Activation of Vinyl Chloride to Covalently Bound Metabolites: Roles of 2-Chloroethylene Oxide and 2-Chloroacetaldehyde†

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ABSTRACT: 2-Chloroethylene oxide, a postulated metabolite of vinyl chloride, was found to rearrange to 2-chloroacetaldehyde and to hydrolyze (and dehydrochlorinate) to glycolaldehyde in aqueous buffer. The hydrolysis was catalyzed by apparently homogeneous preparations of rat liver microsomal epoxide hydratase; purified hydratase also lowered the $t_{1/2}$ of the epoxide in aqueous buffer. 2-Chloroacetaldehyde was reduced by horse liver alcohol dehydrogenase in the presence of NADH and oxidized by yeast aldehyde dehydrogenase in the presence of NAD^+. The NADPH-dependent covalent binding of [^{14}C]vinyl chloride to protein was examined in systems containing rat liver microsomes or highly purified rat liver cytochrome P-450 and NADPH–cytochrome P-450 reductase. Since glycolaldehyde, 2-chloroethanol, and 2-chloroacetic acid are relatively weak alkylating agents, the effects of epoxide hydratase and alcohol and aldehyde dehydrogenases on covalent binding were examined. In a reconstituted system containing cytochrome P-450 and its reductase, >90% of the covalent binding of ^{14}C label from vinyl chloride was blocked by the addition of epoxide hydratase. However, in the microsomal system only 50% of the covalent binding could be blocked by epoxide hydratase but >90% of binding was blocked with either alcohol or aldehyde dehydrogenase. In the microsomal system, neither inhibition of endogenous hydratase with 3,3,3-trichloropropylene oxide nor enhancement of hydratase activity by pretreatment of rats with *trans*-stilbene oxide affected the level of covalent binding of label from [^{14}C]vinyl chloride. The results demonstrate the use of highly purified enzymes in elucidating the chemistry of activation of compounds to reactive metabolites and indicate (1) that cytochrome P-450 activates vinyl chloride, (2) that 2-chloroethylene oxide is formed from vinyl chloride, (3) that 2-chloroethylene oxide is a substrate for epoxide hydratase, (4) that the rearrangement product 2-chloroacetaldehyde is an important alkylating agent derived from vinyl chloride, and (5) that cytochrome P-450 is effectively segregated from epoxide hydratase and highly nucleophilic groups in microsomal membranes in this process. The work suggests that a highly electrophilic metabolite can form a less reactive compound which may be more effective in biological alkylations.

Vinyl chloride[1] has been shown to be toxic and carcinogenic in experimental animals and humans (Maltoni & Lefemine, 1974; Creech & Johnson, 1974; Lee & Harry, 1974; Van Duuren, 1975). These effects are probably the result of metabolism of VC to reactive intermediates, as mixed-function oxidative metabolism is necessary for covalent binding of VC to proteins and nucleic acids and for VC-dependent mutagenesis (Bartsch & Montesano, 1975; Henschler & Bonse, 1977; Bolt & Filser, 1977; Hefner et al., 1975; Malaveille et al., 1976; Bolt et al., 1975; Kappus et al., 1975, 1976; Barbin et al., 1975; Guengerich & Watanabe, 1979).

Several points remain unclear about the chemistry and biochemistry of VC activation. P-450 appears to activate VC (Kappus et al., 1976; Guengerich & Watanabe, 1979), but the involvement of other enzymes in the overall metabolism has not been ascertained. The most commonly postulated reactive metabolite of VC is 2-chloroethylene oxide, which is known to react with model nucleophiles (Barbin et al., 1975). However, 2-chloroethylene oxide can rearrange to form 2-chloroacetaldehyde (Gross & Freiburg, 1969), and both compounds are known alkylating agents and have similar potencies in causing mutation of bacteria (Malaveille et al., 1975); the structures of isolated adducts are consistent with alkylation by either compound (Green & Hathway, 1975, 1977; Watanabe et al., 1976; Jones & Hathway, 1978; Barrio et al., 1972; Laib & Bolt, 1977, 1978).

These studies were carried out to assess the roles of postulated VC metabolites and to identify the enzymes which carry out the transformation of VC and its metabolites.

Experimental Procedure

Chemicals. [1,2-^{14}C]VC was synthesized from [1,2-^{14}C]-dichloroethane (New England Nuclear Corp.) as previously described; chemical and radiochemical purities were >95% (Wagner et al., 1975). 2-Chloroethylene oxide was prepared by photochemical chlorination of ethylene oxide in the presence of *tert*-butyl hypochlorite (Walling & Fredricks, 1962; Guengerich & Strickland, 1977). The material was purified by preparative GC (3% OV-17, 30 °C) and dissolved in distilled tetrahydrofuran for the experiments in which the rearrangement and hydrolysis products were identified; for other experiments, the crude material was diluted with distilled tetrahydrofuran and used without further purification. 2-Chloroacetaldehyde was prepared by acid hydrolysis of the dimethyl acetal and distilled (Secrist et al., 1972). 1,N^6-Ethenoadenosine was purchased from P-L Biochemicals, Milwaukee, WI. TCPO, *trans*-stilbene oxide, 4-(*p*-nitrobenzyl)pyridine, 2-chloroacetaldehyde dimethyl acetal, and glycolaldehyde were purchased from Aldrich Chemical Co., Milwaukee, WI.

