

SRI International

IN VITRO MICROBIOLOGICAL MUTAGENICITY ASSAYS
OF 3M COMPANY COMPOUNDS T-2247 CoC AND T-2248 CoC

Final Report

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SUMMARY

SRI International examined 3M Company Compound T-2247 CoC and T-2248 CoC for mutagenic activity with strains TA1535, TA1537, TA1538, TA98, and TA100 of the bacterium Salmonella typhimurium in the Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3. T-2247 CoC was also tested in desiccators. Each assay was performed in the presence and in the absence of a metabolic activation system. T-2247 CoC and T-2248 CoC were not mutagenic or recombinogenic in any of the assays performed.

INTRODUCTION

SRI International examined 3M Company compounds T-2247 CoC and T-2248 CoC for mutagenicity by in vitro microbiological assays with five strains of the bacterium Salmonella typhimurium (TA1535, TA1537, TA1538, TA98, and TA100) in the standard Ames Salmonella/microsome assay, in desiccators, 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 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 carcinogenicity investigations in animals, neither a positive nor a negative response conclusively proves 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.^{4,6-8} 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 MgCl₂ (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 H₂O.

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.

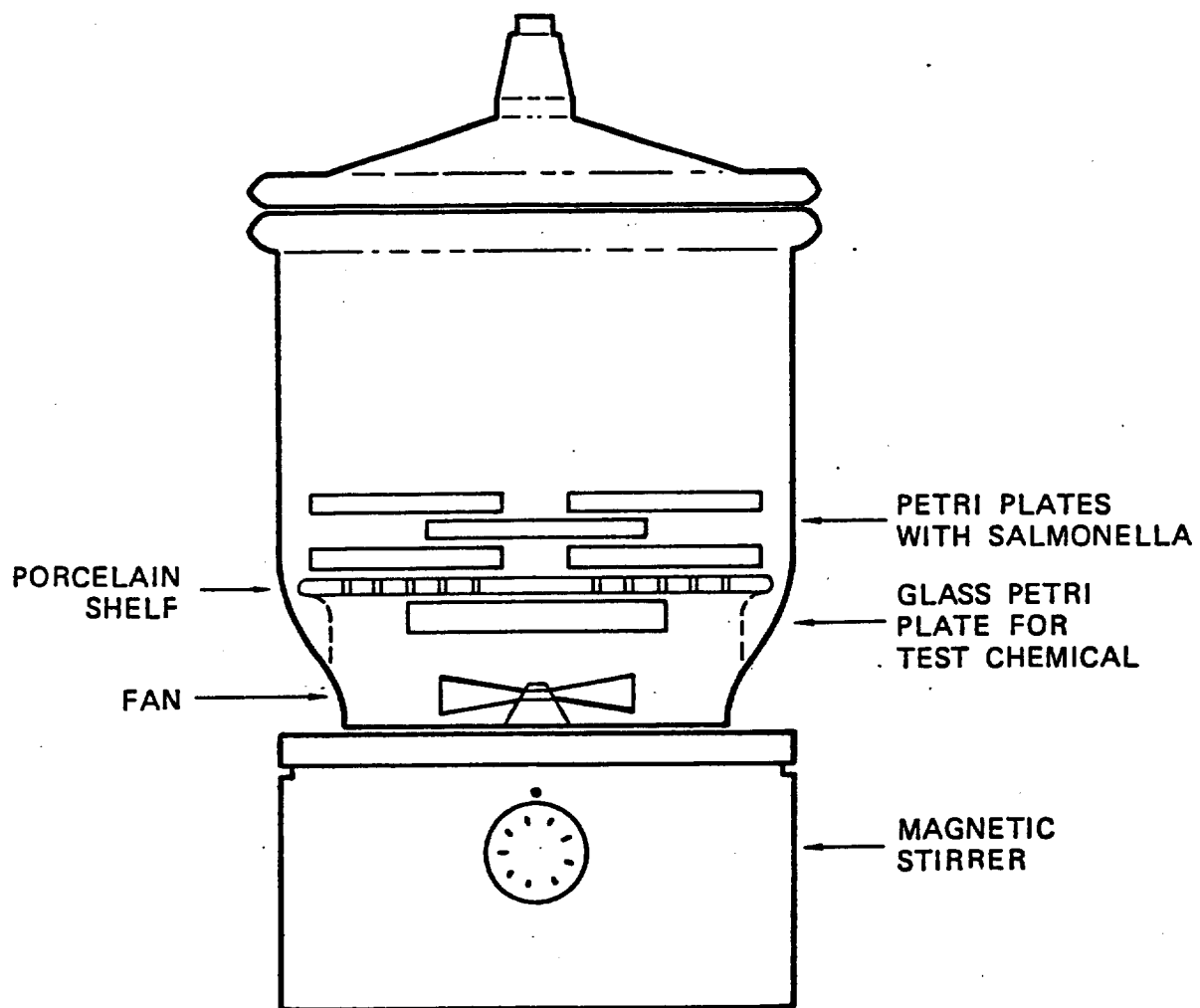
* 0.6 % agar contains 0.05 mM histidine, 0.05 mM biotin, and 0.1 M NaCl.

