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Non-Reversibility of Vinyl Chloride Carcinogenesis in Rodents*

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ABSTRACT

A review of the data obtained from various studies on carcinogenicity of vinyl chloride (VC) in rodents, particularly on the effect of dose, age, duration of exposure and potential reversibility of lesions, revealed that vinyl chloride-induced carcinogenicity in rodents was dose and time related; no recovery occurred in mice even after only 1 month of VC exposures or in rats after 6-month exposures. In addition, younger animals (2 months old) were more susceptible to VC-induced carcinogenicity than animals held for 6 or 12 months prior to exposure. Initial 6 or 12 month exposures were adequate to detect the carcinogenic potential of VC.

The above information was used as a basis for discussion on design of carcinogenicity studies. Possibility of determining the carcinogenic potential of a compound in a shorter period than the traditional 2 year studies in rodents was discussed in consideration with appropriate doses, species, age and exposure duration. Although this approach may be applicable to a strong carcinogen, it was not considered practicable in case of weak or unknown carcinogens.

INTRODUCTION

The data I am going to present today comes from three different studies. Two of these studies in which I was involved, were conducted at Mid-West Research Institute, (1, 2, 3) for the National Institute of Environmental Health Sciences (NIEHS) and the third study was conducted by NIEHS at Research Triangle Park in North Carolina by Drew *et al.* (4) in 1983. The objective of this presentation is not really to report the data on the carcinogenicity of vinyl chloride, which is well published, but the primary objective is to review the data obtained from these studies, from the point of view of study design. As we have seen, from the papers presented here yesterday and also from this morning, the current carcinogenicity bioassay model is the conventional two year studies in two species of rodents. Can we now think of a different design in which we can do these same carcinogenicity studies to determine the carcinogenic potential of the reference compound in a shorter period, possibly six months or a year? Other considerations include selection of appropriate doses, species, appropriate age, and duration of exposure. I would hope that we can look at these data from this point of view and see if we can have some discussion on these areas and how we can best utilize this information.

Vinyl chloride is a very simple chemical ($H_2C = CHCl$). It is a monomer used mainly to make polyvinyl chloride (PVC) which is an essential material in our daily life. Interest in the carcinogenicity of the vinyl chloride was raised by the reports of hepatic angiosarcomas in workers associated with PVC manufacturing (5, 6). Vinyl chloride, like many other carcinogens, is not carcinogenic without metabolic activation. It is activated by the cytochrome P-450 system into an epoxide (chloroethylene oxide) which then can alkylate the macromolecules of cells and leads to neoplastic transformation (7).

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This Symposium section is continued from Volume 11, Number 1, 1983.

FIRST STUDY

In the first study (1, 2) ~~CD mice~~ and ~~Sprague-Dawley~~ rats, were used. The age at initiation of VC exposure was two months. Thirty-six animals of each sex were used in each of control, ~~50, 250 and 1000~~ parts per million (ppm) groups. The exposure was by inhalation, six hours per day, five days per week. The housing was four to six mice per cage, and two rats per cage. They were fed pulverized or pelleted commercial diet at all times except during the exposure. Water was available at all times. Inhalation was done in cubic stainless steel chambers with a volume of 3.5 m^3. Vinyl chloride gas 99.8% purity was obtained from the Matheson Products, New Jersey. The controls were exposed to the filtered air in similar chambers. The parameters were clinical observation, hematology and blood chemistry, cytogenicity, gross and microscopic pathology and other effects. For the purposes of this presentation we will concentrate on clinical observations, particularly the mortality or survival and the pathology. Four animals of each sex per group were terminated at ~~one, two, three, six, nine~~ and at the final termination ~~at 12 months~~.

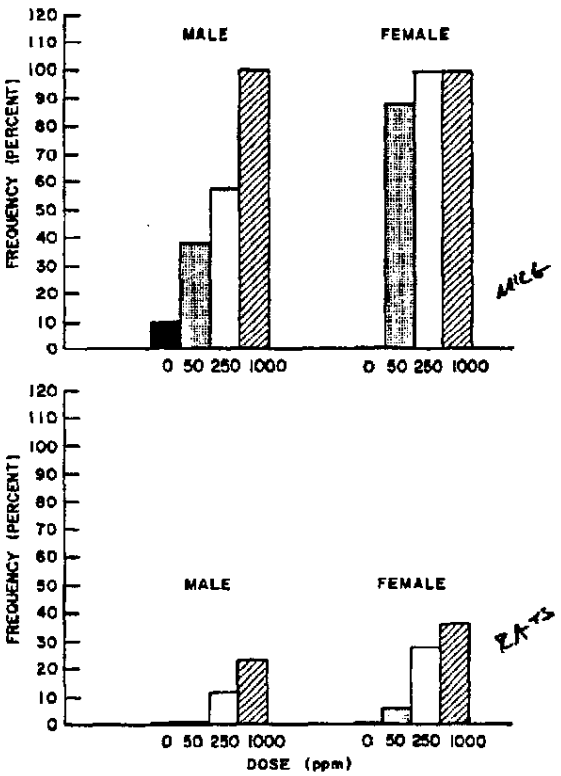

FIG. 1—Mortality in mice (*top*) and in rats (*bottom*) exposed to vinyl chloride for 12 months.

