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Tissue-mediated mutagenicity of vinylidene chloride and 2-chlorobutadiene in *Salmonella typhimurium*

CHLORINATED hydrocarbons, such as vinyl chloride or vinylidene chloride (1,1-dichloroethylene), are produced in large quantities and are present in the environment[1]. Recently, vinyl chloride monomer has been shown to be carcinogenic in animals and man[2–4] and mutagenic in microbial systems[5–7]. Vinylidene chloride (VDC), a structurally related substance and co-polymer of vinyl chloride, is used in the manufacture of plastics[8]; it could also occur as a decomposition product of 1,1,1-trichloroethane[1]. Another chemically related compound, 2-chlorobutadiene (chloroprene), has been used in the manufacture of synthetic rubber since 1930.

We have examined the mutagenicity of VDC and 2-chlorobutadiene in *Salmonella typhimurium* strains, using a tissue-mediated assay which has been found effective in detecting the mutagenicity of various carcinogens, such as nitrosamines[9], vinyl chloride[5] and many others[10,11].

Tests for mutagenicity were carried out according to the procedure developed by Ames *et al.*[11] and modified by Bartsch *et al.*[5] using the histidine-auxotroph strains of *S typhimurium* TA1530 and TA100, provided by Professor B N. Ames. In this procedure, Petri dishes containing 9,000*g* tissue supernatant, an NADPH-generating system and the bacteria in a soft agar layer were exposed to various concentrations of VDC or 2-chlorobutadiene vapour in a desiccator at 37 °C in the dark. The compounds were then replaced by air, and, after further incubation for up to 48 h at 37 °C, the number of histidine-revertant colonies per plate was counted. The concentration of VDC or 2-chlorobutadiene in the incubation medium was determined by gas-liquid chromatography[5]. In the experimental conditions used, the concentrations of VDC in the aqueous phase after 2 h of exposure to 0.2, 2 or 20% VDC in air (v/v) were 3.3×10^{-4} M, 3.3×10^{-3} M or 3.3×10^{-2} M, respectively. The concentrations of 2-chlorobutadiene in the aqueous phase after 2 h of exposure to 0.5, 2 or 8% vapour in air (v/v) were 7×10^{-6} M, 3.7×10^{-4} M or 1.65×10^{-3} M, respectively. No further increases were observed after up to 7 h of exposure.

Fig. 1 shows the mutagenic effect on *S. typhimurium* TA1530 and TA100 of exposure to 0.2, 2 or 20% VDC in air in the presence of 9,000*g* liver supernatant of phenobarbitone-pretreated mice (0.1% in the drinking water for 7 d). When the cofactors ($NADP^+$ and glucose-6-phosphate), required for activity of microsomal mixed-function oxidase, were omitted from the incubation mixture, no mutagenic effect was observed. The mutagenic response, which was greater in the TA100 strain, increased in both strains after exposure to up to 2% VDC in air. The lower mutagenic response observed with a concentration of 20% VDC may result from an inhibitory action of VDC and (or) its metabolite(s) on the microsomal enzymes responsible for the metabolic activation of VDC. 4-Methoxyphenol, which is used as a stabiliser of VDC, when applied at up to 500 μg per plate, was not mutagenic for either strain, whether in the presence or absence of microsomal liver fraction from phenobarbitone-pretreated mice.

Exposure of TA100 strain to 2% VDC in air in the presence of a hepatic microsomal fraction from untreated or phenobarbitone-pretreated mice, caused a linear increase in mutagenic response up to 4 h. Thus, exposure to 2 or 20% VDC in air for 4 h was used to assay rat and mouse liver, kidney and lung fractions (9,000*g* supernatant) for their ability to convert VDC into mutagenic metabolites (Table 1).