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[1] Abbreviations used: VC, vinyl chloride; GC, gas chromatography; TCPO, 3,3,3-trichloropropylene oxide; P-450, liver microsomal cytochrome P-450; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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Animals and Enzymes. Male Sprague-Dawley rats (220–280 g each) were obtained from Harlan Industries, Indianapolis, IN. Phenobarbital treatment was carried out as described elsewhere (Guengerich & Watanabe, 1979). Rats were induced with *trans*-stilbene oxide by daily ip injections (for 3 days) with 1 mL of corn oil containing 0.5 mmol of the epoxide. Microsomes were prepared as described (Guengerich, 1977a).

Cytochrome P-450 ("B" fraction; Guengerich, 1977a, 1978a), NADPH–cytochrome P-450 reductase (Guengerich, 1978b), and epoxide hydratase (Guengerich et al., 1979) were prepared from phenobarbital-treated rats as described elsewhere. Horse liver alcohol dehydrogenase and yeast aldehyde dehydrogenase were purchased from Boehringer-Mannheim, Indianapolis, IN. All enzymes yielded single bands when electrophoresed in the presence of sodium dodecyl sulfate and stained for protein according to Laemmli (1970). An antibody preparation was raised to rat liver cytochrome P-450 in rabbits, and the immunoglobulin G fraction was isolated as previously described (Guengerich, 1978a; Guengerich et al., 1979).

VC Incubations. Incubations contained 2 μM cytochrome P-450, 2 μM NADPH–cytochrome P-450 reductase, 50 μM dilauroylglyceryl-3-phosphorylcholine, 0.1 M potassium phosphate buffer (pH 7.7), 0.5 mM $NADP^+$, 1.0 IU/mL yeast glucose-6-phosphate dehydrogenase, 2 mg/mL bovine serum albumin, and the indicated amount of epoxide hydratase or dehydrogenase in a total volume of 1.0 mL in vials capped with Teflon liners. In microsomal incubations, cytochrome P-450, NADPH–cytochrome P-450 reductase, and dilaurylglyceryl-3-phosphorylcholine were replaced with rat liver microsomes. [1,2-^{14}C]VC (0.3 mCi/mmol) was added to each vial, using a polystaltic pump to give a final concentration of 1.0% (v/v; i.e., 10^4 ppm) in the head space (3.5 mL). Reactions were initiated by the addition of glucose 6-phosphate to 10 mM and proceeded for 45 min at 37 °C. The reactions were stopped by chilling on ice and sweeping away residual VC with N_2. Nonvolatile metabolites were estimated by sweeping incubates with a vigorous stream of H_2O-saturated N_2 for 30 min at 23 °C and counting incubation aliquots in 5 mL of Amersham/Searle ACS cocktail. Bound metabolites were estimated in the manner previously described (Guengerich & Watanabe, 1979; Guengerich, 1977b). All incubations were carried out in duplicate, and each point represents the mean; bound metabolites were corrected for protein recovery (Lowry et al., 1951; Guengerich, 1977b).

Results

Immunological Inhibition of VC Activation. Previous work established that a reconstituted system containing highly purified cytochrome P-450, NADPH–cytochrome P-450 reductase, and synthetic phospholipid could replace liver microsomes in catalyzing the NADPH-dependent metabolism of VC (Guengerich & Watanabe, 1979). An antibody raised to highly purified phenobarbital-treated rat P-450 was added to incubation systems containing phenobarbital-treated rat liver microsomes (Guengerich & Watanabe, 1979) at a level of 7.5 mg of immunoglobulin G per nmol of P-450 (Guengerich & Mason, 1979). This amount of antibody lowered the levels of total nonvolatile and covalently bound metabolites to 33 and 57% of controls, respectively. When a preimmune immunoglobulin G fraction was substituted for the P-450-directed antibody, the respective levels of total nonvolatile and covalently bound metabolites were 84 and 105% of those observed in control experiments.

