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Hepatic Macromolecular Binding Following Exposure to Vinyl Chloride[1]

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Hepatic Macromolecular Binding Following Exposure to Vinyl Chloride. WATANABE, P. G., ZEMPEL, J. A., PEGG, D. G., AND GEHRING, P. J. (1978). *Toxicol. Appl. Pharmacol.*, 44, 571–579. Covalent binding of radioactivity to hepatic macromolecules in rats exposed to ^{14}C-labeled vinyl chloride (VC) was studied to determine if VC-induced carcinogenesis may be related to electrophilic alkylation of macromolecules *in vivo*. Male Sprague–Dawley rats were exposed to 1, 10, 25, 50, 100, 250, 500, 1000, or 5000 ppm of [^{14}C]VC for 6 hr. Following exposure, radioactivity covalently bound to hepatic macromolecules and purified nucleic acids (RNA, DNA) was determined. The total amount of [^{14}C]VC metabolized and hepatic glutathione (GSH) content were also determined. The total amount of radioactivity bound to macromolecules in the liver did not increase proportionately to the increase in the exposure concentration of VC. A disproportionate decrease in macromolecular binding was observed as the concentration of VC increased. The covalent binding to hepatic macromolecules was related to the amount of VC metabolized. At exposures greater than 50 ppm, the amount of ^{14}C bound to macromolecules in the liver correlates with induction of hepatic angiosarcoma. There was no detectable binding of radioactivity to either DNA or RNA in the liver. Hepatic glutathione content was significantly depressed only at exposure concentrations greater than 100 ppm.

Considerable effort has been devoted to research on vinyl chloride (VC) since it was demonstrated to be carcinogenic in man and animals (Creech and Johnson, 1974; Maltoni and Lefemine, 1975). The concept of a reactive metabolite of VC being responsible for the carcinogenic activity (Hefner *et al.*, 1975; Van Duuren, 1975) is supported by evidence of enhanced mutagenic activity of VC to bacteria in the presence of microsomal enzyme-activating systems (Bartsh *et al.*, 1975; Malavielle *et al.*, 1975; Rannug *et al.*, 1974). Metabolites of VC have been identified in the urine of rats as conjugates of cysteine (Green and Hathway, 1975; Watanabe *et al.*, 1976a), suggesting that VC is biotransformed to electrophilic metabolites and that the primary detoxification mechanism for these metabolites is conjugation with hepatic glutathione prior to excretion. Complementary to these data was elucidation of a dose-related reduction of hepatic glutathione in rats exposed to 50 to 2000 ppm of VC for 7 hr (Watanabe *et al.*, 1976b). It was therefore hypothesized that reactive metabolites formed during exposure to low levels of VC (less than 50 ppm) will be readily detoxified

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by reaction with glutathione. However, as the exposure is increased, detoxification will be impaired by the reduction of hepatic glutathione. This will lead to an increase in the level of reactive metabolites and result in an increased reaction of these metabolites with intracellular macromolecules.

Chemical carcinogenesis has been attributed to the reaction of electrophilic metabolites with intracellular macromolecules (Miller and Miller, 1971). Recent reports have demonstrated that liver microsomal enzymes *in vitro* form reactive metabolites from VC which bind to the microsomes (Kappus *et al.*, 1975), protein sulfhydryl groups, RNA (Bolt *et al.*, 1975), and adenosine of DNA (Barbin *et al.*, 1975).

In addition to reduced hepatic glutathione leading to an increase in the binding of reactive metabolites of VC with macromolecules, such an effect may also be associated with other dose-dependent alterations in the fate of VC in the body. The dose dependence of the fate of VC has been elucidated kinetically and attributed to saturable metabolic pathways (Hefner *et al.*, 1975; Green and Hathway, 1975; Watanabe *et al.*, 1976a,c).

