

# Molecular Dosimetry and Repair of $N^2,3$ -Ethenoguanine in Rats Exposed to Vinyl Chloride<sup>1</sup>

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## ABSTRACT

Although the DNA adducts of vinyl chloride (VC) have been well characterized, previous studies have used single concentrations of VC that are well above contemporary human exposures. This study examined the exposure response to VC in male Sprague Dawley rats with respect to the molecular dose of the promutagenic DNA adduct  $N^2,3$ -ethenoguanine ( $N^2,3$ - $\epsilon$ G). Adult rats were exposed by inhalation to 0, 10, 100, or 1100 ppm VC for 1 or 4 weeks (6 h/day, 5 days/week). Weanling rats were similarly exposed for 5 days. The amount of  $N^2,3$ - $\epsilon$ G in hepatocyte (HEP) and nonparenchymal cell (NPC) fractions obtained from the liver was measured with a sensitive immunoaffinity/gas chromatography/high-resolution mass spectrometry assay. Endogenous  $N^2,3$ - $\epsilon$ G was present in HEPs and NPCs from all unexposed rats. The exposure response to VC in each group and cell population was supralinear, with a linear increase from 0 to 100 ppm, and a plateau between 100 and 1100 ppm. There was no statistically significant difference in  $N^2,3$ - $\epsilon$ G concentrations between HEPs and NPCs in any adult exposure group, which suggests that factors other than adduct concentrations contribute to the particular susceptibility of NPCs to VC-induced carcinogenesis. The accumulation of  $N^2,3$ - $\epsilon$ G with respect to time was nearly linear in rats exposed to 600 ppm VC for 1, 2, 4, or 8 weeks (4 h/day, 5 days/week), and no repair of  $N^2,3$ - $\epsilon$ G was detected in rats exposed to VC for 4 weeks and allowed to recover for 1 week.  $N^2,3$ - $\epsilon$ G concentrations in HEPs from weanling rats were 2–3-fold greater than those in adult rats exposed for the same time. Higher adduct concentrations in young rats may contribute to their greater susceptibility to VC-induced hepatic angiosarcoma as well as their particular susceptibility to hepatocellular carcinoma. The molecular dosimetry of  $N^2,3$ - $\epsilon$ G in liver appears to be a sensitive and informative biomarker of genotoxic effect after exposure to VC.  $N^2,3$ - $\epsilon$ G was the predominant etheno adduct measured *in vivo* after exposure to VC, and the saturable nature of VC metabolism was reflected in its molecular dose. The relationships between endogenous  $N^2,3$ - $\epsilon$ G and that formed by low exposures to VC were demonstrated. Conclusions drawn from these exposures may be more relevant for risk assessment purposes than those drawn from high exposures where activation, detoxication, and repair pathways may be saturated or otherwise perturbed. These data are well suited for consideration in future risk assessments of VC that incorporate nontumor mode of action data.

## INTRODUCTION

VC<sup>6</sup> is a major industrial chemical and widespread environmental contaminant whose carcinogenicity to humans and animals is well

established (1). Concern about its carcinogenicity to humans was raised in 1974 after three workers at a VC polymerization plant were diagnosed with hepatic angiosarcoma, a tumor that is otherwise very rare in humans (2–4). Soon thereafter, occupational exposure limits were reduced to 1–5 ppm in most countries. No cases of hepatic angiosarcoma have been reported in VC workers who began employment after that time.

VC-induced carcinogenicity occurs by genotoxic mechanisms. VC is oxidized by CYP450 2E1 in a concentration-dependent manner (5, 6). The metabolic elimination of VC departs from first-order kinetics at 200–300 ppm in Rhesus monkeys (7) and at approximately 220 ppm in Sprague Dawley rats (8). The primary oxidation product of VC, the reactive electrophile 2-chloroethylene oxide, appears to be responsible for its genotoxicity (9, 10). As shown in Fig. 1, 2-chloroethylene oxide reacts with DNA to form 7-(2-oxoethyl)guanine and a variety of exocyclic base adducts (9, 11). Although 7-(2-oxoethyl)guanine is the major adduct formed *in vitro*, it was found to be nonmutagenic when tested with a bacterial DNA polymerase (12). Therefore, the promutagenic exocyclic adducts are believed to be responsible for VC-induced genotoxicity. The formation, role, and detection of these adducts have been reviewed (13).

One such adduct,  $N^2,3$ - $\epsilon$ G, efficiently induces G→A transitions in a variety of test systems (14–17). This mutation was subsequently found to be common in *ras* proto-oncogenes from hepatic angiosarcomas of workers or rats exposed to VC (18–20). Although  $N^2,3$ - $\epsilon$ G is formed in relatively small amounts after exposure to VC, its presence is significant because of its proven mutagenic potential and apparent biological persistence (21, 22). Routine quantitation of this adduct *in vivo* necessitated the development of an ultrasensitive, highly specific GC/ECNCl/HRMS assay (23). The assay was recently improved by the addition of IA chromatography for adduct purification (24).

A low background concentration of  $N^2,3$ - $\epsilon$ G and other exocyclic adducts has consistently been detected in unexposed animals and humans by several laboratories (24–28). It is believed that oxidative metabolic processes are at least partially responsible for this background presence. The formation of  $N^2,3$ - $\epsilon$ G,  $1,N^2$ -ethenoguanine,  $1,N^6$ - $\epsilon$ A,  $3,N^4$ - $\epsilon$ C, and related adducts from the reaction of lipid peroxidation products with nucleosides or DNA has been demonstrated (29–34). It is conceivable that this background mutagenic potential may be involved in the etiology of some spontaneous tumors.

