MOLECULAR PHARMACOLOGY, 13, 993-1004

Metabolism of Vinyl Chloride: Destruction of the Heme of Highly Purified Liver Microsomal Cytochrome P-450 by a Metabolite

F. PETER GUENGERICH AND THOMAS W. STRICKLAND[1]

Department of Biochemistry and Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

(Received March 29, 1977)
(Accepted June 10, 1977)

SUMMARY

GUENGERICH, F. PETER & STRICKLAND, THOMAS W. (1977) Metabolism of vinyl chloride: destruction of the heme of highly purified liver microsomal cytochrome P-450 by a metabolite. *Mol. Pharmacol.*, 13, 993-1004.

The NADPH-dependent, vinyl chloride-mediated destruction of cytochrome P-450 was demonstrated in rat liver microsomes and in highly purified reconstituted enzyme systems containing NADPH-cytochrome P-450 reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) and cytochrome P-450. This loss of cytochrome P-450 could be attributed to heme destruction, but not to lipid peroxidation or binding of electrophiles to free sulfhydryl groups. The system required all components necessary for mixed-function oxidation, including molecular oxygen, and was inhibited by carbon monoxide, suggesting strongly that oxidative metabolism of vinyl chloride by cytochrome P-450 is necessary for the observed destruction. The NADPH-cytochrome P-450 reductase-catalyzed destruction of free and cytochrome P-450-bound heme was also observed in reconstituted systems in the absence of vinyl chloride. Inhibition experiments with carbon monoxide and catalase suggest that the vinyl chloride-mediated destruction of cytochrome P-450 heme differs from these processes. Two proposed metabolites of vinyl chloride, vinyl chloride epoxide and 2-chloroacetaldehyde, do not appear to be responsible for the heme destruction. Evidence for the involvement of free radicals could not be demonstrated when the reaction was examined by EPR spectroscopy or when attempts were made to inhibit cytochrome P-450 destruction with radical-trapping agents.

INTRODUCTION

Exposure to vinyl chloride has been related to hepatic injury and production of liver angiosarcomas in experimental animals (1); a relationship between vinyl chloride exposure and production of such tumors has been established in human beings (2, 3). Evidence has been provided that NADPH-dependent microsomal activation is necessary for vinyl chloride mutagenesis in *Salmonella typhimurium* tester strains (4, 5) and for covalent binding of vinyl chloride to proteins and RNA (6). The hypothesis has been presented that vinyl chloride is metabolized to its epoxide, a highly electrophilic species which is mutagenic (7) and capable of reacting with various nucleophiles (8).

Reynolds *et al.* (9) found that exposure of rats to vinyl chloride results in a selective loss of liver microsomal cytochrome P-450 and in the decreased ability of liver microsomal preparations to metabolize drugs; the authors hypothesized that these losses are due to the reaction of P-450 with an electrophile produced in the metabolism of vinyl chloride by the same

This research was supported by United States Public Health Service Center Grant ES 00267.

[1] Supported by United States Public Health Service Training Grant ES 00112.

ISSN 0026-895X

enzyme system. During the course of our work, Ivanetich *et al.* (10) reported the NADPH-dependent, vinyl chloride-mediated destruction of P-450 in microsomes prepared from phenobarbital-treated rats; these workers also suggested that the loss of P-450 is due to the reaction of electrophilic metabolites with nucleophilic sites of the P-450. Their paper is unclear as to whether the P-450 loss is due to conversion to cytochrome P-420.

In this report, experiments utilizing apparently homogeneous preparations of cytochrome P-450 and NADPH–cytochrome P-450 reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) were used to demonstrate that vinyl chloride-mediated cytochrome P-450 destruction *in vitro* is due to the loss of heme and that the mechanism of such NADPH-dependent heme destruction is clearly distinct from that of the heme destruction observed in lipid peroxidation (11), the "heme oxygenase" reaction (12, 13), and the NADPH–cytochrome P-450 reductase-catalyzed loss of free and cytochrome P-450-bound heme observed here in the absence of added substrates. The metabolite(s) of vinyl chloride responsible for the destruction could not be identified; however, evidence is presented that the epoxide of vinyl chloride and its rearrangement product, 2-chloroacetaldehyde, are not responsible for the observed destruction. A preliminary account of these findings has appeared (14).

MATERIALS AND METHODS

Materials. *d*-Benzphetamine was a gift from Dr. P. W. O'Connell of Upjohn, *N*,*N*-dimethyl-4-aminoazobenzene was donated by Dr. J. A. Miller of the University of Wisconsin, and 1,N^6-ethenoadenosine was a gift from Dr. N. J. Leonard of the University of Illinois. Vinyl chloride was obtained from Matheson Gas Products; ferriprotophorphyrin IX (hemin chloride), superoxide dismutase, and butylated hydroxytoluene, from Sigma; and DNA, RNA, and homogeneous catalase, from Calbiochem. Chloroacetaldehyde (15) and vinyl chloride epoxide (16) were prepared as described; the latter compound was characterized by its mass and NMR spectra (16) and its reaction with *p*-nitrophenylpyridine (8).

Enzyme preparations. Rats were treated with phenobarbital (0.1% in drinking water for 5 days) or 3-methylcholanthrene (20 mg/kg for 3 days), and microsomes were prepared as described (17).

Cytochromes P-450 and NADPH–cytochrome P-450 reductase were purified as described (17); the NADPH–cytochrome P-450 reductase preparations catalyzed the reduction of either 57.5 or 60.4 μmoles of cytochrome *c* per minute per milligram when assayed at 30° in 0.3 M potassium phosphate buffer (pH 7.7) (17). Cytochrome P-450 fractions prepared from phenobarbital- and 3-methylcholanthrene-treated rats contained 15.4 and 14.9 nmoles of cytochrome P-450 per milligram of protein, respectively (these are the "B" fractions described in ref. 17); the rabbit liver microsomal cytochrome $P\text{-}450_{LM\text{-}2}$ fraction (18) contained 12.5 nmoles of cytochrome P-450 per milligram of protein. All enzyme preparations migrated as essentially single Coomassie blue-stained bands (i.e., apparent purity > 95%) upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol according to Laemmli (19).

