SRI International

IN VITRO MICROBIOLOGICAL MUTAGENICITY ASSAYS OF 3M COMPANY'S COMPOUND T-2997CoC

Final Report

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SUMMARY

SRI International examined 3M Company's Compound T-2997CoC for mutagenic activity with strains TA1535, TA1537, TA1538, TA98, and TA100 of Salmonella typhimurium in the standard Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3 using FDA-approved GLP procedures. Each assay was performed in the presence and in the absence of a rat liver metabolic activation system. Compound T-2997CoC was not mutagenic or recombinogenic in any assay performed.

CONTENTS

QUALITY ASSURANCE UNIT

Final Report Statement

SRI International assures the quality and integrity of this study, the Ames Salmonella Microsome assay and the Saccharomyces cerevisiae D3 recombinogenic assay of the 3M Company's compounds T-2997 CoC.

The study was inspected on December 12 and 22, 1980 during the dilution, plating, and plate counting of the two assays. The findings of the Quality Assurance Unit Inspector were reported at the time of each inspection to the Study Director. SRI management was also informed of the inspection results on December 12 and 22, 1980, respectively.

This final report accurately describes the methods and standard operating procedures and reflects the raw data of the study. Any deviations from the approved protocol and standard operating procedures were made with proper authorization and documentation.

Sherry Hansen / QA

Quality Assurance Unit/Title

Jan 21, 1981

Date

INTRODUCTION

SRI International examined 3M Company's Compound T-2997CoC for mutagenicity by *in vitro* microbiological assays 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. 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. The assay procedure with *S. cerevisiae* is about 60% reliable in detecting carcinogens as agents that increase mitotic recombination. However, because the assay 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 carcinogenic or noncarcinogenic to man.

The assays with Compound T-2997CoC were begun on 12 December 1980 and completed on 7 January 1981. Copies of the final report will be kept in our files (Building 28, Room 213) and in SRI's Records Center. The laboratory notebook will be retained in Building 28A, Room 10 for one year after it is filled, and then will be stored in SRI's Record Center. All that is left of Compound T-2997CoC will be kept for six months in our chemical storage room (Building 28, Room 217), then returned to the 3M Company.

MATERIALS

- Test Compounds

  - Name: T-2997CoC.
  - Date Received: 8 December 1980.
  - Description: Waxy amber solid.
  - Stability: Stability assured by client.
  - Storage Condition: Stored at room temperature.
  - Special Testing Conditions: None.

- Indicator Organisms

  - Species: *Salmonella typhimurium* LT2 and *Saccharomyces cerevisiae*.
  - Strains: TA1535, TA1537, TA1538, TA98, and TA100 of *S. typhimurium*; D3 of *S. cerevisiae*.

- Metabolic Activation

  Aroclor 1254-induced rat liver S-9; SRI Batch D7, D8, and NIEHS RLI003, ∿ 30 mg/ml protein.

- Solvent Used

  Dimethylsulfoxide (DMSO).

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 agar 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 visible 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, usually in a dose-related manner.

We obtained our S. typhimurium strains from Dr. Bruce Ames of the University of California at Berkeley. 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 the 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 molecules, thereby increasing the mutagenic effect of these molecules. The uvrB mutation causes decreased 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. In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence

of the plasmid in the cell. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens [e.g., ICR-191, benzo(a)pyrene, aflatoxin B_1, and 7,12-dimethylbenz(a)anthracene]. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. 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 agar plates supplemented with an excess of biotin and histidine. The plates with the plasmid-carrying strains also contain ampicillin (25 µg/ml) to ensure stable maintenance of the plasmid pKM101. New stock culture plates are made every 4 to 6 weeks from single colony isolates that have been 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).

Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic chemicals (e.g., of the aromatic amino type or the 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. 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. In brief, adult male rats (250 to 300 g) are given a single 500 mg/kg intraperitoneal injection of Aroclor 1254 (a mixture of polychlorinated biphenyls). 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

with 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 × 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 experiments consists of, for 10 ml:

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

Assays in Agar

To a sterile 13 × 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) 0.50 ml of metabolic activation mixture (if appropriate)
(4) 0.05 ml of a solution of the test chemical.