In vivo studies are needed to determine whether dose-dependent disproportionate increases in the macromolecular binding of reactive metabolites of VC with increasing exposure concentration may be associated with toxicity and carcinogenicity. Thus, the objective of this study was to characterize the binding of VC to hepatic macromolecules and nucleic acids following exposure to various concentrations of ^{14}C-labeled VC.

METHODS

Materials. ^{14}C-labeled VC was synthesized from [1,2-^{14}C]1,2-dichloroethane (New England Nuclear, Lot Nos. 819–221 and 819–292, 5.0 and 4.8 mCi/mmol, respectively) immediately prior to use (Wagner and Muelder, 1975). Each new batch of [1,2-^{14}C]dichloroethane was analyzed and yielded [^{14}C]VC with a radiochemical purity of 95%. The synthesized [^{14}C]VC has been shown repeatedly in our laboratory to be 95 to 96% radiochemically pure (Wagner *et al.*, 1975). Thus, while each synthesis of [^{14}C]VC prior to each experiment was not analyzed, it was assumed to be of the same purity. Furthermore, no indication during a synthesis which utilizes a gas chromatographic separation suggested that any deviation from previous syntheses had occurred. Nonlabeled VC (Matheson Gas Products) of 99.9% minimum purity was mixed with the [^{14}C]material to obtain the desired specific activity. Typically 40 ml of the [^{14}C]VC–helium gas mixture was injected into a 5 to 10-liter Saran bag (Anspec, Inc.) containing the desired quantity of nonlabeled VC.

Animals. Male Sprague–Dawley rats (Spartan Research Laboratory) weighing 220 to 250 g were used throughout the study. All animals were housed in rooms in which a constant humidity, temperature, and a 12-hr light–dark cycle (7 AM–7 PM, EST) were maintained. Food and water were provided *ad libitum* except during exposure. Groups of rats (three to six animals per group) were exposed to [^{14}C]VC (treated) or room air (controls) for 6 hr between 9:00 AM and 4:00 PM (EST). An additional group of five rats pretreated with phenobarbital (80 mg/kg/day, ip, 3 days prior to exposure) were exposed at the 100-ppm level.

Exposure. Exposures were conducted in 30-liter glass inhalation chambers. ^{14}C-labeled VC was metered into the chamber air flow (~6 liters/min) with a dual syringe

pump. The analytical concentration of VC was monitored continuously by recirculating a fraction of the chamber atmosphere through an infrared spectrophotometer (Wilks) at a wavelength 10.6 μm. In addition, samples (1 ml) of the chamber atmosphere were analyzed at approximately hourly intervals during the exposure by gas chromatography (Watanabe *et al.*, 1976c). At corresponding times, the ^{14}C activity was determined by bubbling 1-ml aliquots of the chamber atmosphere into a scintillation solution containing a mixture of Conciffuor (Mallinckrodt Chemical), 2-methoxyethanol, toluene (6:11:83). The radioactivity was determined by counting in a Mark II or Mark III liquid scintillation spectrometer (Searle Analytic, Inc.).

The target concentrations of VC were 1, 10, 25, 50, 100, 250, 500, 1000, and 5000 ppm. The respective mean analytical concentrations measured by gas chromatography were 1.4 ± 0.3 (SD), 9.3 ± 0.2, 24.7 ± 1.4, 51 ± 2, 109 ± 23, 250 ± 3, 511 ± 11, 1020 ± 13, and 4600 ± 311. The respective specific activities were 132,000, 4801, 3170, 2528, 1750, 837, 217, 301, and 50 dpm/μg of VC. The inhalation chamber was operated in a laboratory fume hood to prevent contamination of the working environment. After transit through the inhalation chamber the [^{14}C]VC was adsorbed on activated charcoal. The charcoal traps were disposed of as radioactive waste according to standard regulations.