Conversion of 2-Chloroethylene Oxide to 2-Chloroacetaldehyde and Glycolaldehyde. 2-Chloroethylene oxide has been reported to rearrange to 2-chloroacetaldehyde (Gross & Freiberg, 1969). This epoxide was synthesized and added to aqueous buffer; rearrangement to 2-chloroacetaldehyde was detected by using GC. In addition, a shoulder on the 2-chloroacetaldehyde peak was detected having a retention time identical with that of glycolaldehyde, expected to arise from hydrolysis and spontaneous dehydrochlorination of 2-chloroethylene oxide (Figure 1A). When the 2-chloroethylene oxide reaction was carried out in the presence of epoxide hydratase, analysis showed that the amount of glycolaldehyde formed exceeded that of 2-chloroacetaldehyde, indicating that 2-chloroethylene oxide is hydrolyzed by the enzyme. When $NaBH_4$ was added to the sample after incubation, both peaks were abolished, in support of the view that the detected compounds were aldehydes. A similar GC experiment was carried out in which products were monitored by using a mass spectrometer in place of the flame ionization detector (Figure 1B). In the presence of either albumin or epoxide hydratase, both glycolaldehyde (m/e 58, 60) and 2-chloroacetaldehyde (m/e 78, 80) were identified by their m/e ratios and retention times. With epoxide hydratase present, ≥80% of the total product was glycolaldehyde; in the absence of this enzyme, ≥80% of the total product was 2-chloroacetaldehyde.

Previous work indicated that rat liver microsomes lower the $t_{1/2}$ of 2-chloroethylene oxide and that this change in $t_{1/2}$ could be blocked by the presence of the epoxide hydratase inhibitor TCPO (Guengerich & Watanabe, 1979). Similar results were obtained when such an experiment was carried out with highly purified epoxide hydratase (Figure 2).

A spectrophotometric assay was devised to measure the epoxide hydratase catalyzed hydration of 2-chloroethylene oxide by quantitating residual substrate with 4-(*p*-nitrobenzyl)pyridine reagent after 30 s.[2] Hydrolysis of 2-chloroethylene oxide was not strictly zero order under such conditions, but the rates observed were linear with respect to protein to an epoxide hydratase concentration of 0.75 mg/mL (when the substrate concentration was 0.5 mM). Such an assay was used to estimate kinetic parameters, which are presented in Table I.

The metabolism of 2-chloroacetaldehyde by dehydrogenases was also investigated. Liver alcohol dehydrogenase catalyzed the NADH-dependent reduction of 2-chloroacetaldehyde, as well as the reverse reaction (Johnson, 1967) (Table I). Yeast aldehyde dehydrogenase catalyzed the NAD^+-dependent oxidation of 2-chloroacetaldehyde.

Inhibition of VC Metabolism in Reconstituted P-450 Systems. The availability of purified enzymes that deactivate different potential VC metabolites provided an opportunity to examine the nature of the reactive metabolites formed from VC, as glycolaldehyde, 2-chloroethanol, and 2-chloroacetic acid are relatively weak alkylating agents and mutagens (Barbin et al., 1975). Reconstituted P-450 systems were set up to activate VC, and to each was added increasing levels of epoxide hydratase or alcohol dehydrogenase. The results indicate that in such systems covalent binding of VC metabolites was readily inhibited by the presence of epoxide hydratase; the level of total nonvolatile metabolites was depressed only upon the addition of a large amount of the enzyme (Figure 3). When a similar experiment was carried out with alcohol dehydrogenase (plus

[2] The history and chemistry of the general reaction have been reviewed by Epstein et al. (1955). The product of the reaction of 2-chloroethylene oxide with 4-(*p*-nitrobenzyl)pyridine was not isolated; the expected product should contain a $CHOCH_2$ group attached to the pyridine nitrogen. The extinction coefficient determined in this work was 24 mM^{-1} cm^{-1} at 565 nm.

