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Int. J. Cancer: 15, 429-437 (1975)

HUMAN, RAT AND MOUSE LIVER-MEDIATED MUTAGENICITY OF VINYL CHLORIDE IN *S. TYPHIMURIUM* STRAINS

by

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Exposure of S. typhimurium *strains TA 1530, TA 1535 and G-46 to vinyl chloride increased the number of His⁺ revertants/plate 16, 12 or 5 times over the spontaneous mutation rate. After 6 h of exposure to vinyl chloride, the mutagenic response for TA 1530 strain was enhanced 7-, 4- or 5-fold when fortified postmitochondrial liver fractions from humans, rats or mice were added. The enzyme-mediated vinyl chloride mutagenicity was dependent on an NADPH generating system and the enzyme activity was localized in a liver microsomal fraction; 9,000 × g liver supernatant was three times more active than microsomes, while liver cytosol or alcohol dehydrogenase did not affect the mutagenicity. Phenobarbitone pretreatment of rats and mice increased the mutagenic response by up to 15-40% as compared to untreated controls. The relative mutagenic activities of VCM, taking the value from mouse liver as 100, for TA 1530 strain mediated by 9,000 × g tissue fractions were: rat liver, 80; mouse and rat kidney, 20 and 16; mouse and rat lung, less than 7; human liver (from four biopsy specimens), 170, 64, 70 and 46. Chloroacetaldehyde and chloroacetic acid, a urinary metabolite of VCM, showed toxic effects, while chloroethanol was weakly mutagenic for TA 1530 strain.*

Vinyl chloride monomer (VCM) is extensively used for the production of polyvinyl chloride and other plastic material, and also as a propellant for aerosols. Carcinogenicity in rats, when exposed to 30,000 ppm VCM in air, was first reported by Viola *et al.* (1971). Subsequently, Maltoni and Lefemine (1974) showed that angiosarcomas of the liver, as well as other tumours, were induced in rats and mice exposed to various doses of VCM by inhalation. Recently, cases of angiosarcoma of the liver have been found in workers exposed to VCM during the polymerization process and a causal relationship between VCM exposure and this type of tumour in man has been established (Creech and Johnson, 1974; Heath *et al.*, 1974; Lee and Harry, 1974; IARC, 1974). The systemic action of this carcinogen and its low chemical reactivity suggest that the biological effects of VCM are dependent upon its metabolic activation. In these studies, we report the mutagenicity of VCM and its presumed metabolites in *S. typhimurium* strains, mediated by liver and other tissue fractions of rat, mouse and human origin.

Received: October 16, 1974.

MATERIAL AND METHODS

Chemicals

VCM (purity 99.9%) was generously provided by Rhône-Progil, Lyons, France, contaminated with ethanol (30 ppm), water (20 ppm), methylchloride (<20 ppm) and non-volatile substances

(>5 ppm). Chloroacetaldehyde (50% aqueous solution), chloroacetic acid (mp 61-63° C) were obtained from Fluka Co., Buchs, Switzerland and chloroethanol (bp 128-130 C) from Merck-Schuchardt, Darmstadt, Fed. Rep. of Germany. Alcohol dehydrogenase (200 U/mg of protein) and glucose-6-phosphate dehydrogenase (450 U/mg of protein) were purchased from Boehringer Mannheim, Fed. Rep. of Germany, or Sigma Chemical Co., St. Louis, Miss., USA. All other products were obtained commercially and were of the purest grade available.

Gas chromatography

VCM concentrations were determined in a Packard model 417 gas chromatograph, fitted with a glass column (0.3 × 400 cm), which was packed with Porapack Q, 100-120 mesh (Packard S.A., Paris, France). A flame ionization detector with N_2 as carrier gas was used at a flow rate of 45 ml/min. For routine use, the column, the detector and the injection port were maintained at 100, 160 and 140° C, whereby VCM had a retention time of 28 min. A freshly prepared solution of VCM in methanol was used as a standard.

Animals and pretreatment

Adult male BD-IV rats (100-130 g), bred in our laboratories, and male OF-1 mice (30-35 g), obtained from Ifa-Credo, St. Germain-sur-L'Arbresle, France, were fed on a Charles River CRF diet. Some rats and mice received phenobarbitone sodium (PB) in their drinking water (1 mg/ml) for 7 days prior to the preparation of the tissue fractions. Human liver samples, with no pathologic lesions, obtained by biopsy from four adult patients, were kindly provided by Drs. G. Della Porta and U. Veronesi (Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy).

