

IN VITRO MICROBIOLOGICAL MUTAGENICITY ASSAYS
OF THREE 3M COMPANY COMPOUNDS

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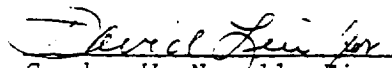
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SUMMARY

SRI International examined 3M Company compounds T-2136 CoC, T-2138 IM, and T-2140 IM for mutagenic activity with strains TA1535, TA1537, TA1538, TA98, and TA100 of the bacterium Salmonella typhimurium in the standard Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3. Each assay was performed in the presence and in the absence of a metabolic activation system.

None of the compounds was mutagenic in either the Salmonella/microsome assay or the S. cerevisiae assay.

INTRODUCTION

SRI International examined compounds T-2136 CoC, T-2138 IM, and T-2140 IM for mutagenicity by in vitro microbiological assays with *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100 and with the yeast *Saccharomyces cerevisiae* D3. An Aroclor 1254-stimulated, rat liver homogenate metabolic activation system was included in the assay procedures to provide metabolic steps that the bacteria either are incapable of conducting or do not carry out under the assay conditions. The purpose of this study was to determine whether these compounds elicited a mutagenic response in microorganisms.

The assay procedure with *S. typhimurium* has proven to be 80 to 90% reliable in detecting carcinogens as mutagens, and it has about the same reliability in identifying chemicals that are not carcinogenic.¹ The assay procedure with *S. cerevisiae* is about 60% reliable in detecting carcinogens as agents that increase mitotic recombination.² The combination of the two assay procedures significantly enhances the probability of detecting potentially hazardous chemicals. However, because the systems do not always provide 100% correlation with the results of carcinogenicity investigations in animals, neither a positive nor a negative response proves conclusively that a chemical is hazardous or nonhazardous to man.

METHODS

Salmonella typhimurium Strains TA1535, TA1537, TA1538, TA98, and TA100

The Salmonella typhimurium strains used at SRI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown on minimal medium petri plates containing a trace of histidine, only those cells that revert to histidine independence (his⁺) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few divisions; in many cases, this growth is essential for mutagenesis to occur. The his⁺ revertants are easily scored as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar the mutation frequency is increased 2- to 100-fold.

We obtained our S. typhimurium strains from Dr. Bruce Ames of the University of California at Berkeley.¹⁻⁵ In addition to having mutations in the histidine operon, all the indicator strains have a mutation (rfa⁻) that leads to a defective lipopolysaccharide coat; they also have a deletion that covers genes involved in the synthesis of vitamin biotin (bio⁻) and in the repair of ultraviolet (uv)-induced DNA damage (uvrB⁻). The rfa⁻ mutation makes the strains more permeable to many large aromatic molecules, thereby increasing the mutagenic effect of these molecules. The uvrB⁻ mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents. Strain TA1535 is reverted to his⁺ by many mutagens that cause base-pair substitutions. TA100 is derived from TA1535 by the introduction of the resistance transfer factor plasmid pKM101. This plasmid is believed to cause an increase in error-prone DNA repair that leads to many more mutations

for a given dose of most mutagens.⁵ In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of the plasmid in the cells. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens (e.g., ICR-191, benzo(a)pyrene, aflatoxin B₁, and 7,12-dimethylbenz(a)anthracene). Strains TA1537 and TA1538 are reverted by many frameshift mutagens. TA1537 is more sensitive than TA1538 to mutation by some acridines and benzanthraces, but the difference is quantitative rather than qualitative. Strain TA98 is derived from TA1538 by the addition of the plasmid pKM101, which makes it more sensitive to some mutagenic agents.

All indicator strains are kept at 4° C on minimal medium plates, supplemented with a trace of biotin, and an excess of histidine. The plates with the plasmid-carrying strains contain in addition ampicillin (25 µg/ml), to ensure stable maintenance of the plasmid pKM101. New stock culture plates are made every two months from single colony reisolates that were checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of the plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37° C in nutrient broth (Oxoid, CM67). After stationary overnight growth, the cultures are shaken for 3 to 4 hours to ensure optimal growth.

Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic chemicals, either of the aromatic amino type or polycyclic hydrocarbon type, are inactive unless they are metabolized to active forms. In animals and man, an enzyme system in the liver or other organs (e.g., lung or kidney) is capable of metabolizing a large number of these chemicals to carcinogens.^{5,6} Some of these intermediate metabolites are very potent mutagens in the S. typhimurium test. Ames has described the liver metabolic activation system that we use.⁶ In brief, adult male rats (250 to 300 g) are given a single 500-mg/kg intraperitoneal injection of a polychlorinated biphenyl, Aroclor 1254. This treatment enhances the synthesis of enzymes involved in the metabolic

conversion of chemicals. Four days after the injection the animals' food is removed but drinking water is provided ad libitum. On the fifth day, the rats are killed, and the liver homogenate is prepared as follows.

The livers are removed aseptically and placed in a preweighed sterile glass beaker. The organ weight is determined, and all subsequent operations are conducted in an ice bath. The livers are washed in an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernatant, referred to as the S-9 fraction, is quickly frozen in dry ice and stored at -80° C.

The metabolic activation mixture for each experiment consists of, for 10 ml:

- 1.00 ml of S-9 Fraction
- 0.20 ml of $MgCl_2$ (0.4 M) and KCl (1.65M)
- 0.05 ml of glucose-6-phosphate (1 M)
- 0.40 ml of NADP (0.1 M)
- 5.00 ml of sodium phosphate (0.2 M, pH 7.4)
- 3.35 ml of H_2O .

Assays in Agar

To a sterile 13 x 100 mm test tube placed in a 43° C heating block, we add in the following order:

- (1) 2.00 ml of 0.6% agar*
- (2) 0.05 ml of indicator organisms
- (3) 0.05 ml of a solution of the test chemical
- (4) 0.50 ml of metabolic activation mixture (optional).

For negative controls, we use steps (1), (2), and (4) (optional) and 0.05 ml of the solvent used for the test chemical. Because the majority of organic compounds are not sufficiently water soluble--particularly at the higher concentrations--we routinely use dimethylsulfoxide (DMSO). Other solvents that are occasionally used are water, ethanol, or benzene. For positive controls, we test each culture by specific mutagens known to revert each strain using steps (1), (2), (3), and (4) (optional).

This mixture is stirred gently and then poured onto minimal agar plates.[†] After the top agar has set, the plates are incubated at 37° C for 2 days. The number of his⁺ revertant colonies is counted and recorded.

* 0.6 % agar contains 0.05 mM histidine, 0.05 mM biotin, and 0.1 M NaCl.

† Minimal agar plates consist of, per liter, 15 g of agar, 10 g of glucose, 0.2 g of MgSO₄·7H₂O, 2 g of citric acid monohydrate, 10 g of K₂HPO₄, and 3.5 g of NaH₂PO₄·4H₂O.

Saccharomyces cerevisiae D3

The yeast S. cerevisiae D3 is a diploid microorganism heterozygous for a mutation leading to a defective enzyme in the adenine-metabolizing pathway.⁹ When grown on medium containing adenine, cells homozygous for this mutation produce a red pigment. These homozygous mutants can be generated from the heterozygotes by mitotic recombination. The frequency of this recombinational event may be increased by incubating the organisms with various mutagens. The degree of mutagenicity of a compound or of its metabolite is determined from the number of red-pigmented colonies appearing on the plates.¹⁰

The S. cerevisiae tester strain is stored at -80° C. For each experiment, the tester strain is inoculated in 1% tryptone and 0.5% yeast extract and grown overnight at 37° C with aeration.

The in vitro yeast mitotic recombination assay in suspension is conducted as follows. The overnight culture is centrifuged, and the cells are resuspended at a concentration of 10^8 cells ml/in a 67 mM phosphate buffer (pH 7.4). To a sterile test tube are added:

- 1.30 ml of the organisms
- 0.50 ml of either the metabolic activation mixture or buffer
- 0.20 ml of the test chemical.

