Fd Chem. Toxic. Vol. 20, pp. 557 to 561, 1982
Printed in Great Britain.

0278-6915/82/050557-05$03.00/0

BACTERIAL MUTAGENICITY STUDIES ON CHLOROFORM *IN VITRO*

N. J. VAN ABBÉ and T. J. GREEN*
Beecham Products Research Department, Randalls Road, Leatherhead, Surrey
and
E. JONES, M. RICHOLD and F. J. C. ROE
Huntingdon Research Centre, Huntingdon, England

(*Received 19 February 1982; revision received 23 March 1982*)

Abstract—Chloroform was tested for mutagenicity in the Salmonella/microsome assay using five strains of *Salmonella typhimurium*. In view of previous reports describing the development of liver and kidney tumours in some experiments involving long-term administration of chloroform to rats and mice, the mutagenicity tests were carried out in the absence of any S-9 microsomal-enzyme preparation and in the presence of S-9 microsomal-enzyme preparations derived from (a) livers and (b) kidneys of rats and mice previously exposed to the microsomal-enzyme inducer Aroclor 1254. No evidence of potential mutagenicity was observed under any of the test conditions. To determine whether the findings might have been influenced by the volatility of the chloroform, the test organisms were exposed to chloroform vapour, but again chloroform gave no indication of potential mutagenicity. Taken in conjunction with already published data from mutagenicity studies with chloroform, it appears unlikely that the tumours observed in some long-term rodent studies are attributable to a genotoxic action of the compound.

INTRODUCTION

In the first published account of tumorigenesis following repeated administration of chloroform to small rodents, Eschenbrenner & Miller (1945) noted that a dose level sufficient to produce liver necrosis seemed to be a prerequisite for hepatoma development. Ilett, Reid, Sipes & Krishna (1973) demonstrated covalent binding of chloroform metabolites to tissue proteins and associated this with the production of necrosis. These authors also showed how the kidney is at risk in circumstances in which the biotransformation of chloroform in the liver may be incomplete. Depletion of glutathione in the liver may be a determining factor in this toxicity, as indicated by Docks & Krishna (1976).

In recent long-term studies, chloroform administration has been associated with the development of liver tumours in B6C3F1 mice of both sexes and of kidney tumours in male Osborne–Mendel rats (National Cancer Institute, 1976); in other studies, kidney tumours have developed in male ICI mice (Roe, Palmer, Worden & Van Abbé, 1979) but not in females of this strain or males of three other strains, nor in male or female Sprague–Dawley rats (Palmer, Street, Roe *et al.* 1979) or beagle dogs (Heywood, Sortell, Noel *et al.* 1979). The lowest dose level at which tumour development has been reported in any species is 60 mg chloroform/kg/day; this produced tumours in the kidneys of male ICI mice, but no excess of tumours occurred when chloroform was given at 17 mg/kg/day to males or females of the same strain.

Although it is now apparent that most known chemical carcinogens increase the numbers of revertant colonies in one or more of the standard test strains of *Salmonella typhimurium* on histidine-deficient medium (Ames, McCann & Yamasaki, 1975), several workers (de Serres & Ashby, 1980; Simmon, Kauhanen & Tardiff, 1977; Uehleke, Werner, Greim & Krämer, 1977) have reported that chloroform does not give positive findings in this type of mutagenicity test. Such studies are usually conducted in the presence and absence of metabolizing enzymes provided by an S-9 microsome mix derived from the livers of rats that have been subjected to liver-enzyme induction by Aroclor 1254. In view of the findings in long-term rodent experiments, however, it seemed of interest to extend the range of bacterial mutagenicity testing to include the use of mouse-liver enzymes as well as rat-liver enzymes and also to examine the effect, if any, of microsomal enzymes derived from the kidneys of both species rather than from the liver. To avoid the possibility of 'false negative' results due to volatilization of the chloroform, an additional experiment was carried out in which chloroform vapour was caused to impinge continuously on the bacterial culture plates; prolonged chloroform exposure on these lines would eventually prove lethal to the organisms, but mutagenic activity, if any, might be detectable in the earlier stages of exposure to the vapour.