Hepatic angiosarcoma originates in the sinusoidal-lining endothelial cells that represent the majority of the NPC population (35). However, the mechanisms that underlie the targeting of VC to the NPCs are not understood. Although VC is activated primarily in HEPs (36), its metabolites can migrate to endothelial cells (37). The base excision repair enzyme MPG, which was shown to remove etheno adducts from DNA *in vitro* (38), was induced in HEPs, but not in

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<sup>6</sup> The abbreviations used are: VC, vinyl chloride;  $N^2,3$ - $\epsilon$ G,  $N^2,3$ -ethenoguanine; GC, gas chromatography; ECNCl, electron capture negative chemical ionization; HRMS,

high-resolution mass spectrometry; IA, immunoaffinity;  $1,N^6$ - $\epsilon$ A,  $1,N^6$ -ethenoadenine;  $3,N^4$ - $\epsilon$ C,  $3,N^4$ -ethenocytosine; NPC, nonparenchymal cell; HEP, hepatocyte; MPG, *N*-methylpurine DNA glycosylase; BHT, butylated hydroxytoluene; PBPK, physiologically based pharmacokinetic; M<sub>1</sub>G, pyrimidol [1,2- $\alpha$ ]purin-10(3H)-one.

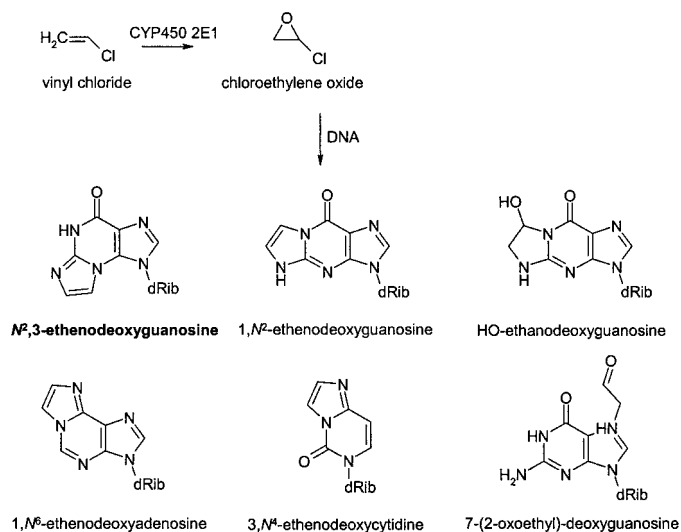


Fig. 1. DNA adducts formed after exposure to VC or its metabolites.

NPCs, after exposure of adult Sprague Dawley rats to vinyl fluoride (39). Based on this evidence, it was speculated that this relative deficiency in DNA repair may be a critical determinant for the particular susceptibility of NPCs to the carcinogenic effects of vinyl halides.

Age-dependent differences in adduct formation are known to correlate with susceptibility to VC-induced carcinogenesis.  $N^2,3$ - $\epsilon$ G has been measured in the livers of preweanling rats at concentrations approximately 4-fold higher than those in adult rats simultaneously exposed to VC (21). It is possible that higher DNA adduct concentrations may play an important role in determining the greater susceptibility of young rats to VC-induced liver tumors (40, 41).

VC is an excellent model for studying the exposure response to chemical carcinogens at the molecular level. Although the formation of DNA adducts in rats exposed to VC has been well characterized, these studies all used exposures to relatively high concentrations of VC that are known to saturate its metabolism. In contrast, the present study used a variety of exposures to determine the molecular dose from high saturating concentrations down to concentrations that approach current occupational exposures. Preliminary results from analyses of a small number of mixed liver cell fractions obtained in this study have been described elsewhere (42, 43). This report examines the mechanisms behind VC-induced carcinogenesis by determining the molecular dosimetry of  $N^2,3$ - $\epsilon$ G separately in HEPs and NPCs, by characterizing the accumulation and repair of  $N^2,3$ - $\epsilon$ G, and by studying the influence of age on DNA adduct dosimetry.

## MATERIALS AND METHODS

**Chemicals.** VC (99% chemically pure) was purchased from Supelco (Bellefonte, PA). *Clostridium histolyticum* type 2 collagenase was obtained from Worthington Biochemical (Freehold, NJ). Nucleic acid purification grade lysis buffer, phenol/chloroform/water, and proteinase K were purchased from PE Biosystems (Foster City, CA). RNases A and  $T_1$  and Percoll were purchased from Sigma (St. Louis, MO). Other chemicals and analytical standards were obtained and characterized as described by Ham *et al.* (24).

**Animal Exposures.** Exposures for the exposure response study were conducted at Huntingdon Life Sciences (East Millstone, NJ), a laboratory that is fully accredited by the Association for Assessment and Accreditation of Laboratory Care International. All procedures were in compliance with the appropriate parts of the Animal Welfare Act. For the adult studies, 11-week-old male Sprague Dawley rats (450–550 g) were purchased from Charles River Laboratories (Portage, MI). The rats were housed in stainless steel cages with

a 12-h light/dark cycle. Certified Rodent Diet No. 5002 (PMI Feeds, St. Louis, MO) was provided *ad libitum* during nonexposure periods, and water was provided at all times. Eight animals per group were acclimated for 1 week and then exposed to target concentrations of 0, 10, 100, or 1100 ppm VC in a whole-body inhalation apparatus for 1 or 4 weeks (6 h/day, 5 days/week). Animals in the recovery group were similarly exposed for 4 weeks and maintained in a control environment for 5 days before they were killed. For the weanling study, 8 females with 8 male pups each were purchased, and 40 pups weaned at day 25 were selected for the study. The weanlings were exposed to VC for 5 days as described above. The atmosphere in each exposure chamber was sampled hourly, and the VC concentration was measured with a Miran 1A infrared spectrophotometer. Each individual measurement was within 20% of the target VC concentration, and the mean for each 6-h exposure period was within 10% of the target concentration.

