Carcinogenesis Vol.4 No.8 pp.1031 – 1038, 1983

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Vinylidene chloride: changes in drug-metabolizing enzymes, mutagenicity and relation to its targets for carcinogenesis

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(Received on 22 March 1983; accepted on 15 June 1983)

Abstract

Results of various studies have shown that male Swiss Webster mice are more susceptible to toxic effects of vinylidene chloride (VDC) than are females of the same mouse strain, females and males of the C57BL mouse strain, Chinese hamsters and rats. The main targets of toxicity are kidney and liver. The kidney of male Swiss Webster mice is the only organ where VDC unambiguously induces tumours. In the present study we have investigated the ability of NADPH-fortified postmitochondrial supernatant fractions (S-9 mix) of kidney and liver from susceptible and non-susceptible animals to activate VDC to a bacterial mutagen. The following sequence of activating potencies was observed: mouse liver (both strains and sexes) and Chinese hamster liver > rat liver > human liver > Chinese hamster kidney > kidney from male mice of both strains > kidney from rats and female mice. The last two preparations only occasionally showed weak activation of VDC. Addition of purified microsomal epoxide hydrolase to S-9 mix did not affect the mutagenicity of VDC; addition of glutathione reduced the mutagenicity up to 50%. Pretreatment of animals (male rats, male and female Swiss Webster mice) with VDC did not potentiate the ability of the subcellular preparations to activate this compound. In fact, in some cases, a weaker activation was observed. Following this treatment, microsomal 7-ethoxycoumarin O-dealkylase was decreased in mouse kidney and in rat liver. The enzyme was not affected in mouse liver and was not measurable in rat kidney. Microsomal epoxide hydrolase activity (with styrene 7,8-oxide as substrate) was not affected in mouse liver and rat kidney. In the kidney of male mice treated with a high concentration of VDC, epoxide hydrolase activity was decreased initially, but after longer treatment, in some cases a weak increase above control was noticed. A stronger increase in activity of epoxide hydrolase was observed in the rat liver and the kidney of female mice. Cytosolic glutathione transferase activity (with 2,4-dinitrochlorobenzene as substrate) was not affected by the VDC treatment in the liver of male mice, but was decreased in the kidney of male mice, and was elevated in the kidney and liver of rats and of female mice. The different effects of VDC on this enzyme may be one of the reasons for the differences in susceptibility towards the toxic and carcinogenic actions of this compound in different species, strains and sexes.

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*Abbreviation: VDC, vinylidene chloride.

Introduction

Vinylidene chloride (1,1-dichloroethylene, VDC*), a reactive chemical substance which is widely used in the production of various copolymers, shows remarkable differences in toxicity in animals depending on species, strain, sex and nutritional state (1–4). For instance, the acute inhalation toxicity is characterized by LC_{50} values which range from < 50 p.p.m. in fasting male mice to > 10 000 p.p.m. in fed female rats. The main targets of acute and chronic toxicity are the kidney and the liver. In addition to toxicity studies, a total of 15 carcinogenicity studies have been performed (2–12). In inhalation experiments with Chinese hamsters, various strains of rats and female mice and in ingestion experiments with rats and mice, either completely negative results were obtained or a few sporadic tumours were found at various sites but they could not be clearly attributed to the action of VDC. The only substance-related tumorigenic effect was observed in male Swiss Webster mice, which developed adenocarcinomas in kidneys after inhalation of VDC (4,5). This effect occurred at exposure to 25 p.p.m. but not to 10 p.p.m. VDC. At 50 p.p.m., mortality was so high that an experiment could not be conducted. Hence, the carcinogenicity of VDC appears rather specific with respect to species, sex, organ and dose. Furthermore, the occurrence of an effect only close to lethal doses and only in animals which are highly susceptible to toxic effects, suggests that there may exist a relationship between toxicity and carcinogenicity, e.g. a common active metabolite responsible for both effects or promotion of the tumour development by toxicity.

An important question arising from these animal data is whether or not VDC can induce tumours in man. Epidemiological studies did not show any correlation between tumour incidences in workers and exposure to VDC during production processes (13–15). An understanding of mechanisms underlying the highly selective carcinogenicity in animals would be a desirable rational complement to the negative empirical carcinogenicity data in man.

In studies with bacteria and yeast, VDC was not mutagenic itself (16–20). However, it was activated to a mutagen by subcellular tissue preparations (16–20). Preparations from the tumour-susceptible species, the mouse, were more effective than from rats (albeit not the same mouse strain as in the positive carcinogenicity study was used) (17–19). Preparations from the target organ, kidney, showed much less activation of VDC to a mutagen than liver preparations (17,19). However, it should be noted that these studies did not take into account potential strain differences in carcinogenicity – which may be expected from the strain differences in toxicity (2,4,12) – and potential changes in the metabolism due to enzyme induction during long-term treatment of animals with VDC.