Because many organic chemicals are not appreciably water soluble, dimethylsulfoxide (DMSO) is used routinely as the solvent for the test chemical. Other solvents that are used occasionally are ethanol, benzene, or water. Several doses of the chemical (up to 5%, w/v or v/v) are tested in each experiment, and appropriate controls are included.

The suspension mixture is incubated at 30° C for 4 hours on a roller drum. The sample is diluted serially in sterile physiological saline, and a volume of 0.2 ml of the 10^{-5} and 10^{-3} dilutions is spread on tryptone-yeast agar plates; five plates are used for the 10^{-3} dilution and three plates are used for the 10^{-5} dilution. The plates are incubated for 2 days at 30° C, followed by 2 days at 4° C to enhance the development of the red pigment indicative of adenine-

deficient homozygosity. Plates of the 10^{-3} dilution are scanned with a dissecting microscope at 10 X magnification, and the number of red colonies or red sectors (mitotic recombinants) is recorded. The surviving fraction of organisms is determined from the number of colonies appearing on the plates of the 10^{-5} dilution.

The number of mitotic recombinants is calculated per 10^5 survivors. A positive response in this assay is indicated by a dose-related increase of more than threefold in the absolute number of mitotic recombinants per milliliter as well as in the relative number of mitotic recombinants per 10^5 survivors.

RESULTS AND DISCUSSION

Tables 1 through 3 present the results of testing T-2136 CoC, T-2138 IM, and T-2140 IM in the Ames Salmonella/microsome assay and with the yeast S. cerevisiae D3.

T-2136 CoC was dissolved in water. T-2138 IM and T-2140 IM were extracted in DMSO overnight; various volumes of the extract were used in the bacterial assay procedure and made up to 100 μ l with DMSO (Tables 2 and 3). The negative control used for these two samples was 100 μ l of DMSO. When known mutagenic compounds were added to the top agar of the positive control plates, the number of mutants above the background count was increased. When various concentrations of T-2138 IM, or T-2140 IM were added to the top agar, no dose-related increase in the number of mutants over the background count was observed, either with or without metabolic activation. These results lead to the conclusion that T-2136 CoC, T-2138 IM, and T-2140 IM are not mutagenic in the Salmonella/microsome assay.

Tables 4 and 5 present the results of testing T-2136 CoC with S. cerevisiae D3. At various concentrations ranging from 0.1% to 5%, this compound did not cause a reproducible, dose-related increase in the number of mitotic recombinants. Therefore, we conclude that this compound is not mutagenic in S. cerevisiae D3. This assay was not conducted on T-2138 IM or T-2140 IM because only limited quantities of these compounds were available.

Table 1
IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
T-2136 CoC

Compound	Metabolic Activation	Micrograms of Compound Added per Plate	Average Histidine Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
Negative control (H ₂ O)	-	50,000	31	7	8	19	132
	+	50,000	23	9	20	29	159
Positive controls							
Sodium azide	-	0.5	279				372
9-Aminoacridine	-	50		623			
2-Nitrofluorene	-	5			841	823	
2-Anthramine	-	2.5	25	5			139
	+	2.5	236	185			1912
	-	1.0			13	21	
	+	1.0			373	368	
T-2136 CoC	-	10	27	6	9	21	150
	-	50	31	5	13	18	156
	-	100	31	7	12	23	146
	-	500	31	8	10	22	154
	-	1000	28	6	14	17	166
	-	5000	22	8	13	23	149
	+	10	21	9	18	33	157
	+	50	27	6	17	26	160
	+	100	26	8	15	19	175
	+	500	27	8	18	32	166
	+	1000	18	7	21	27	191
	+	5000	25	9	22	30	181

