To: J. T. Seaver

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Vorstand: Prof. Dr. D. Henschler

8700 Würzburg, den 5. Mai 1980
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Dear Dr. Watanabe,

Thank you for your letter of April 25, 1980. We have tested purified trichloroethylene, stabilised with triethanolamine (0.0015 %), in E coli K 12 with slightly positive results after addition of fortified microsomes (reprint enclosed). The same sample was found negative in Salmonella typhimurium TA 100 (see other reprint) and in TA 1539 (unpublished).

We have not performed mutagenicity tests with epichlorohydrin stabilised samples of tri. This has been done by Loprieno; he sent us a preliminary report of his findings, I have not (yet) seen a published paper. Our conclusion that epichlorohydrin and epoxibutane might be responsible for the carcinogenicity of technical grade tri, especially in the NCI-bioassay, is based on the testing of these compounds as such, and on the literature report on their mutagenicity and/or carcinogenicity.

Sincerely yours,

D. Henschler
(D,Henschler)

SL 034287

Arch Toxicol 37, 233–236 (1977)

Archives of TOXICOLOGY

Short Communication

Carcinogenicity of Trichloroethylene: Fact or Artifact?

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Abstract. Technical trichloroethylene has been found carcinogenic in mice after high daily doses per os. A GC-MS analysis of this technical sample revealed the presence of considerable amounts of epichlorohydrin and 1.2-epoxibutane as stabilizers. These epoxides are highly mutagenic in the Ames test and are, most probably, responsible for the carcinogenic effect found in mice. The question whether trichloroethylene is carcinogenic or not remains open.

Key words: Trichloroethylene – Epichlorohydrin – 1.2-Epoxibutane – Carcinogenicity – Mutagenicity.

Zusammenfassung. Technisches Trichloräthylen erwies sich nach hohen, täglichen oralen Dosen an Mäusen als carcinogen. Eine GC-MS Analyse des benutzten technischen Präparates ergab die Gegenwart beträchtlicher Gehalte an Epichlorhydrin und 1.2-Epoxibutan, die im Ames Test stark mutagen sind; diese Epoxide tragen höchstwahrscheinlich die carcinogene Wirkung in dem für den Carcinogeneseversuch verwendeten technischen Produkt, wo sie als Stabilisatoren zugesetzt werden. Die Frage, ob Trichloräthylen carcinogen ist oder nicht, bleibt offen.

Trichloroethylene has been reportet to produce high incidences of hepatocellular carcinomas in both male and female mice but not in rats after high daily oral doses in corn oil for 18 months (Memorandum DHEW, 1975). This report resulted in a variety of warnings and precautious measures, especially in the working environment where considerable exposure to this solvent is prevalent, inasmuch as speculations on the similarity with the notorious carcinogen, vinyl chloride with regard to chemical structure and possible metabolic activation have been forwarded (van Duuren and Banerjee, 1976).

Pure trichloroethylene (Tri) has been found slightly mutagenic in a modified Ames mutagenicity testing system (Greim et al., 1975). Mechanistic evaluations of the possible pathways of metabolic activation and deactivation of this compound have, however, revealed that the postulated electrophilic intermediate, trichloroethyl-

Table 1. Contaminants of a technical grade sample of trichloroethylene as identified by GC MS. The typical mass fragments were completely identical for the fractions in the sample and a r. grade samples for comparison

Compound	Main mass peaks m/e					
	% w/w	Molecular ion	Base peak	Other typical peaks I	II	III
Epichlorohydrin	0.22	92	57	27	31	49
Epoxibutane	0.20	72	42	39	41	57
Carbon tetrachloride	0.05	–	117	35	47	82
Chloroform	0.01	118	83	35	47	–
1.1.1-Trichloroethane	0.035	–	97	27	61	117
Diisobutylene (2.2.4 Trimethylpentene-1)	0.020	112	57	41	69	97
Ethylacetate	0.052	88	45	29	43	59
Pentanol-2	0.015	–	45	31	59	60
Butanol-2	0.051	74	45	31	59	73

ene epoxide, may be handled differently in different biological systems: a Lewis acid type catalytic rearrangement to the non-reactive chloral hydrate is likely to occur in the mammalian liver cell (Bonse and Henschler, 1976), a reaction which might not necessarily prevail in microorganisms such as the tester strains used in the in vitro mutagenicity assays.