In order to increase our understanding of events associated with these species, strain, and sex differences, we have now examined the effect of VDC treatment on several drug-metabolizing enzymes in the liver and kidney of tumour-susceptible male Swiss Webster mice and of non-susceptible

female Swiss Webster mice and Sprague Dawley rats. The enzyme activities studied were 7-ethoxycoumarin O-dealkylase, microsomal epoxide hydrolase with styrene oxide as substrate and glutathione transferase with 2,4-dinitrochlorobenzene as substrate. The same enzymes are potentially involved in the metabolism of VDC (21). We also studied the activation of VDC to a bacterial mutagen in parallel. Mutagenicity experiments with metabolizing systems from non-treated animals of some other species and strains which have been used for carcinogenicity and toxicity experiments and from man were included for comparison.

Materials and Methods

Chemicals

VDC (gas chromatographic purity: 99.996% VDC; 27 p.p.m. methylchloride + vinylchloride + chloroacetylene; 6 p.p.m. dichloroacetylene; 4 p.p.m. *trans*-1,2-dichloroethylene), stabilized with 200 p.p.m. 4-methoxyphenol (monomethyl ether of hydroquinone), and 4-methoxyphenol were obtained from BASF AG, Ludwigshafen, FRG.

Animals

Swiss Webster mice and Sprague Dawley rats were generously provided by Prof. C.Maltoni, Bologna, and bred at BASF AG. Chinese hamsters Fue: FUST (bas) were from the same breeding (BASF AG) as used by Prof. C.Maltoni (5). C57BL/6J Han mice were purchased from the Zentralinstitut für Versuchstiere, Hannover, FRG. The male mice used in the induction experiments weighed 30–39 g, the females 27–32 g and the rats 147–179 g.

Treatment with VDC was performed in an inhalation chamber with a daily exposure for 6 h. The 8 day-treatment group was exposed to VDC on 8 sequential days, whereas the control animals were exposed to air. The 1 day-and 3 day-treatment groups were treated with VDC on the last day or on the last 3 days of this 8 day-period and received sham treatments with air during the preceding days. The animals were killed with CO_2 one day after the last exposure. Livers and kidneys were removed under sterile conditions and homogenized using a glass-teflon homogenizer in cold, isotonic KCl solution, which contained 10 mM sodium phosphate buffer pH 7.4. The homogenates were centrifuged for 10 min at 9000 *g*. A part of the resulting postmitochondrial supernatant fraction was then centrifuged for 60 min at 100 000 *g*. The 100 000 *g* supernatant was used for determination of glutathione transferase activity and the microsomal pellet was resuspended in the homogenization buffer and used for determination of epoxide hydrolase and 7-ethoxycoumarin O-dealkylase activities.

Enzyme assays

7-Ethoxycoumarin O-dealkylase activity was measured by the method of Ullrich and Weber (22) at 37°C. The activity of epoxide hydrolase was determined by the method of Oesch *et al.* (23) but without Tween 80 (24) with styrene oxide as substrate at 37°C. Glutathione transferase activity was measured by the method of Habig *et al.* (25) with 2,4-dinitrochlorobenzene as substrate at 30°C. All enzyme assays were checked for linearity with respect to amount of protein and time for each strain of animals. Protein concentrations were measured by the method of Lowry *et al.* (26) using bovine serum albumin as standard.

Mutagenicity experiments

The *his*$^-$ *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA92 were obtained from Dr.B.N.Ames, Berkeley, CA. The *trp*$^-$ strain *Escherichia coli* WP2 *uvrA* was a gift of Dr.R.McMahon, Lilly Research Laboratories, Indianapolis, IN. Stock cultures were stored at −70°C until used. The bacteria were grown overnight in Bacto nutrient broth (8 g/l) which was supplemented with 5 g/l NaCl. Before the experiment, bacteria were centrifuged and resuspended to a titer of $\sim 1.7 \times 10^9$ bacteria (colony forming units) per ml in resuspension medium contained 1.6 g Bacto nutrient broth and 5 g NaCl per liter.

A metabolizing system, called S-9 mix, consisted of the following ingredients: (i) postmitochondrial supernatant fraction from 6.25 to 200 mg tissue made up to a volume of 167 or 333 µl (depending on the experiment) with the buffer used for homogenization; (ii) 167 µl of a solution containing 24 mM $MgCl_2$ and 100 mM KCl; (iii) 167 µl of a solution containing 12 mM NADP, 15 mM glucose-6-phosphate and 150 mM sodium phosphate buffer pH 7.4 and (iv) 100 µl of an appropriate solution of glutathione or epoxide hydrolase (27) in 10 mM sodium phosphate buffer adjusted to pH 7.4 or buffer only (this component was added only in experiments where glutathione or epoxide hydrolase was used).

