

GWUMC

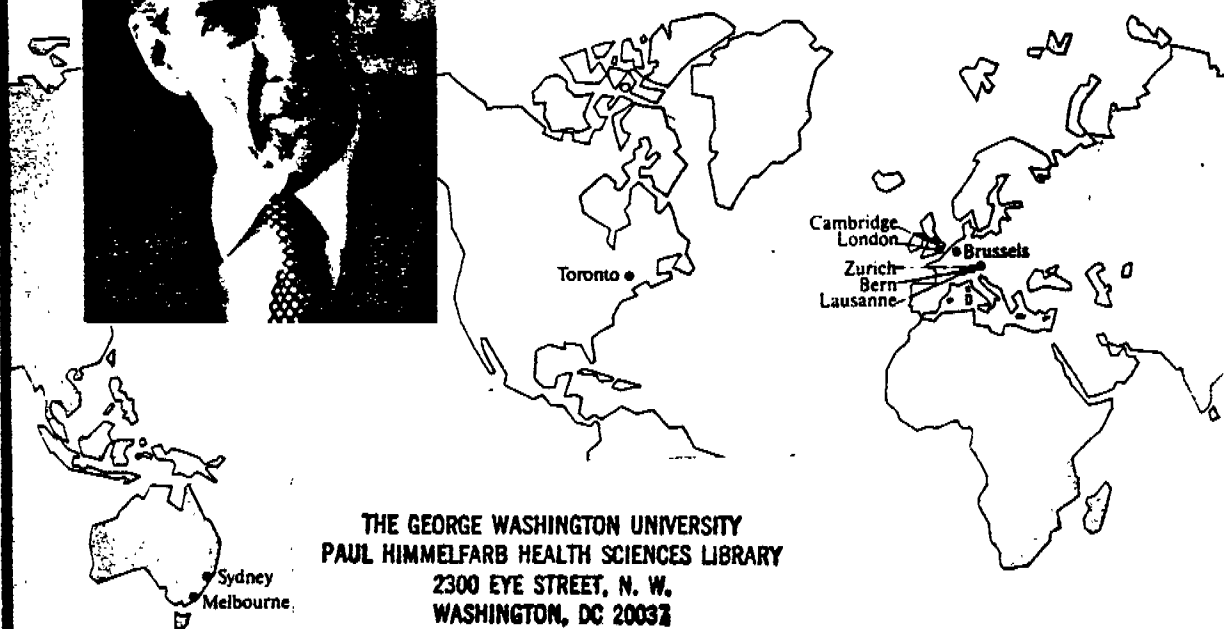


# Cancer Research



VOLUME 45 • NO. 1 CNREA 8 • PP 1-480

January 1985



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JAN. 3 1985



SL 065757

Departments of Pharmacology [D. C. L.] and Biochemistry [M. J. M., F. P. G.] and Center in Molecular Toxicology [D. C. L., M. J. M., F. P. G.], Vanderbilt School of Medicine, Nashville, Tennessee 37232

Oxidation of the vinyl halide carcinogen and hepatotoxin vinylidene chloride (VDC) by microsomal cytochrome P-450 yields 2,2-dichloroacetaldehyde, 2-chloroacetyl chloride, 2-chloroacetic acid, and 1,1-dichloroethylene oxide. The roles of these metabolites in covalent modification of proteins and reduced glutathione (GSH) were examined. 2-Chloroacetyl chloride reacted with model thiols at least  $10^3$ -fold faster than did 1,1-dichloroethylene oxide and at least  $10^4$ -fold faster than did 2,2-dichloroacetaldehyde or 2-chloroacetic acid. Microsomal covalent binding of [ $^{14}\text{C}$ ]VDC was inhibited by GSH but not by lysine, suggesting that protein thiols, rather than amino groups, are major targets. Liver microsomes catalyzed the formation of three GSH:VDC metabolite conjugates, identified as S-(2,2-dichloro-1-hydroxy)ethylglutathione, 2-(S-glutathionyl)acetate, and S-(2-glutathionyl)acetylglutathione, a novel conjugate containing both stable (thioether) and labile (thioester) linkages. The latter two conjugates also were formed in isolated rat hepatocytes and measurable amounts of 2-(S-glutathionyl)acetate were released into the incubation medium. Both 2-(S-glutathionyl)acetate and S-(2-glutathionyl)acetylglutathione were formed with [ $^{35}\text{S}$ ]GSH added to the hepatic medium, indicating that reactive VDC metabolites are capable of crossing the plasma membrane to react with extracellular targets. Unlabeled S-(2-glutathionyl)acetylglutathione underwent carbonyl substitution with added [ $^{35}\text{S}$ ]GSH, suggesting that this conjugate may participate in modification of protein thiols. This conjugate also underwent hydrolysis with a half-life of approximately 3 hr. GSH:VDC metabolite conjugates may serve as accessible models for labile covalent adducts formed between VDC metabolites and protein thiols.

Vinyl halide monomers have afforded investigators an opportunity to study the consequences of procarcinogen bioactivation using relatively simple compounds. Early studies demonstrated that vinyl chloride is oxidized to chloroethylene oxide by microsomal cytochrome P-450 (1, 2, 8), and it was inferred that concomitant production of 2-chloroacetaldehyde was the result of epoxide rearrangement (8, 11). These and other observations contributed to the widely held assumption that epoxides are the

Selectivity of individual vinyl halide metabolites for reaction with particular cellular targets has been observed. Studies with vinyl chloride indicated that dichloroethylene oxide is primarily responsible for modification of DNA *in vivo* and *in vitro*, while 2-chloroacetaldehyde binds primarily to protein (10, 37). The microsomal oxidation of VDC<sup>+</sup> yields VDC oxide, 2-chloroacetaldehyde, 2-chloroacetic acid, and 2,2-dichloroacetaldehyde (17), which may react with a variety of cellular nucleophiles. The variable reactivity of these metabolites may be expected to influence their target selectivity. Relatively low levels of metabolites are bound to DNA isolated *in vivo* (38). Fitch and co-workers (26) suggested that VDC may initiate a tumorigenic response via interaction with cellular components other than DNA. Alternative targets of possible importance for the carcinogenic mechanism may include cellular proteins that regulate DNA transcription, chromatin structure, or cellular growth status (19). Information concerning the disposition of VDC metabolites would therefore be required to establish their role in the initiation of carcinogenic or toxic lesions.

Several reports have suggested that cellular thiols exert a protective influence in VDC intoxication and serve to detoxicate VDC metabolites (13, 16, 27). Accordingly, depletion of cellular GSH by starvation or pretreatment with diethylmaleate or GSH hepatotoxicity in rats (13, 27). Animals administered [ $^{14}$ C]VDC excrete radiolabel in the urine as *N*-acetylcarboxymethylcysteine, thioglycolic acid, thiodiglycolic acid, and unidentified sulfur-containing metabolites (16, 18). The importance of cellular GSH as a detoxicating nucleophile for VDC metabolites implies that protein thiols may themselves be major targets for covalent modification. This suggestion has been verified for other compounds which interact with GSH in a similar manner (22, 31, 32).