Procedure. Following the 6-hr exposure to various concentrations of [^{14}C]VC (1–5000 ppm), the rats were killed immediately by a blow to the head. An aliquot of liver was sampled and used for determining hepatic nonprotein sulfhydryl content by a modification of the method of Sedlak and Lindsay (1968). The remaining liver was frozen immediately on dry ice and stored at −20°C until analyzed. The carcass was analyzed for total radioactivity as described previously (Watanabe *et al.*, 1976a). Previous studies have shown that only a small percentage of radioactivity (<12%) is excreted as metabolites other than [^{14}C]VC during 72 hr following a 6-hr inhalation exposure (Watanabe *et al.*, 1976c). The large proportion of the 12% is comprised of $^{14}CO_2$ excretion 72 hr after exposure. Furthermore, very little urine is excreted during the exposure period. Thus, the nonvolatile radioactivity determined in the tissue immediately after exposure is a good estimate of the total amount of metabolized VC. Macromolecular binding of ^{14}C-labeled VC to hepatic tissue (protein, nucleic acids, and lipid) was determined by the method of Jollow *et al.* (1973). The trichloroacetic acid-precipitable material following exhaustive solvent extraction was digested in 1 N KOH and the radioactivity was determined by liquid scintillation spectrometry. Hepatic nucleic acids (DNA and RNA) were isolated from the 1-, 100-, 250-, and 1000-ppm exposure groups and radioactivity was determined by direct counting of the aqueous fractions by liquid scintillation spectrometry (see below).

Isolation of nucleic acids. RNA and DNA were isolated from rat liver by modification of the techniques described by Okuliara (1970) and Irving and Veazey (1963). Approximately 10 g of frozen tissue was thawed slowly and homogenized in 50 ml of 0.15 M sodium chloride–0.04 M ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0). Sodium dodecyl sulphate (1 g) was added, followed by 1 vol of 90% phenol solution. The mixture was mechanically stirred for 30 min at room temperature and the phenol and water phases were separated by centrifugation. The water phase was collected and sodium acetate was added to a final concentration of 2%. Nucleic acids were precipitated using 1 vol of 95% ethanol and spooled onto a glass rod. The

precipitate was washed with ethanol and dissolved in 20 ml of 0.015 M sodium chloride–0.0015 M sodium citrate buffer (pH 7.0). RNA was precipitated by adding 1 vol of ice-cold 6 M potassium acetate (pH 7.5) and removed by centrifugation. DNA was precipitated from the supernatant by adding 2 vol of 95% ethanol. The resulting crude DNA pellet was dissolved in 20 ml of 0.015 M sodium chloride–0.0015 M sodium citrate buffer (pH 7.0) and the solution was centrifuged at 100,000 *g* for 60 min at 2°C to remove glycogen. RNAase (3 mg/50 ml) was added to the supernatant and the solution was incubated at 37°C for 30 min. One volume of phenol saturated with 0.15 M sodium chloride–0.015 M sodium citrate (pH 7.0) was added and the mixture was stirred for 30 min at room temperature. The aqueous phase was extracted with ether and DNA was precipitated by adding sodium acetate (4 g/100 ml) and 2 vol of 2-ethoxyethanol. The precipitate was redissolved in 10 ml of 0.015 M sodium chloride–0.0015 M sodium citrate at 2°C. The final DNA precipitation was accomplished using 5 ml of ice-cold isopropanol. RNA and DNA pellets were dried at 2°C under reduced pressure and weighed. RNA and DNA were quantified by the orcinol and diphenylamine reactions, respectively (Keleti and Lederer, 1974).

RESULTS

Hepatic macromolecular binding, hepatic nonprotein sulfhydryl content (primarily glutathione, GSH), and the total amount of VC metabolized following various exposure concentrations are summarized in Table 1 and presented graphically in Figure 1. Covalent binding to hepatic macromolecules plotted as a function of the log of the exposure concentration was triphasic and best represented by a sigmoid curve. The linear portion of the binding curve extended from exposure concentrations of approximately 50 to 250 ppm. The lower inflection point appeared to lie between 25 and 50 ppm. The metabolism of VC and binding approach a plateau at concentrations exceeding 250 ppm.