Mouse liver, kidney and lung fractions efficiently converted

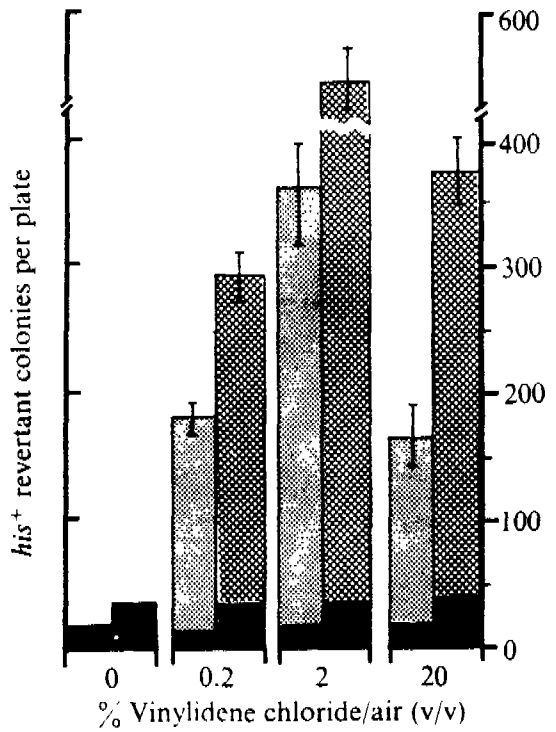

Fig. 1 VDC concentration-dependent induction of reverse mutations in *S. typhimurium* strains TA1530 and TA100. Petri dishes containing 9,000*g* liver supernatant from phenobarbitone-pretreated male OF-1 mice, $NADP^+$ (2 μmol per plate), glucose-6-phosphate (2.5 μmol per plate) and the bacteria in a histidine-deficient medium were exposed to 0, 0.2, 2 or 20% VDC in air (by volume) for 4 h in a desiccator at 37 °C in the dark. VDC was removed under vacuum and replaced by air, and the incubation was continued up to 48 h at 37 °C. Bacterial survival was estimated by seeding TA1530 (10^{-7} dilution) on a histidine-enriched medium. Control assays were carried out by omitting the cofactors ($NADP^+$, glucose-6-phosphate). Mean values ± s.e. from 1–4 series of experiments, each on a pool of five mouse livers, are plotted. Bacteria were plated in triplicate. VDC, containing 0.3% 4-methoxyphenol as antioxidant, was obtained from Merck-Schuchardt (Darmstadt, Federal Republic of Germany). Black, control; stippled, TA 1530; cross-hatched, TA100.

VDC into mutagenic metabolites *in vitro*, activity being greatest in the liver. Phenobarbitone pretreatment of the mice produced an increase in the mutagenic response in assays with all three organ fractions. A much lower mutagenic response was observed in rats than in mice with liver and kidney fractions, and there was only minimal activity with a lung fraction. Rats have been reported to show hepatic and renal damage following inhalation of VDC (ref. 12). When the concentration of VDC in air was raised from 2 to 20%, an increased mutagenic effect was noted only with mouse kidney and lung fractions.

As has been shown previously for vinyl chloride, these results demonstrate that the mutagenic effect of VDC is mediated by microsomal enzymes from various organs, in the presence of an NADPH-generating system and oxygen; these enzymes probably form the alkylating intermediate(s), since *S. typhimurium* TA1530 strain is specifically reverted by monofunctional alkylating agents[11]. Evidence for the participation of a microsomal mixed-function oxidase is further supported by the observation of changes in mutagenicity *in*

Table 1 Mouse and rat tissue mediated mutagenicity of VDC in *S. typhimurium* TA100*