The mutagenicity experiments with *his*$^-$ *S. typhimurium* were performed with minor modification of the methods described by Ames *et al.* (28) and, in case of the volatile VDC, Bartsch *et al.* (17). Experiments with *trp*$^-$ *E. coli* were conducted analogously. A volume (500–767 µl) of S-9 mix (or 500 µl 150 mM KCl), 100 µl of the bacterial suspension and 2000 µl top agar (which consisted of 0.55% agar, 0.55% NaCl, 50 µM histidine, 50 µM biotin, 50 µM tryptophan and 25 mM sodium phosphate buffer pH 7.4, 45°C) were mixed in a test tube and poured onto a Petri dish with minimal agar consisting of 1.5% agar and Vogel-Bonner E medium with 2% glucose. In the case of solid test compounds, the mixture also contained 10–30 µl of a solution of the compound in dimethyl sulfoxide. In the case of VDC, the plates were placed for 4 h in a desiccator with VDC. After a further incubation for 2–3 days in the absence of VDC at 37°C in the dark, the colonies (*his*$^+$ or *trp*$^+$ revertants) were counted.

Results

Mutagenicity in various bacterial strains

Table I shows that metabolically activated VDC was mutagenic in all six bacterial strains used: *S. typhimurium* TA1535, TA100 and TA92, three DNA repair variant strains derived from the substitution mutant *his* G46; *S. typhimurium* TA98 and TA1537, two different frameshift mutants; and *E. coli* WP2 *uvrA*, which possesses a substitution mutation in a gene required for tryptophan synthesis. The stabilizer 4-methoxyphenol could not account for any of these mutations, as it did not show mutagenic effects (Table I). In the absence of an activating system, VDC and 4-methoxyphenol were not mutagenic (data not shown).

Activation of VDC by various metabolizing systems

The liver and the kidney are the main target organs for the toxic and carcinogenic effects of VDC. Subcellular preparations of these organs from various mammalian species were tested for their ability to activate VDC to a mutagen (Table II). Liver S-9 mix from Chinese hamsters and mice were most active. Preparations from male Swiss Webster mice did not differ appreciably from preparations from females of the same strain nor from preparations from C57BL females. Preparations from C57BL males were also similarly active when used in low amount (12.5 mg tissue equivalents/plate), whilst they were less active than those from Swiss Webster mice when used in higher amounts (25 and 50 mg tissue equivalents/plate). Rat liver S-9 mix caused a clearly weaker activation than that from Swiss Webster mouse (see also Table V). Two preparations from human liver were also used. Both had similar activity. When large amounts of postmitochondrial fraction were used, they led to marked mutagenic effects, whereas at smaller concentrations they were much less active than liver preparations from other species.

Preparations from kidney were substantially less active than those from liver. Preparations from Chinese hamsters and from male mice of both strains regularly led to a weak activation, whereas those from female mice and from rats were mostly inactive (see also Table V). In a few experiments, kidney S-9 mix from rats and female mice weakly activated VDC. The reasons for the discrepancies among experiments are not clear, but may, at least in part, be due to the weakness of the mutagenicity.

To investigate the possible roles of microsomal epoxide hydrolase and conjugation with glutathione in the activation and inactivation of VDC, we added epoxide hydrolase (purified from rat liver microsomes (27)) or glutathione to S-9 mix from liver or kidney of Swiss Webster mice. Fifty units of enzyme, a >20-fold excess over the endogenous activity in the S-9 mix, did not affect the mutagenicity of VDC (data not shown). The epoxide hydrolase inhibitor, 1,1,1-trichloro-

Table I. Investigation for liver enzyme-mediated mutagenicity and toxicity of VDC and of its stabilizer 4-methoxyphenol in various bacterial strains.

Test compound	Surviving fraction	Revertant colonies/plate					
		TA100	TA1535	TA92	TA1537	TA98	WP2 uvrA
0 p.p.m. VDC	1.0	105	24	30	17	32	26
375	1.1	740	223	245	42	202	560
2250	1.0	880	490	319	43	247	600
4500	1.0	1270	500	285	52	277	690
10500	1.0	1080	550	295	41	274	740
22500	1.0	1380	660	263	46	314	680
100 mg 4-Methoxyphenol per 20 l desiccator	1.0	125	22	40	25	39	23
0	1.0	102	9	28	17	36	21
3.15–1000 μg 4-Methoxyphenol per plate	0.9–1.0	93–103	8–11	25–32	16–25	34–39	20–25

Minimal agar plates with *his*⁻ *S. typhimurium* or *trp*⁻ *E. coli* strains and with NADPH-fortified liver postmitochondrial supernatant fraction (corresponding to 50 mg tissue/plate) from untreated male adult Swiss Webster mice were kept at 37°C for 4 h in a desiccator with VDC. After further incubation for 2 days at 37°C, colonies (revertants) were counted. The stabilizer 4-methoxyphenol was tested by the same method (whereby no significant sublimation in the desiccator was observed) or by adding it (at 6 different concentrations) directly to the plates. The values are means from 2 to 4 replicate incubations. The individual values deviated by <10% from the mean values, except in some cases where the numbers of colonies were low. Toxicity was estimated by determining the surviving fraction of 600 *his*⁺ mutants which were added as an internal standard to mutagenicity plates (with TA1537).

Table II. Activation of VDC to a mutagen by liver and kidney postmitochondrial supernatant fractions from various species.