Because urinary adducts reflect significant renal as well as hepatic biotransformation, their utility as indicators of intracellular metabolite disposition is limited. In this work, VDC metabolites were compared on the basis of relative reactivities towards sulfhydryl compounds, relative contributions to protein covalent binding *in vitro*, and ability to form GSH conjugates in hepatoma

Received May, 21, 1984; accepted September 27, 1984.

\*The abbreviations used are: VDC, vinylidene chloride (1,1-dichloroethylene); VDC oxide, 1,1-dichloroethylene oxide; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl.

cytes and microsomal systems. The formation of extracellular GSH conjugates was used as an index of the ability of VDC metabolites to cross hepatocyte membranes. The data indicate that multiple VDC metabolites participate in covalent modification of proteins and GSH.

## MATERIALS AND METHODS

**Chemicals.** [ $^{14}\text{C}$ ]VDC was synthesized from 1,1,2-trichloro-[ $^{14}\text{C}$ ]ethane (Amersham, Arlington Heights, IL; 10 mCi/mmol). The labeled starting material was diluted with unlabeled 1,1,2-trichloroethane to a specific activity of approximately 0.2 mCi/mmol and treated with tetracyclo[5.4.0]undec-7-ene for 1 min at 60°. A stream of nitrogen was passed through the mixture to sweep VDC into a tube immersed in dry isopropyl alcohol bath. The [ $^{14}\text{C}$ ]VDC thus collected was of 95% radiochemical purity as assessed by gas chromatography (Tenax, 50°). [ $^{35}\text{S}$ ]Methionine (1170 Ci/mmol) and [ $^{35}\text{S}$ ]GSH (69 Ci/mmol) were from New England Nuclear (Boston, MA).

VDC and thiophenol were purchased from Aldrich (Milwaukee, WI); VDC was distilled before use. Chloroacetyl chloride was from Eastman Rochester, NY and was also distilled before use. GSH, 2-(S-cysteinyl)-acetate, and glutathione-S-transferase were purchased from Sigma St. Louis, MO). This commercial glutathione-S-transferase preparation is composed largely of isozyme B and lesser amounts of other isozymes. Horse liver alcohol dehydrogenase was purchased from Boehringer-Mannheim (Indianapolis, IN). Fetal calf serum was from Grand Island Biological Co. (Grand Island, NY).

Dichloroacetaldehyde and VDC oxide were synthesized as described elsewhere (17). All other chemicals were of the highest purity commercially available.

**Hepatocyte and Microsomal Incubations.** Rat liver and human liver microsomes were prepared as described previously (9, 36). Rat hepatocytes were prepared by collagenase perfusion (29), and viability was assessed by trypan blue exclusion. Fischer's medium, supplemented with 15% fetal calf serum but deficient in sulfur amino acids, was the medium used for all experiments with hepatocytes.

Hepatocytes ( $2$  to  $3 \times 10^6$  cells in a volume of 1 ml) were incubated in sealed 15-ml glass scintillation vials containing Fischer's medium. VDC was added to the suspension from a 1 M stock solution in acetone to a final concentration of 5 mM. [ $^{35}\text{S}$ ]GSH was added to cell suspensions from a 0.4 M stock solution prepared immediately before use. Hepatocytes were incubated for 3 hr in Fischer's medium supplemented with 15 mM [ $^{35}\text{S}$ ]methionine at a specific activity of 2 Ci/mmol to label sulfur pools. The cells were then washed twice and resuspended in fresh medium containing 0.5 mM unlabeled methionine. The specific activity of the hepatocellular [ $^{35}\text{S}$ ]GSH produced was 89 mCi/mmol and was determined from fractions collected from HPLC analyses (23). After incubation with VDC for 60 min at 37°, the cells were rapidly separated from the medium by centrifugation at  $1000 \times g$  for 30 sec, and the medium was removed for analysis of GSH adducts. The pellet was resuspended in 1 ml of 10%  $\text{HClO}_4$  and recentrifuged. The resulting supernatant contained extracellular GSH and GSH:VDC metabolite conjugates and was analyzed by ion-exchange HPLC.

Microsomal incubations contained 100 mM potassium phosphate, pH 7.7, an NADPH generating system consisting of 0.35 IU glucose-6-phosphate dehydrogenase per ml, 10 mM glucose 6-phosphate, and 0.5 mM NADP $^{+}$ , and microsomal protein at a final concentration of 5 to 10 mg/ml. GSH was added from a 40 mM aqueous stock solution; this solution was prepared immediately before use. VDC was added from a 1 M stock solution in acetone or methanol. Incubations were terminated after 60 min at 37° by addition of  $\text{ZnSO}_4$  to a final concentration of 1% (w/v) or  $\text{HClO}_4$  to a final concentration of 3.5% (w/v) and centrifuged at 500 rpm for 5 min. The supernatants were then neutralized with  $\text{KHCO}_3$  for analysis of GSH conjugates.

**Assays.** Depletion of GSH during aqueous incubations with VDC

metabolites was monitored using 5,5'-dithiobis-2,2'-dinitrobenzoic acid (28). VDC metabolites were added directly to solutions containing 0.5 mM GSH in 200 mM potassium phosphate, pH 8.0, at 37°, with an initial metabolite concentration of 50 mM. Aliquots were then removed at various times for assay of residual thiol content. 2-Chloroacetic acid was added as its sodium salt to minimize pH changes. Less than 5% of the GSH was oxidized to GSSG during these incubations. Depletion of thiophenol in  $\text{CHCl}_3$  by VDC metabolites was monitored using 5,5'-dithiobis-2,2'-dinitrobenzoic acid in aqueous acetone. Thirty  $\mu\text{mol}$  of each VDC metabolite were added to 30  $\mu\text{mol}$  thiophenol in 6 ml  $\text{CHCl}_3$  at 25°. Twenty- $\mu\text{l}$  aliquots were then transferred to tubes containing 1 ml of acetone plus 200  $\mu\text{l}$  of 10 mM 5,5'-dithiobis-2,2'-dinitrobenzoic acid in 85% acetone:15%  $\text{H}_2\text{O}$ . Twenty  $\mu\text{l}$  of triethylamine were added, and the absorbance was immediately recorded versus a blank at 490 nm. Second-order rate constants for the reaction of thiophenol with VDC metabolites were determined from plots of reciprocal absorbance versus time.

Covalent binding of [ $^{14}\text{C}$ ]VDC radiolabel to microsomal proteins was assayed by the method of Wallin *et al.* (35). Incubations contained 3 mg microsomal protein (from untreated rats) per ml and 10 mM [ $^{14}\text{C}$ ]VDC (0.03  $\mu\text{Ci}$ ) and were for 30 min at 37°. Proteins were precipitated on 2.4-cm glass fiber filters (Fisher G6). Following 3 washes each with ethanol, methanol, and acetone, the filters were counted using ACS scintillation cocktail (Amersham).