General Observations. In rats, clinical signs observed after five months were lethargy, roughened hair coat, anorexia, rapid weight loss, distended abdomens and death. The mortality in mice exposed to VC for 12 months is shown in Fig. 1. Significant dose-related mortality occurred after 6 months. All high dose mice died by the ninth month. Many deaths occurred during 10–12 months in the mid and low dose groups. Only 2 male

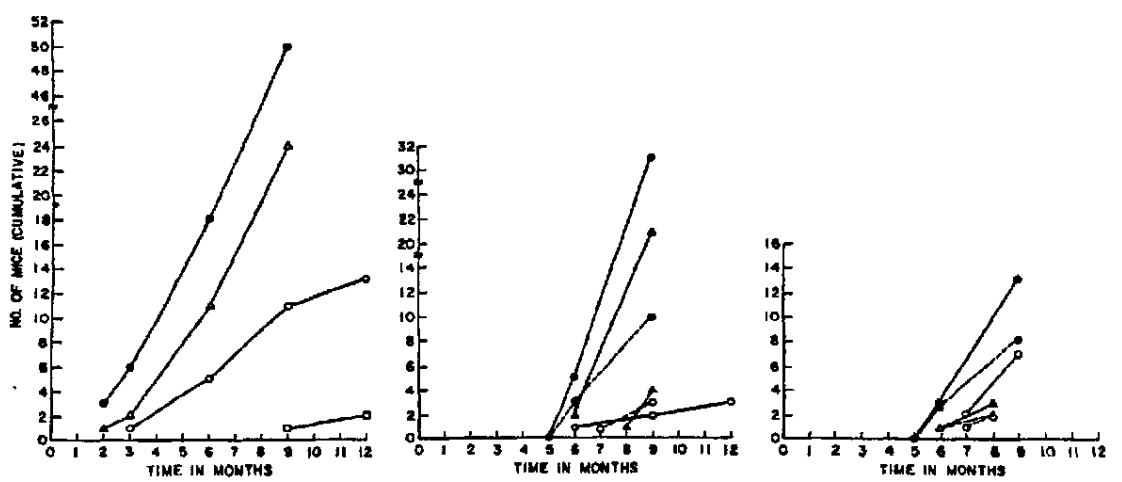

FIG. 2—Incidence of bronchiolo-alveolar adenoma in mice exposed to vinyl chloride (*left*); 72 animals per group. At *center*, the incidence of hemangiosarcoma in mice. Graphs at *center* and *right* depict similar groups and treatments as in the graph at *left*. (*Solid line*: liver; *dashed line*: other organs). *At right*, mammary gland tumor incidence (*solid line*) and lung metastasis (*dashed line*). (● = 1000 ppm; Δ = 250 ppm; ○ = 50 ppm; □ = Øppm).

mice died in the control group which were attributed to injuries from fighting. Females were more susceptible to the carcinogenicity of VC. There were no significant toxic effects other than carcinogenic effects in this study, so, all the mortality was, in fact, related to neoplasia.

Rats were relatively resistant to the effects of VC. Most of the deaths in the high and mid dose groups occurred during 8–12 months. No mortality occurred in controls or low dose males. Females were somewhat more susceptible than males (Fig. 1).

Pathology. The primary neoplasms induced by VC in mice were bronchiolo-alveolar adenoma, hemangiosarcoma of the liver, and mammary gland carcinoma with metastases to lungs. These neoplasms occurred in a dose and time related fashion as shown in Fig. 2 and included bronchiolo-alveolar adenoma, hemangiosarcoma and mammary gland tumor and metastases to lung.

In rats, hemangiosarcoma and hepatocel-

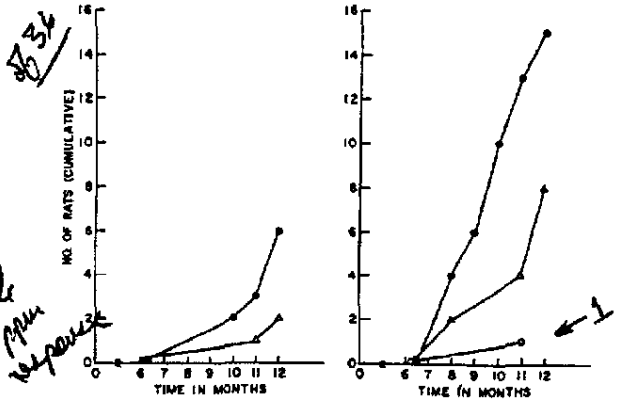

FIG. 3—Incidence of hemangiosarcoma in all tissues of male (*left*) and female (*right*) rats exposed to vinyl chloride. The symbols indicating the treatment groups are the same as in figure 2.

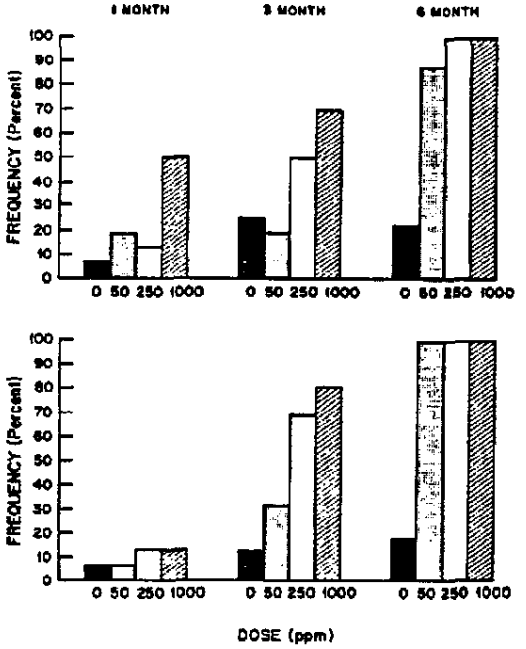

FIG. 4—Mortality in male (*top*) and female (*bottom*) mice exposed to vinyl chloride for 1, 3 or 6 months followed by recovery for 12 months.