**Cell Separations.** After exposure, the rats were anesthetized with i.p. pentobarbital, heparinized i.v., and killed by incision of the abdominal aorta. The liver was perfused *in situ* with collagenase via the portal vein to obtain a mixed liver cell suspension as described previously (44). A portion of the suspension was retained and frozen on dry ice for mixed liver cell analyses. The remaining suspension was centrifuged ( $50 \times g$ , 3 min), and the supernatant was transferred to another tube. The pellet was resuspended in 30 ml of PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , and 1.5 mM  $\text{KH}_2\text{PO}_4$ ), and this process was repeated.

Percoll density medium was diluted to the appropriate concentrations with PBS. HEPs were purified by adding 30 ml of 60% Percoll to the pellet and centrifuging the resulting suspension ( $50 \times g$ , 10 min). The supernatant was discarded, and the tube was wiped clean above the pellet. The HEPs were resuspended in 20 ml of PBS, which was then aliquoted into two 15-ml centrifuge tubes. These tubes were centrifuged ( $350 \times g$ , 5 min), and the supernatant was discarded. The HEP pellets were frozen on dry ice and stored at  $-80^\circ\text{C}$  until analysis.

NPCs were isolated by combining and centrifuging the supernatant fractions that were retained earlier ( $350 \times g$ , 5 min). The supernatant was discarded, and the pellet was redissolved in 20 ml of PBS. NPCs were purified essentially as described by Pertoft and Smedsrød (35). Equal amounts of the suspension were carefully transferred to each of two centrifuge tubes prepared by layering 20 ml of 25% Percoll above 15 ml of 50% Percoll. The tubes were centrifuged without braking ( $800 \times g$ , 20 min), and NPCs were harvested at the interface. The purity of the NPC fraction was estimated to be  $>95\%$  when aliquots of selected samples were stained with trypan blue and examined with a hemocytometer. Each sample was centrifuged again ( $350 \times g$ , 5 min), the supernatant was discarded, and the purified NPCs were frozen on dry ice and stored at  $-80^\circ\text{C}$ .

For the accumulation study, whole liver samples were obtained from male Sprague Dawley rats exposed by inhalation to 500 ppm VC for 1, 2, 4, or 8 weeks (4 h/day, 5 days/week) at IARC (Lyon, France) as described in detail by Guichard *et al.* (45). The samples were transferred on dry ice and stored at  $-80^\circ\text{C}$ .

**DNA Extraction.** Cellular DNA was isolated using a modified phenol/chloroform extraction procedure with 2% BHT in isopropanol (w/v) added to reagents and samples as an antioxidant. The HEP pellet was suspended in 5 ml of lysis buffer containing 0.04% BHT (w/v), and an additional 25  $\mu\text{l}$  of 2% BHT solution were added. RNases A (5 units) and  $T_1$  (270 units) were added, and the samples were incubated for 2 h at  $37^\circ\text{C}$ . Next, proteinase K (45 units) was added, and the samples were incubated for an additional 2 h at  $37^\circ\text{C}$ . The samples were extracted twice with 5 ml of phenol/chloroform/water containing 0.04% BHT (w/v) and once with 5 ml of alumina-purified chloroform containing 0.04% BHT (w/v). DNA was precipitated by the addition of 0.5 ml of 3 M sodium acetate and 10 ml of cold 95% ethanol. The pellet was washed with cold 70% ethanol and redissolved overnight in water at  $4^\circ\text{C}$ . The concentration of each sample was estimated by  $A_{260\text{ nm}}$ , and the DNA was stored at  $-80^\circ\text{C}$  until analysis. The liver samples provided by the IARC group were first homogenized in 10 ml of PBS and centrifuged ( $1000 \times g$ , 15 min). Nuclear DNA was harvested by an automated phenol/chloroform extraction procedure (Applied Biosystems 340A; Applied Biosystems, Foster City, CA). RNA contamination of DNA was measured essentially as described by Boucheron *et al.* (46).

**Quantitation of  $N^2,3$ - $\epsilon$ G/Unmodified Guanine.**  $N^2,3$ - $\epsilon$ G was quantitated with the acid hydrolysis/IA/GC/ECNCl/HRMS method of Ham *et al.* (24),

with minor modifications as described below. Briefly, 70 fmol of [<sup>13</sup>C<sub>4</sub>, <sup>15</sup>N<sub>2</sub>]N<sup>2</sup>,3-εG internal standard (30 μl) were added to 50–400 μg of DNA. Water and 100 μl of 1 N HCl were added to yield a total volume of 1000 μl, and the solution was incubated for 30 min at 70°C. The hydrolysate was transferred to Centricon 10 membrane filters (Millipore, Bedford, MA) and purified by centrifugation (5000 × g, 60 min). A 50–100-μl aliquot of the filtrate was retained for guanine quantitation with high-performance liquid chromatography as described previously (24). Initially, the remaining filtrate was neutralized by adding 0.1 volume of 1 N NaOH and 2 ml of PBS to the retentate, followed by centrifugation (5000 × g, 90 min). To minimize pH variation in later analyses, the filtrates were neutralized by the addition of 2 ml of 500 mM sodium phosphate buffer (pH 7.2) to the retentate, followed by centrifugation (5000 × g, 90 min).