EXPERIMENTAL

Indicator organisms. Salmonella typhimurium strains TA1535, TA100, TA1537, TA1538 and TA98 were used as the histidine-dependent indicator organisms. Strain TA100 was obtained from ICI Pharmaceuticals Ltd, Macclesfield, Cheshire, and the other four from

*Present address: Milk Marketing Board, Veterinary Laboratory, Cleeve House, Lower Wick, Worcester England.

Professor B. N. Ames, University of California, Berkeley, CA, USA. The TA100 strain used was characterized as ampicillin-resistant despite the relatively low spontaneous level of revertants recorded. Stock suspensions in medium were stored frozen in liquid nitrogen and, before use, cultures prepared from the stocks were incubated in nutrient broth for 17 hr at 37°C; resulting suspensions contained approximately 2×10^9 organisms/ml and are referred to as the 'standard bacterial suspension'.

Culture media. Standard liquid medium for all strains was nutrient broth (Oxoid No. 2). The basal layer in plate cultures was Vogel-Bonner minimal agar medium (Difco poured plates); the upper layer, in which the bacteria were suspended, was a 0·6% solution of Difco bacto agar in 0·5% sodium chloride. Histidine-deficient medium was obtained by adding, to each 100 ml of this agar solution, 10 ml of a solution of 0·5 mM-histidine hydrochloride and 0·5 mM-biotin. For histidine-rich medium, 10 ml of a solution of 0·1 M-histidine hydrochloride and 50 mM-biotin was added to each 100 ml of agar solution.

Chemicals. Chloroform (pharmaceutical grade) was obtained from ICI Pharmaceuticals Ltd. The known mutagens used as positive controls were β-naphthylamine and 2-aminoanthracene (from Sigma London Chemical Co., Kingston, Surrey), neutral red (from Raymond A. Lamb Ltd, London) and 2-acetylaminofluorene (from Aldrich Chemical Co., Milwaukee, WI, USA), all of which require oxidation by microsomal enzymes to produce mutagenic metabolites, and sodium azide (from Sigma London Chemical Co.) and 4-nitro-*o*-phenylenediamine (from Aldrich Chemical Co.), which do not require metabolic activation. All chemicals were dissolved in dimethylsulphoxide (DMSO), obtained from Sigma London Chemical Co.

Microsomal-enzyme fraction. Liver S-9 fractions were prepared from specific-pathogen-free rats of the CFY (Sprague–Dawley-derived) strain and mice of the ICI/CFLP strain. The animals were 8–10 wk old and were obtained from Anglia Laboratory Animals, Alconbury, Cambridgeshire. For the kidney S-9 fractions, specific-pathogen-free CD (Sprague–Dawley-derived) rats and CDI (ICR-derived) mice were obtained from Charles River (UK) Ltd, Margate, Kent. Following acclimatization for 1 wk, each animal was injected ip with Aroclor 1254 (200 mg/ml) dissolved in arachis oil, at a dose level of 500 mg/kg body weight, to stimulate microsomal-enzyme activity. Five days after injection, and following a 16-hr period of fasting, the animals were killed by cervical dislocation. Under sterile conditions, the kidneys or livers (as appropriate) were removed and minced in 0·15 M-KCl (5 g tissue in 15 ml KCl) before transfer to a Potter homogenizer (Measuring and Scientific Equipment Ltd, Loughborough, Leics), for homogenization at 4°C. Pooled homogenates were centrifuged at 9000 **g** for 15 min in a refrigerated centrifuge (MSE 25). The supernatant fraction ('S-9') was stored in 3-ml lots at −20°C for not more than 4 wk and was thawed immediately before use for the preparation of S-9 mix following the procedure of Ames *et al.* (1975), 0·3 ml S-9 per ml S-9 mix being used.

Test procedures

Preliminary toxicity test. The same procedure was used for each strain. Minimal agar (15 ml) was poured into 90-mm Petri dishes and allowed to gel. The standard bacterial suspension (0·1 ml) was added to 3 ml histidine-deficient agar at 45°C and the resulting suspension was overlaid on the minimal agar gel. When the upper layer had set, wells 10 mm in diameter were cut in the gel and into these were pipetted 0·1-ml aliquots of a series of DMSO solutions containing 0–10 mg chloroform/ml. Dishes were incubated for 24 hr at 37°C, after which time bacterial toxicity was assessed by measuring the zones of inhibition around each well.