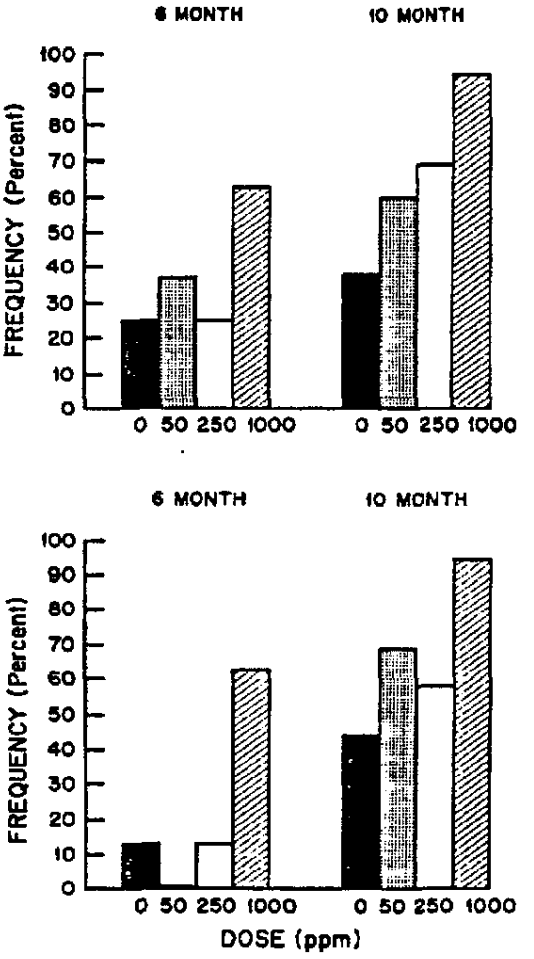

FIG. 5—Mortality in male (*top*) and female (*bottom*) rats exposed to vinyl chloride for 1, 3, 6 and 10 months followed by recovery for 12 months. Exposures of 1 and 3 months are not plotted because of no significant mortality.

lular carcinoma with metastases to the lungs occurred in a dose and time related fashion (Fig. 3).

SECOND STUDY

The second (recovery) study (3) was essentially the same design as the first one. The same strains of mice and rats were used. The exposure, all methods and parameters were similar. Eight to 28 mice of each sex were exposed for a period of 1, 3 and 6 months and four to 16 rats of each sex per group were exposed for 1, 3, 6 and 10 months. At the end of the exposure periods, all animals were maintained in their respective rooms for a 12-month follow-up observation (recovery) period.

All results including clinical observations, and pathologic findings were in line with the first study. The incidence of mortality in mice is summarized in Fig. 4. The incidence of mortality and early terminations increased with increased dose and exposure duration. Most of the mice exposed to VC died during the recovery period. Notice that even those that were just exposed for one month and then allowed to recover, did have a dose-related mortality. However, after 3 months exposure the mortality increased and in those that were exposed for 6 months, 100% mor-

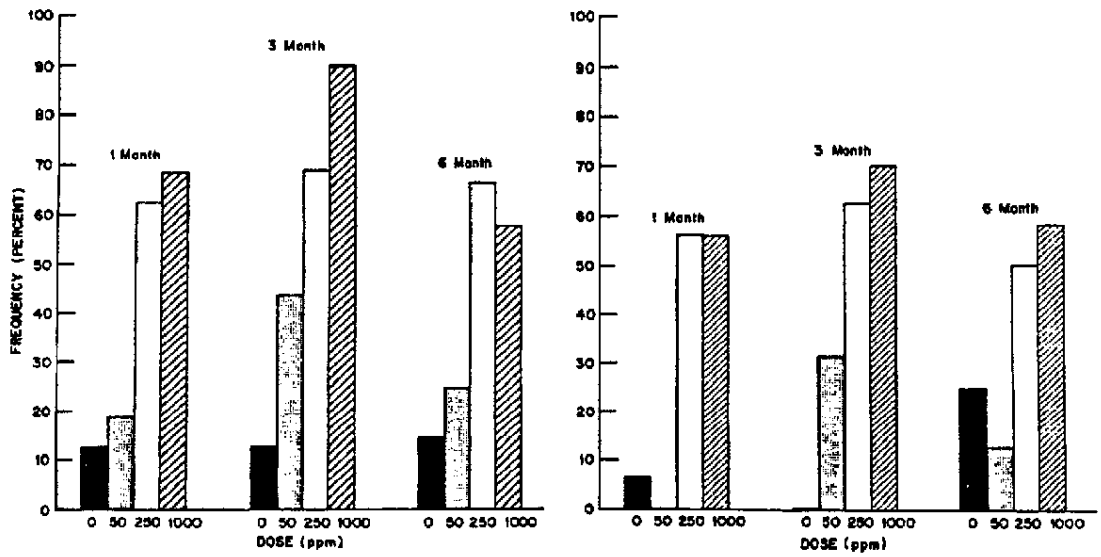

FIG. 6—Incidence of bronchiolo-alveolar adenomas in male (*left*) and female (*right*) mice, exposed to vinyl chloride for 1, 3 or 6 months and followed by 12 months of recovery period.

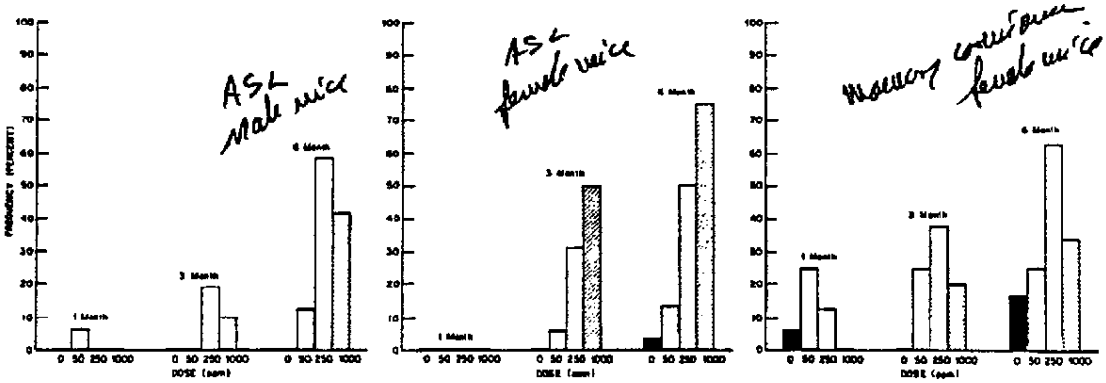

FIG. 7—Hemangiosarcoma incidence in all tissues of male (*left*) and female (*center*) mice, exposed for 1, 3 or 6 months to vinyl chloride and followed by 12 months of recovery. The graph at *right* displays the incidence of mammary gland carcinoma in female mice included in the center graph.

tality occurred in mid and high dose mice of both sexes and low dose females.

Rats were relatively resistant, however, and dose- as well as exposure-duration-related mortality occurred during the recovery period (Fig. 5). After 6 months of exposure, only the high dose rats showed significant mortality. However, after 10 months exposure, dose-related mortality occurred.