Mutagenicity assays

The 9,000 × *g* supernatants or microsomal and soluble fractions were freshly prepared from tissue pools from 3-5 animals, by centrifugation of a homogenate (3 ml of 0.15 M KCl/g wet tissue), as described elsewhere (Bartsch *et al.*, 1975), and utilized in the assays 3-5 h later. The 9,000 × *g* liver supernatant from four humans was prepared under identical conditions, and stored in liquid nitrogen for 3 weeks (Sample A and D), and for 1 week (Sample B and C). The total and microsomal protein content per ml of the 9,000 × *g* supernatants from PB-treated or untreated animals were: rat and mouse liver, 29.7 ± 3 (20), 5.1 ± 1.1 (18); rat and mouse lung, 21.4 ± 3 (9), 1.7 ± 0.3 (8); rat and mouse kidney, 17.4 ± 1.7 (7), 2.1 ± 0.4 (7). (± = SD; number of animal pools analyzed shown in parentheses). *S. typhimurium* strains TA 1530, TA 1535, G-46 and TA 1538, kindly provided by Professor B. Ames, University of California, Berkeley, USA, were grown overnight in nutrient broth and resuspended in saline. Mutagenicity assays were performed in two ways.

Method A

If not otherwise specified, 9,000 × *g* tissue supernatants, fortified with the appropriate cofactors (NADP+, 2 μmoles; glucose-6-phosphate, 2.5 μmoles; $MgCl_2$, 4 μmoles), phosphate buffer pH 7.4 (50 μmoles) and 3-6 × 10^8 bacteria per plate were combined in a soft-agar layer and plated in duplicate or triplicate on Petri dishes with Davis minimal agar, as described (Ames *et al.*, 1973). When microsomal fractions were utilized, the medium was supplemented with 1.0 U of glucose-6-phosphate dehydrogenase. Bacterial survival was determined in parallel by seeding bacteria (10^{-6}-10^{-8} dilutions) on a histidine-enriched medium. Mutagenicity tests with VCM were performed by exposing the Petri dishes to VCM in air in dessicators (volume: 10-15 l) for up to 48 h at 37° C in the dark. For shorter treatments, the VCM was removed under vacuum and replaced by air, and the incubation was continued up to 48 h at 37° C. The concentration of VCM in the Vogel-Bonner medium was determined by gas liquid chromatography of 6 and 48 h following exposure at 37° C to 0.2, 2 and 20% VCM in air (v/v).

Method B

This was carried out by incubation in liquid suspension (Bartsch *et al.*, 1975). The medium (final volume 220 μl) contained 60 μl of 9,000 × *g* liver supernatant from PB-pretreated mice, 1.6 μmol $MgCl_2$, 1 μmol glucose-6-phosphate, 0.8 μmol NADP+, 20 μmol Sorensen phosphate buffer pH 7.4, 20 μl bacterial suspension G-46 or TA 1530 (2-4 × 10^8 cells) and an initial concen-

tration in the medium of 0.083% VCM. The incubation was carried out at 37 °C for 30 min under O_2 and was stopped by dilution with ice-cold saline. The number of His^+ revertants and survivors was determined by plating on selective media as previously described (Bartsch *et al.*, 1975).

RESULTS

Mutagenic response as a function of dose and time of VCM exposure

S. typhimurium strains TA 1530, TA 1535 and G-46, which are sensitive to monofunctional alkylating agents, were specifically reverted by VCM to His prototrophy, following exposure to various concentrations of VCM in air, in the presence of a 9,000 *g* mouse liver supernatant and cofactors as listed under Method A (Table I). Among the strains tested, the TA 1530 showed the highest mutagenic response and the number of His^+ revertants/plate increased approximately six, 12 or 28 times over the spontaneous mutation rate following exposure to 0.2, 2 or 20% VCM in air for 48 h. The G-46 and TA 1535 strains also showed a similar mutagenic dose response, but appeared less sensitive than TA 1530 strain. With TA 1538 strain, which is specifically reverted by frameshift mutagens, no significant increase in the number of His^+ revertants after an exposure to 20% VCM in air for 48 h was noticed. Under these conditions, the concentrations of VCM in the incubation medium after 6 h of exposure to 0.2, 2 or 20% VCM in air were 4×10^{-5} M, 40×10^{-6} M and 400×10^{-5} M, and no further increase was observed up to 48 h. Under these experimental conditions and for all *S. typhimurium* strains utilized, VCM showed no cytotoxic effects. Bacterial survival, even at the concentration of 20% VCM in air, was between 86 and 131% (Table I).