**Separation and Characterization of Glutathione Conjugates of VDC.** Glutathione and cysteine conjugates were analyzed using ion-exchange HPLC as described by Reed *et al.* (23). Aminopropyl silica was prepared from 5  $\mu\text{m}$  Sphersorb silica (Rainin, Woburn, MA), and 0.5- $\times$  30-cm columns were slurry packed as described previously (24). Supernatants from  $\text{ZnSO}_4$ - or  $\text{HClO}_4$ -quenched microsomal incubations were neutralized with  $\text{KHCO}_3$  and treated with ethanolic FDNB for 30 to 60 min before injection. Elution Solvent A contained 80% methanol and 20%  $\text{H}_2\text{O}$  (v/v), and Solvent B contained 3 M sodium acetate, pH 4.5, in 64% methanol. The column was loaded isocratically at 95% Solvent A for 10 min. Conjugates were eluted during a 30-min linear gradient from 5 to 99% Solvent B at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected and counted using ACS scintillation cocktail, while elution of DNP derivatives was monitored at 360 nm. For preparative and some analytical chromatography, ammonium acetate was substituted for sodium acetate in Solvent B.

Effluent fractions containing 3 GSH conjugates (Conjugates A, B, and C) were collected, and several collections were pooled. Methanol was removed from the pooled fractions *in vacuo*, and the fractions were then lyophilized. The residue corresponding to Conjugate A was treated with 2 N  $\text{HCl}$  for 12 hr at 25°. The solution was then extracted with 3 portions of ether; the extracts were concentrated under a stream of nitrogen and then analyzed for 2,2-dichloroacetaldehyde by gas chromatography (17). A portion of the residue corresponding to conjugate B was dissolved in N  $\text{HCl}$  and heated at 110° for 30 min. A second portion was dissolved in 6 N  $\text{HCl}$  and heated under nitrogen at 110° for 30 hr. Both samples were then neutralized with  $\text{KHCO}_3$  and gently gassed with oxygen overnight to oxidize liberated thiols to disulfides. The mixtures were then analyzed by ion-exchange HPLC as described above. Residue containing Conjugate C was hydrolyzed with 6 N  $\text{HCl}$  at 110° for 24 hr. The hydrolysate was reanalyzed for 2-(S-cysteinyl)acetate by ion-exchange HPLC.

## RESULTS

**Reaction of VDC Metabolites with Thiols.** Thiols reacted with major VDC metabolites at measurable rates in aqueous and nonaqueous solutions (Table 1). In aqueous buffer at pH 8, GSH reacted with 2,2-dichloroacetaldehyde and 2-chloroacetate at moderate rates. Despite rapid hydrolysis, 2-chloroacetyl chloride reacted with GSH at least 4 orders of magnitude faster than did 2-chloroacetic acid or 2,2-dichloroacetaldehyde. The rate of reaction of GSH with 2-chloroacetyl chloride was calculated using

### Table 1 Rate constants for reaction of thiols with VDC metabolites

Reactions were started by adding VDC metabolites to solutions of the thiols. Aliquots were then analyzed for residual thiol content as described in "Materials and Methods." Initial VDC metabolite:thiol molar ratios were 1:1 in the  $\text{CHCl}_3$  system and 100:1 in the aqueous system. Pseudo-first-order rate constants for the GSH reaction were determined from plots of the logarithm of absorbance versus time. Second-order rate constants for the thiophenol reaction were determined from plots of reciprocal absorbance versus time.

| Metabolite               | System                                                                     |                                                                                              |
|--------------------------|----------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|
|                          | Thiophenol: $\text{CHCl}_3$ ,<br>25°, k ( $\text{M}^{-1}\text{min}^{-1}$ ) | GSH:200 mM<br>potassium phosphate,<br>pH 8.0, 37°, $k_{\text{obs}}$<br>( $\text{min}^{-1}$ ) |
| VDC oxide                | $90 \pm 20^a$                                                              | — <sup>b</sup>                                                                               |
| 2-Chloroacetyl chloride  | $>7 \times 10^6$                                                           | $>3.8 \times 10^6$                                                                           |
| 2,2-Dichloroacetaldehyde | $<8$                                                                       | $0.024 \pm 0.002$                                                                            |
| 2-Chloroacetic acid      | $<8$                                                                       | $0.006 \pm 0.000$                                                                            |

<sup>a</sup> Mean  $\pm$  S.D. from 3 individual experiments.

<sup>b</sup> —, not determined.

a hydrolysis rate constant of  $4 \text{ s}^{-1}$  for the acyl chloride. This value was estimated from earlier studies comparing the hydrolyses of acetyl chloride and 2-chloroacetyl chloride in aqueous acetone (33) and from the reported hydrolysis rate constant for acetyl chloride in water at  $0^\circ$  (7). The assumptions made were that the ratios of hydrolysis rate constants of the 2 acyl chlorides were similar in water and aqueous acetone, and that the hydrolysis rate constant doubled with each  $10^\circ$  increase in temperature. It also was assumed that the phosphate buffer would not significantly affect the solvolysis rate. VDC oxide was not studied in the aqueous system, because it can be produced only as a dilute solution in chloroform. Addition of the required volume of epoxide solution thus produced a 2-phase mixture unsuitable for the experiment, and attempts to concentrate the epoxide solution were unsuccessful.

To compare the relative reactivities of VDC oxide and 2-chloroacetyl chloride with thiols in the absence of competing hydrolysis, the reaction of each metabolite with an equimolar concentration of thiophenol in chloroform was studied. The acyl chloride reacted with thiophenol at least 800-fold faster than did the epoxide, while neither 2,2-dichloroacetaldehyde nor 2-chloroacetic acid depleted the thiol at a measurable rate in this system (Table 1). The slow reaction of 2-chloroacetic acid relative to 2-chloroacetyl chloride suggests that acylation, rather than alkylation, is the major reaction between 2-chloroacetyl chloride and the thiol. The rate constants presented describe bimolecular reactions, although pseudo-first-order conditions were selected for the aqueous system. High concentrations of VDC metabolites were used in order to achieve rates of GSH conjugation significantly in excess of that of GSH oxidation.