Experiment no.	Species	Phenobarbitone pretreatment	Tissue (9,000*g* supernatant†)	Cofactors‡	2% VDC in air *his*⁺ revertants per plate§	2% VDC in air Relative activity¶	20% VDC in air *his*⁺ revertants per plate§	20% VDC in air Relative activity¶
1		Yes		+	500 ± 23	150	330 ± 29	75
2		Yes		−	23 ± 10	5	7 ± 5	2
3		No	Liver	+	330 ± 49	100	435 ± 46	100
4		No		−	16 ± 4	5	1 ± 3	0
5		Yes		+	147 ± 15	45	173 ± 5	40
6		Yes		−	31 ± 7	9	17 ± 2	4
7	OF-1 mouse♂	No	Kidney	+	67 ± 2	20	125 ± 5	29
8		No		−	20 ± 3	6	16 ± 1	4
9		Yes		+	34 ± 4	10	48 ± 5	11
10		Yes		−	5 ± 4	1	10 ± 1	2
11		No	Lung	+	21 ± 5	6	37 ± 3	8
12		No		−	6 ± 9	2	14 ± 8	3
13		No		+	95 ± 7	30	77 ± 5	18
14		No	Liver	−	0	0	2 ± 2	0
15		No		+	18 ± 4	5	16 ± 2	4
16	BDVI rat ♀	No	Kidney	−	18 ± 2	5	21 ± 4	5
17		No		+	9 ± 2	3	11 ± 2	3
18		No	Lung	−	9 ± 7	3	12 ± 6	3

*Assays carried out as described in Fig. 1.
†Equivalent to 38 mg wet tissue per plate
‡$NADP^+$ (2.0 μmol per plate) and glucose-6-phosphate (2.5 μmol per plate)
§Mean values ± s.e. from 1–4 experiments, each using pooled tissues from 4 mice or 3 rats. The number of spontaneous mutations per plate (49 ± 2) has been subtracted from each value
¶Relative mutagenic activity was expressed by taking the value obtained in experiment 3 as 100.

vitro and (or) the toxicity of VDC following pretreatment of rats or mice with various drugs which are known to modify the activity of drug-metabolising enzymes. As shown in Table 1, phenobarbitone pretreatment of mice caused an increased mutagenic response. In another series of experiments, we examined the effects of pretreating female BD-VI rats with phenobarbitone (0.1% in the drinking water for 7d), pregnenolone-16α-carbonitrile (PCN; 50 mg kg^{-1}; 5 times orally at 12 h intervals) or aminoacetonitrile (AAN: 500 mg kg^{-1}, one subcutaneous injection 24 h before the assay) on the mutagenic effect of exposure to 2% VDC in air on *S. typhimurium* TA1530 strain with liver fractions in assays as described in Fig. 1. Phenobarbitone caused a twofold increase in the mutagenic response, whereas PCN or AAN resulted in reductions of 40 or 60%, respectively, when compared with the corresponding values obtained from untreated rats. Carlson and Fuller[13] reported an increased mortality by VDC in rats following phenobarbitone pretreatment.

The addition of sulphur-containing compounds to *in vitro* mutagenicity assays containing mouse-liver fractions reduced the mutagenic effect of exposure to 2% VDC in air on the *S. typhimurium* TA1530 strain. Addition of N-acetyl-cysteine and N-acetyl-methionine (12 μmol of each per ml soft agar layer) caused an 80% reduction of mutagenic response compared with the appropriate control. These results indicate that the mutagenic VDC metabolite(s) is trapped by nucleophilic sulphur groups, thus competing for binding to bacterial DNA. This observation parallels the findings of Jaeger *et al.*[14] who observed that the toxicity of VDC in rats is correlated with hepatic glutathione concentration, and that cysteine has a protective effect.

Chloroethylene oxide, the suggested primary metabolite of vinyl chloride, has been shown to be an alklylating and mutagenic agent[6]. Since epoxides are now recognised as obligatory intermediates in the metabolism of olefinic compounds by hepatic microsomal mixed function oxidase[15], it can be postulated that 1,1-dichloroethylene oxide may be a primary reactive metabolite of VDC. It is also possible that partial dechlorination[16] of VDC by microsomal enzymes results in vinyl chloride and its metabolic products.