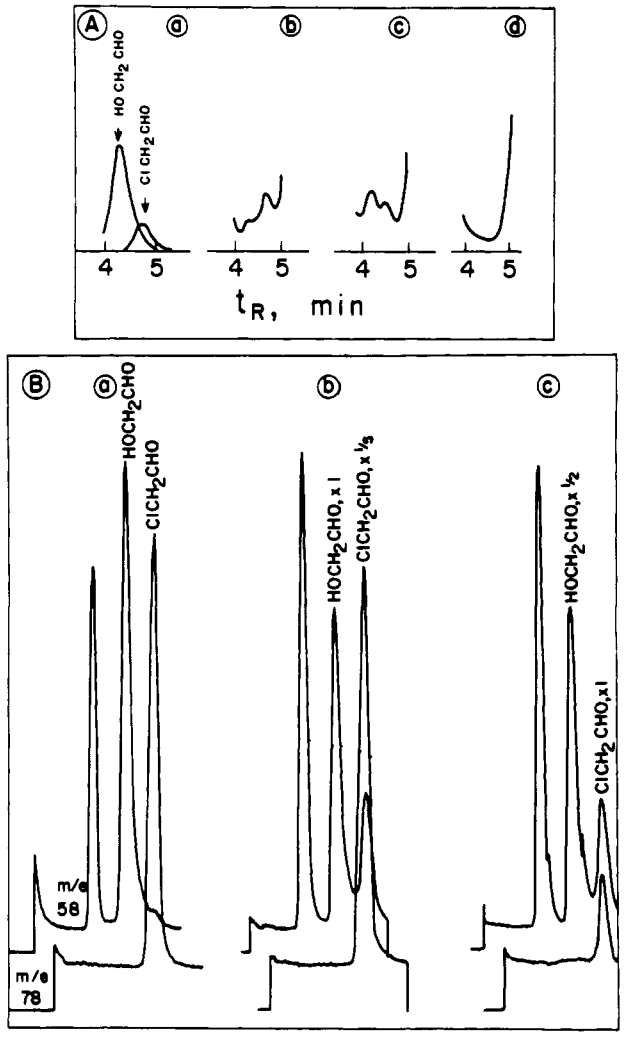

FIGURE 1: Decomposition of 2-chloroethylene oxide to glycolaldehyde and 2-chloroacetaldehyde in aqueous buffer and the effect of epoxide hydratase. 2-Chloroethylene oxide was synthesized as described under Experimental Procedure and added to 0.1 M potassium phosphate buffer (pH 7.7) with the indicated additions. After being mixed immediately, the reaction was allowed to proceed for 10 min at 23 °C and 3-μL aliquots were analyzed directly by gas chromatography on 6 ft × 1/8 in. columns of Porapak QS (80–100 mesh, 150 °C). (A) The column was coupled to a flame ionization detector in a Varian 1800 gas chromatograph. The region between 4- and 5-min retention time (t_R) is shown from each chromatogram. A constant sensitivity setting was used in parts b–d. (a) Standard glycolaldehyde and 2-chloroacetaldehyde (1 M solutions) were injected separately; (b) incubation of the epoxide in buffer; (c) incubation of the epoxide in buffer containing 2.4 mg/mL epoxide hydratase; (d) incubation (c) after the subsequent addition of solid $NaBH_4$ to 20 mg/mL. (B) The column was coupled to a Hewlett-Packard 5920 mass spectrometer. Glycolaldehyde (m/e 58) and 2-chloroacetaldehyde (m/e 78) were monitored simultaneously; the respective retention times were 2.55 and 3.05 min. Data obtained at m/e 60 were identical with those obtained at m/e 58, and data obtained at m/e 80 were identical with those obtained at m/e 78. The basic sensitivity settings were identical for parts b and c with the indicated changes for each peak. (a) Standard glycolaldehyde and 2-chloroacetaldehyde; (b) incubation of the epoxide with 2.5 mg/mL bovine serum albumin; (c) incubation of the epoxide with 2.4 mg/mL epoxide hydratase.

NADH), production of both total nonvolatile and covalently bound metabolites was slightly increased (Figure 4).

Another system was set up in which 2-chloroethylene oxide and adenosine were mixed in aqueous buffer to form 1,N^6-ethenoadenosine (Barrio et al., 1972; Guengerich & Strickland, 1977; Guengerich & Watanabe, 1979). This reaction was also inhibited by epoxide hydratase but not by alcohol dehydrogenase (Figure 5).

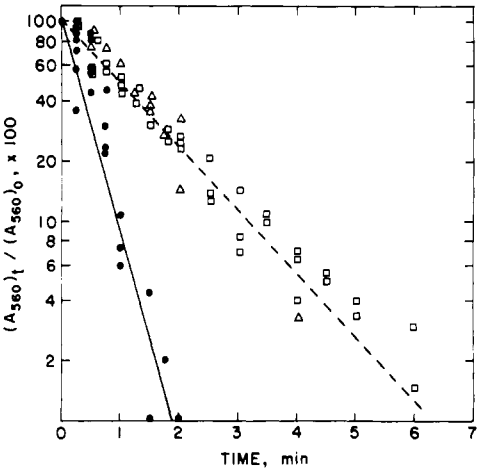

FIGURE 2: Effect of epoxide hydratase on the decomposition of 2-chloroethylene oxide. All incubations were carried out in 0.1 M potassium phosphate buffer (pH 7.7) at 23 °C. 2-Chloroethylene oxide was added to buffer, with mixing, to give an initial concentration of 0.4 mM. Aliquots of 50 μL were withdrawn at various times and added to 0.5 mL of a solution of 50 mM 4-(p-nitrobenzyl)pyridine in acetone, 0.1 M aqueous Tris–acetate (pH 7.4) buffer, and ethylene glycol (1:2:4 v/v/v) with mixing. After 5 min, 0.5 mL of acetone–triethylamine (1:1 v/v) was added with mixing. A_{560} values were read; $(A_{560})_t$ refers to A_{560} at each time point, and $(A_{560})_0$ refers to $A_{(560)}$ at $t = 0$ (~0.6). Data from duplicate experiments are shown for the following systems: buffer only (□); buffer plus epoxide hydratase (●); buffer plus epoxide hydratase plus 0.8 mM TCPO (△).