**Covalent Binding of [ $^{14}\text{C}$ ]VDC Radiolabel in Microsomes.** Rat and human liver microsomes catalyzed covalent binding of [ $^{14}\text{C}$ ]VDC radiolabel to microsomal proteins (Table 2). No radioactivity was bound when NADPH was omitted from the incubation mixtures. Inclusion of 5 mM GSH in the incubation mixtures inhibited roughly half of the binding, but 5 mM lysine inhibited the binding only slightly. These results suggest that protein thiols, rather than protein amino groups, are major targets for covalent modification by VDC metabolites. Inhibition of covalent binding by GSH was not increased when glutathione-S-transferase (0.3 mg/ml) was added to the incubations (data not shown). Covalent

Table 2  
Microsomal covalent binding of [ $^{14}\text{C}$ ]VDC

Complete incubation mixtures contained 100 mM potassium phosphate pH 7.3, 3 mg rat or human liver microsomal protein per ml, 0.35 IU yeast glucose-6-phosphate dehydrogenase per ml, 10 mM glucose 6-phosphate, 0.5 mM NADP, and 10 mM [ $^{14}\text{C}$ ]VDC (0.03  $\mu\text{Ci}$ ). Rat liver microsomes were from untreated rats. Covalent binding of [ $^{14}\text{C}$ ]VDC radiolabel to microsomal protein was as described in "Materials and Methods."

| System                                                     | nmol bound/mg/30 min | % of total system |
|------------------------------------------------------------|----------------------|-------------------|
| Complete                                                   | $22 \pm 3^a$         | 100               |
| -NADPH                                                     | $2 \pm 1$            | 9                 |
| +5 mM GSH                                                  | $12 \pm 1$           | 55                |
| +Alcohol dehydrogenase (0.5 mg/ml):<br>0.5 mM NADH         | $9 \pm 1$            | 41                |
| +Alcohol dehydrogenase (0.5 mg/ml)<br>(boiled):0.5 mM NADH | $23 \pm 3$           | 105               |
| +0.5 mM NADH                                               | $28 \pm 3$           | 127               |
| +5 mM lysine                                               | $18 \pm 1$           | 82                |
| Human liver 31                                             | $11, 10^b$           |                   |
| Human liver 80                                             | $20, 22^b$           |                   |

<sup>a</sup> Mean  $\pm$  S.D. from 3 individual experiments.

<sup>b</sup> Results of duplicate experiments.

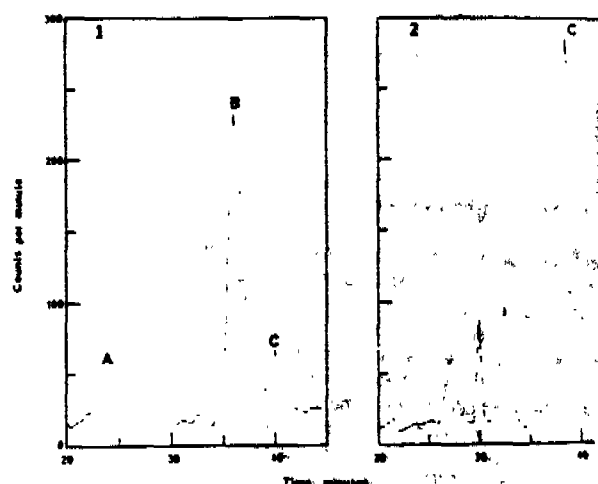


Chart 1. Ion-exchange HPLC of GSH-[ $^{14}\text{C}$ ]VDC metabolite conjugates. Rat liver microsomes (10 mg protein per ml) incubated with [ $^{14}\text{C}$ ]VDC and GSH were derivatized with FDNB as described in "Materials and Methods." Freshly prepared incubation products (1) and products after 48 hr at  $-20^\circ\text{C}$  (2) are shown. The identities of the 3 initial peaks in 2 are unknown.

binding of [ $^{14}\text{C}$ ]VDC radiolabel also was decreased in microsomes supplemented with 0.3 mg alcohol dehydrogenase per ml plus 0.5 mM NADH. Substitution of heat-denatured dehydrogenase returned binding to control levels, while addition of 0.5 mM NADH alone increased binding by 25%. Microsomes prepared from 2 human liver samples catalyzed covalent binding at levels comparable to rat microsomes.

**Production of GSH:VDC Metabolite Conjugates in Microsomes.** Rat liver microsomes supplemented with GSH and [ $^{14}\text{C}$ ]VDC produced 3 GSH conjugates which were analyzed by N-DNP derivatives using ion-exchange HPLC. NADPH and GSH were required for formation of all 3 conjugates. Representative chromatograms of both rat and human liver microsomal products are shown in Charts 1 and 2. Three GSH:VDC metabolite conjugates were designated A, B, and C, in order of elution. Conjugate A (23 min) was present in higher levels in the 2 human samples than in the rat samples, while the relative amounts of Conjugates B (36 min) and C (39 min) were similar in both

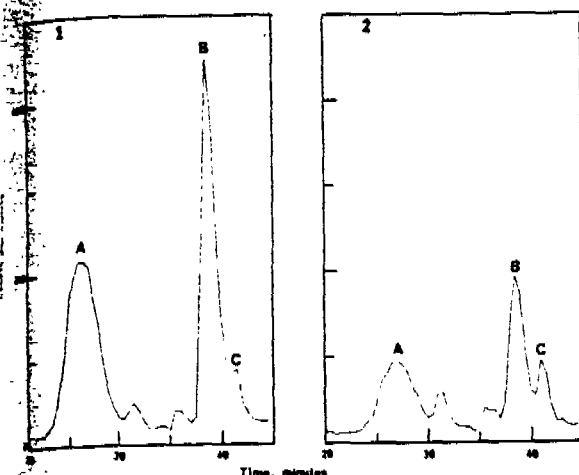


Chart 2. Ion-exchange HPLC of GSH:[ $^{14}\text{C}$ ]VDC metabolite conjugates from human liver microsomes (10 mg protein per ml) incubated with [ $^{14}\text{C}$ ]VDC and GSH. Samples were derivatized with FDNB as described in "Materials and Methods." Systems of incubation products from Samples 80 (1) and 31 (2) from human liver are shown.

Table 3

Formation of GSH:VDC metabolite conjugates by isolated hepatocytes

Isolated rat hepatocytes were incubated at a concentration of  $2$  to  $3 \times 10^6$  cells/ml in a Fischer's medium containing  $5$  mM VDC. After  $1$  hr of incubation, cells were only separated from the medium by centrifugation, and GSH:VDC metabolite conjugates in cells and medium were analyzed separately as described in "Materials and Methods."

| Conjugate | nmol conjugate/ $10^6$ cells/60 min |                     |                                 |                                  |
|-----------|-------------------------------------|---------------------|---------------------------------|----------------------------------|
|           | Cells <sup>a</sup>                  | Medium <sup>a</sup> | Medium: $5$ mM GSH <sup>b</sup> | Medium: $10$ mM GSH <sup>b</sup> |
| A         | $<0.4^c$                            | $<0.4^c$            | $<0.4^d$                        | $<0.4^d$                         |
| B         | $4.8 \pm 0.8^c$                     | $<0.3^c$            | $0.6^d$                         | $3.9^d$                          |
| C         | $12.5 \pm 3.0^c$                    | $1.5 \pm 1.1^c$     | $11.1^d$                        | $45.7^d$                         |

<sup>a</sup>Hepatocytes were preincubated with [ $^{35}\text{S}$ ]methionine to label hepatocyte GSH as described in "Materials and Methods."

<sup>b</sup>Unlabeled hepatocytes were incubated in medium containing  $5$  or  $10$  mM GSH ( $2.5$  mCi/mmol).

<sup>c</sup>Mean ( $\pm$  S.D.) of 3 individual experiments.

<sup>d</sup>Values from a single experiment.