In another series of experiments, TA 1530 strain was exposed to 20% VCM in air for various lengths of time (Fig. 1). In the absence of a 9,000 *g* mouse liver supernatant, VCM exerted a mutagenic action *per se* in TA 1530 strain. The number of His^+ revertants increased as a linear function of the time of exposure to VCM reaching, after 48 h, 20 times the level of the spontaneous mutation rate. In the presence

TABLE I

VCM CONCENTRATION-DEPENDENT INDUCTION OF REVERSE MUTATIONS

Exp. No.	% VCM in air	9,000 *g* sup. ±cofactors [b]	Phenobarbitone pretreatment	No. of His^+ revertants / plate (% bacterial survival [c]) Strain: TA 1530	TA 1535	G-46	TA 1538
1	0	-	Yes or no	10 ± 3 (100)	8 ± 2 (100)	6 ± 3 (100)	23 ± 1 (100)
2	0.2	+	Yes	62 ± 7	44 ± 4 [d]	20 ± 15	
3		-	Yes	41 ± 2 (131)	23 ± 4 (86)	10 ± 1 (111)	
4		+	No	45 ± 10	39 ± 0	11 ± 2	
5		-	No	27 ± 15	21 ± 9	11 ± 8	
6	2	+	Yes	118 ± 23	76 ± 11	25 ± 2	
7		-	Yes	39 ± 26 (116)	40 ± 12 (86)	13 ± 1 (103)	
8		+	No	106 ± 19	64 ± 22	19 ± 1	
9		-	No	70 ± 8	33 ± 2	15 ± 1	
10	20	+	Yes	278 ± 16	179	62 ± 3	
11		-	Yes	131 ± 17 (123)	98 ± 36 (129)	40 ± 0 (119)	32 ± 8 (118)
12		+	No	232 ± 8	161 ± 60	56 ± 0	
13			No	155 ± 11	98 ± 31	30 ± 10	

[a] Strain TA 1530 (bacteria/plate in parentheses) (6.8×10^8); TA 1535 (3.3×10^8); G-46 (5.8×10^8) and TA 1538 (8.3×10^8), were exposed to 0.2, 2 and/or 20% VCM in air (v/v) for 48 h at 37 °C in the presence of an NADPH generating system and a 9,000 *g* liver supernatant from either phenobarbitone-treated or untreated mice.

[b] Bacterial survival for each experiment was determined in parallel; mean values for each exposure group are listed.

[c] Cofactors: $NADP^+$ and glucose-6-phosphate.

[d] Mean values ± SD from two series of experiments, each utilizing a pool of five mouse livers.

(~5 ppm). Chloroacetaldehyde (50% aqueous solution), chloroacetic acid (mp 61-63 C) were obtained from Fluka Co., Buchs, Switzerland and chloroethanol (bp 128-130 C) from Merck-Schuchardt, Darmstadt, Fed. Rep. of Germany. Alcohol dehydrogenase (200 U/mg of protein) and glucose-6-phosphate dehydrogenase (450 U/mg of protein) were purchased from Boehringer Mannheim, Fed. Rep. of Germany, or Sigma Chemical Co., St. Louis, Miss., USA. All other products were obtained commercially and were of the purest grade available.

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Method B

This was carried out by incubation in liquid suspension (Bartsch *et al.*, 1975). The medium (final volume 220 μl) contained 60 μl of 9,000 × *g* liver supernatant from PB-pretreated mice, 1.6 μmol $MgCl_2$, 1 μmol glucose-6-phosphate, 0.8 μmol NADP$^+$, 20 μmol Sorensen phosphate buffer pH 7.4, 20 μl bacterial suspension G-46 or TA 1530 ($2-4 \times 10^7$ cells) and an initial concen-

tration in the medium of 0.083 M VCM. The incubation was carried out at 37 °C for 30 min under O_2 and was stopped by dilution with ice-cold saline. The number of His^+ revertants and survivors was determined by plating on selective media as previously described (Bartsch *et al.*, 1975).

