MUTAGENICITY OF VINYL CHLORIDE, CHLOROETHYLENEOXIDE, CHLOROACETALDEHYDE AND CHLOROETHANOL

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SUMMARY: Exposure of *S. typhimurium* strains TA 1530, TA 1535 and G-46 to vinyl chloride increased the number of *his*$^+$ rev./plate 16, 12 or 5 times over the spontaneous mutation rate. The mutagenic response for TA 1530 strain was enhanced 7, 4 or 5-fold when fortified S-9 liver fractions from humans, rats or mice were added. In TA 1530 strain, chloroacetic acid showed only toxic effects, while chloroacetaldehyde, chloroethanol and chloroethyleneoxide caused a mutagenic response. The latter compound was shown to be a strong alkylating agent.

VCM is extensively used for the production of polyvinyl chloride, other plastic material, and as a propellant for aerosols. The systemic action of this carcinogen in rats [1, 2, 3], mice[2] and humans [4-7], following exposure to VCM, as well as its low chemical reactivity, suggest that the biological effects are dependent upon its metabolic activation. In these studies, we are reporting the mutagenicity of VCM and/or its presumed metabolites in *S. typhimurium* strains, mediated by liver fractions of rat, mouse and human origin.

MATERIAL AND METHODS

Chemicals: VCM (purity 99.9%) was generously provided by Rhône-Progil, Lyon, France. Chloroacetaldehyde (50% aqueous solution), and chloroacetic acid (m.p. 61-63°) (Merck-Schuchardt, Fed. Rep. of Germany), alcohol dehydrogenase (200 U/mg) (Boehringer Mannheim, Fed. Rep. of Germany) were obtained from the sources indicated. All other commercial products were of the purest grade available. The structure and purity

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Abbreviations: VCM, vinyl chloride monomer; PB, phenobarbitone; S-9 9,000 x *g* tissue supernatant; GLC, gas liquid chromatography.

of chloroethyleneoxide [8], b.p. 65-67°C (prepared by chlorination of ethyleneoxide in the gas phase) was ascertained by its NMR-spectrum in $CDCl_3$. The compound which contained traces of chloroacetaldehyde (< 1%) and ethyleneoxide (< 5%) was stored in liquid N_2 and rapidly used for mutagenicity assays. Under these conditions, no change in the NMR-spectrum was noted after 12 days. The synthesis of chloroethyleneoxide according to ref. 9 was unsuccessful.

GLC: VCM concentrations were determined in a Packard model 417 gas chromatograph as described [10].

Animals and pretreatment: Adult female BD-IV rats (100-130 g), bred in our laboratories, and male OF-1 mice (30-35 g, Iffa-Credo, France) were fed a Charles River CRF diet. Some rats and mice received PB sodium 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 Cure e Terapia dei Tumori, Milan, Italy).

Mutagenicity assays: The S-9 microsomal or soluble fractions were prepared from 3-5 pooled animal or individual human tissues, by centrifugation of a homogenate (3 ml of 0.15 M KCl/g wet tissue), as described [10,11]. *S. typhimurium* strains TA 1530, TA 1535, G-46 and TA 1538, kindly provided by Professor B. Ames, were grown overnight in nutrient broth (Difco). If not otherwise specified, the S-9 fraction (or KCl), cofactors ($NADP^+$ and glucose-6-phosphate), 3-8 x 10^8 bacteria/plate and substrate were combined in a soft-agar layer and plated in duplicate or triplicate on Petri dishes with Davis minimal agar, as described [10,11,12]. 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^{-4} dilutions) on a *his* enriched medium. Mutagenicity tests with VCM[10] were performed by