Mutation study (standard procedure). For each indicator strain, 0·1-ml aliquots of the chloroform dilutions were pipetted into bijou bottles, three sets—each of three bottles—being used for each dilution. Similar sets received 0·1-ml aliquots of DMSO or solutions of graded concentrations of the positive control compounds. The standard bacterial suspension (0·1 ml) was added to each bottle. Into each bottle of one of the sets was dispensed 0·5 ml of the rat S-9 mix. This was replaced in the second set by 0·5 ml of the mouse S-9 mix, and in the third by 0·5 ml of sterile 0·9% sodium chloride. The complete series was duplicated for the kidney mixes.

To each bottle, 2·8 ml of histidine-deficient agar was added at 45°C and after thorough mixing the resulting suspension was overlaid on to a previously prepared gel of 15 ml of minimal agar in a 90-mm Petri dish. Triplicate dishes were used to test each concentration of each chemical. After incubation at 37°C for 48 hr, revertant bacterial colonies on each plate were counted with an electronic colony counter (Biotran Mk II, New Brunswick Scientific Co., Inc., Edison, NJ, USA).

Vapour phase study. The procedure differed from the standard technique insofar as the bacterial inoculum and (where appropriate) the microsomal extract (rat-liver S-9 mix) were spread over the surface but the co-factors were incorporated into the top agar. The plates were then placed in an anaerobic jar at 37°C and a stream of chloroform vapour was passed through the jar using a Millipore miniature vacuum/pressure pump (type XX6 122050; Millipore (UK) Ltd, London) to give a flow rate of 0·03 m^3/min. The vapour stream gave a mean passage of 32 ml chloroform/hr in triplicate runs. 'Negative' control plates were treated with a stream of air for comparable periods. After the required exposure time, all plates were taken from the jars and further incubated to give a total 72-hr incubation. As positive controls, ethyl methanesulphonate (2·5%) and 2-acetylaminofluorene (50 μg/plate) were used (applied on discs, not as vapour).

RESULTS

In the preliminary toxicity test, chloroform was apparently non-toxic to all five *S. typhimurium* strains at a concentration of 10,000 μg/well. However, in the bacterial mutagenicity study (Table 1) a concentration of 10,000 μg/plate was toxic for all strains, as shown by the formation of an incomplete bacterial lawn. At and below 1000 μg chloroform/plate the bacterial lawns were satisfactory but there was no significant increase in revertant colonies for any of the indicator

Table 1. *Mutagenicity testing of chloroform in five strains of* Salmonella typhimurium *with/without microsomal-enzyme preparations (standard procedure)*

S. typhimurium strain	Dose of $CHCl_3$ (μg/plate)	No. of revertants/plate*					
		Without activation	With liver S-9 mix		Without activation	With kidney S-9 mix	
			From rat	From mouse		From rat	From mouse
TA1535	0	10	14	12	13	13	10
	10	7	14	12	12	13	16
	100	9	13	13	11	10	15
	1000	7	12	12	14	12	10
	10,000	IL	IL	IL	IL	IL	IL
TA1537	0	6	4	7	7	14	9
	10	2	5	5	7	12	7
	100	3	6	3	9	11	10
	1000	4	5	2	6	11	10
	10,000	IL	IL	IL	IL	IL	IL
TA1538	0	9	14	12	12	20	18
	10	9	12	15	13	22	18
	100	9	14	15	12	18	19
	1000	7	14	14	12	20	15
	10,000	IL	IL	IL	IL	IL	IL
TA98	0	32	45	53	31	30	31
	10	36	42	36	32	30	33
	100	40	45	39	29	33	30
	1000	27	43	45	34	36	30
	10,000	IL	IL	IL	IL	IL	IL
TA100	0	22	28	26	63	70	62
	10	13	22	28	67	74	69
	100	13	25	22	64	67	71
	1000	17	21	25	70	75	69
	10,000	IL	IL	IL	IL	IL	IL

IL = Incomplete bacterial lawn

*Values are means for triplicate plates. Positive-control results are presented in Table 2.

strains, either in the absence of metabolic activators or in the presence of microsomal enzymes from rat or mouse S-9 mix derived from liver or kidney. By contrast, the known mutagens (positive controls) caused large increases in the mutation frequency of the five bacterial strains under these experimental conditions in the presence or absence of metabolic activation, as appropriate (Table 2).