Experiment	Organ	Species	Sex	p.p.m. VDC in mutagenicity experiments	Number of induced revertants/plate				
					Postmitochondrial fraction (tissue equivalents)				
					6.25 mg	12.5 mg	25 mg	50 mg	100 mg
I	Liver	Swiss Webster mouse	male	4500	–[a]	389	688	613	–[a]
			female	4500	–[a]	384	683	719	–[a]
		C57BL mouse	male	4500	–[a]	381	215	296	–[a]
			female	4500	–[a]	362	598	570	–[a]
		Chinese hamster	male	4500	–[a]	312	619	654	–[a]
		Sprague Dawley rat	male	4500	–[a]	162	243	345	–[a]
II	Liver	Human	male	2250	22	87	142	278	584
				4500	41	72	153	305	529
III	Liver	Human	male	2250	4	77	152	345	605
		Swiss Webster mouse	male	2250	133	310	354	475	559
IV	Kidney	Swiss Webster mouse	male	4500	–[a]	–[a]	93	67	85
		Sprague Dawley rat	male	4500	–[a]	–[a]	19	41	15
		Chinese hamster	male	4500	–[a]	–[a]	171	105	138
V	Kidney	C57BL mouse	male	450	–[a]	–[a]	–[a]	126	–[a]
				1250	–[a]	–[a]	–[a]	106	–[a]
				4500	–[a]	–[a]	–[a]	108	–[a]
				12 500	–[a]	–[a]	–[a]	139	–[a]
VI	Kidney	C57BL mouse	male	90	–[a]	–[a]	–[a]	31	–[a]
				450	–[a]	–[a]	–[a]	70	–[a]
				1250	–[a]	–[a]	–[a]	113	–[a]
VII	Kidney	C57BL mouse	female	90	11	–[a]	18	7	–[a]
				450	13	–[a]	6	3	–[a]
				1250	7	–[a]	5	–3	–[a]
				4500	6	–[a]	10	–12	–[a]

Histidine-poor agar plates containing *his*⁻ *S. typhimurium* TA100 and NADPH-fortified postmitochondrial fraction from 6.25 to 100 mg liver or kidney were exposed to VDC for 4 h at 37°C. After a further incubation for 2 days at 37°C, *his*⁺ revertant colonies were counted. Values are means from 2 (Experiment II, V, VI, VII) or 3 (other experiments) replicate incubation minus the corresponding number of mutants in the absence of VDC (98–153). The variation coefficients of the number of colonies on replicate plates were <10%. The two human liver samples were taken during surgical treatment, transported on ice and used for mutagenicity experiments within 3 h after they had been taken. [a]Not determined.

propene 2,3-oxide (29), also had no effect on the activation of VDC by S-9 mix (this experiment was performed with TA98 in presence of 1 mM trichloropropene oxide; data not shown). Glutathione usually reduced the mutagenicity of VDC by between 20 and 50%. Typical examples are shown in Figure 1. However, glutathione increased the mutagenicity of VDC in some experiments, especially when weakly active preparations from kidney or when small amounts of liver preparations were used (data not shown). Kidney S-9 mix from female mice regularly activated VDC to a mutagen in the presence of glutathione, whereas this was usually not the case in its absence. The effect was always weak, maximally 100 colonies above solvent controls of ~150 colonies.

Experiments on the stability of kidney and liver S-9 mix

The weak activation of VDC by kidney preparations in comparison to liver preparations was somewhat surprising. Because extrahepatic subcellular fractions are rarely used for activation in mutagenicity experiments, little is known about their optimal preparation and their stability. Kidney prepara-

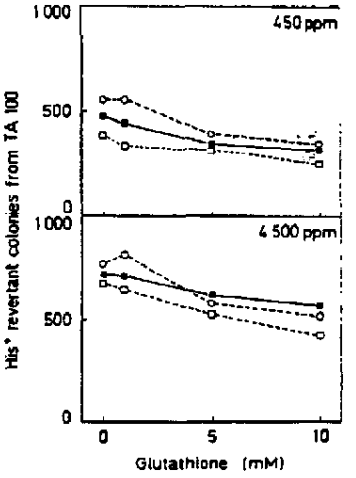

Fig. 1. Effect of glutathione on liver enzyme-mediated mutagenicity of VDC. Minimal agar plates with histidine-auxotrophic *S. typhimurium* TA100, NADPH-fortified, dialyzed liver postmitochondrial supernatant fraction and various concentrations of glutathione were placed for 4 h at 37°C in a desiccator with 0 p.p.m. (data not shown), 450 p.p.m. (upper panel) or 4500 p.p.m. VDC (lower panel). After further incubation for 2 days at 37°C, colonies (revertants) were counted. Values are means from 3 replicate plates. The variation coefficients were <10%. The number of spontaneous revertants varied from 88 to 133 (depending on amount of postmitochondrial fraction and glutathione) and was not subtracted. Dialysis of the postmitochondrial fraction removed >95% of the endogenous glutathione. The concentration of glutathione in the figure is related to the top agar layer. ■, postmitochondrial fraction from 50 mg liver of untreated male C57BL mice; □, postmitochondrial fraction from 50 mg liver of untreated male Swiss Webster mice; ○, postmitochondrial fraction from 100 mg liver of untreated male Swiss Webster mice.

tions used in the present study activated 2-aminoanthracene and benzo[a]pyrene (Table III and data not shown). The activation of 2-aminoanthracene by kidney S-9 mix was even greater than by liver S-9 mix.