<sup>e</sup>Mean of duplicate experiments.

and human samples. Microsomes from Human Liver 80, which produced greater levels of covalent protein binding than microsomes from Human Liver 31 (Table 2), also produced more of each GSH conjugate than microsomes from Human Liver 31. Both the total amount of conjugates formed and the relative proportions of individual conjugates were similar in several incubations with rat liver microsomes and were unaffected by the addition of  $0.3$  mg of purified glutathione-S-transferase per ml to the incubation mixtures.

**Production of GSH:VDC Metabolite Conjugates in Isolated Hepatocytes.** Isolated rat hepatocytes preincubated with [ $^{35}\text{S}$ ]methionine to label cellular GSH ( $24$ ) produced [ $^{35}\text{S}$ ]GSH at a specific activity sufficiently high ( $89$  mCi/mmol) to permit detection of GSH:VDC metabolite conjugates both within the cells and in the surrounding medium. Detectable levels of Conjugates B and C, but not of Conjugate A, were found in [ $^{35}\text{S}$ ]prelabeled cells (Table 3). Conjugate C, the major intracellular conjugate, was found at levels approximately 3-fold higher than Conjugate B. Conjugate C also was found in the incubation medium at approximately 10% of intracellular levels, but Conjugates A and

B were not present in measurable amounts. The presence of measurable levels of a GSH:VDC metabolite conjugate in the hepatocyte incubation medium indicates that conjugates may be released from the hepatocyte. Alternatively, the extracellular conjugates may be formed outside the cells by VDC metabolites which cross cell membranes to react with extracellular GSH. In order to determine if one or both of these processes occur, unlabeled rat hepatocytes were incubated with  $5$  mM VDC in medium containing  $10$  mM [ $^{35}\text{S}$ ]GSH. Cells incubated in this manner accumulated  $0.25$  nmol [ $^{35}\text{S}$ ]GSH per  $10^6$  cells during the incubations, a level which corresponds to approximately 0.5% of the total cellular GSH content and which may represent GSH leakage into a small fraction of nonviable cells. Thus, labeled conjugates would be formed only by VDC metabolites which cross the plasma membrane to react with extracellular GSH. The major conjugate formed extracellularly was Conjugate C (Table 3), although lesser amounts of Conjugate B were also detected. Conjugate A was not formed outside the cells at detectable levels. Conjugate B was formed in the medium at approximately 10% of Conjugate C levels, but the amount of each conjugate formed increased with increasing medium GSH content (Table 3).

**Chemical Characterization of GSH:VDC Metabolite Conjugates.** Chemical characterization of GSH:VDC metabolite conjugates was based on chromatographic properties and chemical degradation of the conjugates. All 3 conjugates required derivatization with FDNB to elute as shown in Chart 1.

Conjugate A, which was produced in microsomal preparations but not in isolated hepatocytes, disappeared from FDNB-treated samples after standing 48 hr at pH 8. Conjugate A was collected from ion-exchange HPLC and subjected to hydrolysis with  $2$  N HCl at  $25^\circ$  for 12 hr. Gas chromatography (electron capture) (17) of ether extracts of the hydrolysate indicated that 2,2-dichloroacetaldehyde was released during hydrolysis. Further, addition of 2,2-dichloroacetaldehyde to neutral aqueous solutions of GSH, followed by S-carboxymethylation of unreacted GSH with iodoacetate and treatment with FDNB, produced (in addition to the expected GSH and GSSG derivatives) a product with a retention time identical to Conjugate A. Both GSH and N-acetylcysteine reacted at a similar rate with 2,2-dichloroacetaldehyde, suggesting that a thiohemiacetal rather than a Schiff base was the reaction product.  $^1\text{H}$  nuclear magnetic resonance of the N-acetylcysteine adduct in  $\text{D}_2\text{O}$  was also consistent with this interpretation ( $5.33$   $\delta$ , multiplet,  $1\text{H}$  [ $\text{CH}_2\text{CH}-$ ];  $4.42$   $\delta$ , triplet,  $1\text{H}$  [ $-\text{CH}(\text{NHCOCH}_3)\text{CO}_2\text{H}$ ];  $3.32$   $\delta$ , multiplet,  $1\text{H}$  [ $-\text{CH}(\text{OH})\text{S}-$ ];  $3.12$   $\delta$ , doublet,  $2\text{H}$  [ $-\text{SCH}_2-$ ];  $2.03$   $\delta$ , singlet,  $3\text{H}$  [ $-\text{NHCOCH}_3$ ]).

Conjugate C coeluted with authentic N-DNP-2-(S-glutathionyl)acetate. Acid hydrolysis of Conjugate C, prepared using microsomes, GSH, and [ $^{14}\text{C}$ ]VDC, followed by analysis of the hydrolysate by HPLC, indicated that Conjugate C was cleaved to 2-(S-cysteinyl)[ $^{14}\text{C}$ ]acetate (Chart 3). The carboxymethylated thiol is an expected VDC metabolite and may be formed by conjugation of GSH with VDC oxide, 2-chloroacetyl chloride, or 2-chloroacetate. This conjugate was stable for at least 48 hr at pH 8.

Conjugate B, the major adduct formed in microsomal incubations, was only moderately stable at pH 8 and had disappeared completely from derivatized samples within 48 hr. Virtually all of the radioactivity initially appearing as Conjugate B eluted as

# VDC ADDUCTS

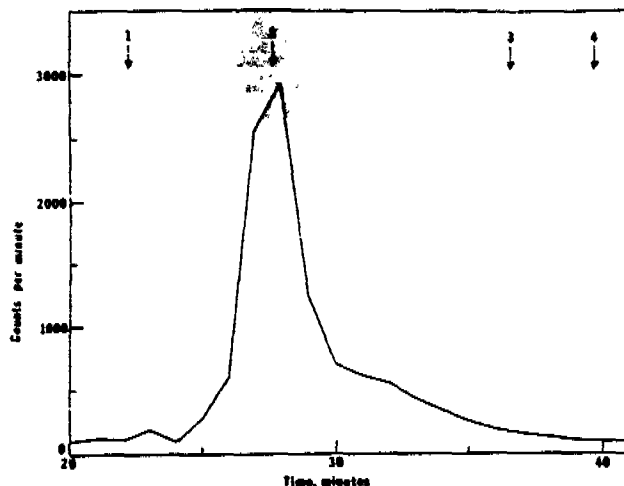


Chart 3. Ion-exchange HPLC of 2-(S-cysteinyl)-[<sup>14</sup>C]acetate released by acid hydrolysis of <sup>14</sup>C-Conjugate C. <sup>14</sup>C-Conjugate C was produced using microsomes, GSH, and [<sup>14</sup>C]VDC and collected using ion-exchange HPLC, following derivatization with FDNB. The collected, derivatized <sup>14</sup>C-Conjugate C was subjected to acid hydrolysis (6 N HCl, 110°, 24 hr), and the products were analyzed using ion-exchange HPLC. Arrows 1, 2, 3, and 4 mark the retention times of N-(2,4-dinitrophenyl) derivatives of cystine, 2-(S-cysteinyl)acetate, 2-(S-glutathionyl)acetate, and glutathione disulfide, respectively.