Pathology. The incidence of tumors in mice was related to the dose and duration of exposure. The incidence of various tumors is summarized in Fig. 6 and 7, including bronchiolo-alveolar adenoma hemangiosarcoma and mammary gland carcinoma. A high incidence of bronchiolo-alveolar adenoma occurred in mid and high dose groups, just after

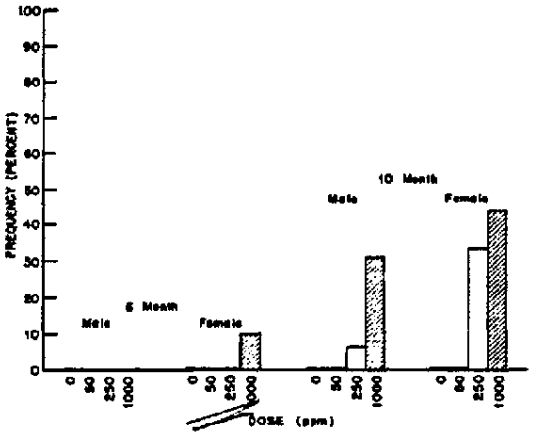

FIG. 8—Graph indicating the incidence of hemangiosarcoma in tissues from male and female rats, exposed for 6 or 10 months and observed during a 12 month recovery period.

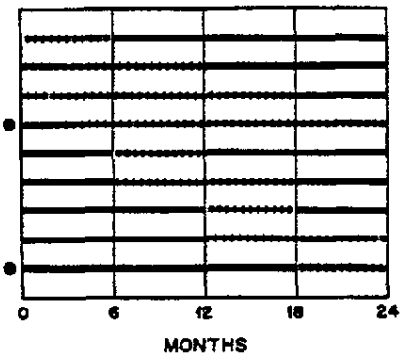

FIG. 9—Protocol design schematic of the vinyl chloride regimen used in the third study. Exposures were 16 hours/day, 5 days/week; mice received 50 ppm, rats 100 ppm and hamsters 200 ppm of vinyl chloride.

TABLE I—Mean Survival of Female Animals Throughout the Study

Exposure Period (Months)	Time (Days)			
	Fischer-344 Rats	Golden Syrian Hamsters	B6C3F1 Mice	Swiss CD-1 Mice
0	703	463	780	474
0–6	682	390	316	340
0–12	634	355	301	347
0–18	575	342	304	321
0–24	622	347	—	—
0–6	682	390	316	340
6–12	703	468	480	472
12–18	688	456	695	521
18–24	708	499	—	—
0–12	634	355	301	347
6–18	659	455	479	443
12–24	717	424	632	472

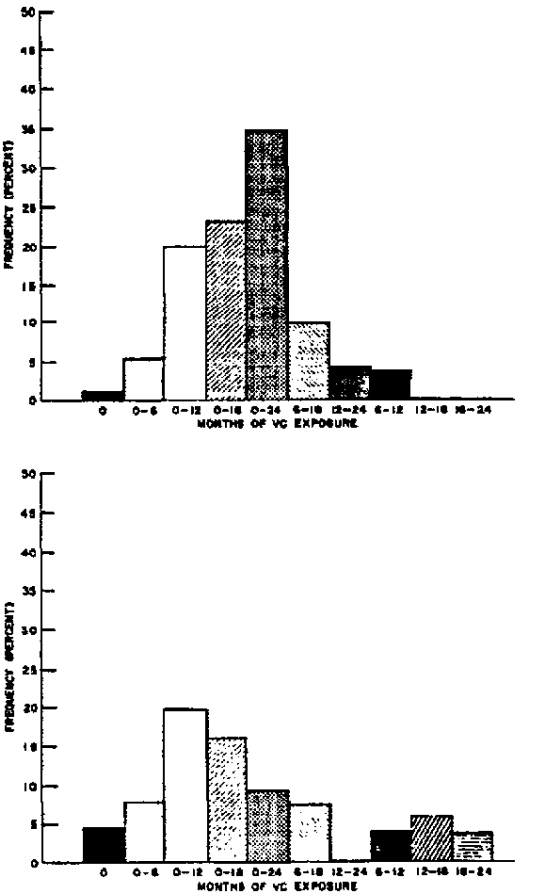

FIG. 10—Histogram describing the incidence of hemangiosarcoma in tissues from Swiss (*top*) and B6C3F1 (*bottom*) mice after vinyl chloride exposures in the third study.

one month exposure. In rats, the primary tumor seen was hemangiosarcoma in liver and other tissues, which occurred in mid and high dose groups after 10 months exposure (Fig. 8).

THIRD STUDY

The third study (4) that was conducted at NIEHS, Research Triangle Park, North Carolina, was designed to determine the effect of age and exposure duration on cancer induction. The study design is shown in Fig. 9. Female mice, rats or hamsters were exposed to 50, 100 or 200 ppm of VC, respectively, for 0, 12, 18 or 24 months and allowed to live their life span thereafter. Other groups of animals were held for 6 or 12 months and then exposed for 6 or 12 months.

The mean survival is shown in Table I. Mean survival was shortened with initial exposure at early age or longer exposure duration. The incidence of VC induced neoplasms in various species of rodents, age and exposure duration groups is shown in Fig. 10–12. The incidence of various neoplasms increased with early initial exposure and longer duration. In all three rodent species an initial 12 month exposure to VC was adequate to detect its carcinogenic potential.

Conclusions. The overall evaluation of these studies indicated that vinyl chloride-induced carcinogenicity in rodents was dose and time related. No recovery occurred in mice even after only 1-month VC exposures or in rats after 6-months exposures. Younger animals (2 months) were more susceptible to

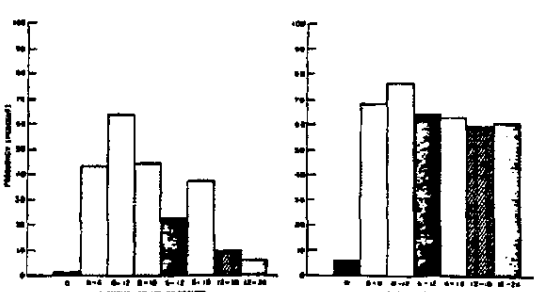

FIG. 11—Mammary gland carcinoma incidence in Swiss (*left*) and B6C3F1 (*right*) in the third study.