Assays. All incubations were carried out at 37°. Absorbance measurements and spectra were recorded using a Varian 635M instrument in the dual-beam mode. The microsomal demethylations of benzphetamine (17, 20, 21) and *N*,*N*-dimethyl-4-aminoazobenzene (17) and hydroxylation of benzo[*a*]pyrene (22) were assayed as described. Heme was assayed using the pyridine hemochrome assay (23). Free P-450 sulfhydryl groups were estimated by allowing the protein to react with 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of 8 M urea for 10 min (24, 25); the value $\Delta\epsilon_{412}$ = 13.6 mM^{-1} cm^{-1} was used in calculations (26). Cytochrome P-450 was assayed according to Omura and Sato (23), and protein assays were carried out according to Lowry *et al.* (27). Formaldehyde (20, 21) and acetaldehyde (28) were assayed as described. 1,N^6-Ethenoadenosine was measured fluorometrically (15,

29) (excitation at 306 nm; emission at 409 nm). Malondialdehyde was assayed as described by Ernster and Nordenbrand (30); H_2O_2 was estimated using *o*-dianisidine (31). Oxygen was removed from solutions (in Thunberg tubes) by alternate cycles (10/sample) of vacuum and equilibration with gas mixtures that had been passed through three 100-ml columns of a solution of 5% $Na_2S_2O_4$ and 0.1% anthraquinone-2-sulfonate in 0.1 N NaOH, followed by one empty column to trap any particles of the solution; the level of oxygen remaining was estimated to be less than 1 nmole in each assay (less than 2 ppm in the gas phase), using the reoxidation of partially photoreduced FMN (32). All statistical analysis utilized *t*-tests for the indicated confidence limits.

RESULTS

Loss of cytochrome P-450 and mixed-function oxidase activity in microsomes in the presence of vinyl chloride and NADPH. Initial experiments indicated that the content of cytochrome P-450 in rat liver microsomes is lowered during incubation with vinyl chloride in the presence of an NADPH-generating system (Table 1). Under these conditions, there were no significant losses of P-450 in the absence of vinyl chloride (with or without NADPH) with any of the microsomal preparations, nor did vinyl chloride destroy P-450 in the absence of NADPH. Loss of P-450 was observed in microsomes prepared from untreated rats and from rats treated with either 3-methylcholanthrene or phenobarbital, but was greatest in the last of these cases. In such experiments, the activity of NADPH–cytohrome P-450 reductase (assayed as NADPH–cytochrome *c* reductase) was not significantly changed during the incubations with vinyl chloride. The levels of cytochrome P-420 were neither raised nor lowered during the incubations.

Microsomes were incubated as above; mixed-function oxidase activities were then measured with several substrates (after removal of vinyl chloride) as described for Table 2. The benzphetamine demethylase activities of microsomes from both untreated and phenobarbital-treated rats were lowered by about one-half. However, the microsomes from untreated or 3-methylcholanthrene-treated rats lost very little of their ability to metabolize *N,N*-dimethyl - 4 - aminoazobenzene; similar findings were made when benzo[*a*]pyrene was used as a substrate. The latter observations were unexpected: two possible explanations are that (a) P-450 is not rate-limiting in the metabolism of these carcinogens under these conditions or (b) there exist differences in the susceptibility of different cytochromes P-450 [which are responsible for the metabolism of different substrates (17, 18)] to vinyl chloride me-

TABLE 1

Vinyl chloride-mediated destruction of cytochrome P-450 in rat liver microsomes

The complete system (2 ml) contained 3 mg of rat liver microsomal protein, an NADPH-generating system consisting of 0.5 mM $NADP^+$ (deleted in the "−NADPH" experiments), 10 mM glucose 6-phosphate, and 0.5 unit/ml of glucose 6-phosphate dehydrogenase, 50 mM potassium HEPES[a] (pH 7.7), 15 mM $MgCl_2$, 20 μM butylated hydroxytoluene (added to contents of the flask in 10 μl of acetone), 1 mM EDTA, and 50% vinyl chloride in the gas phase (10-ml volume). After incubation for 15 min at 37°, the mixtures were diluted 2-fold with cold 0.1 M potassium phosphate buffer (pH 7.4) containing 40% glycerol, 2 mM EDTA, 0.8% Triton N-101, and 0.8% sodium cholate, and nitrogen was bubbled through the samples to remove the poorly soluble vinyl chloride [these detergents abolish mixed-function oxidase activity (33) but do not affect P-450 determinations under these conditions (34)].Samples were stored at −20° until assayed (at 4°). All differences (± NADPH) are statistically significant ($p < 0.025$); experiments were done in triplicate, and results are expressed as means ± standard deviations.

Animal treatment	Specific content of P-450	
	+NADPH	−NADPH
	nmoles/mg protein	
None	0.56 ± 0.02	0.71 ± 0.04
Phenobarbital	1.08 ± 0.22	2.00 ± 0.18
3-Methylcholanthrene	1.04 ± 0.07	1.32 ± 0.06

[a] The abbreviations used in the tables and figures are: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; BHT, butylated hydroxytoluene; Di-12 GPC, dilauroylglyceryl-3-phosphorylcholine.

TABLE 2

Vinyl chloride-mediated loss of mixed-function oxidase activities in rat liver microsomes

Incubation conditions were the same as in Table 1. After incubation and cooling to 0°, nitrogen was bubbled through the samples and $CaCl_2$ was added to 10 mM; microsomes were precipitated by centrifugation at 20,000 × *g* for 20 min. After resuspension and reprecipitation, the protein concentrations and activities were determined as described under MATERIALS AND METHODS. Results are expressed as means of three experiments ± standard deviations.