It has been observed that ethylene, vinyl chloride and some other olefins after metabolic activation destroy cytochrome P-450 (30,31), the enzyme probably required for metabolic activation of VDC. However, rapid inactivation of kidney preparations when they are kept at 37°C or exposed to VDC in mutagenicity experiments is unlikely, because kidney and liver S-9 mix exposed to VDC before a mutagenicity experiment maintained their ability to activate 2-aminoanthracene and VDC (Table III).

Activities of drug-metabolizing enzymes in VDC-treated animals and effects on the activation of VDC

Bartsch *et al.* (17) and Jones and Hathway (19) have demonstrated that enzyme induction by phenobarbital or Aroclor 1254 potentiates the activation of VDC by subcellular preparations. Therefore, possible enzyme inducing effects of VDC itself may be of relevance in carcinogenicity experiments. Female mice and male rats were treated as described in previous carcinogenicity studies (4,5), i.e. they were exposed daily for 6 h to 50 and 200 p.p.m. VDC, respectively. Male mice were treated with 50 p.p.m., a concentration which was lethal for many animals, or with 10 p.p.m., a concentration at which all animals survived. The effects on enzyme activities of these treatments after various periods are

Table III. Exposure of S-9 mix to VDC before the mutagenicity experiment: effect on the ability to activate premutagens.

Source and treatment of S-9 mix	Number of *his*⁺ revertants/plate		
	Control plates	Plates exposed to 4500 p.p.m. VDC	Plates with 1 μg 2-aminoanthracene
Liver S-9 mix			
exposed to VDC at 37°C	142 ± 10	900 ± 50	7800 ± 400
kept at 37°C	128 ± 3	1760 ± 80	5400 ± 400
kept at 0°C	120 ± 15	930 ± 30	4400 ± 300
Kidney S-9 mix			
exposed to VDC at 37°C	205 ± 24	287 ± 14	8300 ± 700
kept at 37°C	152 ± 11	196 ± 16	7000 ± 500
kept at 0°C	151 ± 2	201 ± 14	8700 ± 900

NADPH-fortified postmitochondrial supernatant fractions from organs of untreated male Swiss Webster mice were divided into 3 aliquots. The first aliquot was exposed in a large sealed glass bottle to 9000 p.p.m. VDC at 37°C for 1 h. Meanwhile, the other two aliquots were kept at 37°C and 0°C, respectively. Afterwards, aliquots equivalent to 50 mg tissue together with *his*⁻ *S. typhimurium* TA100 were poured on minimal agar plates. Some plates also received the premutagen 2-aminoanthracene. Others were placed for 4 h at 37°C into a desiccator with VDC. After a further incubation for 2 days at 37°C, *his*⁺ revertant colonies were counted. Values are means and S.D. from 3 replicate incubations.

shown in detail in Table IV and are summarized in Table VI. In the mouse liver, changes in enzyme activity were always very small and may be readily explained by statistical dispersion. However, the weak increase in glutathione transferase activity in female mice after the longest treatment period may be real, since the kidney of the same animals and the liver and the kidney of VDC-treated rats also showed an increase in this enzyme activity. In the rat liver, monooxygenase activity was decreased after the first treatment to less than half of that in control animals and remained low during the observation period, whereas epoxide hydrolase and glutathione transferase activities gradually increased to about 2- and 1.5-fold control activity, respectively.

In the kidneys of mice of both sexes, monooxygenase activity was decreased in animals treated for 1 day with 50 p.p.m. or for 3 days with 10 p.p.m. With both regimens, the activity was below the detection limit after 8 days of treatment except in one experiment (Experiment number 5 in Table IV) with male mice treated with 50 p.p.m. In this aberrant experiment, monooxygenase activity (in control and treated animals) was above the usual control values and a relatively high mortality rate occurred in the longest treatment group. The effects of VDC treatment on renal epoxide hydrolase activity considerably varied among repeat experiment. It appears that two antagonistic processes, a decrease and an increase in activity, competed. An early fall followed by a rise in activity was observed in single experiments in which males and female mice, respectively, were treated with 50 p.p.m. In the repeat experiment with males (with a higher mortality rate than in the first experiment) only the decrease in activity was observed, whereas in the other experiment with females (Experiment number 6 in Table IV) only a pronounced increase was seen. Males exposed to the low VDC concentration showed a weak increase after the 8-day treatment, but no initial decrease in epoxide hydrolase activity. Renal glutathione transferase activity was not markedly altered in male mice treated with the low concentration of VDC, was decreased to less than half of the control activity

Table IV. Effect of VDC treatment on drug-metabolizing enzymes in mouse and rat.