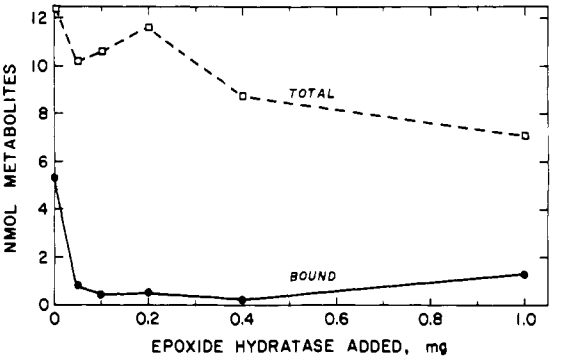

FIGURE 3: Effect of epoxide hydratase on VC metabolism in a reconstituted cytochrome P-450 system. Incubations were carried out as described under Experimental Procedure, and total bound or nonvolatile metabolites per incubation are presented.

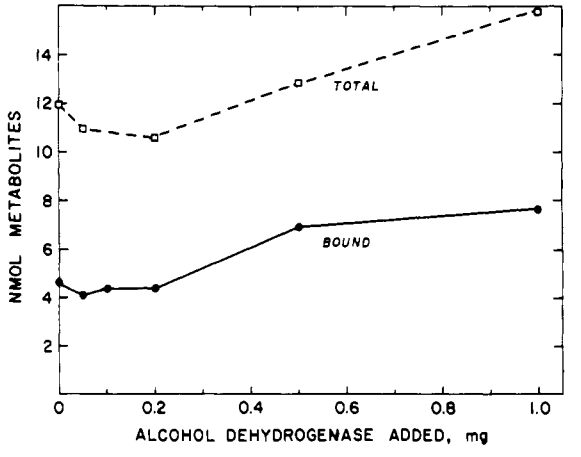

FIGURE 4: Effect of alcohol dehydrogenase on VC metabolism in a reconstituted cytochrome P-450 system. Incubations and assays were carried out as described under Figure 3 with the indicated amounts of alcohol dehydrogenase; 0.5 mM NADH was also present in all incubations.

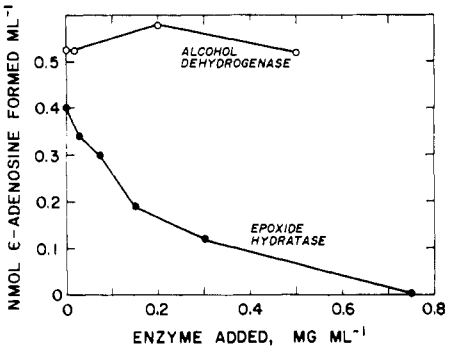

FIGURE 5: Effects of epoxide hydratase and alcohol dehydrogenase on the formation of 1,N^6-ethenoadenosine from adenosine and 2-chloroethylene oxide. All incubations contained 0.1 M potassium phosphate buffer (pH 7.7) and 2 mM adenosine. The indicated levels of epoxide hydratase or alcohol dehydrogenase were present; in the latter case, 0.2 mM NADH was also present. 2-Chloroethylene oxide was added to 1.6 mM, and incubations proceeded for 15 min at 37 °C. 1,N^6-Ethenoadenosine was separated by ion-exchange chromatography and estimated fluorometrically as described elsewhere (Guengerich & Watanabe, 1979). In the incubations containing alcohol dehydrogenase, residual NADH was oxidized prior to chromatography by adding lactate dehydrogenase (10 μg/mL) and pyruvate (0.5 mM) and incubating for an additional 10 min at 37 °C.

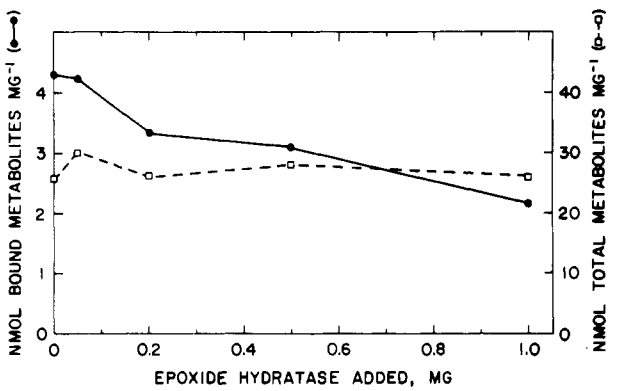

FIGURE 6: Effect of epoxide hydratase on VC metabolism in a microsomal system. Incubations were carried out essentially as described under Figure 4, except that cytochrome P-450, NADPH–cytochrome P-450 reductase, albumin, and dilauroylglyceryl-3-phosphorylcholine were replaced with microsomes prepared from phenobarbital-treated rats (2 mg of protein per mL; 2.0 nmol of cytochrome P-450 per mg).