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.

For negative controls, we use steps (1), (2), and (3) and 0.05 ml of the solvent used for the test chemical. Dimethylsulfoxide (DMSO) was used as the solvent for T-2476ChR. For positive controls, we test each

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

†Minimal agar plates consist of, per liter, 15 g of agar, 10 g of glucose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of citric acid monohydrate, 10 g of K_2HPO_4, and 3.5 g of $NaNH_4HPO_4 \cdot 4H_2O$.

culture by specific mutagens known to revert each strain, using steps (1), (2), (3), and (4).

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. 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 carcinogenic or recombinogenic agents. The recombinogenic activity of a compound or its metabolite is determined from the number of red-pigmented colonies appearing on test plates.

A stock culture of S. cerevisiae is stored at 4°C. For each experiment, broth containing 0.05% $MgSO_4$, 0.15% KH_2PO_4, 0.45% $(NH_4)_2SO_4$, 0.35% peptone, 0.5% yeast extract, and 2% dextrose is inoculated with a loopful of the stock culture and incubated overnight at 30°C with shaking.

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 67 mM phosphate buffer (pH 7.4). To a sterile test tube are added:

- 1.00 ml of the resuspended culture
- 0.50 ml of either the metabolic activation mixture or buffer
- 0.20 ml of the test chemical
- 0.30 ml of buffer.

Several doses of the test chemical 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 then diluted serially in sterile physiologic saline, and 0.2 ml of the 10^{-5} and 10^{-3} dilutions is spread on plates containing the same ingredients as the broth plus 2.0% agar; five plates

are spread with the 10^{-3} dilution and three plates are spread with 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 containing the 10^{-5} dilution are scanned with a dissecting microscope at 10× magnification, and the number of mitotic recombinants (red colonies or red sectors) is recorded. The surviving fraction of organisms is determined from the total 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 3-fold in the absolute number of mitotic recombinants per milliliter as well as in the relative number of mitotic recombinants per 10^5 survivors.

Statistical Method

No statistical method was needed. Results are a tabulation of the number of colonies appearing on the plates after incubation.

References

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RESULTS AND DISCUSSION

Compound T-2997CoC was tested for mutagenicity with the Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3 assay in the presence and in the absence of a metabolic activation system. The compound was tested twice on separate days in both assays. Dimethylsulfoxide (DMSO) was used as the solvent in all assays.

In the Ames Salmonella/microsome assay, Compound T-2997CoC was tested over a wide range of concentrations, from 10 to 5,000 µg/plate (Table 1). Because no toxicity occurred at 5,000 µg/plate, the second test was run using a higher dose range, from 50 to 10,000 µg/plate (Table 2). Still no toxicity or dose-related increase in the number of revertants per plate was observed in either assay.

In the microbiological assays with S. cerevisiae D3, T-2997CoC was tested over a wide range of concentrations, from 0.05 to 5.0% w/v (Tables 3 and 4). No toxicity or dose-related increase in the number of mitotic recombinants was observed in either assay.

We conclude that Compound T-2997CoC is not mutagenic with S. typhimurium or recombinogenic in S. cerevisiae.

Table 1

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

COMPOUND T-2997CoC

Experiment Date: 12 December 1980

Compound	Metabolic Activation	Compound Added (µg/plate)	Histidine Revertants per Plate									
			TA1535		TA1537		TA1538		TA98		TA100	
Negative Control												
DMSO	-		15	16	5	7	12	7	20	16	115	130
	+		16	13	7	3	18	9	38	31	104	110
Positive Controls												
Sodium Azide	-	1.0	336	324							467	442
9-Aminoacridine	-	50.0			508	527						
2-Nitrofluorene	-	5.0					726	738	372	381		
2-Anthramine	-	1.0					17	7	38	37	126	140
	+	1.0					271	294	158	147	519	518
	-	2.5	9	20	7	7						
	+	2.5	252	275	77	101						
Compound T-2997CoC	-	10.0	24	27	3	5	5	7	26	29	99	149
	-	50.0	19	17	3	4	2	13	24	31	124	116
	-	100.0	19	16	5	6	12	8	21	32	115	112
	-	500.0	28	15	5	5	5	9	18	28	99	129
	-	1,000.0	30	15	6	8	5	8	25	24	111	113
	-	5,000.0	20	19	7	3	8	7	18	30	101	97
	+	10.0	7	6	12	12	13	12	36	43	122	135
	+	50.0	15	9	8	5	18	14	38	28	125	125
	+	100.0	3	14	7	7	12	7	26	49	108	112
	+	500.0	9	13	5	9	16	19	37	40	126	134
	+	1,000.0	16	8	9	16	21	26	34	33	129	127
	+	5,000.0	9	16	13	8	7	16	31	24	105	92

