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June 13, 1978

Petition Control Branch
Food and Drug Administration
Department of Health, Education and
Welfare
BF 344
200 "C" Street
Washington, D.C. 20204

Re: FAP 3A2885
Aspartame

Gentlemen:

Attached in triplicate for inclusion as Entries E-99, E-100, and E-101 into our Master File #134 are the reports entitled: "The Metabolism of the Aspartyl Moiety of Aspartame", MRC-751-0032; "The Metabolism of Aspartate in Infant and Adult Mice", MRC-751-0021; and, An Evaluation of the Mutagenic Potential of SC-18862 Employing the Ames Salmonella/Microsome Assay", S.A. 1385. Final Report.

On March 6, 1978 we forwarded to you for inclusion as Entry E-97 into our Master File #134, 'SC-18862: An Evaluation of Mutagenic Potential Employing the Ames Salmonella/Microsome Assay", S.A. 1377. We are herewith submitting an errata for page 7, Results and Discussion and for page 1, Introduction, of the subject report.

Entry E-98, the report entitled "SC-19192: An Evaluation of Mutagenic Potential Employing the Ames Salmonella/Microsome assay", S.A. 1378 submitted March 6, 1978 has also been issued erratum to the footnote on page 5 and are herewith included for correction.

Attached is an updated copy of the Index of Master File #134.

Sincerely,

Robert G. Bost, Ph.D.
Director of Food Products
Regulatory Affairs Department

RGB:mw
Attachments

SC-19192: An Evaluation of Mutagenic Potential
Employing the Ames Salmonella/Microsome Assay; S.A. 1378.

ERRATUM

The last footnote on page 5 should be changed from:
10₂g of K₄HPO to 10g of K₂HPO₄.

*Correction made
3/2/79
B*

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL EMPLOYING
THE AMES SALMONELLA/MICROSOME ASSAY

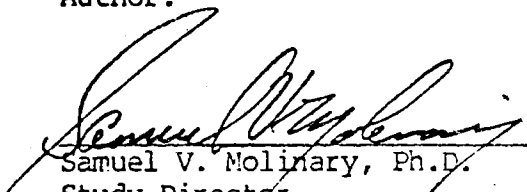
Samuel V. Molinary

Safety Assessment Project Number 1378

Department of Product Safety Assessment

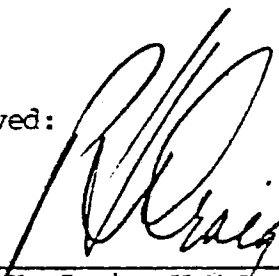
Searle Laboratories
Skokie, Illinois

Author:

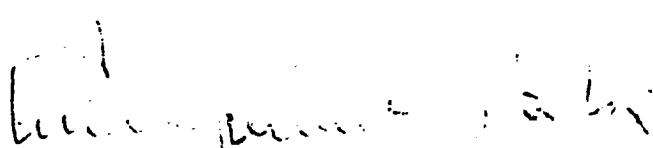

Samuel V. Molinary, Ph.D.
Study Director
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January 30, 1978

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL EMPLOYING
THE AMES SALMONELLA/MICROSOME ASSAY

SUMMARY

SC-19192 was examined for mutagenic activity using the Ames Salmonella/microsome assay with five tester strains of Salmonella typhimurium (TA1535, TA1537, TA1538, TA98 and TA100). The assay was performed in the presence and in the absence of a rat-liver homogenate metabolic activation system.

SC-19192 was not mutagenic in the bacterial system with Salmonella typhimurium either in the presence or in the absence of the metabolic activation system.

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SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL EMPLOYING
THE AMES SALMONELLA/MICROSOME ASSAY

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INTRODUCTION

SC-19192 (Lot #TJT-12-32) was examined for its mutagenic potential in the in vitro microbiological assay employing five strains of Salmonella typhimurium, TA1535, TA1537, TA1538, TA98, and TA100. In order to provide the metabolic steps that the bacteria are incapable of conducting or that they are incapable of carrying out under the assay conditions, an Aroclor 1254-stimulated, rat-liver homogenate metabolic activation system was included in the assay procedure. The purpose of this study was to determine whether this compound is mutagenic in microorganisms.

The Ames Salmonella/microsome assay has proven to be approximately 85-90% accurate in detecting carcinogens as mutagens, and it has about the same accuracy in identifying chemicals that are not carcinogenic.¹ However, because this test is not 100% accurate, neither a positive nor a negative response conclusively proves that a chemical is hazardous or nonhazardous to man.

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METHODS

Salmonella typhimurium strains TA1535, TA1537, TA1538, TA98, TA100.

The Salmonella typhimurium strains used at Searle Laboratories are all histidine auxotrophs. When these histidine-dependent cells are grown on a minimal media petri plate containing a trace of histidine, only those cells that have reverted to histidine prototrophy are able to grow and form colonies. Since DNA replication is usually required for mutagenesis to occur, the small amount of histidine allows all the bacteria on the plate to undergo a few cell divisions. The bacteria that revert to histidine prototrophy (his^+) are easily seen against the slight background growth. The spontaneous mutation, or reversion, frequency of each strain is relatively constant, but when a mutagen is added to the agar, the mutation frequency is increased 3- 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. The rfa^- mutation enhances the permeability of the cells to many large aromatic molecules. These strains also have a DNA deletion that affects the genes involved in biotin synthesis (bio^-) and that affects the DNA polymerase gene which normally allows the repair of ultra-violet (uv) induced DNA damage ($uvrB^-$). The $uvrB^-$ mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents.