Conjugate C [N-DNP:2-(S-glutathionyl)acetate] in samples treated with FDNB and left standing at pH 8 for 48 hr (Chart 1). Lyophilized samples of Conjugate B collected from ion-exchange HPLC were subjected to acid hydrolysis and oxidative work-up to convert liberated thiols to disulfides. Treatment of Conjugate B with N HCl at 110° for 1 hr produced 2-(S-glutathionyl)acetate and GSSG in a 2:1 molar ratio. Hydrolysis of a second sample in 6 N HCl at 110° for 24 hr cleaved Conjugate B to 2-(S-glutathionyl)acetate, cystine, and glutamate. Glycine was not retained on the ion-exchange HPLC column used for these analyses. These results suggest that Conjugate B contains 2 GSH molecules bridged by a stable (thioether) linkage and a labile (thioester) linkage. Such a bisglutathionyl conjugate would be expected to undergo both transesterification and hydrolysis reactions. To determine if Conjugate B would transacylate exogenous thiols, unlabeled Conjugate B was generated in microsomes incubated with VDC and unlabeled GSH. After precipitation of microsomal protein and neutralization to pH 7.5 with KHCO<sub>3</sub>/KOH, [<sup>35</sup>S]GSH was added, and the mixture was allowed to stand 15 min. The mixture was then derivatized with FDNB and analyzed by HPLC. Although some radiolabeled Conjugate C was detected, most of the radiolabel appearing in this region of the chromatogram eluted as Conjugate B (Chart 4). The appearance of radiolabel in Conjugate C may be attributed to S-carboxymethylation of [<sup>35</sup>S]GSH by chloroacetate present in the neutralized supernatant. The presence of radiolabel in Conjugate B indicates that the [<sup>35</sup>S]GSH substituted for unlabeled GSH in the thioester. Further, when unlabeled GSH was omitted from the original microsomal incubation, [<sup>35</sup>S]GSH radiolabel did not elute as Conjugate B. In a separate experiment, <sup>35</sup>S-labeled Conjugate B was derivatized with FDNB, the pH was adjusted to 7.5, and aliquots of the mixture were analyzed periodically by HPLC. Conjugate B underwent a pseudo-first-order decay with a half-life of approximately 3 hr (data not shown).

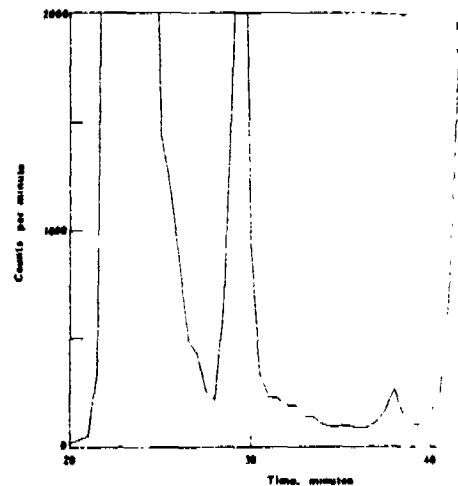


Chart 4. Substitution of [<sup>35</sup>S]GSH into unlabeled Conjugate B. Conjugate B was prepared by incubating rat liver microsomes with VDC and unlabeled GSH. After precipitation of microsomal protein and neutralization to pH 7.5, [<sup>35</sup>S]GSH (5 µCi) was added. After incubation for 30 min, the mixture was derivatized with FDNB and analyzed by ion-exchange HPLC as described in "Materials and Methods." Arrows mark the retention times of Conjugates B and C.

## DISCUSSION

Previous investigators assigned a central role to VDC oxide in the formation of covalent adducts with cellular nucleophiles (16, 25). The data in Table 1 show that 2-chloroacetic acid was considerably more reactive toward thiols than was the epoxide or other metabolites. While the epoxide was stable in aqueous buffer, it is most likely that it would also react with GSH. These data indicate that both the epoxide and acid chlorides may modify thiols despite rapid hydrolysis. 2-Chloroacetic acid and 2,2-dichloroacetaldehyde are considerably more stable in aqueous solution, and while they react more slowly with thiols, their stability may enhance their overall contribution to covalent modification of target macromolecules *in vivo*.

The importance of protein thiols as major targets for covalent modification by VDC metabolites is suggested by the data presented in Table 2. Inhibition of binding by GSH and not by protein amino groups are minor ones. The possible biological significance of modifying even a small population of amino groups cannot be discounted. The comparative chemical properties of thiol and amine nucleophiles may account for the predominance of thiol adducts. Protonation of amines at physiological pH would largely restrict the population of available nitrogen nucleophiles to those lodged in hydrophobic regions of proteins or membranes. These targets may themselves be exposed to lower concentrations of VDC metabolites. The selectivity of VDC metabolites for thiols over other nucleophiles may reflect preference of VDC metabolites for "soft" nucleophiles. The VDC metabolites, 2-chloroacetic acid, 2-chloroacetaldehyde (α-carbon), and VDC oxide, would most likely alkylate via an S<sub>N</sub>2 mechanism and would be expected to prefer the softer nucleophiles (thiols) to the somewhat harder amines.

The data in Table 2 support a role for 2,2-dichloroacetaldehyde in microsomal covalent binding. Approximately 60% of the binding was inhibited by added alcohol dehydrogenase plus NADH, indicating that the aldehyde and not the corresponding alcohol

The reactivity of VDC metabolites toward thiols is indicated by not only the influence of GSH on covalent binding but also the ability of VDC metabolites to form 3 different GSH conjugates. Previous workers have shown that the hepatotoxicity and covalent binding of VDC are potentiated *in vivo* when hepatic GSH levels are reduced by fasting or diethylmaleate pretreatment (13, 17). Other studies have shown that [ $^{14}\text{C}$ ]VDC radiolabel is excreted in the urine as sulfur-containing metabolites apparently derived from GSH conjugates (16, 18). The urinary metabolites contain secondary modifications due to renal biotransformation and enterohepatic recirculation which may mask the identities of specifically formed products. The use of microsomes and isolated hepatocytes to study the formation of GSH conjugates was intended to minimize such secondary modifications. Conjugate A which can be synthesized by adding 2,2-dichloroacetaldehyde to neutral aqueous solutions of GSH, exhibits properties expected of S-(2,2-dichloro-1-hydroxyethyl)glutathione, a thiohemiacetal of the aldehyde and GSH. The assignment of a thiohemiacetal structure, rather than a Schiff base (with the  $\alpha$ -amino group of the  $\gamma$ -glutamyl residue of GSH) is based on several considerations. Both GSH and *N*-acetylcysteine (which cannot form a Schiff base) react with 2,2-dichloroacetaldehyde at similar rates in aqueous buffer (data not shown).  $^1\text{H}$  nuclear magnetic resonance of the *N*-acetylcysteine conjugate also supported a thiohemiacetal structure. Mild acid hydrolysis of Conjugate A released 2,2-dichloroacetaldehyde. While aldehydes do not generally form stable thiohemiacetals, introduction of electron-withdrawing  $\alpha$ -substituents stabilizes thiohemiacetals formed with aldehydes and thiohemiketals formed with ketones (6).