In the vapour phase study, the passage of chloroform vapour for 6–8 hr was highly toxic to strains TA1535 and TA1538. No significant increase in revertant colonies could be detected at any earlier stage,

Table 2. *Mutagenicity of positive-control compounds in five strains of* Salmonella typhimurium *with microsomal-enzyme activation where appropriate (standard procedure)*

S. typhimurium strain	Compound	Dose (μg/plate)	No. of revertants/plate*				
			Without activation	With liver S-9 mix		With kidney S-9 mix	
				From rat	From mouse	From rat	From mouse
TA1535	Sodium azide	5	977	—	—	—	—
	β-Naphthylamine	10	—	136	169	—	—
	2-Aminoanthracene	2	—	—	—	246	151
TA1537	4-NPDA	500	136	—	—	—	—
	Neutral red	10	—	51	51	55	51
TA1538	4-NPDA	500	1559	—	—	—	—
	2-AAF	20	—	106	210	242	174
TA98	4-NPDA	500	1865	—	—	—	—
	2-Aminoanthracene	2	—	318	369	1717	151
TA100	Sodium azide	5	1128	—	—	—	—
	2-Aminoanthracene	2	—	245	333	1146	429

— = Not tested 4-NPDA = 4-Nitro-*o*-phenylenediamine 2-AAF = 2-Acetylaminofluorene

*Values are means for triplicate plates.

Table 3. *Vapour-phase tests for mutagenicity of chloroform in* Salmonella typhimurium *strains TA1535 and TA1538 with/without rat-liver microsomal-enzyme preparations*

Treatment	No. of revertants/plate*			
	TA1535		TA1538	
	Without S-9 mix	With S-9 mix	Without S-9 mix	With S-9 mix
None	22	26	33	45
EMS (2·5%)	*c.* 200	—	*c.* 200	—
2-AAF (50 μg/plate)	—	*c.* 500	—	*c.* 500
$CHCl_3$ vapour—2 hr	27	24	31	42
4 hr	13	27	0	40
6 hr	5	27	0	2
8 hr	0	0	0	3

— = Not tested EMS = Ethyl methanesulphonate 2-AAF = 2-Acetylaminofluorene
*Values are means for triplicate plates.

either in the absence of metabolic activation or in the presence of rat liver S-9 mix (Table 3).

DISCUSSION

The distinction between the apparent non-toxicity of chloroform at 10,000 μg/well in the preliminary toxicity test and the incomplete bacterial lawn noted in the mutagenicity experiments at 10,000 μg chloroform/plate may have been related to volatilization of chloroform from the wells in the preliminary test. Growth of an incomplete lawn at the high dose level in the mutagenicity experiments, however, shows that under these conditions sufficient chloroform remained to exert an antibacterial effect. Table 3 shows that the findings for mutagenicity are identical even when care is taken to make sure that contact with chloroform is not impaired through volatilization. Hence it appears that the mutagenicity findings given in Table 1 correctly reflect the lack of mutagenic activity attributable to chloroform under the various conditions of testing. Whilst confirming the negative findings already reported by other authors, it can now be seen that chloroform is not mutagenic to the five standard indicator strains of *S. typhimurium* in the presence of metabolizing enzymes from either the kidney or the liver, whether obtained from rats or mice.

These findings seem to be fully consistent with those of Diaz Gomez & Castro (1980), who were unable to detect covalent binding of chloroform metabolites to liver DNA or RNA of male Sprague–Dawley rats or of male A/J strain mice after multiple chloroform administration. The development of tumours in small rodents in the course of long-term studies involving repeated intragastric administration of chloroform is therefore unlikely to be a consequence of any direct genotoxic action of the compound. Relevant to the search for an alternative mechanism may be the conclusion reached much earlier by Eschenbrenner & Miller (1945) that chloroform needed to be given at a dose-level sufficient to cause necrosis of the liver in order to provoke the formation of liver tumours in mice.