N<sup>2</sup>,3-εG was isolated from the filtrate by IA chromatography as described previously (24). The adduct was eluted with 3 ml of 100% methanol, and the fraction was dried by Speed-vac vacuum centrifugation. To each sample were added ~25 mg of dry, powdered potassium carbonate, and 500 μl of acetone. Next, 35 μl of 5% pentafluorobenzyl bromide/acetone were added. The tubes were capped and mixed at 50°C for 70 min. The samples were dried by Speed-vac vacuum centrifugation and then extracted and transferred in two 100-μl portions of dichloromethane to disposable silica gel solid-phase extraction columns prepared as described previously (24). The samples were washed with 4 ml of hexane and 6 ml of 5% ethyl acetate/hexane. The derivatives were eluted in 3 ml of ethyl acetate, dried again, redissolved in 15 μl of toluene, and transferred to silanized GC vial inserts for analysis.

The GC/ECN/HRMS analyses were made with a HP 5890 GC coupled to a Varian VG70–250SEQII mass spectrometer as described by Ham *et al.* (24). A 2-μl volume of each sample was injected. Selected ion monitoring at *m/z* = 354.0413 and *m/z* = 360.0489 was used to quantitate the [M-181]<sup>-</sup> fragments of 3,5-PFB<sub>2</sub>-N<sup>2</sup>,3-εG and 3,5-PFB<sub>2</sub>-[<sup>13</sup>C<sub>4</sub>, <sup>15</sup>N<sub>2</sub>]N<sup>2</sup>,3-εG, respectively. The mass resolving power was 7–10 × 10<sup>3</sup>, and the minimum signal: noise ratio used for quantitation was 5:1. The amount of N<sup>2</sup>,3-εG in each sample was calculated from the ratio of the analyte and internal standard peak areas. The N<sup>2</sup>,3-εG concentration in each sample was determined by normalizing the adduct measurement to the amount of guanine measured in the sample by high-performance liquid chromatography. Each result was expressed as the molar ratio between N<sup>2</sup>,3-εG and unmodified guanine in the sample (mol N<sup>2</sup>,3-εG: mol guanine).

**Statistics.** Analyses were performed with the aid of Instat version 3.00 for Windows 95 software (GraphPad Software, San Diego, CA). Differences in N<sup>2</sup>,3-εG concentrations between HEP and NPC populations within exposure groups were compared using the unpaired *t* test with Welch correction. Pairing of HEP and NPC measurements from the same rats was not attempted because fewer NPC than HEP samples were analyzed. Adduct concentrations were compared between exposure regimens with one-way ANOVA followed by multiple comparisons tests performed as follows. N<sup>2</sup>,3-εG concentrations from exposure groups within each exposure regimen were compared with the

matched controls using Dunnett tests. Comparisons between different age groups or exposure regimens were made with the Tukey-Kramer multiple comparisons test. In each case, *P* ≤ 0.05 (two-sided) was considered to be statistically significant. If a sample was analyzed more than once, the result with the strongest internal standard signal (*i.e.*, highest sensitivity) was used.

## RESULTS AND DISCUSSION

Although the DNA adducts induced by VC have been well studied, this is the first comprehensive description of the exposure response to VC across a broad range of exposure concentrations. The formation of N<sup>2</sup>,3-εG in DNA was used as a biomarker of VC exposure down to a concentration that is within an order of magnitude of current occupational exposure limits. Because VC carcinogenicity preferentially targets the sinusoidal-lining endothelial cells, it was important to consider the molecular dosimetry of N<sup>2</sup>,3-εG separately in NPCs and HEPs.

The IA/GC/ECN/HRMS assay used for these determinations is very sensitive and highly specific (24). The lower limit of quantitation was 1–2 fmol N<sup>2</sup>,3-εG/sample, which corresponded to approximately 1 mol N<sup>2</sup>,3-εG/10<sup>8</sup> mol unmodified guanine in 250 μg of DNA. Due to a low yield of NPC from some livers, fewer NPC than HEP samples were available for analysis. Although the rapid DNA extraction procedure (which was designed to minimize the potential for artifactual adduct formation) allowed RNA to remain in some samples, the assay was found to be unaffected by this contamination (data not shown).

A low background amount of endogenous N<sup>2</sup>,3-εG was consistently detected in HEP and NPC DNA from control rats (Table 1; Fig. 2). This observation is consistent with previous measurements of N<sup>2</sup>,3-εG in the liver of unexposed rats (24, 26, 34). This background concentration represents a threshold for the detection of N<sup>2</sup>,3-εG induced by exposure to VC, but it is not known whether this also reflects a threshold for the carcinogenic response to VC. It will be important to assess the biological role of endogenous N<sup>2</sup>,3-εG in future studies because its presence could profoundly influence the estimation of the risk associated with exposure to low concentrations of VC (43).