Hepatic macromolecular binding correlated well with the total amount of VC metabolized as evidenced by a lack of any obvious trend in the ratio of bound VC versus total metabolized VC ($B/A \times 100$, Table 1). This point is further substantiated by the constant fraction of bound versus total radioactivity in the liver with increasing exposures. Although the value for total metabolism appears low for the 1000 ppm exposure, a corresponding reduction in macromolecular binding was not observed. The apparent discrepancy between total metabolism of VC at 500 and 1000 ppm is not understood fully, but it may be due to differences in respiratory parameters causing differences in the uptake of VC.

Hepatic nonprotein sulfhydryl content (primarily GSH) was not depressed significantly at 1, 10, 25, or 50 ppm. Only at concentrations of 100 ppm or greater was a dose-related depression of hepatic GSH evident. Metabolism of VC was not increased in rats exposed to 100 ppm of VC after pretreatment with phenobarbital. Macromolecular binding, however, was increased markedly when compared to nonpretreated animals.

Isolation of RNA and DNA by a nondigestive procedure from the liver of animals exposed to 1, 100, 250, and 1000 ppm of VC failed to reveal any detectable radioactivity. The sensitivity for detecting the radioactivity in DNA and RNA varied at

TABLE 1

TOTAL METABOLISM, HEPATIC MACROMOLECULAR BINDING, AND HEPATIC GLUTATHIONE (GSH) CONCENTRATIONS FOLLOWING INHALATION EXPOSURE (6 HR) TO VINYL CHLORIDE (VC)[a]

Nominal concentration	*A* (μg of VC equivalents metabolized)	*B* (μg of VC equivalents bound per g of protein)	*B/A* × 100	Hepatic GSH (% control)	Percentage of total ^{14}C activity in liver bound to macromolecules
1	29.8 ± 3.2	0.5 ± 0.08	1.78 ± 0.48	104	20 ± 3
10	242 ± 26	3.3 ± 0.2	1.38 ± 0.36	89	21 ± 2
25	557 ± 42	12.2 ± 4.0	2.15 ± 0.60	93	21 ± 2
50	1181 ± 93	23.3 ± 3.4	1.99 ± 0.36	94	20 ± 2
100	2406 ± 173	47.6 ± 4.8	1.99 ± 0.26	81[b]	25 ± 2
250	3826 ± 345	89.6 ± 12.3	2.35 ± 0.28	70[b]	22 ± 2
500	6263 ± 355	98.8 ± 5.0	1.58 ± 0.20	60[b]	25 ± 3
1000	4257 ± 765	106.8 ± 22.2	2.55 ± 0.58	51[b]	22 ± 2
5000	9255 ± 1,467	113.5 ± 10.4	1.12 ± 0.13	39[b]	22 ± 3
Pretreatment with phenobarbital[c]					
100	2160 ± 166	80.0 ± 23.9	3.70 ± 0.82		39 ± 3

[a] Means ± SD.
[b] Statistically different from controls, Student's *t* test ($p < 0.05$).
[c] Rats were injected ip with sodium phenobarbital, 80 mg/kg for 3 days prior to exposure.

each exposure level because the specific activity of the [^{14}C]VC varied with exposure and the different amounts of DNA and RNA analyzed. The sensitivity limits for the 1-, 100-, 250-, and 1000-ppm exposures were 0.007, 0.051, 0.108, and 0.30 μg of VC equivalents/mg of DNA and 0.0001, 0.009, 0.018, and 0.05 μg of VC equivalents/mg of RNA.