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In another series of experiments, TA 1530 strain was exposed to 20% VCM in air for various lengths of time (Fig. 1). In the absence of a 9,000 × *g* mouse liver supernatant, VCM exerted a mutagenic action *per se* in TA 1530 strain. The number of His^+ revertants increased as a linear function of the time of exposure to VCM reaching, after 48 h, 20 times the level of the spontaneous mutation rate. In the presence

TABLE I

VCM CONCENTRATION-DEPENDENT INDUCTION OF REVERSE MUTATIONS

Exp. No.	% VCM in air	9,000 × *g* sup. + cofactors [2]	Phenobarbitone pretreatment	No. of His^+ revertants / plate (% bacterial survival [3]) Strain: TA 1530	TA 1535	G-46	TA 1538
1	0	–	Yes or no	10 ± 3 (100)	8 ± 2 (100)	6 ± 3 (100)	23 ± 1 (100)
2		+	Yes	62 ± 7	44 ± 4	20 ± 15	
3	0.2	–	Yes	41 ± 2 (131)	23 ± 4 (86)	10 ± 1 (111)	
4		+	No	45 ± 10	39 ± 0	11 ± 2	
5		–	No	27 ± 15	21 ± 9	11 ± 8	
6		+	Yes	118 ± 23	76 ± 11	25 ± 2	
7	2	–	Yes	39 ± 26 (116)	40 ± 12 (86)	13 ± 1 (103)	
8		+	No	106 ± 19	64 ± 22	19 ± 1	
9		–	No	70 ± 8	33 ± 2	15 ± 1	
10		+	Yes	278 ± 16	179	62 ± 3	
11	20	–	Yes	131 ± 17 (123)	98 ± 36 (129)	40 ± 0 (119)	32 ± 8 (118)
12		+	No	232 ± 8	161 ± 60	56 ± 0	
13			No	155 ± 11	98 ± 31	30 ± 10	

[1] Strain TA 1530 (bacteria/plate in parentheses) (6.8 × 10^8); TA 1535 (1.3 × 10^9); G-46 (5.8 × 10^8) and TA 1538 (8.3 × 10^8), were exposed to 0.2, 2 and or 20% VCM in air (v/v) for 48 h at 37 °C in the presence of an NADPH generating system and a 9,000 × *g* liver supernatant from either phenobarbitone-treated or untreated mice.

[2] Bacterial survival for each experiment was determined in parallel; mean values for each exposure group are listed.

[3] Cofactors: $NADP^+$ and glucose-6-phosphate.

[4] Mean values ± SD from two series of experiments, each utilizing a pool of five mouse livers.

of a 9,000 × *g* liver supernatant of PB-pretreated mice, the number of His$^+$ revertants increased, after 3, 6, 9 or 48 h of exposure to VCM, 4, 3, 2.5 and 2-fold as compared to the assays without the liver fractions (Fig. 1). When the number of His$^+$ revertants obtained with the liver fractions were corrected by those observed without any metabolic activation system, a plateau was reached after 9 h of exposure.

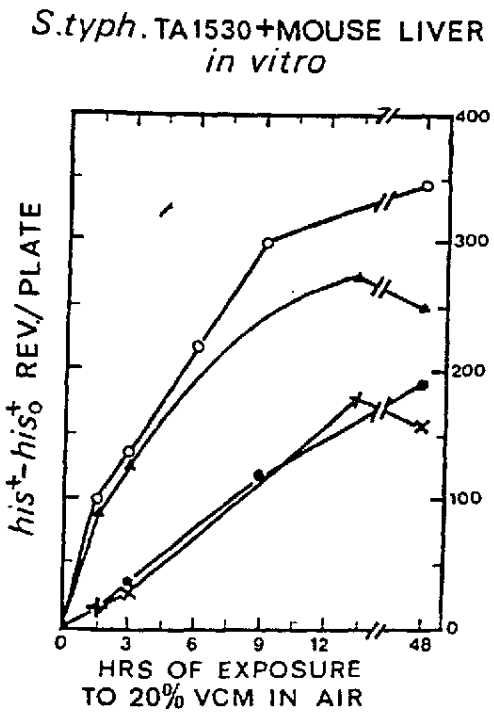

FIGURE 1

Mutagenic response of TA 1530 strain after VCM exposure for various lengths of time.

Mutagenicity assays (procedure A) were performed with TA 1530 and pooled liver fractions from five phenobarbitone-pretreated mice, following exposure to 20% VCM in air at 37° C in the presence of a 9,000 × *g* supernatant and an NADPH generating system (○), or a 9,000 × *g* supernatant, an NADPH generating system, NAD$^+$ (4 μmoles/plate) and alcohol dehydrogenase (5.9 Units/plate) (▲), or a 100,000 × *g* supernatant, an NADPH generating system, NAD$^+$ and alcohol dehydrogenase (×), or KCl and NADP$^+$, only (●).