N<sup>2</sup>,3-εG was clearly induced in HEPs and NPCs after exposure of adult rats to VC for 1 or 4 weeks (Table 1; Fig. 2). The exposure response curves derived from the HEP and NPC populations were supralinear after the 1- and 4-week exposures, with a linear increase from 0 to 100 ppm and a marked plateau between 100 and 1000 ppm (Fig. 3). This response is similar to that previously characterized in mixed liver cells obtained from some of these rats (42, 43). The

Table 1 N<sup>2</sup>,3-εG concentrations measured in male Sprague Dawley rats exposed to VC

VC exposure (ppm)	Exposure duration (wks) <sup>a</sup>	Rat age	No. of rats	HEPs		NPCs	
				mol εG/10 <sup>7</sup> mol guanine (± SE)	No. of rats	mol εG/10 <sup>7</sup> mol guanine (± SE)	No. of rats
0	1	Adult	8	0.55 ± 0.14	3	0.93 ± 0.23	
	4	Adult	7	0.30 ± 0.03		N/A <sup>b</sup>	
	1	Weanling	7	0.16 ± 0.01	4	0.49 ± 0.24	
10	1	Adult	8	1.4 ± 0.4	3	0.79 ± 0.06	
	4	Adult	8	1.5 ± 0.1	5	1.5 ± 0.9	
	1	Weanling	8	4.1 ± 0.8 <sup>c</sup>	5	0.73 ± 0.10	
100	1	Adult	8	3.7 ± 0.4 <sup>c</sup>	4	3.5 ± 0.2	
	4	Adult	8	11 ± 1 <sup>c</sup>	4	7.1 ± 1.1 <sup>d</sup>	
	1	Weanling	8	9.0 ± 0.7 <sup>c</sup>	5	4.3 ± 0.5 <sup>c</sup>	
1100	1	Adult	8	5.5 ± 0.2 <sup>c</sup>	3	6.3 ± 0.2	
	4	Adult	8	11 ± 2 <sup>c</sup>	4	11 ± 2 <sup>d</sup>	
	1	Weanling	8	9.7 ± 0.5 <sup>c</sup>	5	11 ± 1 <sup>c</sup>	

<sup>a</sup> Rats were exposed for the indicated times and VC concentrations in a whole-body inhalation apparatus (6 h/day, 5 days/week).

<sup>b</sup> N/A, not available for analysis.

<sup>c</sup> Statistically significant difference compared with matched controls when tested with the Dunnett post-test (*P* < 0.01).

<sup>d</sup> Statistically significant difference compared with the matched 10 ppm exposure when tested with the Dunnett post-test (*P* < 0.05).

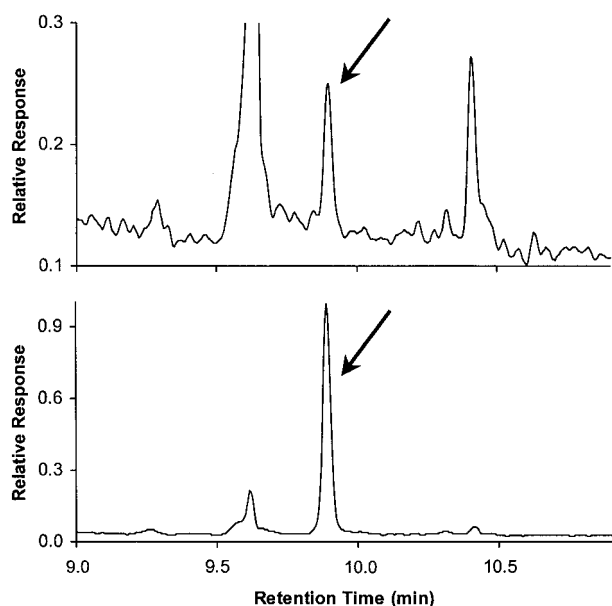


Fig. 2. Representative GC/ECNCl/HRMS chromatograms demonstrating the detection of  $N^2,3$ - $\epsilon$ G in DNA from NPCs isolated from a control weanling rat (*top panel*) and from one exposed to 1100 ppm VC for 5 days (*bottom panel*). Selected ion monitoring at  $m/z = 354.0413$  was used to detect the 3,5-di-pentafluorobenzylated derivative of  $N^2,3$ - $\epsilon$ G. The amount of  $N^2,3$ - $\epsilon$ G in each sample was quantitated by comparing its peak area against that of the [ $^{13}\text{C}_4, ^{15}\text{N}_2$ ] $N^2,3$ - $\epsilon$ G internal standard ( $m/z = 360.0489$ ). The chromatograms corresponding to the internal standard added to each sample are not shown.

supralinear curve is thought to be a consequence of the saturable nature of VC metabolism and is consistent with the pattern of tumor incidences observed in a long-term cancer bioassay (40). These correlations suggest that the molecular dosimetry of  $N^2,3$ - $\epsilon$ G in liver is predictive of cancer risk in this model. By demonstrating the importance of metabolism to the genotoxic response, these data support the relevance of target tissue dose metrics calculated with PBPK models to the cancer risk assessment of VC (47).

Due to the presence of endogenous  $N^2,3$ - $\epsilon$ G, it is important to consider the relative increases in adduct concentrations over controls in addition to their absolute values. When adult rats were exposed to 10 ppm VC for 4 weeks, there was a 5-fold increase in  $N^2,3$ - $\epsilon$ G over the endogenous concentration measured in control rats. If this result is linearly interpolated to a 1 ppm occupational exposure limit, it is estimated that this concentration of VC would increase the biological burden of  $N^2,3$ - $\epsilon$ G by only 50%. In contrast,  $N^2,3$ - $\epsilon$ G concentrations in HEPs were increased 37-fold when rats were exposed to 100 or 1100 ppm VC for 4 weeks. The disparity between the relative increases in adduct concentrations associated with each of these exposures is consistent with the epidemiological observation that VC-induced hepatic angiosarcoma was largely associated with autoclave workers who were routinely exposed to VC concentrations estimated at 500 ppm or more (48, 49).