Technical grade Tri contains several impurities and must be stabilized, for use as a degreasing agent, by antioxidants. Up to now not much attention has been paid to the biological reactivity of these contaminants. We therefore submitted a sample[1]) of the Tri used in the bioassay experiment for carcinogenicity (Memorandum DHEW, 1975) to an extended chemical analysis by gas chromatography – mass spectrometry. The results are presented in Table 1.

The proportion of all identified contaminants amounts to 0.65%. Two of these constitute strong monofunctional alkylating agents, the mutagenicity and/or carcinogenicity of which has previously been described, or suspected, respecitvely: epichlorohydrin (Fishbein, 1976; I.A.R.C. series, 1976) and 1.2-epoxibutane (van Duuren et al., 1967). We reinvestigated the mutagenic potential of these compounds and of another candidate, diisobutylene, in the Ames system (Fig. 1). The results with the most sensitive strain used, *S. typhimurium* TA 100 confirm the high mutagenic activity for both epichlorohydrin and 1.2-epoxibutane, in contrast to a very low activity for diisobutylene, whereas the effect of trichloroethylene is questionable. Carbon tetrachloride, chloroform, and 1.1.1-trichloroethane were found inactive both with and without addition of induced rat liver microsomes.

It is concluded from these results that the carcinogenic effect of the technical sample of Tri used in the bioassay experiment (Memorandum DHEW, 1975) is most probably predominantly, if not exclusively, due to the epoxides which are added to some (Steehrs, 1957) but by no means to all brands of Tri. This view is supported by

[1] Kindly obtained and supplied by the Trichloroethylene Toxicology Subcommittee of the Manufacturing Chemists Associations, USA

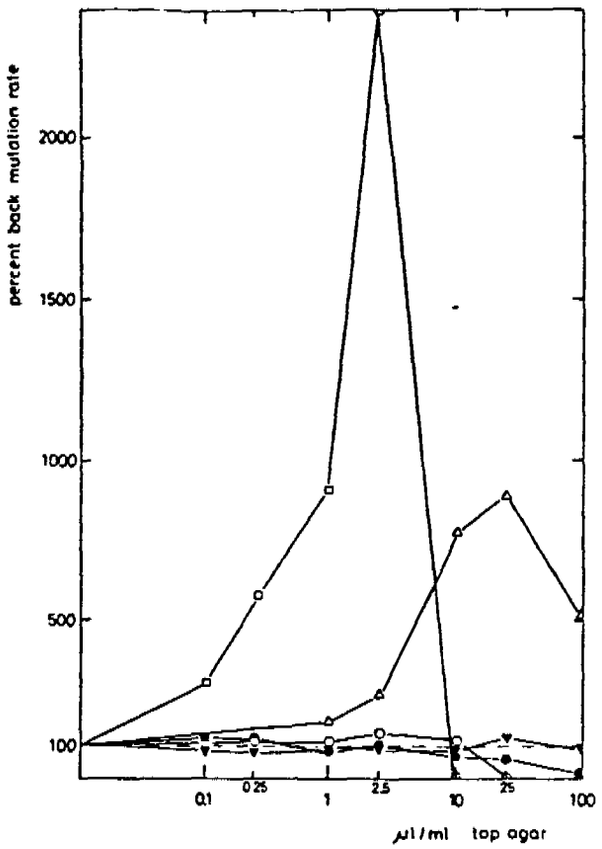

Fig. 1. Mutagenicity in the Ames in vitro system using *S. typhimurium* TA 100 (Ames et al., 1973) of trichloroethylene and contaminants identified in a technical sample. (□) Epichlorohydrin; (△) Epoxibutane; (▲) Diisobutylene; (○) Trichloroethylene. Open symbols: without, filled symbols: with addition of PCB (Aroclor 1254) induced rat liver microsomes. The bacteria were grown in nutrient broth, shaken for 12 h at 37° C, and 0.1 ml was then added to the molten top agar (2 ml), with and without 0.5 ml of "S-9-mix". This mix contained per ml: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose 6 phosphate, 4 mM NADP, 100 mM sodium phosphate (pH 7.4) and 0.3 ml of liver homogenates (S 9) (9000 × g supernatant) from male Wistar rats (of about 250 g each) which were induced by a single i.p. injection of a polychlorinated biphenyl (PCB) mixture (Aroclor 1254), diluted in corn oil to a concentration of 200 mg/ml. A dosage of 500 mg/kg was given to each rat 5 days before sacrifice. The solutions tested were added directly to the top agar. Triplicate petri plates containing Vogel-Bonner E medium were overlayed with this mixture and incubated at 37° C. After 48 h the revertant colonies were counted. Spontaneous back mutation rate 138 ± 8 (without microsomes) and 242 ± 10 (with microsomes) colonies per plate