No increase over the spontaneous mutation rate with strains TA 1530, TA 1535 and G-46 was detected when the incubation was performed for 30 min in liquid suspension (Method B) in the presence of a fortified 9,000 × *g* liver supernatant from PB-pretreated mice and at an initial co ncentration of 0.083 M VCM in the medium.

Mutagenic response as a function of animal species and tissues, including human tissues

Rat or mouse liver, kidney and lung fractions (9,000 × *g* supernatant) were assayed, following exposure to 20% VCM for 6 h at 37° C, for their capability to generate VCM metabolites mutagenic for TA 1530 strain (Table II). Fortified liver fractions from PB-pretreated or untreated mice and rats showed an enzymatic capacity to convert VCM into mutagenic metabolites *in vitro*, which was equally high in both species (Table II, exp. Nos. 2 and 14). The mutagenic response in the liver of PB-pretreated or untreated animals was, respectively, four and three times higher than that of the appropriate control (Table II, exp. Nos. 2, 14, 4 and 16 *versus* No. 1). With kidney and lung fractions from mice or rats, only a marginal mutagenic response, or none at all, was observed with either PB-pretreated or untreated animals (Table II, exp. Nos. 6-13 and 18-25 *versus* No. 1).

Postmitochondrial liver supernatants from four adult humans showed a great variation in their capacity to convert VCM into mutagenic metabolites (Tables II, exp. Nos. 26-33). Sample A caused a mutagenic response which was seven times higher than that of the appropriate control (Table II, exp. No. 26 *versus* No. 1) and twice as high as that obtained with rat or mouse liver. Samples B, C and D showed moderate activity (Table II, exp. Nos. 28, 30 and 32) and samples C and D caused a mutagenic response which appeared to be independent of the added co-factors, NADP$^+$ and glucose-6-phosphate (Table II, exp. Nos. 30-33).

Attempts to characterize enzyme(s) and metabolite(s) involved in VCM mutagenicity

Fortified postmitochondrial (9,000 × *g*) liver supernatant from PB-pretreated mice, but not the soluble proteins (100,000 × *g* supernatant), significantly increased the number of His$^+$ revertants of TA 1530 strain by 7, 4, 3, 2.5 and 2 times, following exposure to VCM for 1.5, 3, 6, 9 or

48 h, respectively, as compared to assays containing KCl and $NADP^+$ only (Fig. 1). Addition of 5.9 U of alcohol dehydrogenase and 4 μmol of NAD^+ to either the fortified 9,000 × *g* or 100,000 × *g* liver supernatant did not increase the mutation rate (Fig. 1). When the $NADPH$-generating system ($NADP^+$ and glucose-6-phosphate) was omitted from the 9,000 × *g* liver supernatants, the number of His^+ revertants was reduced to control levels (Table II, exp. Nos. 2-5 and 14-17 *versus* No. 1). In another series of experiments, the subcellular localization of enzyme activity(ies), which converts VCM into mutagenic metabolites, was examined. A hepatic

TABLE II

HUMAN, RAT AND MOUSE TISSUE-MEDIATED MUTAGENICITY OF VCM IN *S. TYPHIMURIUM* TA 1530

Exp. No.	Species		Phenobarbitone pretreatment	Organ [1] 9,000 × *g* sup.	±cofactors	No. of ($His^+ - His_0^+$) [2] revertants/plate	Relative activity
1	—		—	None	±	53 ± 32 (4) [3]	(100)
2			Yes		+	242 ± 61 (3) [3]	457
3	Mouse		Yes	Liver	−	129 ± 88 (3) [3]	243
4			No		+	150 ± 43 (4) [3]	283
5			No		−	46 ± 12 [4]	87
6			Yes		+	67 ± 1	126
7			Yes		−	62 ± 9	117
8			No	Lung	+	63 ± 15	117
9			No		−	55 ± 7	104
10			Yes		+	90 ± 8	170
11			Yes		−	93 ± 3	175
12			No	Kidney	+	67 ± 11	126
13			No		−	80 ± 21	151
14			Yes		+	203 ± 13	383
15	Rat		Yes	Liver	−	76 ± 7	143
16			No		+	183 ± 6	345
17			No		−	47 ± 18	87
18			Yes		+	66 ± 21	124
19			Yes	Lung	−	66 ± 11	124
20			No		+	75 ± 7	141
21			No		−	49 ± 9	92
22			Yes		+	84 ± 20	158
23			Yes	Kidney	−	86 ± 23	162
24			No		+	90 ± 13	170
25			No		−	92 ± 19	173
26		A			+	380 ± 19	717
27					−	93 ± 6	175
28		B			+	174 ± 41	328
29					−	99 ± 4	187
30	Human	C	No	Liver	+	185 ± 20	349
31	sample				−	234 ± 15	441
32		D			+	157 ± 4	266
33					−	115 ± 29	217

[1] Equivalent to 38 mg of wet tissue/plate.

