m$ for the low-affinity site was increased significantly by both of these inducing agents (Table 2). Neither inducing agent significantly affected either of the $V_{max}$ values (Table 2). The values of $V_{max}$ per nmole cytochrome P-450 were not affected by β-naphthoflavone induction, but were decreased by 2- to 3-fold following phenobarbital induction.

*NADH as electron donor for the hepatic microsomal metabolism of vinylidene chloride.* Incubation of reaction mixtures containing hepatic microsomes from phenobarbital-treated rats, vinylidene chloride (6.9 mM), EDTA (0.2 mM) and NADH (2 mM) resulted in the production of 0.23 nmole monochloroacetate · (mg microsomal protein)$^{-1}$ · (20 min)$^{-1}$ and 0.03 nmole dichloroacetaldehyde · (mg microsomal protein)$^{-1}$ · (3 min)$^{-1}$, corresponding to 13 and 19% of the rates of production of these metabolites from vinylidene chloride with NADPH as the electron donor.

*Effect of inhibitors on vinylidene chloride metabolism.* The inhibitors of hepatic cytochrome P-450, viz. SKF-525A (200 mM) and CO:$O_2$ (80:20, v/v), each inhibited the production of monochloroacetate by 60% (±5%) and of dichloroacetaldehyde by 55% (±5%) in reaction mixtures containing vinylidene chloride (6.9 mM), hepatic microsomes (4 mg protein/ml) from phenobarbital-pretreated rats, EDTA (0.2 mM) and NADPH-generating system. Incubations were at 30° for 20 min for monochloroacetate production and 3 min for dichloroacetaldehyde production (data not shown).

*Assay for the covalently bound chlorinated acetyl moiety.* The treatment of reaction mixtures containing vinylidene chloride, NADPH-generating system, EDTA and hepatic microsomes (4 mg protein/ml) from phenobarbital-induced rats, which had been incubated at 30° for 30 min, with 20% $H_2SO_4$ for 16 hr at 100° [8] resulted in no significant increase in the levels of monochloroacetic acid or dichloroacetic acid (data not shown).

*Hepatic microsomal $H_2O_2$ production.* Incubation for 5 min at 30° of hepatic microsomes (2 mg protein/ml) from phenobarbital-treated rats, NADPH-generating system, EDTA (0.1 mM)

Table 3. Effect of vinylidene chloride on the levels of hepatic microsomal cytochrome P-450*

| Inducing agent | Vinylidene chloride (mM) | Cytochrome P-450 (loss/initial level) | Heme (loss/initial level) |
|---|---|---|---|
| None | 0 | 0.06/(0.83 ± 0.08) | 0.04/(1.44 ± 0.07) |
| | 6.9 | 0.10/(0.77 ± 0.07) | 0.11/(1.43 ± 0.11) |
| β-Naphthoflavone | 0 | 0.10/(1.24 ± 0.06) | 0.08/(1.84 ± 0.38) |
| | 6.9 | 0.10/(1.05 ± 0.18) | 0.12/(1.80 ± 0.32) |
| Phenobarbital | 0 | 0.11/(2.10 ± 0.28) | 0.13/(2.84 ± 0.31) |
| | 6.9 | 0.12/(1.99 ± 0.20) | 0.18/(2.73 ± 0.27) |

* 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, where indicated, vinylidene chloride (6.9 mM). Losses of cytochrome P-450 and heme in nmoles/mg microsomal protein are reported for samples incubated at 30° for 15 min with shaking, relative to unincubated samples.

and vinylidene chloride (6.9 mM) resulted in the production of 3.2 ± 1.6 nmoles $H_2O_2 \cdot (\text{mg protein})^{-1} \cdot \text{min}^{-1}$.

*Effect of vinylidene chloride on the levels of hepatic microsomal cytochrome P-450 and heme.* Incubation of vinylidene chloride, hepatic microsomes, EDTA and an NADPH-generating system resulted in no significant alteration in the levels of cytochrome P-450 or heme in hepatic microsomes from untreated and β-naphthoflavone- or phenobarbital-treated rats (Table 3).