The identity of Conjugate C with 2-(S-glutathionyl)acetate was almost immediately evident. This product is an expected metabolite and would, upon renal biotransformation, give rise to several of the reported urinary metabolites, including 2-[S-(N-acetylcysteinyl)acetate, thiodiglycollic acid, dithioglycollic acid, thiolglycollic acid, and possibly N-acetyl-S-(2-hydroxyethyl)cysteine (16, 18). Conjugate C is also the most stable of

The relative amounts of GSH:VDC metabolite conjugates differed in microsomal and hepatocyte incubations. The production of conjugates in the microsomal system is probably limited only by the amounts of metabolites generated, their stability, and the concentration of GSH. In contrast to the microsomal system, the formation of GSH:VDC metabolite conjugates in isolated hepatocytes is subject to additional factors present in whole cells. Conjugate C was the major GSH conjugate found in the hepatocytes, and Conjugate B was found at approximately one-fourth the level of Conjugate C (Table 3). Conjugate A was not found in the hepatocytes, an indication that 2,2-dichloroacetaldehyde produced in the cells is so efficiently oxidized or reduced by cellular dehydrogenases that only negligible amounts are conjugated. The relative ratio of Conjugate B to Conjugate C in hepatocytes is nearly the opposite of that seen in microsomes (Chart 1; Table 3). The reduced levels of Conjugate B in hepatocytes may be due to reduced availability of 2-chloroacetyl chloride or VDC oxide. A more attractive alternative explanation for the inversion of Conjugate B:C ratios in microsomes versus hepatocytes is a possible contribution of enzymatic hydrolysis of Conjugate B in the hepatocytes. However, the ability of Conjugate B to serve as a substrate for hepatocyte esterases is entirely speculative.

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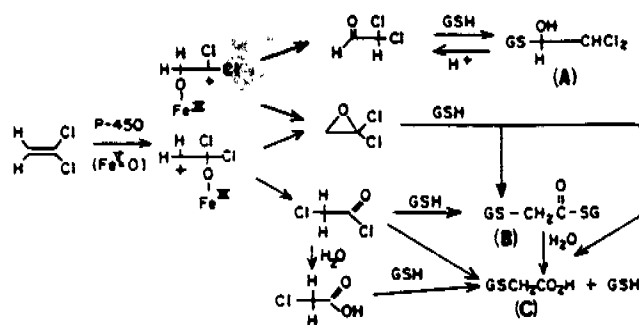


Chart 5. General scheme for oxidative and conjugative metabolism of VDC. See text for discussion.

of metabolite cell-to-cell migration has been offered as an explanation for formation of DNA:vinyl chloride metabolite adducts in hepatic nonparenchymal cells, which do not efficiently metabolize vinyl chloride (10). The covalent modification of macromolecules within target cells which do not produce VDC metabolites may be a consequence of metabolite migration.

An integrated scheme for the major oxidative and conjugative pathways of VDC metabolism is presented in Chart 5. The pathways presented represent major reactions related to covalent modification of GSH (and protein thiols). Secondary oxidative and reductive pathways for 2,2-dichloroacetaldehyde have been omitted as have possible reactions of VDC metabolites with DNA, which will be a subject of further study in this laboratory. The data presented here argue that multiple VDC metabolites participate in the covalent modification of cellular proteins and that protein thiols are major targets. Formation of GSH:VDC metabolite conjugates offers a model for VDC metabolite-protein thiol interaction that presents advantages over the isolation of amino acid adducts via protein hydrolysis. The GSH conjugates may be isolated and quantitated rapidly, and individual conjugates contain characteristic metabolite:thiol linkages. Stability of these conjugates may reasonably approximate the stability of protein thiol:metabolite adducts. Interestingly, Jaeger *et al.* (14) observed that hepatic [<sup>14</sup>C]VDC covalent binding, measured as trichloroacetic acid-insoluble radioactivity, decayed with a half-life of 2 to 3 hr. This figure closely approximates the half-life of Conjugate B. In contrast, hepatic covalent binding of acetaminophen, which apparently forms stable phenylthioether adducts with protein thiols (32), decays with a half-life of approximately 12 hr (15). These data suggest that a significant fraction of the covalent binding of VDC metabolites consists of labile adducts which are subsequently lost to hydrolysis or dissociation. The consequences of formation of such adducts have not been addressed, but it may be hypothesized that such metastable adducts play a significant role in the initiation of tumorigenic and toxic processes.

## REFERENCES

- Barbin, A., Bresil, H., Croisy, A., Jacquignon, P., Malaveille, C., Montesano, R., and Bartsch, H. Liver microsome-mediated formation of alkylating agents from vinyl chloride and vinyl bromide. *Biochem. Biophys. Res. Commun.*, 67: 596-603, 1975.
- Bartsch, H., Malaveille, C., Barbin, A., and Planche, G. Mutagenic and alkylating metabolites of haloethylenes, chlorobutadienes, and dichlorobutenes produced by rodent or human liver tissues: evidence for oxirane formation by cytochrome P-450-linked monooxygenases. *Arch. Toxicol.*, 41: 249-277, 1979.

