

INTRODUCTION

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. Complimentary to these data was elucidation of a dose-related reduction of hepatic glutathione in rats exposed to 50-2000 ppm VC for 7 hours (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 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 electrophiles 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 dependency of the fate of VC has been elucidated kinetically and attributed to saturable metabolic pathways (Hefner, et al., 1975; Green and Hathway, 1975; and 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 ¹⁴C-labeled VC.

METHODS

Material. ^{14}C -labeled VC was synthesized from (1,2- ^{14}C) 1,2-dichloroethane (New England Nuclear, Lot #819-221 and 819-292, 5.0 and 4.8 mCi/mmol, respectively) immediately prior to use (Wagner and Muelder, 1975). The synthesized ^{14}C -VC has been reported to be 95-96% radiochemically pure (Wagner, et al., 1975). Non-labeled 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 5-10 liter Saran bag (Ansbec, Inc.) containing the desired quantity of non-labeled VC.

Animals. Male Sprague-Dawley rats (Spartan Research Laboratory) weighing 220-250 g were used throughout the study. All animals were housed in rooms in which a constant humidity, temperature and a 12 hour light-dark cycle (7 AM - 7 PM, EST) were maintained. Food and water were provided ad libitum except during exposure. Exposures were conducted between 9:00 AM and 4:00 PM. Groups of rats (3-6) were exposed to 1, 10, 25, 50, 100, 250, 500, 1000, or 5000 ppm ^{14}C -VC for 6 hours. Control animals used for hepatic nonprotein sulfhydryl determinations (5/group) were exposed concomitantly to room air. An additional group of 5 rats pretreated with phenobarbital (80 mg/kg/day, ip, 3 days prior to exposure) were exposed at the 100 ppm level.

Exposure. The rats were exposed under dynamic conditions to varying concentrations of ^{14}C -VC (treated) or room air (control) in 30 l glass inhalation chambers. ^{14}C -labeled VC was metered into the chamber air flow (~6 l/min) with a dual syringe pump. The nominal concentration of VC was the ratio of the rate at which the VC gas was dispersed to the total chamber air flow. 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 μ . In addition, samples (1 ml) of the chamber atmosphere were analyzed at approximate hour 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 Concifluor (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 nominal 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 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 hour exposure to varying 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). The radioactivity determined in the tissue and carcass was non-volatile, therefore this radioactivity represented the total amount of metabolized VC. Protein binding of ^{14}C -labeled VC to hepatic tissue was determined by the method of Jollow, et al., (1973). 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

Okuiiara (1970) and Irving and Veazey (1968). Approximately 10 g frozen tissue was thawed slowly and homogenized in 50 ml 0.15M sodium chloride - 0.04M ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0). Sodium dodecyl sulphate (1 g) was added, followed by 1 volume 90% phenol solution. The mixture was mechanically stirred for 30 minutes at room temperature and the phenol and water phases separated by centrifugation. The water phase was collected and sodium acetate added to a final concentration of 2%. Nucleic acids were precipitated using 1 volume 95% ethanol and spooled on a glass rod. The precipitate was washed with ethanol and dissolved in 20 ml 0.015M sodium chloride - 0.0015M sodium citrate buffer (pH 7.0). RNA was precipitated by addition of 1 volume ice cold 6M potassium acetate (pH 7.5) and removed by centrifugation. DNA was precipitated from the supernatant by addition of 2 volumes 95% ethanol. The resulting crude DNA pellet was dissolved in 20 ml 0.015M sodium chloride-0.0015M sodium citrate buffer (pH 7.0) and the solution centrifuged at 100,000 x g for 60 minutes at 2°C to remove glycogen. RNAase (3 mg/50 ml) was added to the supernatant and the solution incubated at 37°C for 30 minutes. One volume of phenol saturated with 0.15M sodium chloride-0.015M sodium citrate (pH 7.0) was added and the mixture stirred for 30 minutes at room temperature. The aqueous phase was extracted with ether and DNA was

precipitated by addition of sodium acetate (4 g/100 ml) and 2 volumes 2-ethoxyethanol. The precipitate was redissolved in 10 ml 0.015M sodium chloride-0.0015M sodium citrate at 2°C. The final DNA precipitation was accomplished using 5 ml 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 non-protein 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 non-protein 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 VC after pretreatment with phenobarbital. Macromolecular binding, however, was increased markedly when compared to non-pretreated animals.

Isolation of RNA and DNA by a non-digestive procedure from the liver of animals exposed to 1, 100, 250, and 1000 ppm VC failed to reveal any detectable radioactivity. The sensitivity was such that if the hepatic radioactivity was distributed uniformly by weight throughout the tissue, measureable radioactivity would have been detected in the isolated nucleic acids.

DISCUSSION

The results indicated that the total amount of radioactivity bound to macromolecules in the liver did not increase proportionately with the increase in the exposure concentration of VC. Instead, covalent binding of metabolites of VC to hepatic macromolecules decreased disproportionately with the increase in exposure. Macromolecular binding was related directly to the total amount of VC which was metabolized over exposure concentrations ranging from 1 to 5000 ppm 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 percent 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 can not 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 observation Bolt, et al., (1976a) reported covalent binding of radioactivity to DNA and RNA following a 5 hour static exposure to 145 ppm ^{14}C -VC. The specific activity used by Bolt, et al., (1976a) was higher than used in our study and this may account for the difference in the two observations. However, the amount of binding to nucleic acids reported by Bolt, et al., (1976a) was not greater than that which could be predicted to be due to one carbon fragment incorporated into native nucleotides. The authors present data supporting the concept that the radioactivity associated with nucleic acids following exposure to ^{14}C -VC is not due to incorporation into native nucleotides. However, due to the very small degree of binding observed, until the bound species can be identified in vivo this will remain a critical question.

Both our results and that 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) has 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 protein binding was increased markedly. The true significance of the increased protein 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 exclusion of 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. To date essentially all studies of metabolic and clinical parameters have been either conducted on or related to the hepatocyte.

The hepatocyte 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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TABLE 1

Total Metabolism, Hepatic Macromolecular Binding and Hepatic Glutathione (GSH) Levels
Following Inhalation Exposure (6 Hours) To Vinyl Chloride (VC)^a

Nominal Conc.	A	B	B/A x 100	Hepatic GSH (% Control)	¹⁴ C-Activity in Liver Bound to Macromolecules
	µg VC Equivalents Metabolized	µg VC Equivalents Bound Per g Protein			
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	1,181±93	23.3±3.4	1.99±0.36	94	20±2
100	2,406±173	47.6±4.8	1.99±0.26	81 ^b	25±2
250	3,826±345	89.6±12.3	2.35±0.28	70 ^b	22±2
500	6,263±355	98.8±5.0	1.58±0.20	60 ^b	25±3
1000	4,257±765	106.8±22.2	2.55±0.58	51 ^b	22±2
5000	9,255±1,467	113.5±10.4	1.12±0.13	39 ^b	22±3
pretreatment with phenobarbital ^c					
100	2,160±166	80.0±23.9	3.70±0.82		39±3

^a Means ± standard deviation

^b Statistically different from controls, Student t-test (p < 0.05)

^c Rats were injected ip with sodium phenobarbital, 80 mg/kg for 3 days prior to exposure

LEGEND

Figure 1 Hepatic protein binding and glutathione (GSH) depression expressed as a function of the logarithm of the exposure concentration to vinyl chloride (VC). Protein binding (●) μg equivalents VC bound per g protein (mean $\pm$ S.D.) GSH (▲) percent of control.

FIGURE 1