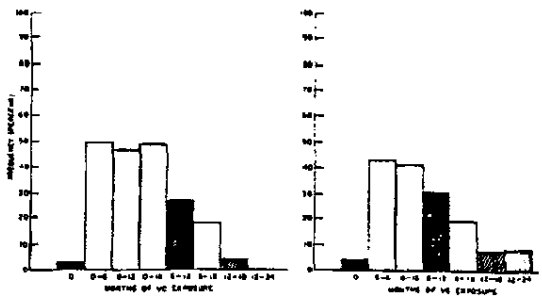

FIG. 12—Time distribution of liver hemangiosarcoma (*left*) and mammary gland carcinoma (*right*) in Fischer 344 rats exposed to vinyl chloride in the third study.

the VC-induced carcinogenicity than animals held for 6 or 12 months prior to exposure, and initial 6 or 12 month exposures were adequate to detect the carcinogenic potential of the compound.

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DISCUSSION OF THE PAPER

DR. DIENER: You have given us quite a "slug" of information and also your conclusions, but I wonder if you would tell us how this relates to any possible protocols that you would devise for a drug or chemical that, let's say, is a weak carcinogen? I don't quite know what you're driving at. What are you advocating?

DR. BHANDARI: Maybe I'll change the compound and call it a hypolipidemic compound (from the last presentation), which induces liver tumors in rodents. The only thing that mattered was the dose. At lower doses, it may take a year or more to produce tumors. But if that dose was, say, ten times the lower dose, which qualifies maximum tolerated dose or minimum toxic dose, because animals will not die because of toxicity. At this high dose if you can produce adenomas by 6 months and, hepatocellular carcinomas by 12 months, why do we have to wait for two years? Of course it is only true if we have a positive finding. Say, if we did a study—we went with the high doses and we don't see anything by one year. Does it mean that we don't need to go anymore? There we do have a problem, and we do have to go up to two years to express the full carcinogenic potential of the test compound. On the other hand if we did have a positive result as early as six months or you were convinced at that time, that there is no sense going on to two years to prove that the compound is a carcinogen, you can save the time and the resources.

DR. VESSELINOVITCH: Once you know the potency of a carcinogen, this is an important carcinogen, but if you don't know, how can you guess the time and the dose?

DR. BHANDARI: True. That brings us to my question of yesterday to Dr. Diener, where we talked about the maximum tolerated dose or the minimum toxic dose. If we can think of changing some of those criteria for MTD, such as a dose that does not effect the survival because of the toxicity for at least one year. As I mentioned in my presentation, the animals did not die of toxicity, but the survival was affected due to carcinogenicity. Generally, industry-wise, we try to stay as low as possible, because we don't want to have a carcinogenic compound. I'm advocating that if we're going to have a carcinogen, why not find out earlier? Why do we have to wait a year or two at lower dosing?

[CANCER RESEARCH 45, 186–193, January 1985]

Formation of Glutathione Conjugates by Reactive Metabolites of Vinylidene Chloride in Microsomes and Isolated Hepatocytes[1]

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ABSTRACT

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 10^6-fold faster than did 2,2-dichloroacetaldehyde or 2-chloroacetic acid. Microsomal covalent binding of [^{14}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}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}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.

INTRODUCTION

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, 6), 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 principal products of microsomal vinyl halide oxidation, which, as such, occupied a position of central importance in covalent modification of cellular macromolecules and production of more stable metabolites (3). Recent mechanistic studies in this laboratory have challenged this view. Epoxides are not obligate intermediates in vinyl halide biotransformation but are formed together with carbonyl products (haloacetaldehydes and haloacyl halides) via partitioning of a common catalytic intermediate (17, 20).

Selectivity of individual vinyl halide metabolites for reaction with particular cellular targets has been observed. Studies with vinyl chloride indicated that chloroethylene 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[4] yields VDC oxide, 2-chloroacetyl chloride, 2-chloroacetic acid, and 2,2-dichloroacetaldehyde (4, 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 radiolabel are bound to DNA isolated from rats given [^{14}C]VDC, and Reitz *et al.* (26) suggested that VDC may initiate a tumorigenic response via interaction with cellular components other than DNA. Alternative targets of possible importance in this epigenetic mechanism may include cellular proteins that regulate DNA transcription, chromatin structure, or cellular metabolic status (19). Information concerning the disposition of VDC metabolites would therefore be required to establish their roles 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 hepatic GSH by starvation or pretreatment with diethylmaleate enhanced VDC hepatotoxicity in rats (13, 27). Animals administered [^{14}C]VDC excrete radiolabel in the urine as *N*-acetylcarboxymethylcysteine, thioglycollic acid, thiodiglycollic 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 hepato-

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[4] 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.

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

...TERIALS AND METHODS

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

...DC and thiophenol were purchased from Aldrich (Milwaukee, WI); ...C was distilled before use. Chloroacetyl chloride was from Eastman ...chester, NY) and was also distilled before use. GSH, 2-(*S*-cystei...acetate, and glutathione-*S*-transferase were purchased from Sigma ...Louis, MO). This commercial glutathione-*S*-transferase preparation ...mposed largely of isozyme B and lesser amounts of other isozymes. ...se liver alcohol dehydrogenase was purchased from Boehringer-...nheim (Indianapolis, IN). Fetal calf serum was from Grand Island ...ogical Co. (Grand Island, NY).