- Bolt, H. M., Laib, R. J., and Fiser, J. G. Reactive metabolites and carcinogenicity of halogenated ethylenes. *Biochem. Pharmacol.*, 37: 1-4, 1982.
- Costa, A. K., and Ivanetich, K. M. Vinylidene chloride: its metabolism by hepatic microsomal cytochrome P-450 *in vitro*. *Biochem. Pharmacol.*, 37: 2083-2092, 1982.
- Fariss, M. W., and Reed, D. J. Measurement of glutathione and glutathione disulfide efflux from isolated rat hepatocytes. In: R. A. Harris and N. C. Conner (eds.), *Isolation, Characterization, and Use of Hepatocytes*, pp. 34-56. New York: Elsevier North-Holland Excerpta Medica, 1983.
- Field, L., and Sweetman, B. Biologically oriented organic sulfur chemistry. Reactions of thiols with highly reactive carbonyl compounds. *J. Org. Chem.*, 34: 1799-1803, 1969.
- Gold, V., and Hilton, J. The hydrolysis of acetic anhydride. Part IV. Catalysis by hydrochloric acid and the hydrolysis of acetyl chloride. *J. Chem. Soc.*, 842, 1955.
- Guengerich, F. P., Crawford, W. M., Jr., and Watanabe, P. G. Activation of vinyl chloride to covalently bound metabolites: roles of 2-chloroethylenoxide and 2-chloroacetaldehyde. *Biochemistry*, 18: 5177-5182, 1979.
- Guengerich, F. P., and Martin, M. W. Purification of cytochrome P-450 NADPH-cytochrome P-450 reductase, and epoxide hydratase from a single preparation of rat liver microsomes. *Arch. Biochem. Biophys.*, 205: 365-379, 1980.
- Guengerich, F. P., Mason, P. S., Stott, W. T., Fox, T. R., and Watanabe, P. G. Roles of 2-haloethylenoxide and 2-haloacetaldehydes derived from vinyl bromide and vinyl chloride in irreversible binding to protein and DNA. *Cancer Res.*, 41: 4391-4398, 1981.
- Guengerich, F. P., and Watanabe, P. G. Metabolism of [<sup>14</sup>C]- and [<sup>3</sup>H]-labeled vinyl chloride *in vivo* and *in vitro*. *Biochem. Pharmacol.*, 28: 589-595, 1979.
- Hanschler, D., and Bartsch, H. Metabolic activation of chlorinated ethylenes: dependence of mutagenic effect on electrophilic reactivity of the metabolite. *Arch. Toxicol.*, 38: 7-12, 1977.
- Jaeger, R. J., Conolly, R. B., and Murphy, S. D. Effect of 18 hr fast on glutathione depletion on 1,1-dichloroethylene-induced hepatotoxicity and lethality in rats. *Exp. Mol. Pathol.*, 20: 187-198, 1974.
- Jaeger, R. J., Stoner, L. G., and Coffman, L. 1,1-Dichloroethylene hepatotoxicity: proposed mechanism of action and distribution and binding of radioactivity following inhalation exposure in rats. *Environ. Health Perspect.*, 27: 113-119, 1977.
- Jollow, D. J., Mitchell, J. R., Potter, W. Z., Davis, D. C., Gillette, R. J., Brodie, B. B. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. *J. Pharmacol. Exp. Ther.*, 187: 195-202, 1973.
- Jones, B. K., and Hathway, D. E. The biological fate of vinylidene chloride. *Chem.-Biol. Interact.*, 20: 27-41, 1978.
- Lieber, D. C., and Guengerich, F. P. Olefin oxidation by cytochrome P-450: evidence for group migration in catalytic intermediates formed with vinylidene chloride and trans-1-phenyl-1-butene. *Biochemistry*, 22: 5482-5489, 1983.
- McKenna, M. J., Zampal, J. A., Madrid, E. O., Braun, W. H., and Gehring, J. Metabolism and pharmacokinetic profile of vinylidene chloride in rats following oral administration. *Toxicol. Appl. Pharmacol.*, 45: 821-835, 1978.
- Miller, E. C., and Miller, J. A. Searches for ultimate chemical carcinogens: their reactions with cellular macromolecules. *Cancer (Phila.)*, 47: 2327-2337, 1981.
- Miller, R. E., and Guengerich, F. P. Oxidation of trichloroethylene by microsomal cytochrome P-450: evidence for chlorine migration in a transition state not involving trichloroethylene oxide. *Biochemistry*, 21: 10-15, 1982.
- Miller, R. E., and Guengerich, F. P. Metabolism of trichloroethylene by isolated hepatocytes, microsomes, and reconstituted enzyme systems containing cytochrome P-450. *Cancer Res.*, 43: 1145-1152, 1983.
- Mills, G. T., West, S. B., Walsh, J. S., Wolf, F. J., and Lu, A. Y. H. C. Nitro residue formation from ronidazole, a 5-nitroimidazole. III. Studies on the mechanism of protein alkylation *in vitro*. *Chem.-Biol. Interact.*, 41: 297-311, 1982.
- Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., and Potter, D. W. High-performance liquid chromatography analysis of nanomolar levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal. Biochem.*, 100: 55-62, 1980.
- Reed, D. J., and Orrantius, S. The role of methionine in glutathione biosynthesis by isolated hepatocytes. *Biochem. Biophys. Res. Commun.*, 77: 157-164, 1977.
- Reichert, D., Werner, H. W., Metzler, M., and Hanschler, D. Molecular mechanism of 1,1-dichloroethylene toxicity: excreted metabolites reveal different pathways of reactive intermediates. *Arch. Toxicol.*, 42: 159-169, 1979.
- Reitz, R. H., Watanabe, P. G., McKenna, M. J., Quast, J. F., and Gehring, J. Effects of vinylidene chloride on DNA synthesis and DNA repair in the rat and mouse: a comparative study with dimethyl nitrosamine. *Toxicol. Appl. Pharmacol.*, 52: 357-370, 1980.
- Reynolds, E. S., Moslen, M. T., Boor, P. J., and Jaeger, R. G. 1,1-Dichloroethylene hepatotoxicity: time course of GSH changes and biochemical alterations. *Am. J. Pathol.*, 101: 331-344, 1980.
- Sedlack, J., and Lindsay, R. H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.*, 195-205, 1968.

# VDC ADDUCTS

32. Segen, P. O. Preparation of rat liver cells. II. Effects of ions and chelators on tissue dispersion. *Exp. Cell Res.*, 78: 25-30, 1973.
33. Shen, A. L., Fahl, W. E., and Jefcoate, C. R. Metabolism of benzo(a)pyrene by isolated hepatocytes and factors affecting covalent binding of benzo(a)pyrene metabolites to DNA in hepatocytes and microsomal systems. *Arch. Biochem. Biophys.*, 204: 511-523, 1980.
34. Smith, C. V., Todd, E. L., Hughes, H. L., and Mitchell, J. R. Isolation and identification of specific products of alkylation of hepatic protein *in vivo* by reactive metabolites of acetaminophen and bromobenzene. *Fed. Proc.*, 43: 381, 1984.
35. Streeter, A. J., Dahlin, D. C., Nelson, S. D., and Balke, T. A. Mechanistic studies on the covalent binding of acetaminophen to protein: evidence for cysteine residues as major sites of arylation *in vitro*. *Chem.-Biol. Interact.*, 148: 349-366, 1984.
36. Ugi, L. and Beck, F. Reaktion von Carbonsäurehalogeniden mit Wasser und Aminen. *Chem. Ber.*, 94: 1839-1850, 1961.
37. Umhauer, D. R., and Pegg, A. E. Alkylation of intracellular and extracellular DNA by dimethylnitrosamine following activation by isolated rat hepatocytes. *Cancer Res.*, 41: 3471-3474, 1981.
38. Walkin, H., Schelin, C., Tunek, A., and Jergil, B. A rapid and sensitive method for determination of covalent binding of benzo(a)pyrene to proteins. *Chem.-Biol. Interact.*, 38: 109-118, 1981.
39. Wang, P. P., Beaune, P., Kaminsky, L. S., Dannan, G. A., Kadlubar, F. F., Larrey, D., and Guengerich, F. P. Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. *Biochemistry*, 22: 5375-5383, 1983.
40. Zapata, F., Croisy, A., Barbin, A., Malaveille, C., Tomatis, L., and Bartsch, H. Carcinogenicity of chloroethylene oxide, an ultimate reactive metabolite of vinyl chloride, and bis(chloromethyl)ether after subcutaneous administration and in initiation-promotion experiments in mice. *Cancer Res.*, 40: 352-356, 1980.