Inhibition of VC Metabolism in Microsomal Systems. The experiments described in Figures 3 and 4 were repeated by using phenobarbital-treated rat liver microsomes in place of the reconstituted P-450 system. The addition of epoxide hydratase did not affect the level of VC metabolism to total nonvolatile metabolites and lowered the level of covalently bound metabolites by 50% (Figure 6). On the other hand, alcohol dehydrogenase lowered the level of covalently bound metabolites by 98% (Figure 7). In this experiment, the level of total nonvolatile metabolites was also lowered ~70%. This observation is explained by the subsequent finding that sweeping of incubations with N_2 to determine total nonvolatile metabolites) under these experimental conditions removed 60–70% of added 2-chloroethanol (bp 121 °C), the expected product of the enzymatic reduction of 2-chloroacetaldehyde. In another experiment, yeast aldehyde dehydrogenase blocked 93% of the covalent binding but did not affect the levels of total nonvolatile metabolites (Figure 8).

Effects of Modulation of Endogenous Epoxide Hydratase Activity upon Metabolism. The above experiments all involved adding epoxide hydratase to microsomes or reconstituted systems. Varying levels of the epoxide hydratase inhibitor TCPO

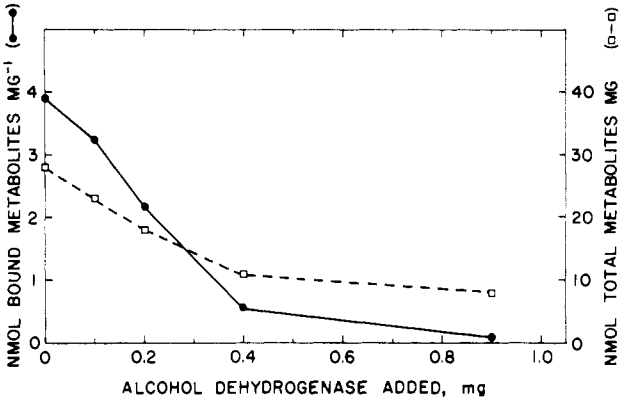

FIGURE 7: Effect of alcohol dehydrogenase on VC metabolism in a microsomal system. Incubations were carried out as described under Figure 6 with modifications as noted under Figure 4.

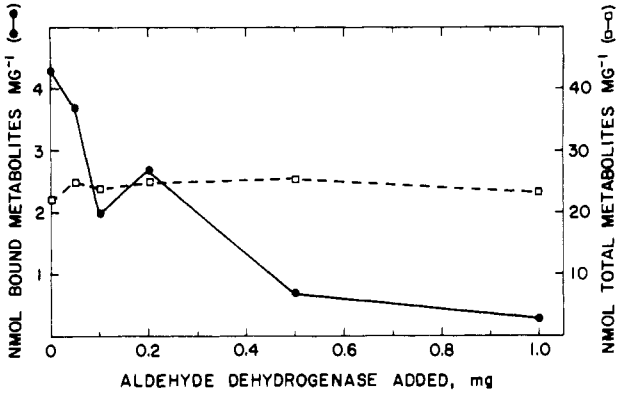

FIGURE 8: Effect of aldehyde dehydrogenase on VC metabolism in a microsomal system. Incubations were carried out as described under Figure 6 with varying levels of aldehyde dehydrogenase in the presence of 0.5 mM NAD^+.

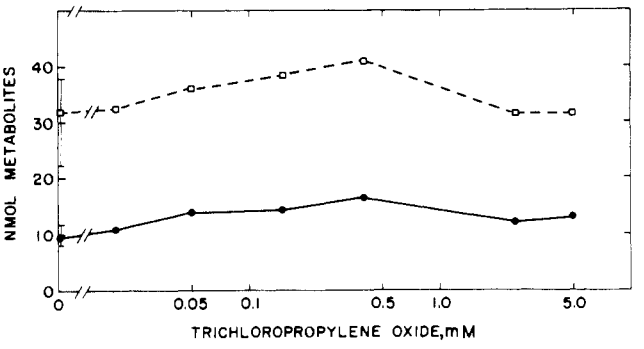

FIGURE 9: Effects of varying levels of TCPO on the microsomal metabolism of VC. Incubations were carried out as described under Figure 6 in the presence of varying levels of TCPO; the final concentration of acetone (used to add TCPO) was 1% (v/v) in all cases.