Exposure of *S. typhimurium* TA100 strain to 0.5% up to 8% of 2-chlorobutadiene in air in the absence of any metabolic activation system caused a linear increasing mutagenic response, as a function of the 2-chlorobutadiene concentration, which, at a concentration of 8%, reached three times the spontaneous mutation rate (Table 2). Exposure to higher concentration (20%) caused a strong toxicity in the bacteria. This mutagenic and (or) toxic effect could be caused by a direct action of 2-chlorobutadiene or, more likely, by one of its enzymic (bacteria) or non-enzymic breakdown products. A three or twofold increased mutagenic response, respectively, was observed when a fortified 9,000*g* liver supernatant from either phenobarbitone or untreated mice was added to such assays (Table 2). These results support an enzymic formation of a mutagenic metabolite(s) from 2-chlorobutadiene, probably an epoxide, as it was strongly implicated for vinyl chloride[5].

Most chemical carcinogens have now been found to be mutagens, when assayed in one of the mutagenicity tests that combine microbial or mammalian cell systems as genetic targets with an *in vitro* or *in vivo* metabolic activation system[17]. The increasing evidence of a possible correlation between mutagenicity and carcinogenicity does not mean that one biological effect may be equated with another. Thus, mutagenicity in microorganisms cannot be automatically assumed to imply a possible carcinogenic effect in man. Mutagenicity tests are at present an excellent method for screening environmental chemicals to be submitted to a more careful set of bioassays. While there are no criteria at present for extrapolation from experimental data to man, the available evidence indicates that precautions are justifiable on the basis of experimental evidence of carcinogenicity. There is, in fact good indication that experimental evidence of carcinogenicity can in certain cases predict a similar effect in man; as was the case of diethylstilboestrol, *bis*-chloromethylether and vinyl chloride[18].

Recently, VDC has been found to be carcinogenic in the rat, (Viola, Bigotti and Caputo, personal communication) and 2-chlorobutadiene has been suggested as being responsible for an increased incidence of skin and lung cancer among exposed workers[19]. A high incidence of chromosome aberration in lymphocytes from the peripheral blood of workers exposed to 2-chlorobutadiene has been reported[20]. These findings, combined with the present results, indicate that an epidemiological survey of workers exposed to these substances may be necessary, as well as measures to prevent, or greatly reduce, exposure of humans.

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Table 2 Mutagenicity of 2-chlorobutadiene in *S. typhimurium* TA100*

Experiment no.	2-chlorobutadiene† in air (%)	Phenobarbitone pretreatment	*his*⁺ revertants/plate 9,000*g* liver supernantant +cofactors‡	KCl§
1	0	Yes or no	35 ± 3	35 ± 2
2	0.5	Yes	175 ± 15	49 ± 3
3		No	101 ± 4	
4	2	Yes	232 ± 17	70 ± 4
5		No	154 ± 15	
6	8	Yes	331 ± 30	117 ± 6
7		No	306 ± 25	

*Bacteria were exposed to 0.5, 2 and 8% 2-chlorobutadiene in air (v/v) for 4 h at 37 °C in presence of a NADPH generating system and a 9,000*g* liver supernatant from either phenobarbitone-treated or untreated male OF-1 mice as described in Fig. 1.

†2-Chlorobutadiene (purity 98.94%) was provided by Distugil, Le Pont de Claix, France, contaminated with 0.98% 1-chlorobutadiene, 370 p.p.m. butadiene and 280 p.p.m. vinylacetylene. *Tertiary* butylcatechole which was present in 2-chlorobutadiene as an antioxidant (200 p.p.m.) was not mutagenic up to 500 μg per plate for the TA100 strain either in the presence or absence of a microsomal liver fraction of phenobarbitone pretreated mice.

‡When the cofactors ($NADP^+$ and glucose-6-phosphate) were omitted, the number of *his*⁺ revertants per plate was identical with assays containing KCl only. Mean values ± s.e. from 2 series of experiments, each on pooled livers from 4 mice.

§9,000*g* supernatant + cofactors was replaced by a 0.9% KCl solution.

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