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IN VITRO MICROBIOLOGICAL MUTAGENICITY ASSAYS OF
3M COMPANY'S COMPOUND T-3290CoC

Final Report

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SUMMARY

SRI International examined 3M Company's Compound T-3290CoC for mutagenic activity with strains TA1535, TA1537, TA1538, TA98, and TA100 of bacterium Salmonella typhimurium in the standard Ames Salmonella/microsome in vitro mutagenicity assay. Compound T-3290CoC was also screened for recombinogenic activity with the yeast Saccharomyces cerevisiae D3 assay. Both assays were performed in the presence and absence of a rat-liver metabolic activation system. Compound T-3290CoC was found to be neither mutagenic nor recombinogenic when tested using these procedures.

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INTRODUCTION

SRI International examined 3M Company's Compound T-3290CoC 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 microorganisms 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.

MATERIALS

- Test Compound

  - Name: T-3290CoC
  - Date Received: 8 October 1982
  - Description: Yellow-amber liquid
  - Storage Conditions: Room temperature
  - Special Testing Conditions: None

- Indicator Organisms

  - Species: *Salmonella typhimurium* LT2
    Saccharomyces cerevisiae

  - Strains: TA1535, TA1537, TA1538, TA98, and TA100 for *S. typhimurium*; D3 for *S. cerevisiae*

- Metabolic Activation

  Aroclor 1254-induced rat-liver S-9; SRI Batch E-8;
  ~ 31 mg/ml protein.

- Solvent Used

  Sterile water

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 renders the bacteria unable to use the accurate excision repair mechanism to remove certain chemically or physically induced DNA lesions 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 frozen in nutrient broth supplemented with 10% sterile glycerol at -80°C in 1-ml samples containing about 10^9 cells. New frozen stock cultures are made every 3 months 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, the frozen 1-ml samples are allowed to thaw at room temperature before inoculation in 50 ml of glucose minimal liquid medium supplemented with an excess of biotin and histidine. The cultures are grown at 37°C, unshaken for 4 hours, then gently shaken (100 rpm) for 11 to 14 hours. All strains are genetically analyzed whenever experiments are performed.

Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic chemicals (e.g., of the aromatic amine 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 Sprague-Dawley rats (200 to 250 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 minced with sterile surgical scissors in three volumes of 0.15 M KCl, (3 ml/g of wet organ), 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 on 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 (1.65 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.

Plate Incorporation Assay

Prior to testing, the test article is serially diluted from an initial stock. The dose levels are based on the results of a preliminary range-finding experiment. The article is usually tested over a minimum of six dose levels, the highest nontoxic dose level being 10 mg/plate unless solubility, mutagenicity, or toxicity dictates a lower upper limit. All assays are repeated at least once on a separate day.

The plate incorporation assay is performed in the following way. To a sterile 13 × 100 mm test tube placed in a 43°C heating block we add:

(1) 2.00 ml of 0.6% agar containing 0.6% NaCl, 0.05 mM biotin and 0.05 mM histidine
(2) 0.05 ml of indicator organisms (about 10^8 bacteria)
(3) 0.05 ml of a solution of the test article
(4) 0.50 ml of metabolic activation mixture (if appropriate).

This mixture is stirred gently and then poured on plates containing about 25 ml of minimal glucose agar. After the top agar has set, the plates are incubated for 48 hours at 37°C. The number of his^+ revertant colonies is counted using a BioTran II automated colony counter when possible. When accurate counts cannot be obtained (e.g., because of precipitate), the plates are counted manually using an electric probe colony counter.

Concurrent sterility, negative (solvent), and positive controls are run with every experiment. Sterility controls include plating out separately steps (3) and (4). For negative controls, we use steps (1), (2), (4), and 0.05 ml of the solvent used for the test article. For positive controls, we test each bacterial culture with the following mutagens using steps (1), (2), (3), and (4):

- Sodium azide for the base-pair substitution mutants TA1535 and TA100.
- 9-Aminoacridine for the frameshift mutant TA1537.
- 2-Nitrofluorene for the frameshift mutants TA1538 and TA98.
- 2-Anthramine for all tester strains, in the presence of metabolic activation.

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 3 days at 30°C, followed by 1 day at 4°C to enhance the development of the red pigment indicative of adenine-deficient homozygosity. Plates containing the 10^{-3} 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.

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RESULTS AND DISCUSSION

3M Company's Compound T-3290CoC was screened for mutagenic activity with the standard Ames Salmonella/microsome in vitro mutagenicity assay using the five standard Ames strains of Salmonella typhimurium: TA1535, TA1537, TA1538, TA98, and TA100. This compound was assayed on two separate days, 11 October and 18 October 1982, each time over a dose range of 10 to 5,000 µg/plate, with two plates per dose level, using sterile water as the solvent. All assays were performed both in the presence and in the absence of a rat-liver metabolic activation system. This compound foamed when vortexed; however, this did not appear to interfere with the testing. No dose-related increase in the number of histidine-independent revertants was observed in either of the two assays. Therefore, we conclude that Compound T-3290CoC is nonmutagenic when tested by these procedures. Data from these assays are presented in Tables 1 and 2.

Compound T-3290CoC was also assayed for recombinogenic activity using the yeast Saccharomyces cerevisiae D3 assay for mitotic recombination. This assay was performed on two separate days, 11 October and 18 October 1982. This compound was tested twice over the dose range of 0.05% to 5.0%, both with and without a rat-liver metabolic activation system. Compound T-3290CoC was found to be reproducibly nonrecombinogenic when tested by these procedures. No dose-related increase in the number of mitotic recombinants per 10^5 survivors was observed. Data from these assays are presented in Tables 3 and 4.