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

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

Hepatocytes (2 to 3 × 10^6 cells in a volume of 1 ml) were incubated ...sealed 15-ml glass scintillation vials containing Fischer's medium. VDC ...as added to the suspension from a 1 M stock solution in acetone to a ...al concentration of 5 mM. [^{35}S]GSH was added to cell suspensions ...rom a 0.4 M stock solution prepared immediately before use. Hepato...ytes were incubated for 3 hr in Fischer's medium supplemented with ...5 mM [^{35}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}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 × *g* for 30 sec, and the medium was removed for analysis of GSH adducts. The pellet was resuspended in 1 ml of 10% $HClO_4$ and recentrifuged. The resulting supernatant contained intracellular 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 $ZnSO_4$ to a final concentration of 1% (w/v) or $HClO_4$ to a final concentration of 3.5% (w/v) and centrifuged at 3500 rpm for 5 min. The supernatants were then neutralized with $KHCO_3$ 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 $CHCl_3$ by VDC metabolites was monitored using 5,5′-dithiobis-2,2-dinitrobenzoic acid in aqueous acetone. Thirty μmol of each VDC metabolite were added to 30 μmol thiophenol in 6 ml $CHCl_3$ at 25°. Twenty-μl aliquots were then transferred to tubes containing 1 ml of acetone plus 200 μl of 10 mM 5,5′-dithiobis-2,2′-dinitrobenzoic acid in 85% acetone:15% H_2O. Twenty μl 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}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}C]VDC (0.03 μCi) 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 μm Spherisorb silica (Rainin, Woburn, MA), and 0.5- x 30-cm columns were slurry packed as described previously (24). Supernatants from $ZnSO_4$- or $HClO_4$-quenched microsomal incubations were neutralized with $KHCO_3$ and treated with ethanolic FDNB for 30 to 60 min before injection. Elution Solvent A contained 80% methanol and 20% H_2O (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 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 HCl and heated at 110° for 30 min. A second portion was dissolved in 6 N HCl and heated under nitrogen at 110° for 30 hr. Both samples were then neutralized with $KHCO_3$ 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 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 ... did 2-chloroacetic acid or 2,2-dichloroacetaldehyde. The rate ... reaction of GSH with 2-chloroacetyl chloride was calculated ...ing

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 $CHCl_3$ 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:$CHCl_3$, 25°, k (M^{-1} min^{-1})	GSH:200 mM potassium phosphate, pH 8.0, 37°, k_{app} (min^{-1})
VDC oxide	90 ± 20[a]	—[b]
2-Chloroacetyl chloride	>7 × 10⁴	>3.6 × 10³
2,2-Dichloroacetaldehyde	<6	0.024 ± 0.002
2-Chloroacetic acid	<6	0.005 ± 0.000

[a] Mean ± S.D. from 3 individual experiments.
[b] —, not determined.

Table 2

Microsomal covalent binding of [^{14}C]VDC

Complete incubation mixtures contained 100 mM potassium phosphate, pH 7.7 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}C]VDC (0.03 μCi). Rat liver microsomes were from untreated animals. Covalent binding of [^{14}C]VDC radiolabel to microsomal protein was assayed as described in "Materials and Methods."

System	nmol bound/mg/30 min	% of complete system
Complete	22 ± 3[a]	100
−NADPH	2 ± 1	9
+5 mM GSH	12 ± 1	55
+Alcohol dehydrogenase (0.5 mg/ml): 0.5 mM NADH	9 ± 1	41
+Alcohol dehydrogenase (0.5 mg/ml) (boiled):0.5 mM NADH	23 ± 3	105
+0.5 mM NADH	28 ± 3	127
+5 mM lysine	18 ± 1	82
Human liver 31	11, 10[b]	
Human liver 80	20, 22[b]	

[a] Mean ± S.D. from 3 individual experiments.
[b] Results of duplicate experiments.

a hydrolysis rate constant of 4 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° (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° 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 600-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}C]VDC Radiolabel in Microsomes. Rat and human liver microsomes catalyzed covalent binding of [^{14}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 binding of [^{14}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.

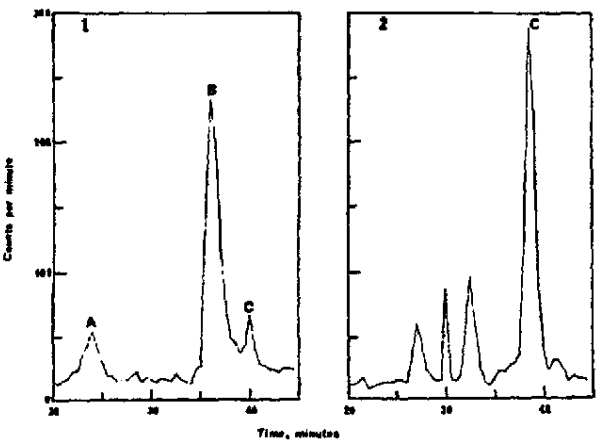

Chart 1. Ion-exchange HPLC of GSH:[^{14}C]VDC metabolite conjugates from rat liver microsomes (10 mg protein per ml) incubated with [^{14}C]VDC and GSH. Samples were derivatized with FDNB as described in "Materials and Methods." Analyses of freshly prepared incubation products (*1*) and products after 48 hr at pH 8 (*2*). The identities of the 3 initial peaks in *2* are unknown.

Production of GSH:VDC Metabolite Conjugates in Microsomes. Rat liver microsomes supplemented with GSH and [^{14}C]VDC produced 3 GSH conjugates which were analyzed as *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 rat

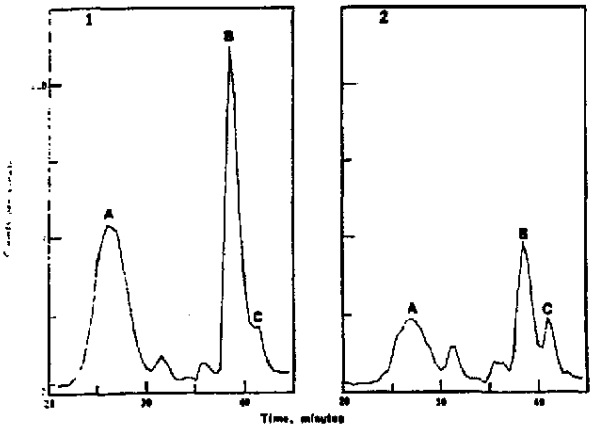

Chart 2. Ion-exchange HPLC of GSH:[^{14}C]VDC metabolite conjugates from rat liver microsomes (10 mg protein per ml) incubated with [^{14}C]VDC and GSH. Samples were derivatized with FDNB as described in "Materials and Methods." Traces of incubation products from Samples 80 (1) and 31 (2) from human 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 × 10^6 cells/ml in Fischer's medium containing 5 mM VDC. After 1 hr of incubation, cells were rapidly separated from the medium by centrifugation, and GSH:VDC metabolite conjugates in cells and medium were analyzed separately as described in "Materials and Methods."