[2] Mutagenicity assays (procedure A), 6 h of exposure to 20% VCM in air at 37° C. Number of spontaneous mutations/plate (his_0) from each value subtracted.

[3] Mean values ± SD and number of different experiments in parenthesis, each utilizing pooled tissues from five mice.

[4] Mean values ± SD from three mutagenicity assays for experiments Nos. 5 to 33, utilizing pooled tissues from five mice or three rats or individual human samples.

microsomal fraction from PB-pretreated mice increased the number of His$^+$ revertants in the presence of an NADPH generating system (Table III, exp. Nos. 4-5). The mutagenic response, however, was lower when compared to the 9,000 × *g* supernatant or to the recombined microsomal and cytosol fraction (Tables III, exp. Nos. 2, 3, 8 and 9). Liver cytosol did not increase the mutagenic response of VCM (Table III, exp. Nos. 6 and 7 and Fig. 1).

At various VCM concentrations and in all three bacterial strains (TA 1530, TA 1535 and G-46), a 15-40% enhancement of the mutagenic response was observed when liver fractions (9,000 × *g* supernatant) from PB-pretreated mice were compared to untreated controls (Table I). In rats, PB pretreatment caused a similar increase of the mutation rate (Table II, exp. Nos. 14 and 16). Chloroacetaldehyde and chloroethanol, two presumed metabolites, and chloroacetic acid, a urinary metabolite of VCM (Hefner *et al.*, 1974; Grigorescu and Toba, 1966), were assayed in equimolar concentrations for their mutagenic action in TA 1530 strain (Table III). Chloroacetaldehyde and chloroacetic acid caused a high toxicity on the bacteria. Chloroethanol, at the highest concentration utilized (108 μmoles/plate) increased the number of His$^+$ revertants 10 times over the spontaneous mutation rate (Table IV, exp. Nos. 26-28 *versus* No. 1). A five-fold increase of the number of His$^+$ revertants over the spontaneous level was observed at 10.8 μmoles of the chloroacetaldehyde/plate (Table IV, exp. No. 14). Chloroacetic acid was toxic at all concentrations, but did not cause a direct or tissue-mediated mutagenic response in TA 1530 strain (Table IV, exp. Nos. 2-10).

DISCUSSION

S. typhimurium strains TA 1530, TA 1535 and G-46 were reverted by VCM to His prototrophy (Fig. 1), which indicates that the mutagenic effect is induced by base-pair substitution. For this type of mutation, a covalent interaction of VCM or its metabolite(s) with the bacterial DNA is required. The inability of TA 1538 strain to revert in the presence of VCM to His$^+$ confirms that VCM or its metabolite(s) do not act as frameshift mutagens. The highest mutagenic response was seen with TA 1530 strain, which is excision-repair-deficient (*uvrB*$^-$) and a much lower response was noticed with G-46, which is *uvrB*$^+$ (Ames *et al.*, 1973). During 48 h of exposure to 0.2-20% VCM in air, no cytotoxicity was noted for any of the four strains (Table I).

Exposure of TA 1530 strain to 20% VCM in air in the absence of any metabolic activation

TABLE III

EFFECT OF VARIOUS SUBCELLULAR TISSUE FRACTIONS AND COFACTORS ON THE MUTAGENIC EFFECT OF VCM

Exp. No.	Mouse liver [1] fraction	±cofactors	No. of (His$^+$ − His_0^+) [2] revertants/plate	Relative activity
1	None (KCl)	—	96 ± 38 [3]	(100)
2	9,000 × *g* sup.	+	310 ± 18	323
3		−	128 ± 88	133
4	Microsomal fraction	+	175 ± 20	182
5		−	86 ± 2	90
6	100,000 × *g* sup.	+	138 ± 12	144
7	(cytosol)	−	90 ± 16	94
8	Microsomal fraction	+	501 ± 25	522
9	+ cytosol	−	95 ± 10	100

[1] Equivalent to 38 mg of wet tissue/plate.

[2] Mutagenicity assays (procedure A): 6 h of exposure to 20% VCM in air at 37°C; number of spontaneous mutations/plate (his_0) from each value subtracted.