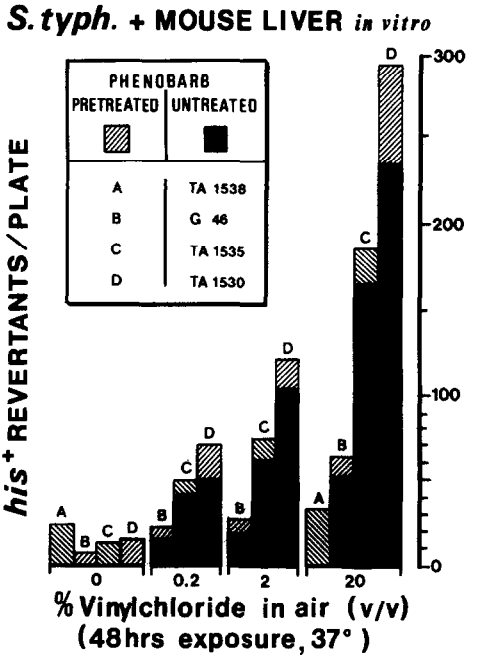

Chart 1. VCM concentration dependent induction of reverse mutations.

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 hrs at 37°C in the presence of a NADPH generating system and a S-9 liver fraction from either PB treated or untreated mice. Mean values are plotted from two series of experiments each utilizing a pool of 5 mouse livers. Bacterial survival for each experiment was determined in parallel; mean values for each exposure group are listed.

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exposing the Petri dishes to VCM in air in dessicators (10-15 litres) for up to 48hrs at 37°C. in the dark. For shorter treatments, the VCM was removed under vacuum, replaced by air and the incubation was continued up to 48 hrs at 37°C. The concentration of VCM in Vogel-Bonner medium, as determined by GLC after exposure at 37 to 0.2, 2 and 20% VCM in air (v/v) for 6 or 48 hrs, was 4×10^{-5} M; 4×10^{-4} M or 4×10^{-3} M.

RESULTS AND DISCUSSION

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 [12], were specifically reverted by VCM to *his* prototrophy, following exposure to various concentrations of VCM in air, in the presence of a fortified S-9 mouse liver fraction (Chart 1). Among the strains tested, the TA 1530 showed the highest mutagenic response and the number of *his*$^+$ rev./plate increased approximately 6, 12, or 28 times over the spontaneous mutation rate following exposure to 0.2, 2 or 20% VCM in air for 48 hours. With TA 1538 strain, which is specifically reverted by frameshift mutagens, no significant mutagenic response was noticed. For all *S. typhimurium* strains utilized, VCM at (4-400) x 10^{-5} M concentration showed no strong cytotoxic effects. Bacterial survival, under these experimental conditions, was between 86 and 131%. At various VCM concentrations and in all three bacterial strains (TA 1530, TA 1535 and G-46), PB pretreatment caused a 15-40% enhancement of the mutagenic response when S-9 liver fractions were compared to untreated controls (Chart 1).

When TA 1530 strain was exposed to 20% VCM in air for various lengths of time, VCM exerted a mutagenic action *per se*. The number of *his*$^+$ revertants increased as a linear function of the time of exposure to VCM, reaching after 48 hrs, 20 times the level of the spontaneous mutation rate (10 *his*$^+$ rev./plate). However, after 1.5, 3, 6, 9, or 48 hrs of exposure to VCM and in the presence of a S-9 mouse liver fraction of PB pretreated mice, the number of *his*$^+$ revertants increased 4, 3, 2.5 and 2-fold respectively as compared to the assays without the liver fractions. Addition of 5.9 U of alcohol dehydrogenase and 4 µmol of NAD^+ to either the fortified 9,000 x *g* or 100,000 x *g* liver supernatant did not increase the mutation rate.

Mutagenic response as a function of animal species and tissues including humans: Fortified S-9 liver fractions from PB pretreated or untreated mice and rats showed an enzymatic capacity to convert VCM into mutagenic metabolites *in vitro*, which was similar in both species (Table 1). With

TABLE 1. Human, rat and mouse liver mediated mutagenicity of VCM.

Exp. No.	SPECIES	S-9 [a]	No. of *his*$^+$ rev./plate [b]	
			PB pretreated	untreated
1		None		53 ± 32 [c]
2	MOUSE	Liver	242 ± 61 [c]	150 ± 43 [c]
3	RAT	Liver	203 ± 13 [d]	183 ± 6 [d]
4	HUMAN A	Liver		380 ± 19
5	B			174 ± 41
6	C			185 ± 20
7	D			157 ± 4

[a] Equivalent to 38 mg of wet tissue/plate + NADPH generating system.