Based upon previous analyses of a small number of HEP and NPC fractions from vinyl fluoride-exposed rats with an earlier version of this assay (42), we hypothesized that  $N^2,3$ - $\epsilon$ G would be present in NPCs at higher concentrations than in HEPs after exposure to VC. However, the response of the HEP and NPC populations to VC was similar in adult rats in this study. This suggests that differences in adduct concentrations between HEPs and NPCs are not indicative of the susceptibility of each population to VC-induced carcinogenicity. Due to the presence of other cell types in the NPC fraction, the possibility of a small difference in  $N^2,3$ - $\epsilon$ G between HEPs and endothelial cells cannot be excluded, however.

Important differences between HEPs and NPCs that were not

characterized in this experiment might also contribute to the susceptibility of the latter population to VC-induced carcinogenesis. Because the basal proliferation rate of NPCs is more than 2-fold greater than that of HEPs (50), a similar adduct concentration may cause more mutations to be fixed in the NPC DNA. Another potential factor is the demonstrated difference in DNA repair capacity between HEPs and NPCs (39). However, the rates of accumulation in the two cell populations would be expected to be different if significant differences in repair existed. The parallel nature of the molecular dosimetry curves between HEPs and NPCs at 1 and 4 weeks supports a similar rate of repair.

There was a 2–3-fold accumulation of  $N^2,3$ - $\epsilon$ G between 1 and 4 weeks in both cell populations (Table 1; Fig. 3).  $N^2,3$ - $\epsilon$ G concentrations in HEP and NPC DNA from rats exposed for 4 weeks and allowed to recover for 1 week were similar to those measured in rats exposed for 4 weeks and killed immediately (Table 1; Fig. 4). The lack of a decrease in  $N^2,3$ - $\epsilon$ G concentrations in the recovery group is consistent with a previous half-life estimate of more than 30 days in preweanling rats (21). There appeared to be a small but consistent increase in  $N^2,3$ - $\epsilon$ G concentrations in HEPs after the recovery period, although this difference was only significant in rats exposed to 1100 ppm VC. Although the underlying mechanisms are not understood, there is precedence for an increase in adduct concentrations during a recovery period; a similar phenomenon was reported in a study of the malondialdehyde-induced guanine adduct  $M_1G$  (51).

To further examine the repair of  $N^2,3$ - $\epsilon$ G, tissues from a collaborative study with the IARC were examined. Whole liver DNA samples from male Sprague Dawley rats exposed to 500 ppm VC for 1, 2, 4,

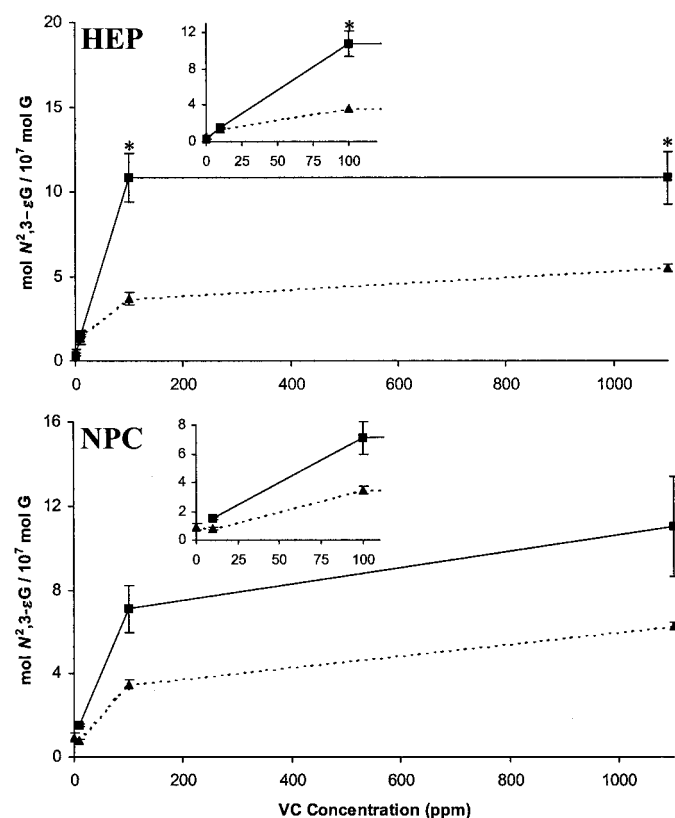


Fig. 3. Molecular dosimetry of  $N^2,3$ - $\epsilon$ G in HEPs (*top panel*) and NPCs (*bottom panel*) from adult rats exposed to VC for 1 ( $\blacktriangle$ ) or 4 ( $\blacksquare$ ) weeks (6 h/day, 5 days/week). The lower exposure concentrations are detailed in the *insets*. NPC data from the 4 week controls are not available. Results are shown as the mean  $\pm$  SE. \* indicates that a statistically significant difference exists with the corresponding 1 week result with the Tukey-Kramer multiple comparisons test ( $P < 0.01$ ).

or 8 weeks (4 h/day, 5 days/week) were analyzed using the present method. The high degree of linearity ( $r^2 > 0.99$ ) in the resulting curve (Fig. 5) is consistent with the slow repair of  $N^2,3$ - $\epsilon$ G in this model. It is important to note that these results contradict the previous finding of a plateau in  $N^2,3$ - $\epsilon$ G concentrations between 4 and 8 weeks of exposure when this DNA was analyzed with a less sophisticated version of this assay (42), and the accumulation of  $N^2,3$ - $\epsilon$ G now appears to be similar to that of  $1,N^6$ - $\epsilon$ A and  $3,N^4$ - $\epsilon$ C in these liver samples (45). The concentration of  $N^2,3$ - $\epsilon$ G measured in these rats was much greater than the concentrations of  $1,N^6$ - $\epsilon$ A and  $3,N^4$ - $\epsilon$ C