Experiment number, animals and treatment	Number of treated and surviving animals	Enzyme activities (nmol product/min/mg protein)					
		Liver			Kidney		
		Monooxygenase	Epoxide hydrolase	Glutathione transferase	Monooxygenase	Epoxide hydrolase	Glutathione transferase
1. Male Swiss Webster mice							
Control	5/5	1.27 ± 0.24	2.23 ± 0.39	3150 ± 360			
10 p.p.m., 1 day	5/5	1.12 ± 0.13	1.98 ± 0.18	3390 ± 490			
10 p.p.m., 3 days	5/5	1.54 ± 0.34	2.41 ± 0.51	3520 ± 1080			
10 p.p.m., 8 days	5/5	1.52 ± 0.17	2.53 ± 0.29	2920 ± 440			
2. Male Swiss Webster mice							
Control	25/25	1.69	1.74	3980	0.05	0.14	441
10 p.p.m., 1 day	25/25	1.16	1.59	4580	0.04	0.15	314
10 p.p.m., 3 days	25/25	1.19	1.67	4180	<0.01	0.16	359
10 p.p.m., 8 days	25/25	1.09	1.50	4020	<0.01	0.24	427
3. Male Swiss Webster mice							
Control	5/5	1.19 ± 0.30	2.29 ± 0.43	3360 ± 410			
50 p.p.m., 1 day	10/10	1.44 ± 0.12	2.58 ± 0.27	2980 ± 340			
50 p.p.m., 3 days	10/8	1.30 ± 0.46	2.45 ± 0.06	2890 ± 500			
50 p.p.m., 8 days	10/7	1.42 ± 0.18	2.24 ± 0.52	2760 ± 550			
4. Male Swiss Webster mice							
Control	25/25	2.49	2.01	4170	0.06	0.12	554
50 p.p.m., 1 day	50/50	1.60	1.88	4470	<0.01	0.03	256
50 p.p.m., 3 days	50/16	1.83	1.77	4420	<0.01	0.10	253
50 p.p.m., 8 days	50/17	3.10	1.53	3500	<0.01	0.16	270
5. Male Swiss Webster mice							
Control	25/25	1.64	2.27	4300	0.14	0.07	597
50 p.p.m., 1 day	30/30	1.32	1.90	5340	0.47	0.01	226
50 p.p.m., 3 days	35/16	1.69	1.72	4630	0.13	0.02	231
50 p.p.m., 8 days	40/7	2.22	1.71	5870	0.12	<0.01	289
6. Female Swiss Webster mice							
Control	10/10	2.90	1.99	1090	0.04	0.13	461
50 p.p.m., 1 day	15/15	3.17	1.91	1210	0.02	0.50	509
50 p.p.m., 3 days	15/15	2.04	1.99	1000	<0.01	0.53	439
50 p.p.m., 8 days	15/15	2.41	2.04	1270	<0.01	0.50	595
7. Female Swiss Webster mice							
Control	25/25	2.07	1.68	1440	0.04	0.11	670
50 p.p.m., 1 day	25/25	2.39	1.75	1830	0.01	<0.02	990
50 p.p.m., 3 days	25/25	1.74	1.12	1730	0.02	0.21	830
50 p.p.m., 8 days	25/25	1.94	1.54	2030	<0.01	0.22	880
8. Male Sprague Dawley[a] rats							
Control	10/10	0.89	8.31	817	<0.01	0.94	156
200 p.p.m., 1 day	10/10	0.36	8.24	543	<0.01	2.43	249
200 p.p.m., 3 days	10/10	0.39	9.32	779	<0.01	1.19	245
200 p.p.m., 8 days	10/8	0.56	15.69	1110	<0.01	1.18	256
9. Male Sprague Dawley[a] rats							
Control	12/12	0.55	7.42	1330	<0.01	1.13	506
200 p.p.m., 1 day	12/12	0.24	7.71	1490	<0.01	0.96	572
200 p.p.m., 3 days	12/12	0.29	13.40	2010	<0.01	1.37	694
200 p.p.m., 8 days	12/12	0.23	13.63	2290	<0.01	1.20	775

Animals were exposed to an atmosphere containing VDC for 6 h on 1–8 subsequent days and killed one day after the last treatment. A monooxygenase (7-ethoxycoumarin O-dealkylase) and an epoxide hydrolase activity (with styrene 7,8-oxide as substrate) were determined in the microsomal fractions. Glutathione transferase activity with 2,4-dinitro-chlorobenzene was measured in the cytosolic fractions. Values are means and S.D. of 3 individually measured animals or activities of the pooled fractions from all surviving or at least 10 animals. [a]Two different stocks of Sprague Dawley rats were used. The animals of experiment 8 were from a commercial source (WIGA, Sulzfeld, FRG) whereas those of experiment 9 were bred from animals obtained from Prof. C.Maltoni.

throughout the treatment in males at the high concentration of VDC, and was slightly increased in treated females.

In rat kidney, monooxygenase activity was below the detection limit in control and in treated animals. Epoxide hydrolase activity was not measurably affected and glutathione transferase activity was slightly enhanced by the treatment.