[b] Mutagenicity assays with TA 1530; 6 hrs of exposure to 20% VCM in air at 37°C. No. of spontaneous mutations/plate (10 ± 3) from each value subtracted.

[c] Mean values ± S.D. of 3 or 4 different experiments, each utilizing pooled tissues from 5 mice.

[d] Mean values ± S.D. from 3 mutagenicity assays, utilizing pooled tissues from 3 rats or individual human samples.

TABLE 2. Effect of various subcellular tissue fractions on the mutagenic response by VCM.

Exp. No.	Mouse liver fraction plus NADPH generating system [a]	No. of *his*$^+$ rev./ plate [b]
1	None (KCl)	96 ± 38 [c]
2	9,000 x *g* sup. (S-9)	310 ± 18
3	Microsomal fraction	175 ± 20
4	Cytosol	138 ± 12
5	Micr. fraction + cytosol	501 ± 25

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

[b] Mutagenicity assays with strain TA 1530; 6 hrs of exposure to 20% VCM in air at 37°C. No. of spontaneous mutations/plate (10 ± 3) from each value subtracted.

[c] Mean values ± S.D. of 3 mutagenicity assays, each utilizing pooled liver tissue from 5 PB pretreated mice.

S-9 kidney and lung fractions from mice or rats, a marginal or no mutagenic response was observed with either PB pretreated or untreated animals. The S-9 liver fractions from 4 adult humans showed a great variation to convert VCM into mutagenic metabolites (Table 1). Sample A caused a mutagenic response which was 7 times higher than a control (Exp. No. 1), and twice as high as that obtained with rat or mouse liver. Samples B, C and D showed moderate activity.

Attempts to characterise enzyme(s) and metabolite(s) involved in VCM mutagenicity: Fortified S-9 liver fractions from PB pretreated mice, but not the soluble proteins (100,000 x *g* supernatant), significantly increased the number of *his*$^+$ rev. of TA 1530 strain, following exposure to VCM, as compared to a control (Table 2, Exp. No. 1). A hepatic microsomal fraction from PB pretreated mice increased the number of *his*$^+$ rev. only in the presence of a NADPH generating system. The mutagenic response, however, was lower when compared to the 9,000 x *g* supernatant, or to the recombined microsomal and cytosol fraction (Table 2). Chloroacetaldehyde, a presumed metabolite, and chloroacetic acid, a urinary metabolite of VCM [13, 14], showed a high toxicity in the bacteria (Table 3). In the presence of a S-9 mouse liver fraction, chloroethanol increased the number of *his*$^+$ rev. 10 times (Exp. No. 2), and chloroacetaldehyde 6 times (Exp. No. 9) over the spontaneous mutation rate. The two compounds also exhibited a direct mutagenic action (Table 3 and ref. 15). Chloroacetic acid did not cause a direct or tissue-mediated mutagenic response in TA 1530 strain. Chloroethyleneoxide, a presumed intermediary metabolite, caused a mutagenic response (Exp. Nos 15-17), paralleled by a relatively low toxicity, when compared with chloroacetaldehyde (Exp. Nos 12 and 13).

We have also demonstrated an alkylating activity of chloroethyleneoxide, but not of chloroacetaldehyde, by its fast reaction with 4-(p-nitrobenzyl)pyridine [16]. The half-life of the epoxide (0.32 mM concen-

TABLE 3. MUTAGENIC EFFECT OF CHLOROETHANOL, CHLOROACETALDEHYDE, CHLOROACETIC ACID AND CHLOROETHYLENEOXIDE IN STRAIN TA 1530