Table 2

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

COMPOUND T-2997CoC

Experiment Date: 15 December 1980

Compound	Metabolic Activation	Compound Added (µg/plate)	Histidine Revertants per Plate									
			TA1535		TA1537		TA1538		TA98		TA100	
Negative Control												
DMSO	-		24	15	14	8	8	13	25	24	100	110
	+		6	8	7	8	25	8	18	27	99	108
Positive Controls												
Sodium Azide	-	1.0	224	276							317	347
9-Aminoacridine	-	50.0			721	604						
2-Nitrofluorene	-	5.0					691	863	337	420		
2-Anthramine	-	1.0					9	15	15	18	93	72
	+	1.0					154	149	153	161	383	357
	-	2.5	13	14	16	8						
	+	2.5	257	209	64	51						
Compound T-2997CoC	-	50.0	16	9	5	13	13	12	12	25	108	125
	-	100.0	17	8	8	15	9	12	17	19	79	108
	-	500.0	15	9	7	3	9	4	20	16	88	112
	-	1,000.0	16	17	2	2	8	8	17	16	80	113
	-	5,000.0	13	12	6	8	4	6	7	12	72	97
	-	10,000.0	9	15	1	6	4	7	14	20	54	57
	+	50.0	6	9	5	9	5	19	24	37	91	114
	+	100.0	7	7	8	8	14	14	18	32	86	123
	+	500.0	6	12	7	6	17	9	19	19	108	103
	+	1,000.0	5	15	6	5	15	13	16	22	93	105
	+	5,000.0	4	5	4	4	15	9	20	21	98	104
	+	10,000.0	6	7	5	6	12	14	17	16	88	97

Table 3

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE D3

COMPOUND T-2997CoC

Experiment Date: 17 December 1980

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						
DMSO	-		7.4	100	2.0	2.8
	+		6.5	100	2.0	3.0
Positive Control						
1,2,3,4-Diepoxybutane	-	0.025	6.6	89	1100.0	1700.0
	+	0.025	5.2	80	530.0	1000.0
Compound T-2997CoC	-	.05	6.5	88	1.0	1.5
	-	.1	7.0	95	1.0	1.4
	-	.5	6.2	84	1.0	1.6
	-	1.0	6.4	86	1.0	1.6
	-	5.0	6.5	88	3.0	4.6
	+	.05	7.0	108	1.0	1.4
	+	.1	5.9	91	1.0	1.7
	+	.5	6.4	98	1.0	1.6
	+	1.0	6.2	95	1.0	1.6
	+	5.0	6.5	100	2.0	3.1

Table 4

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE D3

COMPOUND T-2997CoC

Experiment Date: 7 January 1981

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						
DMSO	-		6.8	100	5.0	7.5
	+		6.5	100	3.5	5.4
Positive Control						
1,2,3,4-Diepoxybutane	-	0.025	5.1	75	900.0	1800.0
	+	0.025	5.6	86	1000.0	1800.0
Compound T-2997CoC	-	.05	6.4	94	2.0	3.1
	-	.1	6.4	94	4.0	6.3
	-	.5	6.9	101	3.0	4.3
	-	1.0	6.7	99	1.0	1.5
	-	5.0	5.0	74	6.0	12.0
	+	.05	6.1	94	2.0	3.3
	+	.1	6.7	103	8.0	12.0
	+	.5	6.5	100	5.0	7.7
	+	1.0	5.4	83	3.0	5.6
	+	5.0	6.3	97	7.0	11.0