(Oesch, 1973) were added to microsomal incubations to block the endogenous enzyme; production of both total nonvolatile and covalently bound VC metabolites was stimulated slightly by TCPO, but the ratio of bound to total products did not appear to be significantly altered at any TCPO concentration examined (Figure 9).

Schmassmann & Oesch (1978) have reported that administration of *trans*-stilbene oxide to rats selectively induces liver microsomal epoxide hydratase. Mukhtar et al. (1978), in contrast, found alteration of certain mixed-function activities as well. In this laboratory, *trans*-stilbene oxide enhanced liver microsomal epoxide hydratase activity 2.8-fold (measured using styrene oxide;[3] $p < 0.01$) but did not significantly affect

Table I: Parameters for the Metabolism of Possible VC Metabolites[a]

reaction	enzyme	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	pH opt.
hydrolysis of 2-chloroethylene oxide[b]	epoxide hydratase	0.64	0.74	7.7
NAD^+ oxidn of $ClCH_2CH_2OH$[c]	alcohol dehydrogenase	4.0	0.89	7.7
NADH redn of $ClCH_2CHO$[d]	alcohol dehydrogenase	0.36	66	6.5-7.5
NAD^+ oxidn of $ClCH_2CHO$[e]	aldehyde dehydrogenase	0.015	0.20	

[a] All K_m and V_{max} data were obtained in the presence of 0.1 M potassium phosphate buffer (pH 7.7). At least eight points (each in duplicate) were used for each reaction over the indicated concentration ranges; data were analyzed by using a previously described computer program (Guengerich, 1978a). pH optima were obtained in 0.1 M potassium phosphate (pH 6.0–8.0) and Tris-HCl (pH 7.7–9.7) buffers. [b] Data were obtained from incubations carried out using 37 μg of epoxide hydratase in 50-μL total volume at 23 °C. Reactions were initiated by adding 2-chloroethylene oxide while mixing and quenched by adding reaction aliquots to 4-(*p*-nitrobenzyl)pyridine reagent (Figure 2). Final tetrahydrofuran concentrations were 6% (v/v), and incubation times were 30 s. Blank incubations were carried out with boiled (15 min) enzyme at each concentration of 2-chloroethylene oxide, and the resulting values were subtracted from the values obtained using epoxide hydratase. 2-Chloroethylene concentrations of 15 μM–3 mM were used. [c] Incubations were carried out by measuring the 2-chloroethanol-dependent rate of NAD^+ reduction (at 340 nm) in the presence of 50 μg of alcohol dehydrogenase at 37 °C. The NAD^+ concentration was 0.5 mM and the 2-chloroethanol concentration was varied over the range 0.5–100 mM. [d] The 2-chloroacetaldehyde-dependent rate of NADH oxidation (measured at 340 nm) was measured in the presence of 0.5 μg of alcohol dehydrogenase at 37 °C. The NADH concentration was 0.10 mM, and the 2-chloroacetaldehyde concentration was varied over the range 10 μM–2 mM. [e] The 2-chloroacetaldehyde-dependent rate of NAD^+ reduction (at 340 nm) was measured in the presence of 100 μg of aldehyde dehydrogenase at 37 °C. The NAD^+ concentration was 0.5 mM, and the 2-chloroacetaldehyde concentration was varied over the range 3 μM–4 mM.

the specific content of P-450 or the specific activities of NADPH–cytochrome *c* reductase, benzo[*a*]pyrene hydroxylase, or *d*-benzphetamine *N*-demethylase ($p > 0.1$, $n = 5$ individual rats). The microsomal preparations derived from stilbene oxide treated rats were compared to those prepared from untreated rats in parallel experiments. Both bound and total nonvolatile metabolite levels were lowered after treatment of the animals, decreasing from 4.39 ± 1.14 to 2.68 ± 0.27 nmol/mg of protein in the former case and from 41 ± 2.5 to 19.5 ± 1.0 nmol/mg of protein in the latter case. The reason for the decrease in the rate of metabolism is not immediately obvious; a small population of the P-450 may have a high activity toward VC and be particularly sensitive to the effect of stilbene oxide as a repressor or bound inhibitor. The ratio of covalently bound to total nonvolatile VC metabolites, which should be sensitive to alteration of enzymes processing VC metabolites produced by P-450, was 0.11 ± 0.03 with untreated rat microsomes and 0.14 ± 0.02 with *trans*-stilbene oxide treated rat microsomes (mean of three experiments ± SD in each case).

[3] Multiple forms of epoxide hydratase have been purified from both untreated and *trans*-stilbene oxide treated rats; however, the ratio of styrene oxide hydratase to 2-chloroethylene oxide hydratase activities is similar in all fractions separated from both sets of animals (Guengerich et al., 1979).