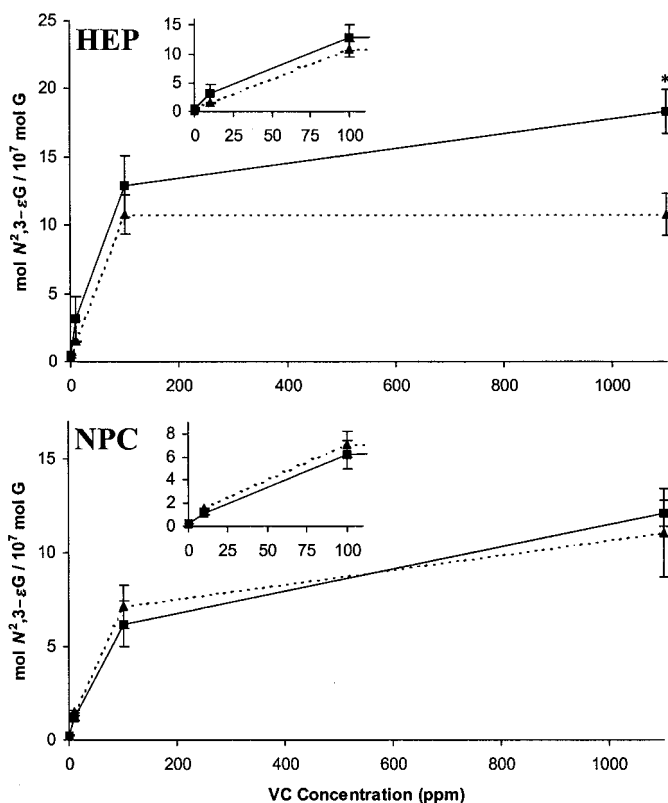


Fig. 4. Molecular dosimetry of  $N^2,3$ - $\epsilon$ G in HEPs (top panel) and NPCs (bottom panel) from rats exposed to VC for 4 weeks (6 h/day, 5 days/week) and killed immediately ( $\blacktriangle$ ) and from those exposed for 4 weeks and allowed to recover for 5 days ( $\blacksquare$ ). The lower exposure concentrations are detailed in the insets. NPC data from the 4 week controls are not available. Results are shown as the mean  $\pm$  SE. \* indicates that a statistically significant difference exists when compared with the corresponding 4 week result with the Tukey-Kramer multiple comparisons test ( $P < 0.01$ ).

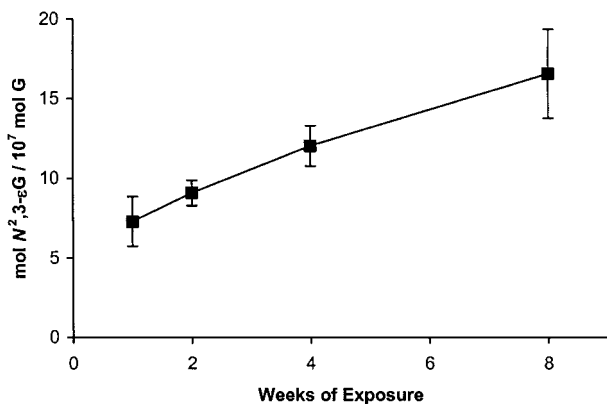


Fig. 5. Accumulation of  $N^2,3$ - $\epsilon$ G in whole liver DNA from rats exposed to 500 ppm VC for the indicated times (4 h/day, 5 days/week). The rats were killed immediately after the exposures. The results from 5 rats/group are shown as the mean  $\pm$  SE.

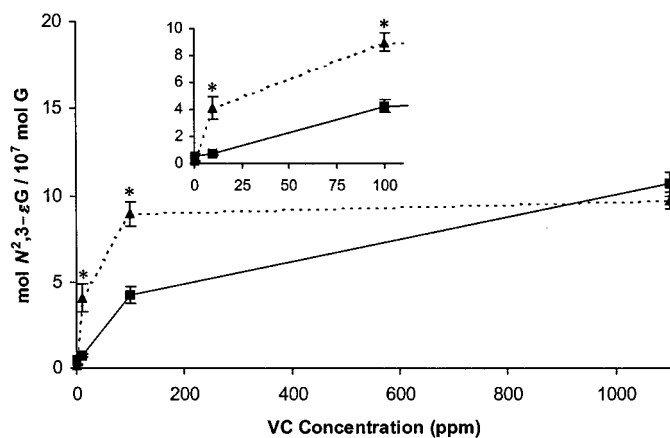


Fig. 6. Comparison of  $N^2,3$ - $\epsilon$ G concentrations measured in HEPs ( $\blacktriangle$ ) and NPCs ( $\blacksquare$ ) from weanling rats exposed to VC for 5 days (6 h/day). Results are shown as the mean  $\pm$  SE. \* indicates that a statistically significant difference exists when compared with the corresponding NPC result using the unpaired  $t$  test with Welch correction ( $P < 0.01$ ).

previously measured in the livers of the same rats with an IA- $^{32}$ P postlabeling assay (45). After 8 weeks of exposure to 500 ppm VC, the concentration of  $N^2,3$ - $\epsilon$ G was approximately 30-fold greater than that of  $1,N^6$ - $\epsilon$ A and 20-fold greater than that of  $3,N^4$ - $\epsilon$ C. The remaining etheno adduct,  $1,N^2$ -ethenoguanine, was not detected in HEPs from rats exposed to VC and thus is not believed to play a significant role in VC-induced carcinogenesis (34).