Animal treatment	Substrate	Oxidase specific activity	
		+NADPH	−NADPH
		nmoles product/min/mg protein	
None	Benzphetamine[a]	1.86 ± 0.21[b]	4.03 ± 0.49
Phenobarbital	Benzphetamine[a]	7.2 ± 1.7[b]	18.3 ± 1.3
None	*N,N*-Dimethyl-4-aminoazobenzene[a]	0.37 ± 0.11[c]	0.41 ± 0.04
3-Methylcholanthrene	*N,N*-Dimethyl-4-aminoazobenzene[a]	5.24 ± 0.25[c]	6.91 ± 0.97
3-Methylcholanthrene	Benzo[*a*]pyrene[d]	0.65 ± 0.02[c]	0.68 ± 0.04

[a] The product determined was formaldehyde.

[b] Significantly different from "−NADPH" control ($p < 0.01$).

[c] Not significantly different from "−NADPH" control ($p > 0.10$).

[d] Fluorescent metabolites were assayed according to Nebert and Gelboin (22) and standardized against 3-hydroxybenzo[*a*]pyrene.

tabolites. The losses of P-450 and mixed-function oxidase activity appeared to be most related in microsomes prepared from phenobarbital-treated rats (Tables 1 and 2); the remainder of the work presented in this paper focuses mainly on the use of such microsomes and enzymes derived from them.

Evidence that lipid peroxidation does not play a role in vinyl chloride-mediated cytochrome P-450 destruction. The peroxidation of microsomal lipids occurs in the presence of NADPH and trace amounts of iron (30, 35, 36). Since evidence exists that certain alkyl halides such as CCl_4 and $CBrCl_3$ exert their destructive effects by promoting lipid peroxidation (37, 38), the possibility was considered that a similar phenomenon occurs in the case of vinyl chloride. However, the losses of P-450 and benzphetamine demethylase activity observed in the presence of vinyl chloride and NADPH could be demonstrated in the presence of high concentrations of EDTA and butylated hydroxytoluene, potent inhibitors of microsomal lipid peroxidation (11, 30, 39) (Tables 1 and 2). No real increase in the rate of malondialdehyde production (an index of lipid peroxidation) was observed in the presence of both vinyl chloride and NADPH, and the total amounts of malondialdehyde formed were quite low in the presence of butylated hydroxytoluene and EDTA (Table 3). In the absence of these inhibitors, lipid peroxidation occurred much more rapidly, but vinyl chloride did not increase this rate.

The possibility was considered that the double bond of vinyl chloride was being attacked by oxygen species in a manner resembling the attack on unsaturated fatty acyl moieties in lipid peroxidation (30). By analogy, such a process would produce formaldehyde (or possibly acetaldehyde) instead of malondialdehyde. However, under the conditions used in Table 3 (either with or without EDTA and butylated hydroxytoluene present), neither formaldehyde nor acetaldehyde could be detected.

Vinyl chloride-mediated cytochrome P-450 destruction in reconstituted enzyme systems. To clarify the role of cytochrome P-450 in catalyzing its own destruction in the presence of vinyl chloride, a series of experiments was carried out with highly purified enzyme systems containing cytochrome P-450 and NADPH–cytochrome P-450 reductase purified to apparent homogeneity; vinyl chloride-mediated P-450 destruction was readily demonstrated (Table 4). All components were required for maximal destruction; the significant loss of P-450 observed in the absence of vinyl chlo-

TABLE 3

Malondialdehyde production by rat liver microsomes in the presence and absence of vinyl chloride

The complete system containing vinyl chloride was the same as in Table 1 (microsomes from phenobarbital-treated rats were used), except that 0.2 mM $FeCl_3$ and 2 mM ADP were added. Where noted, butylated hydroxytoluene and EDTA were omitted. Results are expressed as means of three experiments ± standard deviations.

System	Malondialdehyde produced	
	+BHT, +EDTA	−BHT, −EDTA
	nmoles/min/mg protein	
Complete	0.05 ± 0.01[a]	3.66 ± 0.75[a]
−Vinyl chloride (control)	0.04 ± 0.01	4.49 ± 0.46
−NADPH	0.02 ± 0.01	0.24 ± 0.09
−vinyl chloride, −NADPH	0.01 ± 0.01	0.11 ± 0.02

[a] Not significantly different from "−vinyl chloride" control ($p > 0.10$).

TABLE 4

Vinyl chloride-mediated cytochrome P-450 destruction in reconstituted enzyme systems

The complete system contained (in 1.5 ml) 1.2 nmoles of P-450 (prepared from phenobarbital-treated rats), 25.5 μg of NADPH–cytochrome P-450 reductase, 75 nmoles of dilauroylglyceryl-3-phosphorylcholine, 0.38 μmole of deoxycholate, 75 μmoles of potassium HEPES (pH 7.7), 25 μmoles of $MgCl_2$, 0.75 μmole of NADPH, 15 nmoles of butylated hydroxytoluene, 1.5 μmoles of EDTA, and 50% vinyl chloride in the gas phase (10-ml volume). Incubations proceeded for 10 min at 37°; after termination as in Table 1, cytochrome P-450 was assayed. All experiments were done in triplicate, and results are expressed as means ± standard deviations.

System	P-450
	μM
Complete	0.11 ± 0.05
−NADPH	0.75 ± 0.07[a]
−Reductase	0.65 ± 0.07[a]
−Vinyl chloride	0.43 ± 0.05[a]
−Vinyl chloride, −reductase, −NADPH	0.78 ± 0.06[a]
−Di-12 GPC, −deoxycholate	0.43 ± 0.09[b]

[a] Significantly different from complete system ($p < 0.01$).

[b] Significantly different from complete system ($p < 0.025$).

ride is discussed below. The vinyl chloride-mediated destruction was dependent upon the presence of added phospholipid (dilauroylglyceryl-3-phosphorylcholine) and deoxycholate, as are the oxidations of other compounds with these enzyme systems (17, 18, 34). In other experiments, similar P-450 losses were observed when the P-450 prepared from phenobarbital-treated animals was replaced with enzyme prepared from 3-methylcholanthrene-induced rats.