† 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.

Assays in Desiccators for Volatile Compounds

The standard Ames plate test is not entirely suitable for the testing of highly volatile chemicals, so we have modified the procedure to conduct such testing. The Salmonella plates are prepared as described for the assays in agar, but no test chemical is added. The plates, the lids having been removed, are placed side by side on a perforated shelf in a 9-liter desiccator. A known volume of the test chemical is added to a glass petri plate that is placed in the center of and attached to the bottom of the shelf. A control chemical is tested similarly in each experiment. The desiccator is sealed and placed on a magnetic stir plate in a room maintained at 37° C. A magnetic stirrer with vanes, placed in the base of each desiccator, ensures adequate dispersion of the chemical. After incubation for 8 hours, the plates are removed from the desiccators, their lids are replaced, and they are incubated at 37° C for an additional 42 hours. The number is his⁺ revertants is counted and recorded.

DESICCATOR ASSAY



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

Table 1 presents the results of testing T-2247 CoC in the Ames Salmonella/microsome assay. The data are an average of two assays performed on separate days. The compound was tested over a wide range of concentrations, from 10 to 5000 µg/plate, both with and without metabolic activation. Because no dose-related increase in the number of mutants over the background count was observed, we conclude that T-2247 CoC was not mutagenic in S. typhimurium.

As Table 2 shows, T-2248 was tested at dose levels of 10 to 5000 µg/plate. Toxicity was observed in strain TA1538 at 1000 µg/plate and in strains TA1535, TA1537, TA1538, TA98, and TA100 at 5000 µg/plate without metabolic activation. T-2248 CoC was toxic at 1000 µg/plate in strain TA1537 and at 5000 µg/plate in all strains when tested with activation. No mutagenic activity was observed with T-2248 CoC in the Ames Salmonella/microsome assay.

Table 3 presents the averaged results of testing T-2247 CoC in the desiccator assay on strains TA98 and TA100. The assay was conducted in duplicate (two plates per strain per dose) with an exposure of 8 hours. The sample was exposed to a wide range of doses of from 0.1 to 5.0 ml per desiccator. No mutagenicity or toxicity was observed.

Tables 4 and 5 present the results of assays on T-2247 CoC with S. cerevisiae D3. T-2247 CoC was tested at concentrations of from 0.1 to 5.0% (Table 4) and at concentrations of from 1.0 to 5.0% (Table 5). The compound was not toxic in these assays and did not cause a dose-related increase in the number of mitotic recombinants. Therefore, we conclude that T-2247 CoC was not recombinogenic in S. cerevisiae D3.

Tables 6 and 7 present the results of testing T-2248 CoC with S. cerevisiae D3. A slight toxic and apparent mutagenic response was seen at 5.0% without metabolic activation (Table 6). T-2248 CoC was retested at concentrations from 1.0 to 5.0% (Table 7). No dose-

related increase in the number of mitotic recombinants was observed; therefore, we conclude that T-2248 CoC was not recombinogenic to S. cerevisiae D3.

We conclude that T-2247 CoC is not toxic or mutagenic in either S. typhimurium or S. cerevisiae D3. T-2248 CoC is toxic at higher doses in the Salmonella/microsome assay and is not mutagenic in either S. typhimurium or S. cerevisiae D3.

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Table 1
IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
T-2247 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)	-		11	8	18	15	89
	+		9	5	27	24	83
Positive controls							
Sodium azide	-	0.5	141				268
9-Aminoacridine	-	50.0		63			
2-Nitrofluorene	-	5.0			802	553	
2-Anthramine	-	1.0			20	16	81
	-	2.5	11	19			
	+	1.0			254	251	497
	+	2.5	186	138			
T-2247 CoC	-	10	19	8	17	14	89
	-	50	14	10	21	17	93
	-	100	18	11	25	14	81
	-	500	16	13	11	19	111
	-	1000	18	12	17	17	91
	-	5000	17	12	14	17	99
	+	10	7	5	21	21	92
	+	50	10	7	28	23	97
	+	100	9	6	16	22	97
	+	500	8	11	16	27	103
	+	1000	8	5	23	23	106
	+	5000	13	5	14	29	88