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Strain TA1535 is reverted to his^+ by mutagens that cause base-pair substitutions. Strain 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 Plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of the plasmid in the cells. It has been shown that TA100 can detect base-substitution mutagens such as benzyl chloride, 2-(2-furyl)3-(5-nitro-2-furyl)acrylamide (AF₂) and other nitrofurans ⁶ carcinogens in plate assays more easily than can TA1535. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens, i.e. aflatoxin B₁, and 7,12-dimethylbenz(a)anthracene. Strains TA1537 and TA1538 are reverted by 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 pKM101 plasmid which makes it more sensitive to some mutagenic agents.

All the indicator strains are routinely checked for their genotypic characteristics (his^- , rfa^- , $uvrB^-$, bio^-) and for the presence of the plasmid as described by Ames, et al. ⁶ Cultures are then stored in 10% sterile glycerol at -80° C. For each experiment, an inoculum from the stock cultures is grown overnight at 37° C in nutrient broth (Difco). 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 carcinogens or mutagens unless they are metabolized to active form. In animals and man, an enzyme system in the liver or other organs is capable of metabolizing a large number of the chemicals to carcinogens.^{4,6-8} Some of these metabolic intermediates are very potent mutagens in the S. typhimurium test. The liver metabolic activation system is prepared and used as described by Ames, et al.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.

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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 (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, the following were added in order:

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

For negative controls, steps (1), (2), and (3) (optional) and 0.05 ml of the solvent are used for the test chemical. Because the majority of organic compounds are not sufficiently water soluble, particularly at the higher concentrations, dimethylsulfoxide (DMSO) is routinely used as the solvent. For positive controls, each culture is tested by specific mutagens known to revert each strain using steps (1), (2), (3) (optional), and (4).

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 and 0.05 mM biotin.

[†]Minimal agar plates consist of, per liter, 15 g of agar, 20 g of glucose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of citric acid monohydrate, 10g of K_2HPO_4 , and 3.5 g of $NaH_2PO_4 \cdot 4H_2O$.

Data storage

The final report, protocol and raw data will be stored at Searle Laboratories, Skokie, Illinois.

Standard operating procedures

For additional technical details and more extensive descriptions of procedures, see the Product Safety Assessment Standard Procedures File.

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RESULTS AND DISCUSSION

The data presented in the following pages in Tables 1 and 2 represent two assays conducted on different days. In both assays, SC-19192 was dissolved in DMSO; no solubility problems were encountered. The assays were conducted in the presence and in the absence of the rat-liver activation system. In each assay, SC-19192 was tested at six doses ranging from 10 to 5000 µg/plate. No evidence of toxicity was observed at any dose level in either assay.

No dose-related increase in the number of revertant colonies was observed in these assays either in the presence or in the absence of the metabolic activation system. The positive and negative control data in both assays suggest that all bacterial strains were competent to reveal the presence of mutagens.

The results of these two assays lead to the conclusion that SC-19192 is not mutagenic in this test system.

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TABLE 1

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL EMPLOYING
THE AMES SALMONELLA/MICROSOME ASSAY

Compound	Metabolic activation ^a	Micrograms per plate	Average histidine revertants per plate				
			TA1535	TA1537	TA1538	TA98	TA100
Negative controls	-		9	3	5	9	60
	+		8	6	15	18	62
Positive controls							
^b							
MNNG	-	2	858				
9-aminoacridine	-	100		1755			
2-nitrofluorene	-	10			1287		
AF ₂	-	0.02				190	491
2-anthramine	-	2.5	8	6	4	10	73
	+	2.5	57	52	396	344	484
SC-19192							
	-	10	11	3	4	5	71
	-	50	7	3	8	9	62
	-	100	7	2	5	8	57
	-	500	9	4	7	8	59
	-	1000	8	3	8	7	65
	-	5000	3	2	3	7	54
	+	5	5	4	11	13	67
	+	50	8	5	11	14	65
	+	100	6	4	13	15	70
	+	500	7	3	12	17	59
	+	1000	5	3	11	16	55
	+	5000	4	5	14	16	57

a

The symbol (-) indicates the absence of the metabolic activation system and the symbol (+) indicates its presence.

b

N-methyl-N-nitro-N-nitrosoguanidine

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TABLE 2

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL EMPLOYING
THE AMES SALMONELLA/MICROSOME ASSAY

Compound	Metabolic activation ^a	Micrograms per plate	Average histidine revertants per plate				
			TA1535	TA1537	TA1538	TA98	TA100
Negative controls	-		8	4	9	11	68
	+		5	8	17	20	68
Positive controls							
^b							
MNNG	-	2	801				
9-aminoacridine	-	100		1693			
2-nitrofluorene	-	10			1468		
AF ₂	-	0.02				154	580
2-anthramine	-	2.5	7	5	10	16	88
	+	2.5	129	45	416	388	518
SC-19192							
	-	10	6	5	4	7	59
	-	50	11	3	6	9	60
	-	100	11	2	7	9	63
	-	500	6	2	3	10	69
	-	1000	12	2	5	11	48
	-	5000	11	4	5	13	64
	+	10	6	8	11	18	81
	+	50	5	11	12	20	72
	+	100	7	8	11	17	66
	+	500	6	6	16	17	77
	+	1000	6	5	11	18	81
	+	5000	7	4	20	19	71

^a

The symbol (-) indicates the absence of the metabolic activation system and the symbol (+) indicates its presence.

^b

N-methyl-N-nitro-N-nitrosoguanidine

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REFERENCES

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