Both the P-450 and NADPH–cytochrome P-450 reductase preparations were prepared by procedures that reduce the level of phospholipid to less than 0.25 nmole/nmole of enzyme (40); thus the use of the synthetic saturated phospholipid permits the reconstitution of a system virtually free of unsaturated phospholipid. Since this system readily catalyzes P-450 destruction, even in the presence of EDTA and butylated hydroxytoluene, the role of lipid peroxidation must be considered negligible.

Loss of heme during vinyl chloride-mediated cytochrome P-450 destruction. Loss of cytochrome P-450 during metabolism has been observed with several substrates, including parathion and several other thionsulfur compounds (41), secobarbital (11), fluorexene (42), and allylisopropylacetamide (43). At least two mechanisms appear to be involved. With thionosulfur compounds a reactive sulfur is produced that binds covalently to cytochrome P-450 (to convert cysteinyl residues to hydropersulfides) (44); while the level of detectable P-450 falls during metabolism, the heme content does not (41). With allylisopropylacetamide, fluorexene, and secobarbital, the heme appears to be actually destroyed (11, 42, 43).

Since it has been postulated that vinyl chloride is metabolized to a strong electrophile (its epoxide), the possibility that P-450 is lost via reaction of an electrophile with protein nucleophiles was considered. However, preliminary experiments with microsomes indicated that neither exogenous cysteine nor reduced glutathione, when added at 1 mM, could protect P-450 against the vinyl chloride-mediated destruction. It was felt that the suspected

metabolite might be too reactive to leave the P-450 enzyme; if reaction with protein nucleophiles occurred, the most likely nucleophiles would probably be the cysteinyl residues. The results presented in Table 5 indicate that there were no detectable decrease in the number of free sulfhydryl groups of the P-450 during the vinyl chloride reaction under the conditions used.

However, the loss of heme was found to parallel the loss of detectable cytochrome P-450 (Table 5). The heme destruction was inhibited approximately 75% by the addition of 20% CO to the gas phase, suggesting that P-450 (i.e., its ferrous state) is involved in the metabolism of vinyl chloride to a destructive metabolite. This loss of heme, and its inhibition by CO, was also demonstrated in intact microsomes prepared from phenobarbital-treated rats.

TABLE 5

Effects of vinyl chloride on heme content and free sulfhydryl residues of cytochrome P-450 in reconstituted enzyme systems

Complete systems were essentially the same as in Table 4; cytochrome P-450 from phenobarbital-treated rats was used. Free sulfhydryls and heme were assayed as described under MATERIALS AND METHODS. CO was added as 20% of the gas phase where indicated and was removed by 10 cycles of alternate vacuum and equilibration with nitrogen prior to the heme assays [experiments with equilibration of other gases (see MATERIALS AND METHODS) indicated that more than 99% of the CO should be removed after such treatment; in control experiments no CO bound to P-450 heme was detected in the reduced vs. oxidized difference spectra]. Results are expressed as averages of duplicate experiments for free sulfhydryls and as means ± standard deviations of triplicate experiments for heme.

System	Free sulfhydryls/cytochrome P-450	Heme
		μM
Complete	5.3	0.27 ± 0.04
−Vinyl chloride (control)	4.0	0.70 ± 0.06[a]
−Reductase	4.0	0.92 ± 0.02[a]
+CO	−[b]	0.59 ± 0.12[c]

[a] Significantly different from complete system ($p < 0.005$).

[b] Not determined.

[c] Not significantly different from "−vinyl chloride" control ($p > 0.10$).

NADPH-mediated cytochrome P-450 destruction in the absence of vinyl chloride. In preliminary work with the vinyl chloride-mediated destruction of P-450, it was found that a significant level of destruction occurred in the absence of vinyl chloride (Table 4). Further experiments (Table 6) indicated that this destruction of cytochrome P-450 involved the loss of heme. Such destruction was also observed with a highly purified rabbit liver microsomal cytochrome P-450 preparation (P-450_{LM-2}).

The destruction of P-450 in the absence of vinyl chloride could be blocked with a saturating concentration of substrate benzphetamine or by the addition of catalase (Table 6). The addition of either CO (20%) or ethanol (3 mM), a trapping agent for hydroxyl radicals (45), had no effect on this system. In contrast, the vinyl chloride-mediated destruction was strongly inhibited by CO (see above) but not inhibited by catalase. Benzphetamine (1 mM) did not inhibit the vinyl chloride-mediated destruction. Two possible explanations are that (a) the substrate binding site for benzphetamine is saturated with vinyl chloride

TABLE 6

Destruction of cytochrome P-450 and cytochrome P-450-bound heme in reconstituted enzyme systems in the absence of vinyl chloride

The complete system contained 0.90 μM cytochrome P-450 (from phenobarbital-treated rats) and other components as in Table 4, with the exception of vinyl chloride; incubation was carried out for 20 min at 37°, and P-450 and heme were assayed as described under MATERIALS AND METHODS. When indicated, catalase and benzphetamine were present at concentrations of 800 units/ml and 1.0 mM. Results are expressed as means of triplicate experiments ± standard deviations.

System	P-450	Heme
	μM	μM
Complete	0.32 ± 0.04[a]	0.26 ± 0.04[a]
−Reductase, −NADPH	0.88 ± 0.08	0.98 ± 0.04
−Reductase (control)	0.80 ± 0.05	0.90 ± 0.06
+Benzphetamine	0.85 ± 0.02[b]	
+Catalase	0.71 ± 0.10[b]	

[a] Significantly different from "−reductase" control ($p < 0.01$).

[b] Not significantly different from "−reductase" control ($p > 0.10$).

under the assay conditions, or (b) two or more forms of P-450 are present in the P-450 preparation, and different forms of the cytochrome bind and metabolize benzphetamine and vinyl chloride.

Only a low level of NADPH-mediated destruction of P-450 could be demonstrated in microsomes (in the presence of EDTA and butylated hydroxytoluene and absence of vinyl chloride), presumably because of the presence of endogenous catalase. However, when NaN_3 (1 mM) was added to inhibit catalase, about one-half the heme was destroyed in 15 min in the presence of an NADPH-generating system.