In Table V, results of mutagenicity experiments using subcellular fractions from VDC-treated and control animals are compared. A summary of these data is also included together with biochemical data in Table VI. With liver preparations as activating systems, the effects of VDC treatment were rather weak. This is in agreement with the weakness of the effects on the activities of drug-metabolizing enzymes. Kidney preparations from control as well as from VDC-treated rats and female mice did not activate VDC to a mutagen or had, at most, marginal effects. With male mice, kidney preparations from control animals showed a weak but definite activation

Table V. Activation of VDC to a mutagen by liver and kidney postmitochondrial supernatant fractions from VDC-treated animals.

Experiment number, animals and treatment	Number of VDC-induced *his*$^+$ revertant colonies			
	Liver		Kidney	
	25 mg	100 mg	25 mg	100 mg
1. Male Swiss Webster mice				
Control	490*	610*	89*	105*
10 p.p.m., 1 day	315*	518*	100*	102*
10 p.p.m., 3 days	294*	469*	39*	37*
10 p.p.m., 8 days	294*	508*	82*	108*
4. Male Swiss Webster mice				
Control	544*	746*	62*	109*
50 p.p.m., 1 day	456*	565*	28 NS	60*
50 p.p.m., 3 days	300*	475*	10 NS	43*
50 p.p.m., 8 days	446*	556*	4 NS	87*
5. Male Swiss Webster mice				
Control	413*	814*	28*	34*
50 p.p.m., 1 day	328*	408*	0	12NS
50 p.p.m., 3 days	370*	640*	4 NS	−7NS
50 p.p.m., 8 days	302*	982*	1 NS	31*
6. Female Swiss Webster mice				
Control	163*	381*	12 NS	13NS
50 p.p.m., 1 day	186*	453*	12 NS	−14NS
50 p.p.m., 3 days	198*	388*	−1 NS	−20NS
50 p.p.m., 8 days	236*	545*	−2 NS	2NS
7. Female Swiss Webster mice				
Control	398*	795*	−7 NS	−5NS
50 p.p.m., 1 day	409*	633*	16 NS	−8NS
50 p.p.m., 3 days	306*	681*	28 NS	18NS
50 p.p.m., 8 days	362*	751*	−2 NS	20*
8. Male Sprague Dawley rats				
Control	50*	267*	16 NS	−7NS
200 p.p.m., 1 day	73*	83*	30*	46*
200 p.p.m., 3 days	111*	196*	7 NS	24NS
200 p.p.m., 8 days	28*	190*	8 NS	−11NS
9. Male Sprague Dawley rats				
Control	19*	110*	22*	42NS
200 p.p.m., 1 day	50*	97*	2 NS	15NS
200 p.p.m., 3 days	61*	16*	14*	46*
200 p.p.m., 8 days	17*	67*	7 NS	33*

Histidine-poor agar plates containing *his*$^-$ *S. typhimurium* TA100 and NADPH-fortified postmitochondrial fraction from 12.5 to 100 mg liver or kidney of control and VDC-treated animals were exposed at 37°C to 4500 p.p.m. VDC for 4 h. After a further incubation for 2 days at 37°C, *his*$^+$ revertant colonies were counted. Values with liver preparations are means from 4 parallel incubations minus the corresponding number of mutants in the absence of VDC (95–168). Values with kidney preparations are means from 5 VDC-exposed plates minus means from 3 corresponding non-exposed plates (112–197). Asterisks indicate statistically significant ($p < 0.05$, t-test) differences from non-exposed controls. NS, not significantly different from controls. Activities of drug metabolizing enzymes are shown in Table III using the same numbering of the experiments.

of VDC to a mutagen. Preparations from VDC-treated animals, especially at the higher treatment concentration, activated VDC less effectively than controls.

Discussion

In the present study, we have investigated VDC with respect to mutagenicity, effects on drug-metabolizing enzymes and the relationship between these two effects. Our results confirm earlier studies (17,19) which described reversion of bacterial strains with the *his* G46 substitution mutation; moreover, we found that VDC causes various other substitution and also frameshift mutations. Along with mutagenicity studies in yeast (20) and in *E. coli* K12 (32) these results indicate that VDC can induce a wide spectrum of different mutations. As was observed in previous studies, metabolic activation was required for mutagenicity to occur and preparations from mice showed stronger activation than preparations from rats and liver was more active than kidney, respectively. In contrast to previous studies, we investigated the same strains of animals which had been used for carcinogenicity experiments. Interestingly, kidney preparations from male mice activated VDC, whereas preparations from female mice and from rats were practically inactive, which is in line with the results of the carcinogenicity studies. However, in spite of negative carcinogenicity results, Chinese hamster kidney preparations activated VDC even more than did preparations from male mice. Together with the relatively potent activation of VDC by hepatic preparations, this indicates that formation of mutagenic metabolites is not sufficient (but probably necessary) for carcinogenesis in a tissue to occur. Indeed, in previous studies it had been noticed that tumorigenic concentrations of VDC resulted in massive tissue damage (12), but caused only minimal DNA alkylation and DNA repair synthesis (33). Hence, non-genetic toxic effects may have been the decisive additional factor that tumours were formed only in the kidney in male mice, but not in other tissues and species which are able to activate VDC to a mutagen.