## DISCUSSION

The proposal that hepatic microsomal cytochrome P-450 metabolizes vinylidene chloride [15] is confirmed by the results reported herein. First, vinylidene chloride appeared to bind to the active site of hepatic microsomal cytochrome P-450 since, in the presence of hepatic microsomes, it produced a Type I difference spectrum and stimulated CO-inhibitable NADPH oxidation (see Results, Tables 1 and 2) [33]. Furthermore, vinylidene chloride was shown to be converted to dichloroacetaldehyde and to monochloroacetic acid by hepatic microsomes plus NADPH-generating system. The conversion of vinylidene chloride to these metabolites was supported by NADH, but at approximately 15% of the rate of the NADPH-supported reaction (see results). Finally, the inhibitors of cytochrome P-450, SKF-525A and CO [34], effectively inhibited the conversion of vinylidene chloride to monochloroacetate and dichloroacetaldehyde *in vitro* (see Results).

The form of cytochrome P-450 elevated by β-naphthoflavone, viz. cytochrome P-448, does not appear to bind or metabolize vinylidene chloride. β-Naphthoflavone induction did not affect the values of $K_s$ and did not alter, or decreased, $\Delta A_{max}$ and $\Delta A_{max}$ per nmole cytochrome P-450 for the binding of vinylidene chloride. The rates of conversion of vinylidene chloride to monochloroacetate and dichloroacetaldehyde per mg microsomal protein and per nmole cytochrome P-450 were also not affected following β-naphthoflavone induction (Tables 1 and 2, see Results).

The phenobarbital-inducible form of cytochrome P-450 appeared to play a minor role in the binding and metabolism of vinylidene chloride. Phenobarbital induction resulted in significant increases in $\Delta A_{max}$ and the rate of monochloroacetate production per mg microsomal protein, but it did not affect, or resulted in decreases in, the values of these parameters per nmole cytochrome P-450 (Tables 1 and 2, see Results). The conversion of vinylidene chloride to dichloroacetaldehyde does not appear to be catalyzed by the phenobarbital-inducible form of cytochrome P-450 since phenobarbital induction did not increase the rate of dichloroacetaldehyde production per mg of microsomal protein and decreased its rate of production per nmole cytochrome P-450 by 2- to 3-fold (Table 2). Thus, one or more of the forms of cytochrome P-450 present in microsomes from untreated rats may be more efficient in the metabolism of vinylidene chloride to chloroacetate and particularly to dichloroacetaldehyde than is the phenobarbital-inducible form of the enzyme (see Results).

It would appear that vinylidene chloride may be a partial uncoupler of cytochrome P-450 since the rates of vinylidene chloride stimulated CO-inhibitable NADPH oxidation greatly exceeded the rates of production of dichloroacetaldehyde and monochloroacetate. This proposal is supported by the observation that vinylidene chloride stimulated the hepatic microsomal production of $H_2O_2$ and that, in the presence of vinylidene chloride, the rate of $H_2O_2$ production was equal to that of NADPH oxidation ($P > 0.1$) (Tables 1 and 2, see Results). Tetrachloroethylene also appears to be a partial uncoupler of cytochrome P-450 since the rate of tetrachloroethylene stimulated hepatic microsomal CO-inhibitable NADPH oxidation greatly exceeded its rate of metabolism, while for another analogue, trichloroethylene, these rates are equivalent [31, 35].

The results reported herein have provided the first unequivocal identification of dichloroacetaldehyde as a metabolite of vinylidene chloride *in vitro* or *in vivo*.* The identification of dichloroacetaldehyde as

* The unidentified volatile halogenated metabolite of vinylidene chloride, reported by Leibman and Ortiz [15] to be produced by post-mitochondrial supernatant fraction and NADPH-generating system *in vitro*, would be expected to be dichloroacetaldehyde.

Table 4. Relationship between the metabolism of selected chlorinated ethylenes and the ability to degrade hepatic microsomal cytochrome *in vitro*

| Chlorinated ethylene | Metabolism by cytochrome P-450* | | Ability to degrade hepatic microsomal cytochrome P-450* | Reference |
|---|---|---|---|---|
| | Product | Rate† | | |
| Vinylidene chloride | {Monochloroacetate, Dichloroacetaldehyde} | 0.2 | No | (see Results) |
| Trichloroethylene | Chloral hydrate | 5.8 | Yes | [31] |
| Tetrachloroethylene | Trichloroacetate | 0.5 | No | [33] |

* Experiments were carried out using hepatic microsomes from phenobarbital-treated rats.
† Expressed in nmoles · $min^{-1}$ · (mg microsomal protein)$^{-1}$.

a metabolite of vinylidene chloride from the hepatic microsomal cytochrome P-450 enzyme system is fully consistent with the proposed pathways for the metabolism of vinylidene chloride *in vivo* (see Fig. 1) and indicates that cytochrome P-450 catalyzes the first step in the metabolism of this compound *in vivo*. It is anticipated that dichloroacetaldehyde may undergo a series of reactions, such as Schiff base formation with protein side chains or oxidative or reductive reactions *in vitro* and *in vivo*. This proposal is supported by the very short time period for which dichloroacetaldehyde production is linear, with microsomes from phenobarbital-treated rats *in vitro*, viz. 3 min, relative to the 30-min time period over which chloroacetate production is linear (see Results). An ability of dichloroacetaldehyde to undergo side reactions might explain, in part, why this compound has not been identified as a metabolite of vinylidene chloride *in vivo*.