Table 1

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

Compound T-3290CoC

Experiment Date: 11 October 1982

Compound	Metabolic Activation	Compound Added per plate	Histidine Revertants per Plate									
			TA1535		TA1537		TA1538		TA98		TA100	
Negative Controls												
Sterile water	-		21	20	4	11	10	12	19	24	176	154
	+		7	13	7	9	16	17	25	33	179	139
Positive Controls												
Sodium Azide	-	1 µg	528	509							507	464
9-Aminoacridine	-	50			173	166						
2-Nitrofluorene	-	5					605	595	285	283		
2-Anthramine	-	1					17	20	19	19	173	184
	+	1					111	116	129	101	392	441
	-	2.5	21	18	8	9						
	+	2.5	105	115	69	65						
Compound T-3290CoC	-	10	25	24	7	13	16	14	18	19	164	155
	-	50	17	11	9	6	17	10	18	19	170	165
	-	100	24	15	6	6	18	20	21	24	164	161
	-	500	25	14	7	8	11	13	17	23	159	177
	-	1000	17	21	8	5	10	11	18	18	171	164
	-	5000	18	20	6	5	11	12	14	21	153	139
	+	10	15	9	8	6	18	29	28	27	162	160
	+	50	8	9	9	6	19	26	31	28	144	171
	+	100	8	7	9	7	18	19	36	21	177	167
	+	500	7	9	5	7	17	14	39	39	131	170
	+	1000	7	9	5	3	18	21	33	26	176	164
	+	5000	7	6	2	10	16	18	24	21	166	151

Table 2

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM

Compound T-3290CoC

Experiment Date: 18 October 1982

Compound	Metabolic Activation	Compound Added per plate	Histidine Revertants per Plate									
			TA1535		TA1537		TA1538		TA98		TA100	
Negative Controls												
Sterile water	-		21	16	4	6	12	6	17	13	152	122
	+		6	5	5	6	13	18	24	20	128	137
Positive Controls												
Sodium Azide	-	1 µg	377	445							381	411
9-Aminoacridine	-	50			94	103						
2-Nitrofluorene	-	5					450	462	292	277		
2-Anthramine	-	1					10	12	22	14	122	116
	+	1					158	171	187	170	387	445
	-	2.5	16	15	4	6						
	+	2.5	100	127	61	47						
Compound T-3290CoC	-	10	20	19	6	4	13	16	24	13	111	125
	-	50	29	18	5	3	9	8	19	19	117	101
	-	100	18	16	4	8	8	17	14	18	110	137
	-	500	11	17	8	6	9	12	11	19	146	125
	-	1000	16	24	6	5	10	10	20	17	116	160
	-	5000	15	15	12	7	11	12	21	19	137	126
	+	10	10	13	11	9	24	20	31	21	130	124
	+	50	7	9	6	6	20	26	20	19	116	145
	+	100	8	7	6	8	20	16	29	30	101	134
	+	500	8	6	7	4	27	29	25	28	122	116
	+	1000	4	6	7	5	18	15	31	20	137	117
	+	5000	5	14	12	7	20	14	14	19	135	131

Table 3

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE D3

Compound T-3290CoC

Experiment Date: 11 October 1982

Compound	Metabolic Activation	Percent Concentration weight/volume	Survivors		Mitotic Recombinants	
			Cells per ml ($\times 10^{-7}$)	Percent	Per ml ($\times 10^{-3}$)	Per 10^5 Survivors
Negative Controls						
Sterile water	-		4.8	100	4	8.3
	+		5.3	100	6	11.3
Positive Controls						
1,2,3,4-Diepoxybutane	-	0.025	5.2	100	877	1686
Sterigmatocystin	-	0.0005	4.9	100	5	10.2
	+	0.0005	5.7	100	399	700
Compound T-3290CoC	-	0.05	4.6	96	2	4.3
	-	0.1	4.4	92	2	4.5
	-	0.5	5.1	100	4	7.8
	-	1	4.9	100	4	8.2
	-	5	4.5	94	1	2.2
	+	0.05	4.8	91	3	6.3
	+	0.1	4.7	89	4	8.5
	+	0.5	4.7	89	2	4.3
	+	1	5.0	94	3	6
	+	5	4.0	75	6	15

Table 4

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE D3

Compound T-3290CoC

Experiment Date: 18 October 1982

Compound	Metabolic Activation	Percent Concentration weight/volume	Survivors Cells per ml ($\times 10^{-7}$)	Survivors Percent	Mitotic Recombinants Per ml ($\times 10^{-3}$)	Mitotic Recombinants Per 10^5 Survivors
Negative Controls						
Sterile water	-		4.0	100	3	7.5
	+		4.1	100	6	14.6
Positive Controls						
1,2,3,4-Diepoxybutane	-	0.025	4.6	100	946	2057
Sterigmatocystin	-	0.0005	4.3	100	3	7.0
	+	0.0005	4.3	100	414	963
Compound T-3290CoC	-	0.05	3.8	95	4	10.5
	-	0.1	3.6	90	6	16.7
	-	0.5	3.6	90	5	13.9
	-	1	3.5	88	5	14.3
	-	5	3.8	95	2	5.3
	+	0.05	4.4	100	4	9.1
	+	0.1	4.3	100	4	9.3
	+	0.5	4.2	100	2	4.8
	+	1	4.7	100	4	8.5
	+	5	3.6	88	3	8.3