Table 2

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
T-2248 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)	-		11	8	18	15	89
	+		9	5	27	24	83
Positive controls							
Sodium azide	-	0.5	141				268
9-Aminoacridine	-	50.0		63			
2-Nitrofluorene	-	5.0			802	553	
2-Anthramine	-	1.0			20	16	81
	-	2.5	11	19			
	+	1.0			254	251	497
	+	2.5	186	138			
T-2248 CoC	-	10	19	13	17	30	98
	-	50	12	12	13	14	87
	-	100	24	13	20	30	98
	-	500	15	10	10	22	83
	-	1000	10	7	T*	20	28
	-	5000	9T	T	T	T	T
	+	10	8	12	25	16	88
	+	50	11	6	24	22	94
	+	100	8	9	28	29	105
	+	500	12	9	19	28	91
	+	1000	8	7T	13	29	58
	+	5000	T	T	T	T	T

*T, toxic.

Table 3

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM IN DESICCATORS
T-2247 CoC; 8-HOUR EXPOSURE

<u>Compound</u>	<u>Metabolic Activation</u>	<u>Amount of Compound in Desiccator (ml)</u>	<u>Revertants/Plate</u>	
			<u>TA98</u>	<u>TA100</u>
Negative control (H ₂ O)	-		13	112
	+		27	129
Positive control 1,1-Dichloroethylene	-	1.0	31	524
	+	1.0	35	445
T-2247 CoC	-	0.1	12	121
	-	0.5	11	118
	-	1.0	15	115
	-	5.0	7	97
	+	0.1	33	97
	+	0.5	18	116
	+	1.0	27	104
	+	5.0	19	115

Table 4

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE

T-2247 CoC

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)	-		6.2	100	3.5	5.7
	+		6.1	100	3.5	5.7
Positive control 1,2,3,4-Diepoxybutane	-	0.025	6.8	111	460	680
	+	0.025	6.1	98	350	570
T-2247 CoC	-	0.1	5.8	94	1.0	1.7
	-	0.5	6.9	111	3.0	4.4
	-	1.0	5.6	90	5.0	8.9
	-	5.0	7.5	121	-0-	-0-
	+	0.1	6.5	107	3.0	4.6
	+	0.5	6.9	113	3.0	4.4
	+	1.0	6.4	105	2.0	3.1
	+	5.0	5.1	84	2.0	3.9

Table 5

IN VITRO ASSAYS WITH *SACCHAROMYCES CEREVISIAE*
T-2247 CoC

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	3.5	5.2
	+		8.3	100	2.5	3.0
Positive control 1,2,3,4-Diepoxybutane	-	0.025	6.9	101	610	880
	+	0.025	7.0	84	570	810
T-2247 CoC	-	1.0	6.9	701	2.0	2.9
	-	2.0	5.7	84	1.0	1.8
	-	4.0	4.9	72	3.0	6.1
	-	5.0	7.6	112	3.0	4.0
	+	1.0	8.4	101	2.0	2.4
	+	2.0	5.3	64	2.0	3.8
	+	4.0	5.7	69	3.0	5.3
	+	5.0	7.4	89	2.0	2.7

Table 6

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE
T-2248 CoC

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)	-		6.2	100	3.5	5.7
	+		6.1	100	3.5	5.7
Positive control 1,2,3,4-Diepoxybutane	-	0.025	6.8	111	460	680
	+	0.025	6.1	98	350	570
T-2248 CoC	-	0.1	5.6	90	3.0	5.4
	-	0.5	6.5	105	3.8	5.9
	-	1.0	5.0	81	6.0	12
	-	5.0	3.1	50	10.0	32
	+	0.1	7.0	115	1.3	1.9
	+	0.5	7.3	120	-0-	-0-
	+	1.0	6.5	107	1.0	1.5
	+	5.0	6.1	100	3.0	4.9

Table 7

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE
T-2248 CoC

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)	-		6.6	100	2.5	4.0
	+		8.6	100	1.0	1.2
Positive control 1,2,3,4-Diepoxybutane	-	0.025	4.6	70	520	1100
	+	0.025	5.6	65	560	1000
T-2248 CoC 21	-	1.0	6.4	97	1.0	1.6
	-	2.0	7.1	108	1.0	1.4
	-	3.0	5.6	85	5.0	8.9
	-	4.0	5.8	88	7.0	12
	-	5.0	7.0	106	3.0	4.3
	+	1.0	5.2	60	6.0	12
	+	2.0	6.5	76	1.0	1.5
	+	3.0	6.4	74	5.0	7.8
	+	4.0	7.5	87	1.0	1.3
	+	5.0	5.9	69	2.0	3.4