[3] Mean values ± SD of three mutagenicity assays, each utilizing pooled liver tissue from five phenobarbitone-pretreated mice.

system caused a linear increasing mutagenic response, as a function of incubation time, which was 20 times the spontaneous mutation rate at 48 h (Fig. 1). This mutagenic effect could be caused by a direct action of VCM or, more likely, by one of its enzymic (bacteria) or non-enzymic break-down products.

A much higher mutagenic response was observed when a 9,000 × *g* liver supernatant from mice or rats was added to such an assay. The number of His$^+$ revertants increased, after 3, 6, 9, 12 or 48 h, by 4, 3, 2.5 or 2 times when compared to the assays without a tissue fraction (Fig. 1). These results strongly support an enzymic formation of VCM intermediates which are mutagenic for TA 1530 strain. To study the subcellular localization of these enzymes, various liver fractions from PB-pretreated animals were assayed. A microsomal fraction in the presence of an NADPH generating system significantly increased the number of His$^+$ revertants over controls, where the co-factors had been omitted (Table III). A 100,000 × *g* liver supernatant, in the presence or absence of NADP$^+$, did not modify the mutagenic response. The recombined microsomal and cytosol fractions gave a response

TABLE IV

MUTAGENIC EFFECT OF CHLOROACETIC ACID, CHLOROACETALDEHYDE AND CHLOROETHANOL IN STRAIN TA 1530

Exp. No.	Compound (μMoles/plate)		±9,000 × g sup. [a]	±cofactors	No. of His$^+$ revertants/plate [b]	% bacterial survival [b]
1	None		+	−	14±4	(100)
2	Chloroacetic acid	(1.1)	+	+	13±2 [d]	
3			+	−	13±6	
4			−	−	16±4	100
5		(10.8)	+	+	14±2	
6			+	−	13±2	
7			−	−	8±2	<0.004
8, 9, 10		(108)	±	±	0	0
11	Chloroacetaldehyde	(1.1)	+	+	16±3	
12			+	−	23±6	
13			−	−	30±8	<0.004
14		(10.8)	+	+	60±8	
15, 16			±	−	0	<0.004
17, 18, 19		(108)	±	±	0	0
20	Chloroethanol	(1.1)	+	+	14±4	
21			+	−	17±1	
22			−	−	18±6	100
23		(10.8)	+	+	26±2	
24			+	−	28±6	
25			−	−	27±1	100
26		(108)	+	+	155±8	
27			+	−	120±7	
28			−	−	72±0	100

[a] Equivalent to 39 mg of pooled wet liver tissue from five phenobarbitone-pretreated mice per plate.
[b] Mutagenicity assays (procedure A), following incubation for 48 h at 37° C. Survival was determined with 1.6×10^8 bacteria/plate.
[c] Direct plating test in 2.2 ml of soft agar.
[d] Mean values ± sd of two mutagenicity assays.

similar to that observed with the original 9,000 × *g* supernatant (Table III). These results strongly imply that the microsomal mixed function oxidase plays a role in VCM biotransformation. This is further supported by the requirement of an NADPH generating system for the enzymic formation of mutagenic metabolites by liver fractions (Table II), as well as by the increase of His^+ revertants, when mice and rats were pretreated with PB. The addition of soluble liver proteins to a microsomal fraction significantly increased the mutagenic response (Table III). Consequently, a two-step activation mechanism, each being localized in a different cell compartment, could be involved. On the other hand, the protective effect of liver cytosol against lipid peroxidation (Kamataki *et al.*, 1974) may prolong the viability of the microsomal enzymes and thus increase the yield of mutagenic VCM metabolites.

When the liver-mediated mutagenic response was corrected by the corresponding values obtained without any tissue fractions (Fig. 1), the resulting curve indicated a viability of the enzyme system involved in VCM biotransformation up to 9 h after incubation. Although the VCM concentration was not rate-limiting, the number of His^+ revertants caused by enzymic formation did not increase further (Fig. 1).

Our inability to find a mutagenic effect, when bacteria strains G-46 and TA 1530 were incubated with an initial concentration of 0.083 M of VCM in liquid suspension (Method B), may be due to the rapid loss of VCM in the liquid phase by diffusion into the atmosphere, as well as to the slowness of the enzyme-mediated formation of mutagenic metabolites within the first hour of incubation.