the fact that carcinomas were not produced in rats but only in mice, a species with a comparatively low activity of epoxide hydrase (Oesch, 1973), the enzyme which detoxifies epoxides. Further studies in intact animals with exposure to nonepoxide stabilized Tri, both by inhalation and gavage, are needed to substantiate or reject the above assumption. Epidemiological investigations should also take into consideration the possibility that epoxide additives of high vapour pressure might be involved as carcinogenic factors. Until otherwise proven, positive evidence for a carcinogenic risk can only be based on epoxide-stabilized samples of trichloroethylene.

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Received April 20, 1977

Biochemical Pharmacology, Vol. 24, pp. 2013-2017. Pergamon Press, 1975. Printed in Great Britain.

MUTAGENICITY IN VITRO AND POTENTIAL CARCINOGENICITY OF CHLORINATED ETHYLENES AS A FUNCTION OF METABOLIC OXIRANE FORMATION

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(Received 10 July 1975; accepted 19 August 1975)

All chlorinated ethylenes undergo biotransformation in mammalian organisms, the main pathway being an oxidation to oxiranes, as the first step. The stability of these oxiranes varies widely and depends on the number of chlorine substitutions and on the relative position of the substituents in the molecule. Symmetrically substituted oxiranes from the tetrachloro- and the isomeric 1,2-dichloroethylenes seem to be rather stable [1),2)]. In the case of vinyl chloride and trichloroethylene the polarity of the unsymmetrical oxiranes exerts lesser stability and induces intramolecular rearrangement[3),4)]. Attempts to prepare the oxirane with the highest polarity from 1.1-dichloroethylene (1.1-DCE) remained unsuccessful till now. In the reaction of 1.1-DCE with m-chloroperoxybenzoic acid, the corresponding oxirane could not be detected; instead, the chloroacetyl chloride was isolated as the product of oxirane-rearrangement[5)].

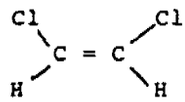
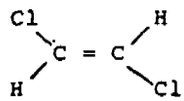
However, no direct relationship exists between chemical reactivity and biological effects because the thermal rearrangement of the oxiranes leads to different chemical species: either acyl chlorides or aldehydes[4)] which are suspect of causing quite different biological effects. Besides rearrangement, oxiranes may react directly with biologic nucleophiles. Thus, the mutagenic

activity of vinyl chloride (VCM) has been claimed to be exerted by a direct alkylating action of the oxirane[6),7)].

VCM has been demonstrated to be carcinogenic in animals[8),9)] and in man (for references, see[10)]).Quite recently, a carcinogenic activity of trichloroethylene (TRI) has been reported in mice after long-term administration of rather high daily oral doses (0.5 or 1.0 g/kg). This report[11)] in connection with the above considerations prompted us to determine the mutagenicity of the whole series of chlorinated ethylenes with a metabolic activating microsomal enzyme system in vitro.