An epoxide has been proposed as important intermediate in the mutagenicity of VDC (16,17,32,34). Urinary metabolites suggest the formation of glutathione conjugates (34–38), one of them probably derived from the epoxide. We therefore studied the effect of microsomal epoxide hydrolase and glutathione on the mutagenicity of VDC. Since addition of purified epoxide hydrolase from rat liver microsomes or an inhibitor of microsomal epoxide hydrolase, 1,1,1-trichloropropene 2,3-oxide (29), did not affect the mutagenicity of VDC, this enzyme does not seem to be important for the metabolic control of the mutagenic species derived from VDC. Glutathione reduced the mutagenicity of VDC as expected from available biochemical data (34–38) and from a mutation-decreasing effect by other thiols (17). We suspect that the stimulating effect which was observed in some experiments with low concentrations of subcellular preparations in the presence of glutathione was due to a stabilization of activating enzymes, e.g. by inhibition of lipid peroxidation.

To date there are no reports about the effects of VDC on drug-metabolizing enzymes. Such effects may be important in chronic exposures, e.g. in carcinogenicity experiments, because they could potentiate or protect against toxic effects. Various increases and decreases in enzyme activities occurred after treatment of animals with VDC (Table VI). In the mouse, effects were found in the kidney whilst the enzyme activities remained virtually unchanged in the liver. In kidney of male mice, decreases in all three enzyme activities were observed after short exposure. An indication of an increase was seen only with epoxide hydrolase after prolonged treatment. In contrast, in female mice, only the monooxygenase activity was decreased and the activities of the other two enzymes were increased (with the exception of a short intermediate decrease in epoxide hydrolase activity in one experiment). We suggest that (i) the decreases in activity were caused by cytotoxic effects and, therefore, were predominant in the males, which are more susceptible than females, and (ii)

Table VI. Summary of the effects observed in animals after treatment with VDC.

Animals, treatment	Mortality (in 8-day treatment group)	Changes in activities							
		Liver				Kidney			
		Monooxygenase	Epoxide hydrolase	Glutathione transferase	Activation of VDC	Monooxygenase	Epoxide hydrolase	Glutathione transferase	Activation of VDC
Male Swiss Webster mice, 10 p.p.m.	0%	=	=	=	(=)	↓	(↑)	=	(↑)
Male Swiss Webster mice, 50 p.p.m.	69%	=	=	=	=	↓*	↑*	↓	↓
Female Swiss Webster mice, 50 p.p.m.	0%	=	=	(↑)	=	↓	↑*	↓	0
Male Sprague Dawley rats, 200 p.p.m.	9%	↓	↓	↑	(↑)	0	=	↓	0

↑, increase; ↓, decrease; =, no change; 0, not measurable or extremely weak activities. Brackets indicate that the effect was weak in comparison with the variation and that, therefore, the interpretation must be considered as tentative. Asterisks signify that the indicated changes were clearly predominant, but that the effects were complex and also included opposite changes at some stages of the time course. The results are shown in detail in the Tables IV and V.

the mechanism(s) which increases enzyme activities, possibly enzyme induction, is not related to toxicity, but directly to the VDC concentration; furthermore, the two mechanisms may counteract each other; thus, toxicity may inhibit enzyme induction while increases in activities such as glutathione transferase may protect against toxicity. Hence, differences in susceptibility in the uninduced state may be potentiated by increases in the activities of protecting enzymes in the less susceptible animals.

The rat tolerates higher concentrations of VDC than mice (of both sexes) and was therefore treated with 200 p.p.m. VDC compared with 50 p.p.m. for mice. Enzyme activities in the rat were affected in the kidney and also in the liver. A moderate decrease in hepatic monooxygenase activity was observed after the first treatment and, later, a gradual increase of hepatic epoxide hydrolase and glutathione transferase activity was seen. In the kidney, an increase in glutathione transferase, but not in epoxide hydrolase activity occurred; monooxygenase activity was not measurable either in control or in treated animals. Since 7-ethoxycoumarin is a broad spectrum substrate which is dealkylated by various cytochromes P-450, it is likely that enzymes which activate VDC also metabolize 7-ethoxycoumarin. Thus, low monooxygenase activity may be one contributing factor for the low susceptibility of rats, compared to mice, to VDC.

In conclusion, in this comparative biochemical study we have made various observations which can be associated with the susceptibility of tissues to the carcinogenic action of VDC. The results indicate relatively complex relationships between various enzyme activities, formation of mutagenic metabolites, toxicity, induction of potentially inactivating enzymes and carcinogenicity. Altogether, our data support the notion that two effects are necessary for the formation of tumours by VDC: (i) formation of mutagens and (ii) tissue damage. The exceptional sensitivity of male Swiss Webster mice to the toxic action of VDC appears to be due to a special biochemical situation.

Acknowledgements

We thank Dr.C.Maltoni, Bologna, for Swiss Webster mice and Sprague Dawley rats, Dr.B.N.Ames, Berkeley, for *S. typhimurium* strains, Dr.R.E.McMahon, Indianapolis, for *E. coli* WP2 *uvrA*, and Ms.U.Brewi and Ms.C.Clagau for excellent technical assistance.

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