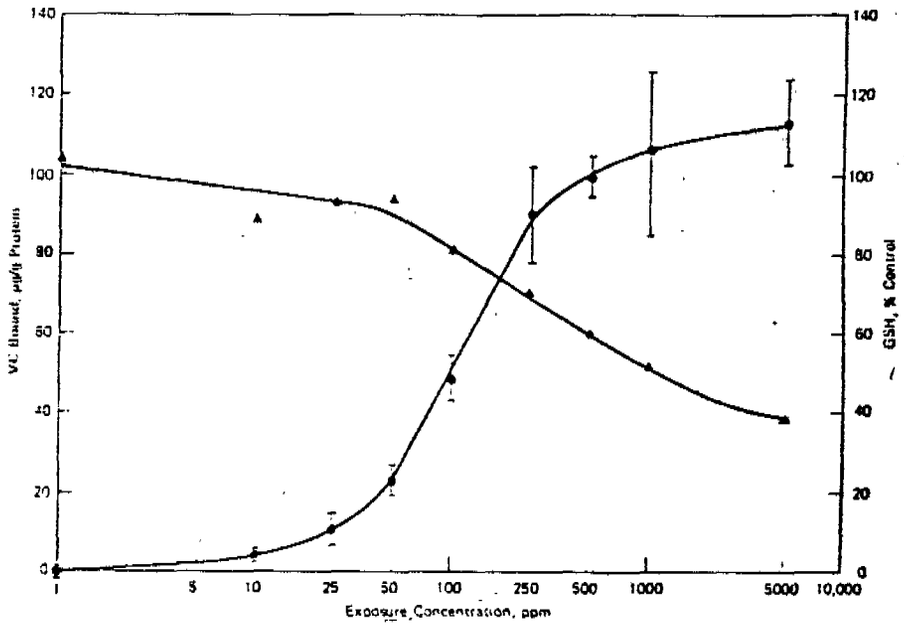

FIG. 1. Hepatic macromolecular binding and glutathione (GSH) depression expressed as a function of the logarithm of the exposure concentration to vinyl chloride (VC). Macromolecular binding (●) is expressed as microgram equivalents of VC bound per gram of protein (mean ± SD) GSH (▲) is expressed as percentage of control.

DISCUSSION

The results indicated that the total amount of radioactivity bound to macromolecules in the liver did not increase proportionately to the increase in the exposure concentration of VC. Macromolecular binding was related directly to the total amount of VC which was metabolized over exposure concentrations ranging from 1 to 5000 ppm of VC. Macromolecular binding plotted as a function of the log of the exposure concentration gave a sigmoid-shaped curve. The linear portion of the curve is bounded by low and high inflection points below 50 and above 250 ppm, respectively. Above 500 ppm binding appeared to plateau. Below 100 ppm binding was approximately proportional to the increase in exposure.

It is particularly significant that available data (Maltoni, 1975) indicate that the percentage induction of hepatic angiosarcoma in rats is linear between 50 and 500 ppm

when expressed as the log of the exposure concentration. Above 2500 ppm tumor incidence is constant. This correlates with the plateau effect observed in total metabolism and hepatic macromolecular binding above 500 ppm in the present study. One mechanism of chemical carcinogenesis is believed to be reaction of electrophilic metabolites with intracellular macromolecules (Miller and Miller, 1971). The covalent binding to hepatic macromolecules reflects the activation of VC to reactive metabolites. Therefore, both total metabolism of VC and covalent binding appear to correlate with the induction of hepatic angiosarcoma in rats exposed to concentrations greater than 50 ppm.

The toxicologic significance of the metabolism and covalent binding of VC at levels below 50 ppm is not clear. Deviation in these parameters from the log–linear relationship at higher levels indicates that the carcinogenic response of the population may be changed at lower level exposures. However, this hypothesis cannot be validated until the results of carcinogenesis bioassays currently being conducted at 25, 10, and 1 ppm become available.

The reactive metabolites of VC are detoxified presumably by reaction with GSH (Watanabe *et al.*, 1976b). Therefore, it is important that significant depression of hepatic GSH was dose related only at levels of 100 ppm and greater. This is consistent with previous studies which showed that a single 50 ppm exposure to VC was in the threshold zone for depression of hepatic GSH (Watanabe *et al.*, 1976b). This suggests that carcinogenicity of VC is related to the decreased ability to detoxify the reactive metabolites of VC.