The results reported herein on the hepatic microsomal metabolism of vinylidene chloride do not aid in clarifying the controversy surrounding the relationship between the metabolism and toxicity of vinylidene chloride *in vivo*. Phenobarbital pretreatment, which decreases the toxicity of vinylidene chloride *in vivo*, would be expected to enhance the metabolism of this compound *in vivo* as a consequence of its ability to enhance the proliferation of the endoplasmic reticulum.* This situation is consistent with the proposal of Reynolds *et al.* [1] that the toxicity of vinylidene chloride is a property of the parent compound and not of a metabolite thereof. However, it is possible that a metabolite of vinylidene chloride mediates its toxicity, provided that the detoxification of reactive metabolites of vinylidene chloride is enhanced by phenobarbital to a greater extent than is the metabolic activation of vinylidene chloride. It is not clear, either in view of the proposed metabolic pathways or the reported rates of metabolism, why the toxicity of vinylidene chloride greatly exceeds that of the other chlorinated ethylenes (Table 4) (see, for example, Refs. 1 and 8).

Vinylidene chloride would appear to require metabolism by cytochrome P-450 in order to be converted into a mutagenic species. Phenobarbital treatment, which slightly enhanced the metabolism of vinylidene chloride per mg microsomal protein *in vitro*, also slightly increased the mutagenicity of vinylidene chloride, while inhibitors of cytochrome P-450 decreased both the metabolism and the mutagenicity of vinylidene chloride *in vitro* [7] (see Results). The reactive mutagenic species produced from vinylidene chloride may be 1,1-dichloroethylene oxide, as proposed by Bonse *et al.* [8] or may be a rearrangement product thereof, viz. chloroacetyl chloride or dichloroacetaldehyde.

The metabolism of vinylidene chloride by hepatic cytochrome P-450 did not result in decreased levels of microsomal cytochrome P-450 or heme *in vitro* (Table 3). This observation is consistent with the findings of Reynolds *et al.* [1] that the endoplasmic reticulum is spared following the exposure of rats to vinylidene chloride, but it is in contrast to the abilities of other chlorinated ethylenes (viz. vinyl chloride and trichloroethylene) that are metabolically activated by hepatic cytochrome P-450 to modify the heme of this enzyme. The relative abilities of the chlorinated ethylenes to modify the heme of cytochrome P-450 does, however, correlate with their total rates of metabolism by hepatic microsomal cytochrome P-450 and with the way in which the expected epoxide derivatives of the chlorinated ethylenes rearrange (see Table 4). The chlorinated ethylenes which are slowly metabolized by hepatic microsomal cytochrome P-450, viz. vinylidene chloride and tetrachloroethylene, do not modify the heme of this enzyme, while trichloroethylene, which has a far greater rate of metabolism, does (Table 4). The ability of the chlorinated ethylenes to modify the heme of cytochrome P-450 also correlates with the chemical nature of the final rearrangement products of the expected epoxide derivatives. The epoxides that rearrange exclusively to aldehydes *in vitro* correspond to chlorinated ethylenes that modify the heme of cytochrome P-450, viz. vinyl chloride and trichloroethylene [31, 37], while the epoxides that rearrange to an acyl chloride or to both an aldehyde and an acyl chloride *in vitro* are characteristic of chlorinated ethylenes that do not modify the heme of cytochrome P-450, viz. vinylidene chloride and tetrachloroethylene (see Results) (Table 4) [33].

* Phenobarbital pretreatment, which slightly increased (per mg microsomal protein) the cytochrome P-450 mediated conversion of vinylidene chloride to monochloroacetate but not to dichloroacetaldehyde *in vitro* (see Results, Table 2), would be expected to increase the rate of conversion of vinylidene chloride to both of these metabolites *in vivo* as a consequence of the ability of phenobarbital to increase the proliferation of the endoplasmic reticulum *in vivo* [36].

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