NADPH-cytochrome P-450 reductase-catalyzed destruction of free heme. In order to define the activating role of P-450 in the vinyl chloride-mediated destruction of P-450 heme, experiments were carried out to examine the possibility of NADPH-cytochrome P-450 reductase-catalyzed destruction of free heme in the presence of vinyl chloride. It was found that the destruction of free heme proceeded in the absence of vinyl chloride and was not enhanced by its presence (Table 7, experiment I); thus a role for P-450 in the vinyl chloride-dependent destruction of (cytochrome P-450) heme is further indicated, as a mixture of NADPH, the reductase, vinyl chloride, and air did not produce the vinyl chloride-dependent destruction of free heme. This system was investigated further, and destruction of heme in the absence of vinyl chloride was found not to be greatly enhanced by the addition of either of the major rat liver cytochromes P-450; the destruction was routinely inhibited about 50% by the addition of catalase but was insensitive to inhibition by EDTA, butylated hydroxytoluene, benzphetamine, ethanol, or CO.

Mechanistic studies on heme destruction in the presence of vinyl chloride. The results discussed above suggest that a metabolite(s) of vinyl chloride is responsible for the observed destruction of P-450 heme. Some evidence has been presented that the initial product of the metabolism of vinyl chloride is the epoxide, which rearranges to form chloroacetaldehyde with a half-life of 1.6 min at neutral pH (8). Both these compounds were prepared; however, neither was particularly effective in destroying either free or P-450-bound heme (Fig. 1). Neither catalase (800 units/ml) nor superoxide dismutase (80 μg/ml) significantly inhibited vinyl chloride-mediated P-450 destruction in the reconstituted system. The vinyl chloride-mediated destruction of P-450 was found to be completely dependent upon oxygen; substitu-

TABLE 7

Destruction of free heme (ferriprotoporphyrin IX) by NADPH and NADPH-cytochrome P-450 reductase

The complete system in experiment I (in 1.5 ml) contained 17 μg of NADPH-cytochrome P-450 reductase, 1.5 nmoles of heme, 75 μmoles of potassium HEPES (pH 7.7), 75 μg of dilauroylglyceryl-3-phosphorylcholine, 150 μg of deoxycholate, 0.3 μmole of NADPH, and (where indicated) 50% vinyl chloride in the gas phase (10-ml volume); incubations were carried out for 15 min at 37°, and the mixtures were assayed for heme as described under MATERIALS AND METHODS. All experiments were done in triplicate, and results are expressed as means ± standard deviations. The complete system of experiment II was the same as in experiment I, except that 3.0 nmoles of heme were used. Where noted, the following components were added: butylated hydroxytoluene, 10 mM; EDTA, 1 mM; benzphetamine, 1 mM; catalase, 800 units/ml; ethanol, 3 mM; cytochrome P-450 from phenobarbital-treated rats, 0.2 μM; and cytochrome P-450 from 3-methylcholanthrene-treated rats, 0.2 μM.

System	Heme
	μM
Experiment I	
Complete	0.29 ± 0.02[a]
+Vinyl chloride	0.25 ± 0.05[b]
−Reductase (control)	0.96 ± 0.10
Experiment II	
Complete	1.28 ± 0.03[a]
−Reductase (control)	2.09 ± 0.09
+BHT, +EDTA	1.02 ± 0.14
+Benzphetamine	1.15 ± 0.09
+Catalase	1.64 ± 0.09[c]
+Ethanol	1.10 ± 0.01
+P-450 (phenobarbital)	0.99 ± 0.14
+P-450 (3-methylcholanthrene)	1.19 ± 0.02

[a] Significantly different from "−reductase" control ($p < 0.005$).

[b] Not significantly different from complete system ($p > 0.10$).

[c] Significantly different from both complete system and "−reductase" control ($p < 0.02$).

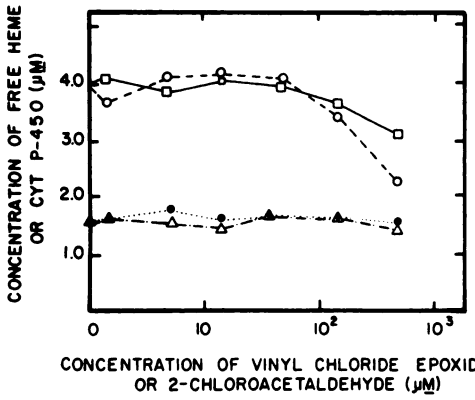

FIG. 1. *Attempted destruction of free and cytochrome P-450-bound heme with vinyl chloride epoxide and 2-chloroacetaldehyde*

Ferriprotoporphyrin IX was incubated with various concentrations of either vinyl chloride epoxide (□——□) or 2-chloroacetaldehyde (○- - -○) in 50 mM HEPES buffer (pH 7.7) at 37° (2-ml volume) for 15 min; the remaining heme was determined (23). Cytochrome P-450 (from phenobarbital-treated rats) was also incubated with vinyl chloride epoxide (△-·-·△) or 2-chloroacetaldehyde (●·····●) under the same conditions, and the remining cytochrome P-450 was assayed as described in Table 1.

tion of a 1:1 anaerobic mixture of nitrogen and vinyl chloride for the 1:1 mixture of air and vinyl chloride abolished all of the NADPH-dependent loss of P-450 in liver microsomes from phenobarbital-treated rats. (A control experiment established that after deoxygenation was carried out in the usual manner and air and vinyl chloride were readmitted to the tubes prior to incubation, the same amount of P-450 was lost as in the usual complete destructive system.)