Exp. No.	Compound	µmoles/ml of soft agar layer	No. of *his*$^+$ rev./plate[a] fortified S-9 fraction added[c]	fortified S-9 fraction omitted	% bacterial survival[b]
1	none	-	10 ± 2[d]	10 ± 2	100
2		40	130 ± 9	60 ± 6	100
3	ClH_2C-CH_2OH	4	21 ± 2	27 ± 1	100
4		0.4	14 ± 3	18 ± 4	100
5		40	0	0	0
6	$ClH_2C-COOH$	4	14 ± 2	8 ± 2	<0.004
7		0.4	13 ± 2	16 ± 3	100
8		40	0	0	0
9	ClH_2C-CHO	4	62 ± 4	0	<0.004
10		0.4	17 ± 1	30 ± 3	<0.004
11		4		0[e]	0
12	ClH_2CHO	1		150 ± 22	<0.004
13		0.4		37 ± 10	<0.004
14		4		0	0
15	$ClHC\overset{O}{\diagdown\diagup}CH_2$	1		90 ± 11	<0.4
16		0.4		61 ± 2	11
17		0.04		10 ± 2	90

[a] Mutagenicity assays following incubation for 48 hrs at 37°C.
[b] Determined in the absence of a fortified S-9 fraction, following 48 hrs exposure at 37°C.
[c] Equivalent to 38 mg of pooled wet liver tissue from 5 PB pretreated mice/plate (Exp. Nos. 1-10).
[d] Mean values ± SE (Exp. Nos. 1-17).
[e] Direct plating test in 2.2 ml of soft agar (Exp. Nos. 11-13) or in soft agar containing 4.5% acetone (v/v) (Exp. Nos. 14-17).

tration) in acetone - 0.1 M Tris buffer, pH 7.4 (30% v/v) - was determined as 1.6 min., using the same colorimetric method [16] after different times of preincubation at 37°C. These data support a high electrophilic reactivity of chloroethyleneoxide, an example of halogen substituted aliphatic epoxides. The chemical and biological properties of this class of compounds have not yet been reported in detail.

Our studies [10] and those of others [17] strongly support that a microsomal mixed function oxidase of human, mouse and rat liver, which is

the major target organ for the carcinogenicity of VCM in humans, as well as in animals, efficiently convert this carcinogen into a mutagenic metabolite(s) *in vitro*. The role of these compounds in VCM carcinogenesis is under study.

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References

1. Viola, P.L., Bigotti, A. and Caputo, A. Cancer Res., *31*, 516-519, 1971
2. Maltoni, C. and Lefemine, G. Environmental Res., *7*, 387-405, 1974
3. Caputo, A., Viola, P.L. and Bigotti, A. IRCS, *2*, 1582, 1974
4. Creech, J.L. and Johnson, M.N. J. occup. Med., *16*, 150-151, 1974
5. Heath, C.W.Jr., Falk, H. and Creech, J.L. Ann. N.Y. Acad. Sci. USA, in press, 1974
6. Lee, F.I. and Harry, D.S. Lancet, 1316-1318, 1974
7. International Agency for Research on Cancer, Internal Technical Report, No. 74/005. Lyon 24-25 June, 1974
8. Gross, H. and Freiberg, J. J. für praktische Chemie, *311*, 506-510, 1969
9. Zief, M. and Schramm, C.H. Chemistry and Industry, 660-661, 1964
10. Bartsch, H., Malaveille, C. and Montesano, R. Int. J. Cancer, in press, 1975
11. Bartsch, H., Malaveille, C. and Montesano, R. Cancer Res., in press, 1975
12. Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. Proc. Nat. Acad, Sci. USA, *8*, 2281-2285, 1973
13. Grigorescu, I. and Toba, Gh. Rev. Chim. Rom., *17*, 499-501, 1966
14. Hefner, R.E.Jr., Watanabe, P.G. and Gehring, P.J. Ann. N.Y. Acad. Sci. USA, in press, 1974
15. Rosenkranz, S., Carr, H.S. and Rosenkranz, H.S. Mutation Res., *26*, 367-370, 1974
16. Swaisland, A.J., Grover, P.L. and Sims, P. Biochem. Pharmacol., *22*, 1547-1556, 1973
17. Rannug, U., Johansson, A., Ramel, C. and Wachtmeister, C.A. Ambio, *23*, 194-197, 1974