Materials and Methods. Tetrachloroethylene, trichloroethylene, cis- and trans-1,2-dichloroethylene, 1,1-dichloroethylene were obtained from Merck & Co., Darmstadt, as a.g. reagents; vinylchloride as a purified gas (> 99,9%) from BASF, Ludwigshafen. Mutagenic activity of the derivatives formed during microsomal activation was tested in a metabolizing in vitro system[12)] with E coli K 12. One can use this bioauxotrophic strain in four mutation systems to test mutagenic agents: In the three back mutation systems gal^+, arg^+, and nad^+, and in the MTR system, where forward mutation leads to resistance to 5-methyl-DL-tryptophane[13)]. For the experiments 6 to 9 x 10^8 cells of an overnight culture were suspended in 1.5 ml incubate containing 5 mg microsomal protein, isolated from mouse livers, and the NADPH generating system 5 mM $MgCl_2$, 16 mM DL-isocitrate-Na_3, 0.66 mM NADP-Na_3, 20 µl isocitrate-dehydrogenase (20 milliunits/µl) in 0.1 M phosphate buffer pH 7.4, as well as different concentrations of the test compounds. These concentrations were selected from preliminary experiments so that they did not reduce cell survival by more than 20 per cent (Table 1). After 2 hours of incubation in a shaking water bath at 37°, the reaction was terminated in ice. The incubate was diluted in saline and plated on appropriate selective media as described previously[14)]. Survival of the E coli K 12 strain was determined by plating on the complete medium. Mutagenicity is expressed as colony-forming units (cfu) that were counted on the appropriate selective media per cfu counted on the complete medium. Liver microsomes were isolated from male mice pretreated for 10 days with 0.1 per cent phenobarbital in the drinking water to increase microsomal enzyme activity[12)].

Tab.1: Mutagenicity of chlorinated ethylenes after incubation in a metabolic activating microsomal system

	concentration in the medium[x] at 37° C [mM]	%survival of bacteria	% of spontaneous mutation rate in different operons of E coli K_{12}			
			gal$^+$	arg$^+$	MTR	nad$^+$
$Cl_2C = CCl_2$ Tetrachloroethylene	0.9	99±1	100	100	100	100
$Cl_2C = CHCl$ Trichloroethylene	3.3	76±4	123±23	232±36	114±18	100
$Cl_2C = CH_2$ 1.1-Dichloroethylene	2.5	74±7	120±14	229±26	100	100
 cis-1.2-Dichloroethylene	2.9	88±5	100	100	100	100
 trans-1.2-Dichloroethylene	2.3	90±3	100	100	100	100
$ClCH = CH_2$ Vinyl chloride	10.6	72±3	231±20	663±141	172±35	148±24

x) determined by GC analyses after injection of 5 µl of the liquid compounds; except vinyl chloride where the gas was introduced by bubbling through the liquid at 15° C.

Results and Discussion. The results of the experiments are listed in Table 1. Cytotoxicity of the chlorinated ethylenes varies widely. To obtain 80-100 per cent survival of the tester strain, only 1 mM of trichloroethylene could be used but 10 mM of vinyl chloride.

No mutagenic activity of the chlorinated ethylenes was detected in the test system without microsomal enzyme activity. When the complete incubate with metabolically active microsomes was used, conversion of VCM, 1.1-DCE as well as TRI induced mutations, the latter compounds being less mutagenic than VCM. The highest mutation rates were detected in the arginine genes, whereas reversibili-

ty in the gal$^+$ and nad$^+$ systems and the forward mutation to MTR resistance were less sensitive to the mutagenic effects of the metabolites. Tetrachloroethylene and the cis- and trans-isomers of dichloroethylene were not metabolized to mutagens at all.

Direct comparison of the mutagenic activity of the chlorinated ethylenes is not possible since different substrate concentrations had to be used to minimize cell death of the tester strain. However it is evident that mutagenicity of VCM is several times higher than that of 1.1-DCE and TRI.

Our results are indicative of a conspicuous correlation between the stability of the oxiranes, as outlined earlier, and the mutagenicity of all six chlorinated ethylenes: those forming very unstable oxiranes (VCM, 1.1-DCE,TRI) induce mutations in the test system, whereas the others (Per, cis- and trans-1.2-DCE) forming much mor stable oxiranes, do not.

The mutagenicity of trichloroethylene, though only slight in extent in the gal$^+$ system, which is known as very sensitive, has not been anticipated. Trichloroethylene is metabolized *in vitro* and *in vivo* to the scarcely reactive chloral hydrate and furtheron to trichloroethanol and trichloracetic acid[15),16)]. The latter compounds are not known to induce cytotoxic or genetic effects. Conversion of TRI-oxirane to chloral *in vivo* is a quite unexpected reaction because thermal rearrangement *in vitro* entirely forms dichloroacetyl chloride[17)]. This different behaviour should be further investigated. The results of such experiments may contribute to the better understanding of the mutagenic and potentially carcinogenic properties of trichloroethylene.

Acknowledgement: The skilful technical assistence of Mrs. Hesse and Steinhilber is gratefully acknowledged.

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