Table 2
IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
T-2138 IM

Compound	Metabolic Activation	Amount of Compound Added per Plate	Average Histidine Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
Negative control (DMSO)	-	100 μ l	21	4	10	19	121
	+	100	17	6	21	25	131
Positive controls							
Sodium azide	-	0.5 μ g	322				405
9-Aminoacridine	-	50		654			
2-Nitrofluorene	-	5			1197	758	
2-Anthramine	-	2.5	16	8			145
	+	2.5	285	194			2055
	-	1.0			17	18	
	+	1.0			780	1430	
T-2138 IM	-	2	22	5	11	11	141
	-	5	21	4	11	15	141
	-	10	26*	7	13	13	139
	-	25	26*	6	14	17	150
	-	50	29*	5	15	15	133
	-	100	30*	4	10	15	145
	+	2	12	5	21	24	134
	+	5	17	6	15	21	160
	+	10	12	4	28	26	168
	+	25	19*	8	21	24	174
	+	50	23*	5	17	27	167
	+	100	25*	7	26	26	162

* Average of three experiments.

Table 3
IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
T-2140 IM

Compound	Metabolic Activation	Amount of Compound Added per Plate	Average Histidine Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	TA100
Negative control (DMSO)	-	100 μ l	18	4	10	19	121
	+	100	16	6	21	25	131
Positive controls							
Sodium azide	-	0.5 μ g	322				405
9-Aminoacridine	-	50		654			
2-Nitrofluorene	-	5			1197	758	
2-Anthramine	-	2.5	16	8			145
	+	2.5	285	194			2055
	-	1.0			17	18	
	+	1.0			780	1430	
T-2140 IM	-	2	14	6	9	13	105
	-	5	16	5	10	18	114
	-	10	17	8	11	15	113
	-	25	19	4	12	11	128
	-	50	15	4	10	18	128
	-	100	15	5	10	23	132
	+	2	17	6	20	26	118
	+	5	13	9	18	24	138
	+	10	16	7	19	28	113
	+	25	11	5	15	21	151
	+	50	14	6	19	25	148
	+	100	12	6	17	31	147

Table 4

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE

T-2136 CoC

Experiment 1

Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Survivors		Mitotic Recombinants	
			Cells per ml ($\times 10^{-7}$)	Percent	Per ml ($\times 10^{-3}$)	Per 10^5 Survivors
Negative control (H ₂ O)	-		7.9	100	5.0	6.3
	+		7.4	100	5.0	6.8
Positive control 1,2,3,4-Diepoxybutane	-	0.025	5.9	75	880.0	1484.0
	+	0.025	5.0	68	807.5	1608.6
T-2136 CoC	-	0.1	6.8	86	6.0	8.8
	-	0.5	7.0	89	2.0	2.9
	-	1.0	6.8	86	4.0	5.9
	-	5.0	5.7	72	5.0	8.8
	+	0.1	6.7	91	9.0	13.4
	+	0.5	8.3	112	3.0	3.6
	+	1.0	6.5	88	3.0	4.6
	+	5.0	5.8	78	5.0	8.6

Table 5

IN VITRO ASSAYS WITH *SACCHAROMYCES CEREVISIAE*
T-2136 CoC

Experiment 2

Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Survivors		Mitotic Recombinants	
			Cells per ml ($\times 10^{-7}$)	Percent	Per ml ($\times 10^{-3}$)	Per 10^5 Survivors
Negative control (H ₂ O)	-		6.8	100	9.0	13.2
	+		9.6	100	5.0	5.2
Positive control 1,2,3,4-Diepoxybutane	-	0.025	6.9	101	677.0	981.2
	+	0.025	6.5	68	605.0	933.6
T-2136 CoC	-	1.0	7.0	103	2.0	2.9
	-	2.0	6.9	101	8.0	11.6
	-	4.0	10.0	147	7.0	7.0
	-	5.0	8.5	125	3.0	3.5
	+	1.0	10.8	113	5.0	4.6
	+	2.0	8.8	92	2.0	2.3
	+	4.0	9.5	99	7.0	7.4
	+	5.0	10.6	110	7.0	6.6

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