The possibility was considered that radicals might be involved in the vinyl chloride-mediated P-450 destruction. A system containing 17 μM P-450, 3 μM NADPH-cytochrome P-450 reductase, 150 μM dilauroylglycerol-3-phosphorylcholine, and NADPH, vinyl chloride, and other components as in Table 4 was incubated for 4 min at 37° and then rapidly cooled. No free radicals ($g = 2$ region) were observed when such a mixture was examined by EPR spectroscopy (46) over a wide range of temperatures (from room temperature to −170°) and modulation settings. The vinyl chloride-mediated destruction of P-450 in rat liver microsomes was not inhibited by the presence of either 50 μM diphenylpicrylhydrazyl radical, 20 μM butylated hydroxytoluene (Table 1), or 5% acrylamide [in the last case, a considerable conversion of cytochrome P-450 to cytochrome P-420 (about 40% in the presence or absence of vinyl chloride and NADPH) complicated the experiment].

Studies on the mechanism of heme destruction in the absence of vinyl chloride. Studies with inhibitors suggested that the NADPH–cytochrome P-450 reductase-mediated destructions of free and P-450-bound heme are similar processes. Both reactions are insensitive to CO at the concentrations used, suggesting that the heme or hemoprotein does not go through a cycle involving its reduced state and that reactive oxygen species are produced by the flavoprotein instead of the heme. Neither reaction was inhibited by the addition of superoxide dismutase (80 μg/ml). Both reactions were inhibited by catalase, although the destruction of P-450-bound heme was inhibited more than the destruction of free heme.

H_2O_2 is produced during the aerobic oxidation of NADPH by either ferriprotoporphyrin IX or cytochrome P-450 (47, 48) in the presence of NADPH–cytochrome P-450 reductase. The rates of NADPH oxidation were measured to be 1.2 and 5.0 nmoles/ml/min under the experimental conditions involving the complete systems of Tables 7 (experiment II) and 6, respectively. The rate of production of H_2O_2 was estimated to be 20% and 50% of the rate of NADPH oxidation, respectively. Concentrations of H_2O_2 equivalent to the total levels of NADPH oxidized (during the entire 15-min incubation periods) were added to the complete systems containing all components except NADPH, since the above *o*-dianisidine estimates might not account for H_2O_2 that might be consumed in heme destruction. Only 0.11 nmole/ml of ferriprotoporphyrin IX and 0.04 nmol/ml of P-450 heme were destroyed under such conditions (cf. 0.81 and 0.64 nmol/ml in Tables 7 and 6, respectively); these values are not consistent with a primary role for free

H_2O_2 in the heme destruction.

The possibility was further considered that NADPH–cytochrome P-450 reductase might reduce heme and that the reoxidation of ferriprotoporphyrin IX by oxygen under such conditions would result in heme destruction; however, no significant heme loss was observed (as judged by the hemochrome assay) when ferriprotoporphyrin IX was reduced under anaerobic conditions with a stoichiometric amount of $Na_2S_2O_4$ and then oxidized in the presence of air.

Further studies on vinyl chloride: cytochrome P-450 binding spectra, and metabolism to products bound to adenine derivatives. The addition of vinyl chloride to rat liver microsomes produced a trough at 423 nm and a peak at 393 nm in the oxidized difference spectrum; however, the addition of vinyl chloride to purified cytochrome P-450 produced only a definite trough at 423 nm in the difference spectrum. When vinyl chloride was added to $Na_2S_2O_4$-reduced samples of either microsomes or purified cytochrome P-450, a peak at 423–426 nm and a small trough at 410 nm were observed in the difference spectra in each case. At the present time, the significance of these spectral changes in relationship to binding of vinyl chloride is not clear.

Ivanetich *et al.* (10) have suggested that these spectral changes and also vinyl chloride-enhanced rates of microsomal NADPH oxidation are related to vinyl chloride metabolism. However, these measurements should be interpreted with caution, as several alkyl halides, most notably perfluorohexane (49) and several other halogenated hexane derivatives,[2] bind to P-450 and increase its NADPH oxidase activity (in the presence of NADPH–cytochrome P-450 reductase) without being metabolized.

Other workers have provided evidence for the transformation of adenosine to 1,N^6-ethenoadenosine by microsomes in the presence of vinyl chloride and NADPH (8). The finding of such a derivative is consistent with activation of vinyl chloride to either vinyl chloride epoxide or 2-chloroacetaldehyde. We have attempted to use formation of such a fluorescent derivative (15, 29) as an assay of vinyl chloride activation. Rat liver microsomes catalyzed the formation of such a derivative (at the rate of about 0.5 nmole/mg of protein per 30 min); this activity was induced about 3–4-fold by treatment of the animals with either phenobarbital or 3-methylcholanthrene. The reaction was inhibited 45% upon the addition of 20% CO to the gas phase of the incubations, suggesting cytochrome P-450 involvement; however, we have been unable to demonstrate such a reaction using any of the purified cytochrome P-450 fractions prepared from either phenobarbital- or 3-methylcholanthrene-treated rats (17), or with rabbit liver cytochrome $P\text{-}450_{LM\text{-}2}$, in the presence of NADPH–cytochrome P-450 reductase and other components necessary for mixed-function oxidative metabolism (17, 34). The conversion of adenosine to 1,N^6-ethenoadenosine may not be related to physiological activation, as the presence of such derivatized residues in isolated nucleic acids (rather than adenosine alone) could not be detected when vinyl chloride, microsomes, and NADPH were incubated with either native calf thymus DNA, denatured thymus DNA (prepared from native DNA by heating to 100° and rapidly cooling), or soluble *Escherichia coli* RNA. Since evidence has been presented (6) for the microsomal activation of [^{14}C]vinyl chloride to species covalently bound to RNA, this binding apparently takes place to form adducts other than 1,N^6-ethenoadenosyl residues.

DISCUSSION

The results presented here provide evidence that the heme of cytochrome P-450 is destroyed by a metabolite of vinyl chloride produced by the enzyme. This process was demonstrated both with rat liver microsomes and with highly purified reconstituted enzyme systems. Such a process is postulated to account for the specific loss of liver microsomal cytochrome P-450 and its metabolic activities observed after exposure of animals to vinyl chloride (9).

[2] G. N. Nordblom, F. P. Guengerich, and M. J. Coon, unpublished observations.