Agustin & Lim-Sylianco (1978) recently reported that in mutagenicity tests against *S. typhimurium* TA1537 positive findings were obtained with urine concentrates from male mice treated with chloroform at a dose level of 700 mg/kg. At and above a similar dose level, these authors also obtained positive results in a micronucleus test, but dose levels of chloroform up to 400 mg/kg were inactive. Although these findings suggest that mutagenic metabolites are formed when chloroform is given to male mice at very high dose levels, they probably do not account for the enhanced renal-tumour risk encountered in earlier studies in response to chloroform at dose levels of only 60 mg/kg/day (Roe *et al.* 1979).

The mechanism by which chloroform may lead to the development of tumours in the liver or kidneys of mice or rats, when given repeatedly at dose levels insufficient for detectable mutagenic effect, remains to be elucidated. The dose levels at which excess tumours developed when chloroform was given repeatedly to rodents greatly exceeded the dose levels to which humans are exposed when they use products incorporating chloroform as a preservative or flavouring agent. It seems likely that a non-genotoxic mechanism was involved under the experimental conditions that gave rise to these tumours.

REFERENCES

Agustin, J. S. & Lim-Sylianco, C. Y. (1978). Mutagenic and clastogenic effects of chloroform. *Bull. phil. Biochem. Soc.* **1**, 17.

Ames, B. N., McCann, J. & Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Res.* **31**, 347.

de Serres, F. J. & Ashby, J. (Editors) (1980). *Short-term Tests for Carcinogens: Report of the International Collaborative Program.* Elsevier/North-Holland Biomedical Press, Amsterdam.

Diaz Gomez, M. I. & Castro, J. A. (1980). Covalent binding of chloroform metabolites to nuclear proteins—no evidence for binding to nucleic acids. *Cancer Lett.* **9**, 213.

Docks, E. L. & Krishna, G. (1976). The role of glutathione in chloroform-induced hepatotoxicity. *Expl molec. Path.* **24**, 13.

Eschenbrenner, A. B. & Miller, E. (1945). Induction of hepatomas in mice by repeated oral administration of chloroform, with observations on sex differences. *J. natn. Cancer Inst.* **5**, 251.

Heywood, R., Sortwell, R. J., Noel, P. R. B., Street, A. E.,

Prentice, D. E., Roe, F. J. C., Wadsworth, P. F., Worden, A. N. & Van Abbé, N. J. (1979). Safety evaluation of toothpaste containing chloroform III. Long-term study in beagle dogs. *J. envir. Path. Toxicol.* **2,** 835.

Ilett, K. F., Reid, W. D., Sipes, I. G. & Krishna, G. (1973). Chloroform toxicity in mice: correlation of renal and hepatic necrosis with covalent binding of metabolites to tissue macromolecules. *Expl molec. Path.* **19,** 215.

National Cancer Institute (1976). Report on Carcinogenesis Bioassay of Chloroform. 1 March.

Palmer, A. K., Street, A. E., Roe, F. J. C., Worden, A. N. & Van Abbé, N. J. (1979). Safety evaluation of toothpaste containing chloroform II. Long-term studies in rats. *J. envir. Path. Toxicol.* **2,** 821.

Roe, F. J. C., Palmer, A. K., Worden, A. N. & Van Abbé, N. J. (1979). Safety evaluation of toothpaste containing chloroform I. Long-term studies in mice. *J. envir. Path. Toxicol.* **2,** 799.

Simmon, V. F., Kauhanen, K. & Tardiff, R. G. (1977). Mutagenic activity of chemicals identified in drinking water. *Devl. Toxicol. envir. Sci.* **2,** 249.

Uehleke, H., Werner, T., Greim, H. & Krämer, M. (1977). Metabolic activation of haloalkanes and tests *in vitro* for mutagenicity. *Xenobiotica* **7,** 393.