Although no loss of  $N^2,3$ - $\epsilon$ G from DNA was observed after the 1-week recovery period, the accumulation of this adduct was disproportional to the time of the exposure. For example, there was only a 2–3-fold accumulation of  $N^2,3$ - $\epsilon$ G between 1 and 4 weeks of exposure in HEP DNA from the present study as well as liver DNA from the IARC collaboration. These results strongly suggest that  $N^2,3$ - $\epsilon$ G is slowly repaired *in vivo*. Furthermore, the persistence of  $N^2,3$ - $\epsilon$ G observed in these studies does not necessarily contradict the published data regarding the repair of this adduct by MPG. Although it was shown that etheno adducts were released from DNA in the presence of MPG,  $N^2,3$ - $\epsilon$ G was released to a much smaller extent than  $1,N^6$ - $\epsilon$ A (38). It will be necessary to measure  $N^2,3$ - $\epsilon$ G after a longer recovery period to determine whether differences in repair might contribute to the greater susceptibility of NPC to VC-induced carcinogenesis. In any case, the persistence of  $N^2,3$ - $\epsilon$ G effectively amplifies the mutagenic potential of this adduct *in vivo*. These findings demonstrate the value of molecular dosimetry to the study of DNA repair. As was reported with the  $M_1G$  adduct (51), a repair mechanism demonstrated *in vitro* appeared to be inefficient in the whole organism.

$N^2,3$ - $\epsilon$ G was detected as both an endogenous and VC-induced lesion in HEPs and NPCs harvested from weanling rats (Table 1; Fig. 6).  $N^2,3$ - $\epsilon$ G was measured at significantly higher concentrations in weanling rats than in adult rats exposed for the same time. This is thought to be a consequence of greater CYP450 2E1 protein concentrations in the livers of young rats.<sup>7</sup> In this study, the concentration of  $N^2,3$ - $\epsilon$ G measured in HEPs and NPCs of weanling rats was approximately 2–3-fold greater than that in similarly exposed adults. This finding is consistent with the 4-fold difference reported between preweanling rats and their dams after exposure to 600 ppm VC for 5 days (21). (These adduct measurements are expected to be slightly underestimated due to dilution as a consequence of the relatively high cell proliferation rate in the livers of young rats.) A greater burden of adducts, coupled with the high cell proliferation rate in the livers of

<sup>7</sup> F. P. Guengerich, unpublished data.

young rats, may play a role in determining the higher susceptibility of young rats to VC-induced carcinogenesis compared with adults (41). These findings provide mechanistic support for the decision to include an additional protection factor for young populations in the recent United States Environmental Protection Agency VC risk assessment.<sup>8</sup>

In contrast to adults, a difference in adduct concentrations was detected between the HEP and NPC populations from the weanlings. The concentration of N<sup>2</sup>,3-εG in HEPs was significantly greater than that measured in NPCs after the 10 and 100 ppm VC exposures ( $P < 0.01$ ), although the adduct concentration in both populations was similar at 1100 ppm. This differential response of the HEP and NPC populations may contribute to the particular susceptibility of young rats to VC-induced hepatocellular carcinoma (40, 41).

After exposure to genotoxic carcinogens, DNA adducts are sensitive biomarkers that provide information about promutagenic effects on the target molecule. When the concentration of a DNA adduct is measured, aspects of absorption, transport, activation, and detoxication are incorporated into the result. This can greatly simplify extrapolations and help define differences between species, strains, and routes of exposure. Furthermore, this end point makes it possible to characterize not only the formation but also the repair of a DNA adduct. This allows the study of DNA repair in the presence of all of the repair pathways that are present under relevant conditions in the whole organism. Finally, advances in technology now allow the study of the effects of doses at or near concentrations commonly experienced by humans. Conclusions drawn from these exposures may be more relevant for risk assessment purposes than those drawn from high exposures, at which activation, detoxication, and repair pathways may be saturated or otherwise perturbed (26, 52).

The United States Environmental Protection Agency made provisions in the 1996 Proposed Guidelines for Carcinogen Risk Assessment (53) for the consideration of nontumor data in chemical risk assessments. These results provide mode of action evidence that serves to increase confidence in the agency's recent VC risk assessment, which relied largely on the PBPK model of Clewell *et al.* (47). If appropriate human dosimetry data become available in the future, these results could be incorporated into the PBPK model to extrapolate the genotoxic effects of VC metabolites across species. Such a model might also consider the influence of endogenous DNA adducts on the risk posed by exposure to VC. It is hoped that the inclusion of such experimental data may improve our ability to evaluate carcinogenic risk in the future, particularly at the low concentrations that are relevant to most contemporary occupational and environmental exposures.

The findings detailed in this report strongly support a causal role for N<sup>2</sup>,3-εG in VC-induced carcinogenesis. The exposure-response curve correlates well with the kinetics of VC metabolism as well as tumor incidences observed in cancer bioassays. N<sup>2</sup>,3-εG appears to be the predominant etheno adduct present in the livers of rats exposed to VC and is readily induced in the NPC population that is targeted by VC. The long persistence and potent mutagenicity of N<sup>2</sup>,3-εG suggest that it would effectively miscode *in vivo*. Taken together, these findings implicate N<sup>2</sup>,3-εG as an important precursor lesion to carcinogenesis induced by VC.

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