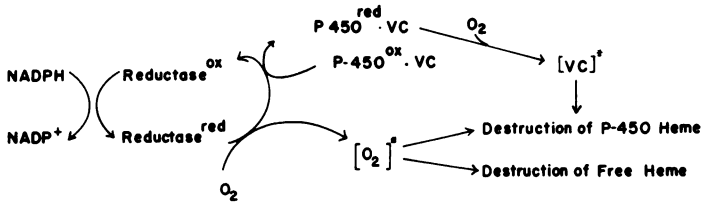

FIG. 2. *Possible mechanisms for NADPH-dependent destruction of free and cytochrome P-450-bound heme in the presence and absence of vinyl chloride (VC)*

See the text for discussion. The superscripts "ox" and "red" refer to the oxidized and reduced forms, respectively, of P-450 and NADPH–cytochrome P-450 reductase.

Both free and P-450-bound heme were destroyed by the flavoprotein NADPH–cytochrome P-450 reductase in the absence of vinyl chloride, and both processes appear to be similar (if not identical), as both are partially inhibited by catalase and are insensitive to CO. It is postulated that a partially reduced oxygen species produced by the reductase is responsible for such destruction, although evidence could not be demonstrated for the involvement of free H_2O_2, superoxide anion, or a hydroxyl radical in these studies. The vinyl chloride-mediated destruction of cytochrome P-450 heme appears to be a clearly distinct process, as judged by its sensitivity to CO and insensitivity to catalase under the conditions used here and the requirement for P-450 in vinyl chloride-dependent heme destruction. A working scheme is presented in Fig. 2, where the brackets and asterisks are used to denote unidentified activated species of vinyl chloride and oxygen, and, on the basis of the experiments discussed above, the vinyl chloride-dependent and -independent destructions of heme are viewed as separate pathways, in contrast to a hypothesis in which vinyl chloride stimulates a destructive process that occurs only in its absence.

During the course of our work, Masters and Schacter reported destruction of heme by purified NADPH–cytochrome *c* reductase (a protease-solubilized fragment of NADPH–cytochrome P-450 reductase) (50); our work confirms these findings. Those workers postulated that biliverdin and bilirubin are products of the reaction; our spectral studies suggest that this is not the case, and efforts are in progress to characterize the product(s). This metabolite may be an intermediate in the overall reaction of the heme oxygenase system, which metabolizes heme to biliverdin, contains NADPH–cytochrome P-450 reductase, and is highly sensitive to CO (12, 13).

NADPH-dependent P-450 destruction in microsomes has also been observed with the substrates secobarbital (11), allylisopropylacetamide (43), and fluorexene (42), all of which contain unsaturated double bonds.[3] The possibility arises that these compounds destroy heme via similar mechanisms. All these compounds could be metabolized to their epoxides; however, such epoxides would be rather electrophilic species and the heme moiety would not be expected to act as a strong nucleophile. In this work, the epoxide of vinyl chloride and its rearrangement product, 2-chloroacetaldehyde, were prepared and were not found readily to destroy free or cytochrome P-450-bound heme when added at concentrations that could conceivably be produced under the incubation conditions used here (Fig. 1). A destructive mechanism involving free radicals might seem more likely. Radicals were not detected by EPR spectroscopy; however, coupling might prevent observation of any radicals at detectable levels. Oster (56) has interpreted the production of turbidity by microsomes in the presence of 7% acrylonitrile and NADPH as evidence for radical production in the presence of a vinyl

[3] A number of other substrates are metabolized by microsomes, in the presence of NADPH and oxygen, to species that appear to bind to cytochrome P-450 (and probably the heme moiety) and inhibit further metabolism (51–55); however, actual destruction of heme has not been demonstrated in any of these cases.

compound; however, we found considerable P-450 destruction (i.e., conversion to cytochrome P-420) in the presence of acrylamide monomer and feel that such a turbidometric assay does not accurately reflect radical production. Neither acrylamide, butylated hydroxytoluene, nor diphenylpicrylhydrazyl radical inhibited microsomal P-450 destruction in the presence of vinyl chloride; however, if radicals are produced and are too reactive to leave the area of the heme, such trapping agents would not be effective. Thus a radical destructive mechanism should probably not be ruled out.

The chemical mechanisms of the microsomal activation of vinyl chloride to species that destroy cytochrome P-450-bound heme and to species that are responsible for mutagenesis (and possibly carcinogenesis) are both still unclear. We postulate that different mechanisms are involved, as the former process is localized to the activating enzyme, consistent with high reactivity of the activated species; mutagenesis would appear to require a metabolite stable enough to leave the site of activation and interact with nucleic acids or proteins that are more closely involved in such a process.

ACKNOWLEDGMENT

We wish to express our gratitude to Dr. T. Martin, Department of Chemistry, Vanderbilt University, for his help with the EPR spectroscopy.