	nmol conjugate/10^6 cells/60 min			
Conjugate	Cells[a]	Medium[a]	Medium: 5 mM GSH[b]	Medium: 10 mM GSH[b]
A	<0.4[c]	<0.4[c]	<0.4[d]	<0.4[e]
B	4.8 ± 0.8[c]	<0.3[c]	0.6[d]	3.9[e]
C	12.5 ± 3.0[c]	1.5 ± 1.1[c]	11.1[d]	45.7[e]

[a] Hepatocytes were preincubated with [^{35}S]methionine to label hepatocyte GSH as described in "Materials and Methods."
[b] Unlabeled hepatocytes were incubated in medium containing 5 or 10 mM [^{35}S]GSH (2.5 mCi/mmol).
[c] Mean (± S.D.) of 3 individual experiments.
[d] Values from a single experiment.
[e] 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}S]methionine to label cellular GSH (24) produced [^{35}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}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}S]GSH. Cells incubated in this manner accumulated 0.25 nmol [^{35}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° 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}H nuclear magnetic resonance of the *N*-acetylcysteine adduct in D_2O was also consistent with this interpretation (5.33 δ, multiplet, 1H [$Cl_2C\underline{H}$—]; 4.42 δ, triplet, 1H [—$C\underline{H}(NHCOCH_3)CO_2H$]; 3.32 δ, multiplet, 1H [—$C\underline{H}(OH)S$—]; 3.12 δ, doublet, 2H [—$SC\underline{H}_2$—]; 2.03 δ, singlet, 3H [—$NHCOC\underline{H}_3$]).

Conjugate C coeluted with authentic *N*-DNP:2-(*S*-glutathionyl)acetate. Acid hydrolysis of Conjugate C, prepared using microsomes, GSH, and [^{14}C]VDC, followed by analysis of the hydrolysate by HPLC, indicated that Conjugate C was cleaved to 2-(*S*-cysteinyl)[^{14}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

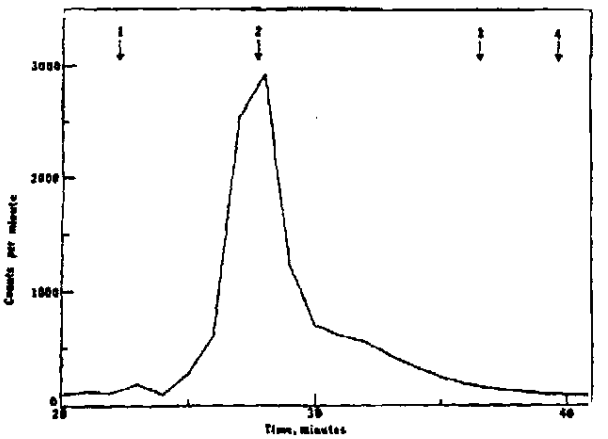

Chart 3. Ion-exchange HPLC of 2-(*S*-cysteinyl)-[^{14}C]acetate released by acid hydrolysis of ^{14}C-Conjugate C. ^{14}C-Conjugate C was produced using microsomes, GSH, and [^{14}C]VDC and collected using ion-exchange HPLC, following derivatization with FDNB. The collected, derivatized ^{14}C-Conjugate 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.

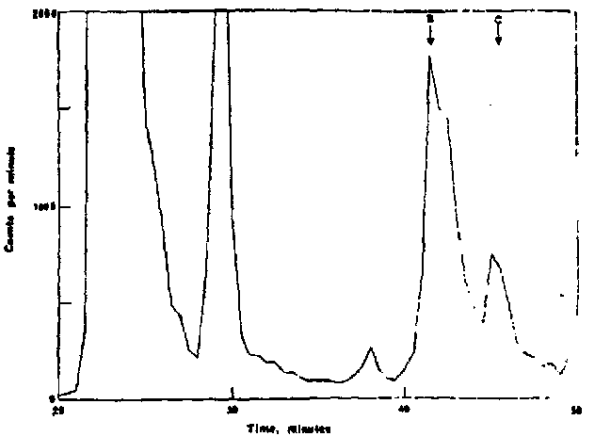

Chart 4. Substitution of [^{35}S]GSH into unlabeled Conjugate B. Unlabeled Conjugate B was prepared by incubating rat liver microsomes with VDC and unlabeled GSH. After precipitation of microsomal protein and neutralization to pH 7.5, [^{35}S]GSH (5 μCi) was added. After incubation for 30 min, the samples were 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.

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 $KHCO_3$:KOH, [^{35}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 [^{35}S]GSH by chloroacetate present in the neutralized supernatant. The presence of radiolabel in Conjugate B indicates that the [^{35}S]GSH substituted for unlabeled GSH in the thioester. Further, when unlabeled GSH was omitted from the original microsomal incubation, [^{35}S]GSH radiolabel did not elute as Conjugate B. In a separate experiment, ^{35}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).

DISCUSSION

Previous investigators assigned a central role to VDC oxide in the formation of covalent adducts with cellular nucleophiles (12, 16, 25). The data in Table 1 show that 2-chloroacetyl chloride was considerably more reactive toward thiols than was the epoxide or other metabolites. While the epoxide was not studied in aqueous buffer, it is most likely that it would also react with GSH. These data indicate that both the epoxide and acyl chloride 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 lysine would suggest that protein thiols are major targets and that protein amino groups are minor ones. The possible toxicological significance of modifying even a small population of amino targets 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 an overall preference of VDC metabolites for "soft" nucleophiles. Three of the VDC metabolites, 2-chloroacetic acid, 2-chloroacetyl chloride (α-carbon), and VDC oxide, would most likely alkylate via an S_N2 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, indiating that the aldehyde and not the corresponding alcohol

forms adducts. These observations also suggest that cytosolic and mitochondrial dehydrogenases capable of reducing or oxidizing 2,2-dichloroacetaldehyde may afford partial protection against cellular damage due to aldehyde production.