Covalent binding of radioactivity to isolated nucleic acids (both RNA and DNA) was not detectable in any of the exposure groups tested. The detection limit in all groups was sufficient to detect the ^{14}C activity if it were equally distributed by weight throughout the components of the liver. Therefore, it was concluded that VC does not preferentially react with intracellular nucleic acids. In contrast to this, initial studies by Bolt *et al.* (1976a) reported covalent binding of radioactivity to DNA and RNA following a 5-hr static exposure to 145 ppm of [^{14}C]VC. The specific activity used by Bolt *et al.* (1976a) was higher than that used in our study and this may account for the difference in the two observations. However, more recently it has been reported by this group (Laib and Bolt, 1977) that greater than 95% of the radioactivity associated with RNA following inhalation exposure in rats to 1300 ppm of [^{14}C]VC for 5 hr is due to one carbon fragment incorporation of ^{14}C into the native nucleotides.

Both our results and those of Bolt *et al.* (1976a) confirm the conclusion that VC does not preferentially react with hepatic nucleic acids. It is important to emphasize that the methodologies employed only detect covalent binding and exclude any other more subtle interactions. Therefore, a very small degree of covalent binding to nucleic acids does not exclude the possibility of other interactions which result in the loss of the ability to control cellular replication.

Pretreatment with phenobarbital did not increase the total metabolism of VC. Bolt *et al.* (1976b) have reported similar data showing that phenobarbital pretreatment failed to stimulate the uptake of VC following a static inhalation exposure. Although total metabolism was not affected by phenobarbital, the macromolecular binding was increased markedly. The true significance of the increased binding is difficult to interpret since phenobarbital increases total protein in the liver, and the increase in

binding may reflect a nonspecific interaction merely due to an increase in the available protein binding sites.

In summary, the results of the studies reported herein do not associate the carcinogenic effect of VC with a disproportionate increase in binding of electrophilic metabolites of VC to hepatic macromolecules as the exposure concentration is increased. Even more significantly, there was no evidence for any preferential binding of electrophilic metabolites to nucleic acids of hepatocytes. This suggests that the carcinogenic activity of VC may not be associated directly with this commonly accepted mechanism for carcinogenesis. Before excluding this mechanism entirely, additional experiments are needed to show whether binding to nucleic acids may occur after repeated exposure since repeated exposure may induce preferentially alternate metabolic pathways. Another aspect relating to this is whether the administration of phenobarbital may enhance binding to nucleic acids. This is important because phenobarbital does increase the hepatic macromolecular binding of ^{14}C activity to macromolecules *in toto* in rats exposed to 100 ppm even though it did not increase the total amount of VC metabolized. These aspects are being explored.

Even more important, before excluding alkylation of nucleic acids as the mechanism for VC-induced carcinogenesis, is the need to determine the absence of such activity in target tissue rather than hepatocytes. Essentially all studies of metabolic and clinical parameters to date have been either conducted on or related to the hepatocyte. The hepatocytes may constitute primarily a means for detoxification since they are not particularly susceptible to VC induced toxicity. Toxicity may be induced in tissues with smaller capacity to detoxify the reactive metabolites of VC. The induction of tumors of the nervous system by ethylnitrosourea has been correlated with the persistence of *O*-6-ethylguanine in the nervous system (Goth and Rajewsky, 1974). Although other metabolizing organs such as the liver produce *O*-6-ethylguanine, the turnover rate of DNA mediated by repair mechanisms is sufficient to prevent induction of cancer. Likewise, the mechanism of carcinogenesis of VC may be due to an inability of the target tissue, in this case endothelium, to repair lesions whether the reaction be with nucleic acids or critical proteins.

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