REFERENCES

1. Maltoni, C. & Lefemine, C. (1974) *Environ. Res.*, 7, 387–405.
2. Creech, J. L. & Johnson, M. N. (1974) *J. Occup. Med.*, 16, 150–151.
3. Lee, F. I. & Harry, D. S. (1974) *Lancet*, 1, 1316–1318.
4. Bartsch, H., Malaveille, C. & Montesano, R. (1975) *Int. J. Cancer*, 15, 429–437.
5. Malaveille, C., Bartsch, H., Barbin, A., Camus, A. M., Montesano, R., Croisy, A. & Jacquingon, P. (1975) *Biochem. Biophys. Res. Commun.*, 63, 363–370.
6. Kappus, H., Bolt, H. M., Buchter, A. & Bolt, W. (1975) *Nature*, 257, 134–135.
7. Rannug, U., Göthe, R. & Wachtmeister, C. A. (1976) *Chem.-Biol. Interactions*, 12, 251–263.
8. Barbin, A., Brésil, H., Croisy, A., Jacquignon, P., Malaveille, C., Montesano, R. & Bartsch, H. (1975) *Biochem. Biophys. Res. Commun.*, 67, 596–603.
9. Reynolds, E. S., Moslen, M. T., Szabo, S. & Jaeger, R. J. (1975) *Res. Commun. Chem. Pathol. Pharmacol.*, 12, 685–694.
10. Ivanetich, K. M., Aronson, I. & Katz, I. D. (1977) *Biochem. Biophys. Res. Commun.*, 74, 1411–1418.
11. Levin, W., Lu, A. Y. H., Jacobson, M., Kuntzman, R., Poyer, J. L. & McCay, P. B. (1973) *Arch. Biochem. Biophys.*, 158, 842–852.
12. Tenhunen, R., Marver, H. S. & Schmid, R. (1969) *J. Biol. Chem.*, 244, 6388–6394.
13. Schacter, B. A., Nelson, E. B., Marver, H. S. & Masters, B. S. S. (1972) *J. Biol. Chem.*, 247, 3601–3607.
14. Strickland, T. W. & Guengerich, F. P. (1977) *Fed. Proc.*, 36, 991.
15. Barrio, J. R., Secrist, J. A. & Leonard, N. J. (1972) *Biochem. Biophys. Res. Commun.*, 46, 597–604.
16. Walling, C. & Fredericks, P. S. (1962) *J. Am. Chem. Soc.*, 84, 3326–3331.
17. Guengerich, F. P. (1977) *J. Biol. Chem.*, 252, 3970–3979.
18. Haugen, D. A., van der Hoeven, T. A. & Coon, M. J. (1975) *J. Biol. Chem.*, 250, 3567–3570.
19. Laemmli, U. K. (1970) *Nature*, 227, 680–685.
20. Nash, T. (1953) *Biochem. J.*, 55, 416–421.
21. Cochin, J. & Azelrod, J. (1959) *J. Pharmacol. Exp. Ther.*, 125, 105–110.
22. Nebert, D. W. & Gelboin, H. V. (1968) *J. Biol. Chem.*, 243, 6242–6249.
23. Omura, T. & Sato, R. (1964) *J. Biol. Chem.*, 239, 2370–2378.
24. Yu, C.-A. & Gunsalus, I. C. (1974) *J. Biol. Chem.*, 249, 107–110.
25. Vatsis, K. P., Chiang, Y. L. & Coon, M. J. (1977) *Fed. Proc.*, 36, 832.
26. Habeeb, A. F. S. A. (1972) *Methods Enzymol.*, 25, 457–469.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
28. Lieber, C. S. & DeCarli, L. M. (1970) *J. Biol. Chem.*, 245, 2502–2512.
29. Secrist, J. A., Barrio, J. R., Leonard, N. J. & Weber, G. (1972) *Biochemistry*, 11, 3499–3506.
30. Ernster, L. & Nordenbrand, K. (1967) *Methods Enzymol.*, 10, 574–580.
31. Webster, D. A. (1975) *J. Biol. Chem.*, 250, 4955–4958.
32. Foust, G. P., Burleigh, B. D., Jr., Mayhew, S. G., Williams, C. H., Jr. & Massey, V. (1969) *Anal. Biochem.*, 27, 530–535.
33. Lu, A. Y. H., Levin, W. & Kuntzman, R. (1974) *Biochem. Biophys. Res. Commun.*, 60, 266–272.
34. van der Hoeven, T. A. & Coon, M. J. (1974) *J.*

Biol. Chem., 249, 6302-6310.
35. Poyer, J. L. & McCay, P. B. (1971) *J. Biol. Chem.*, 246, 263-269.
36. Pederson, T. C., Buege, J. A. & Aust, S. D. (1973) *J. Biol. Chem.*, 248, 7134-7141.
37. Gillette, J. R., Mitchell, J. R. & Brodie, B. B. (1974) *Annu. Rev. Pharmacol.*, 14, 271-288.
38. Koch, R. R., Glende, E. A., Jr. & Rechnagel, R. O. (1974) *Biochem. Pharmacol.*, 23, 2907-2915.
39. Welton, A. F. & Aust, S. D. (1972) *Biochem. Biophys. Res. Commun.*, 49, 661-666.
40. Guengerich, F. P. & Coon, M. J. (1975) *Fed. Proc.*, 34, 622.
41. Hunter, A. L. & Neal, R. A. (1975) *Biochem. Pharmacol.*, 24, 2199-2205.
42. Ivanetich, K. M., Marsh, J. A., Bradshaw, J. J. & Kaminsky, L. S. (1975) *Biochem. Pharmacol.*, 24, 1933-1936.
43. De Matteis, F. (1971) *Biochem. J.*, 124, 767-777.
44. Catignani, G. L. & Neal, R. A. (1975) *Biochem. Biophys. Res. Commun*, 65, 629-636.
45. Fong, K.-L., McCay, P. B., Poyer, J. L., Keele, B. B. & Misra, H. (1973) *J. Biol. Chem.*, 248, 7792-7797.
46. Lu, A. Y. H., Junk, K. W. & Coon, J. J. (1969) *J. Biol. Chem.*, 244, 3714-3721.
47. Nordblom, G. D. & Coon, J. J. (1976) *Fed. Proc.*, 35, 281.
48. Werringloer, J., Hildebrandt, A. G. & Estabrook, R. W. (1976) *Fed. Proc.*, 35, 1535.
49. Ullrich, V. & Diehl, H. (1971) *Eur. J. Biochem.*, 20, 509-512.
50. Masters, B. S. S. & Schacter, B. A. (1976) *Ann. Clin. Res.*, 8, 18-27.
51. Franklin, M. R. (1971) *Xenobiotica*, 1, 581-591.
52. Schenkman, J. B., Wilson, B. J. & Cinti, D. L. (1972) *Biochem. Pharmacol.*, 21, 2373-2384.
53. Werringloer, J. & Estabrook, R. W. (1973) *Life Sci.*, 13, 1319-1330.
54. Ullrich, V. & Schnabel, K. H. (1973) *Arch. Biochem. Biophys.*, 159, 240-248.
55. Franklin, M. R. (1974) *Mol. Pharmacol.*, 10, 975-985.
56. Oster, G. (1975) *Ann. N. Y. Acad. Sci.*, 246, 149.