The reactivity of VDC metabolites toward thiols is indicated by only the influence of GSH on covalent binding but also the ity of VDC metabolites to form 3 different GSH conjugates. vious workers have shown that the hepatotoxicity and covalent binding of VDC are potentiated *in vivo* when hepatic GSH els are reduced by fasting or diethylmaleate pretreatment (13, . Other studies have shown that [^{14}C]VDC radiolabel is excted in the urine as sulfur-containing metabolites apparently rived from GSH conjugates (16, 18). The urinary metabolites ntain secondary modifications due to renal biotransformation d enterohepatic recirculation which may mask the identities of initially formed products. The use of microsomes and isolated patocytes to study the formation of GSH conjugates was ended to minimize such secondary modifications. Conjugate which can be synthesized by adding 2,2-dichloroacetaldehyde neutral aqueous solutions of GSH, exhibits properties expected of *S*-(2,2-dichloro-1-hydroxy)ethylglutathione, a thiohemacetal of the aldehyde and GSH. The assignment of a thiohemacetal structure, rather than a Schiff base (with the α-amino roup of the γ-glutamyl residue of GSH) is based on several onsiderations. Both GSH and *N*-acetylcysteine (which cannot orm a Schiff base) react with 2,2-dichloroacetaldehyde at similar ates in aqueous buffer (data not shown). ^{1}H nuclear magnetic esonance 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 α-substituents stabilizes thiohemiacetals formed with aldehydes and thiohemiketals formed with ketones (6).

Conjugate B is identified as *S*-(2-glutathionyl)acetylglutathione, a bisglutathionyl conjugate formally derived from one molecule of 2-chloroacetyl chloride and 2 of GSH. This conjugate is appropriately labile for a thioester and undergoes carbonyl substitution with added [^{35}S]GSH at neutral pH (Chart 4). The characteristic reactions of the acyl moiety of Conjugate B are essentially the same as those for 2-chloroacetyl chloride, *i.e.*, acylation and hydrolysis. However, the conjugate hydrolyzes much more slowly than the highly reactive acyl chloride and is thus available to a greater number of targets for a greater period of time. In this case, conjugation serves to prolong the life of a reactive VDC metabolite, albeit as a metabolite of somewhat lesser reactivity. While Conjugate B formally derives from 2-chloroacetyl chloride, attack of GSH on the methylene carbon of VDC oxide, followed by acylation of a second thiol by the resulting intermediate, would also yield Conjugate B. However, previous studies of VDC oxidation demonstrated that VDC oxide is formed in much smaller amounts than 2-chloroacetyl chloride (17) and lead to the conclusion that the epoxide is only a minor contributor to the production of Conjugate B.

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*-acetyl)cysteinyl]acetate, thiodiglycollic acid, dithioglycollic acid, thioglycollic acid, and possibly *N*-acetyl-*S*-(2-hydroxy)-ethylcysteine (16, 18). Conjugate C is also the most stable of the 3 conjugates, due most likely to the stable thioether linkage between the VDC-derived carbon and cysteine sulfur. Conjugate C may be formed by the direct carboxymethylation of GSH by 2-chloroacetate, or by alkylation of GSH by 2-chloroacetyl chloride or VDC oxide, followed by hydrolysis of the intermediate product. Conjugate B hydrolysis also may provide a significant source of Conjugate C.

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 speculatory.

Hepatocytes that were incubated with VDC released measurable amounts of Conjugate C but not Conjugates A or B into the medium. The extracellular conjugate in ^{35}S-prelabeled cells may represent conjugate efflux from the cells or conjugate formed outside the cells by VDC metabolites that cross the plasma membrane to react with labeled GSH released into the medium. To determine the extent to which the latter process occurred, unlabeled cells and VDC were incubated in medium containing [^{35}C]GSH. Because hepatocytes cannot accumulate GSH from the medium, only extracellularly formed conjugates contain radiolabel. Labeled Conjugates B and C were recovered from the medium (Table 3), demonstrating extracellular formation of both conjugates. Formation of extracellular conjugates increased with increasing medium GSH content. Cells that were incubated without added GSH would release approximately 2.5 nmol/hr/10^6 cells into the medium (5). Assuming all glutathione efflux as GSH, the maximum amount of GSH present outside the cells during a 1-hr incubation would be 5 to 10 μM. In view of the fact that the levels of extracellularly formed conjugates with 5 mM medium [^{35}S]GSH were approaching the limits of detection, it is likely that levels of extracellularly formed conjugates in ^{35}S-prelabeled cells were substantially below detection limits. The conjugates observed in the medium of the ^{35}S-prelabeled cells therefore represented efflux of Conjugate C from the cells. Extracellular formation of Conjugate B demonstrates that 2-chloroacetyl chloride and VDC oxide are stable enough to migrate a considerable distance through cells and perhaps within a tissue. This phenomenon has been demonstrated previously for metabolites of benzo(a)pyrene (30), dimethylnitrosamine (34), vinyl chloride (10), and trichloroethylene (21). The phenomenon

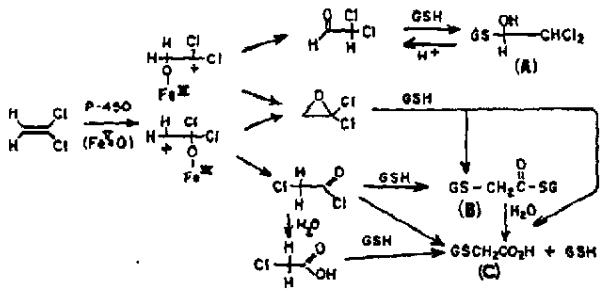

Chart 5. General scheme for oxidative and conjugative metabolism of VDC. See text for discussion.

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 [^{14}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.

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