Chloroethylene oxide, a possible reactive intermediate, which may be formed from VCM by the hepatic microsomal mixed-function oxidase, rearranges spontaneously to chloroacetaldehyde (Zief and Schramm, 1964). Thus, two conceivable metabolites, chloroacetaldehyde and chloroethanol, and a urinary excretion product, chloroacetic acid (Grigorescu and Toba, 1966; Hefner *et al.*, 1974), were assayed for their mutagenic action at equimolar concentrations. At the concentration of 10.8 μmoles/plate, chloroacetaldehyde and chloroacetic acid showed a strong toxicity in the TA 1530 strain. Only chloroethanol, at a 40 mM concentration (108 μmoles/plate), caused a significant increase in the number of His^+ revertants in TA 1530 strain (Table IV). Rosenkranz *et al.* (1974) also reported a weak direct mutagenic action of chloroethanol in the same strain. Chloroethylene oxide caused a mutagenic effect in the TA 1530 strain and it was shown to be a strong alkylating agent (Malaveille *et al.*, 1975).

Among the various tissues of mice and rats, only hepatic postmitochondrial supernatants were highly efficient in the presence of an NADPH generating system in converting VCM into metabolites mutagenic for TA 1530 strain. One human liver sample showed an activity twice as high as that of rat or mouse liver in converting VCM into mutagenic metabolites. The three other samples showed a moderate activity (Table II).

The extrapolation of the results obtained in our *in vitro* mutagenicity studies to the mechanism of tumour induction by VCM *in vivo* is difficult to make at present. Although it becomes more evident that the majority of chemical carcinogens, after metabolic activation, show a mutagenic effect (Miller and Miller, 1971), it is not yet clear which of the VCM metabolite(s) is(are) responsible for the mutagenic action and whether it(they) is(are) identical with those responsible for the carcinogenicity of VCM. However, our studies have indicated that human, mouse and rat liver, which are the major target organs for the carcinogenicity of VCM in humans as well as in animals, efficiently convert this carcinogen into mutagenic metabolites. We have applied this mutagenicity test to a structural analogue of VCM, vinylidene chloride (1,1-dichloroethylene), which is used in the production of plastic materials. Our results show that the mutagenic effect is higher than that observed with VCM, when the compound is metabolically activated by rat or mouse microsomal liver enzymes (unpublished data).

ACKNOWLEDGEMENTS

This study was partially supported by the National Cancer Institute, USA, Contract NOI-CP-55630.

The authors are greatly indebted to Drs. G. Della Porta, U. Veronesi and M. Boiocchi for providing the samples of human tissues. The excellent technical assistance of Mrs. C. Gabet, Miss A. M. Camus and Mr. A. Barbin is gratefully acknowledged. The authors wish to thank Miss A. Pickett for typing this manuscript.

MUTAGÉNICITÉ DU CHLORURE DE VINYLE DANS DES SOUCHES DE *S. TYPHIMURIUM* EN PRÉSENCE DE FOIE HUMAIN, DE RAT OU DE SOURIS

L'exposition au chlorure de vinyle des souches TA 1530, TA 1535 et G-46 de S. typhimurium *fait augmenter le nombre de révertants His⁺/boîte de Pétri respectivement de 16, 12 et 5 fois par rapport au taux de mutation spontanée. Après six heures d'exposition de la souche TA 1530 au chlorure de vinyle, l'effet mutagène est multiplié par 7, 4 et 5, lorsque l'on ajoute la fraction postmitochondriale de foie humain, de rat ou de souris et les cofacteurs enzymatiques requis. L'enzyme impliquée dans la transformation du chlorure de vinyle en mutagène requiert un système générateur de NADPH, et se situe dans la fraction microsomale. Le surnageant à 9,000 g présente une activité enzymatique trois fois plus importante que celle des microsomes, tandis que le cytosol ou l'alcool déshydrogénase n'affecte pas la mutagénicité. Le prétraitement de rats et de souris au phénobarbital augmente de 15 à 40% l'effet mutagène par comparaison avec les animaux témoins. Les activités mutagènes relatives du chlorure de vinyle, exprimées en pourcentage (foie de souris = 100%), déterminées par les fractions tissulaires obtenues à 9,000 g et révélées avec la souche TA 1530 sont: foie de rat, 80%; rein de souris et de rat, 20 et 16%; poumon de souris et de rat, <7%; foie humain (quatre biopsies), 170, 64, 70 et 46%. La chloroacétaldéhyde et l'acide chloroacétique, un métabolite du chlorure de vinyle excrété dans l'urine, se révèlent très toxiques, tandis que le chloroéthanol est faiblement mutagène pour la souche TA 1530.*

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