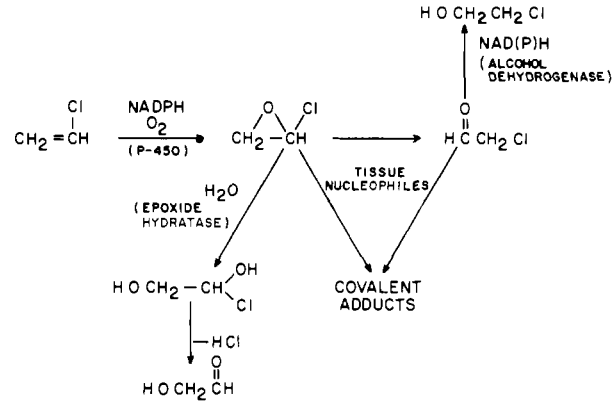

FIGURE 10: Postulated pathways for metabolism of vinyl chloride.

Discussion

The P-450 antibody experiments, coupled with data obtained with reconstituted P-450 systems, indicate that most of the microsomal covalent binding of VC label can be attributed to P-450's. The lack of complete inhibition is not unexpected, as inhibition by P-450-directed antibodies is a function of several variables, including the antibody, the particular substrate, and the source of microsomes (Thomas et al., 1977; Guengerich & Mason, 1979). However, we cannot definitively state at this time that other pathways are not involved in VC metabolism and activation.

2-Chloroethylene oxide appears to be the alkylating agent involved in the conversion of adenosine to 1,N^6-ethenoadenosine by 2-chloroethylene oxide (Figure 5) and the covalent binding of metabolites produced in reconstituted P-450 systems (Figures 3 and 4), as epoxide hydratase could abolish essentially all alkylation in each case. However, in microsomal incubations, dehydrogenases were more effective than epoxide hydratase in blocking covalent binding (Figures 6–8). Further, neither inhibition (by TCPO) nor enhancement (with *trans*-stilbene oxide) of endogenous microsomal epoxide hydratase activity affected the portion of VC metabolites that become covalently bound (Figure 9).

These observations are consistent with the scheme presented in Figure 10. In aqueous buffer or in reconstituted P-450 systems, both added nucleophiles and epoxide hydratase have ready access to 2-chloroethylene oxide. In microsomal incubations, 2-chloroethylene oxide is somehow protected from *both* endogenous and exogenous epoxide hydratase as well as nucleophilic sites and rearranges to 2-chloroacetaldehyde, which is the effective alkylating agent. The nature of the shielding of 2-chloroethylene oxide from epoxide hydratase is not clear. The data are not consistent with a hypothesis in which P-450 and epoxide hydratase are tightly coupled catalytically in microsomal membranes (Oesch, 1973). The epoxide cannot be so reactive as to bind only to P-450, as [^{14}C]-VC can be metabolized by microsomes to give label covalently bound to non-P-450 proteins, added nucleic acids, and cysteine derivatives (Guengerich & Watanabe, 1979). 2-Chloroacetaldehyde is more stable than 2-chloroethylene oxide in aqueous solution and probably more capable of migrating to various subcellular organelles and possibly other cells to alkylate.

These results exemplify biochemical techniques which can be used to identify the chemical nature of reactive products formed in situ which are too unstable to isolate. Moreover, this work indicates that a milder reactive metabolite may be more important in damaging critical cellular targets than a stronger metabolite and that organization of metabolically important enzymes into a membrane environment can influence the nature of the metabolites involved in such damage.

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Primary Structure of Rabbit α-Lactalbumin†

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ABSTRACT: Rabbit α-lactalbumin was purified from the milk of New Zealand White rabbits. It was found to exist predominantly as a glycoprotein, containing 5 mol of glucosamine per mol of protein, as well as other sugars. The amino acid sequence of the protein was determined by sequenator analysis and carboxypeptidase digestion. There are 122 amino acids in the protein and a single carbohydrate moiety, probably attached to an asparagine residue at position 45. The C terminus of rabbit α-lactalbumin is one residue shorter than that of the other α-lactalbumins. Sequence comparisons indicate that the α-lactalbumin gene has been undergoing more frequent mutation than had previously been thought. A new method of preparative peptide mapping using 2,5-diphenyloxazole (PPO) fluor to detect peptides is described.

α-Lactalbumin is one of the two protein subunits of lactose synthase (EC 2.4.1.22). Sequence studies have shown it to be homologous to the antibacterial glycosidase lysozyme (Brew et al., 1967).

The primary structures of several α-lactalbumins are known, including human (Findlay & Brew, 1972), cow (Brew et al., 1970), goat (MacGillivary et al., 1979), and guinea pig (Brew, 1972). A partial sequence of kangaroo α-lactalbumin has been reported (Brew et al., 1973). Rabbit α-lactalbumin is interesting in that it exists predominantly as a glycoprotein, while

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