Haworth (1988)

Rotenone Reregistration

EPA Administrative Number 6704-Q

Toxicology Data

(Guideline No. 84-2)

April 24, 1987

VOLUME 3 SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENESIS ASSAY (ROTENONE)

(Guideline No. 84-2)

26 pages

Study Title

Salmonella/Mammalian-Microsome Plate Incorporation Mutagenesis Assay (Rotenone)

Data Requirement

Guideline No. 84-2

Authors

Study Director: Steve R. Haworth, Ph.D.

Study Completed On

November 3, 1978

Performing Laboratory

EG&G Mason Research Institute
1530 East Jefferson Street
Rockville, Maryland 20852

Laboratory Project ID

Study #019-563-165-1

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1)(A), (B), or (C).

Agency U.S. Fish and Wildlife Service

Agency Agent: Fred P. Meyer Date: April 24, 1987

Director
National Fisheries Research Center
La Crosse, Wisconsin

Signature

GOOD LABORATORY PRACTICE STATEMENT

This study meets the requirements for 40 CFR Part 160

Submitter U.S. Fish and Wildlife Service

Sponsor U.S. Fish and Wildlife Service

Study Director Steve R. Haworth

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SALMONELLA/MAMMALIAN-MICROSOME PLATE
INCORPORATION MUTAGENESIS ASSAY
STUDY #019-563-165-1

Notebook #MRI-WL-21 Page 236, 240, 242, 248
Archived at EG&G Mason Research Institute, 1530 East Jefferson Street, Rockville, Maryland 20852

Sponsor: U. S. Fish and Wildlife Service
Box 818
La Crosse, Wisconsin 54601

Test Article I.D.: Retenone Crystalline Code #864200

Test Article Lot No.: 735-RAP-1502

Compound Description: White Crystalline Solid

Storage Conditions: Room Temperature in Carcinogen Room under Desiccation

Date Received: 11/3/78

Study Coordinator: John L. Allen
U. S. Fish and Wildlife Service

Study Director: Steve R. Haworth, Ph.D.
EG&G Mason Research Institute

Report Date: 6-29-79

Steve R. Haworth 6/29/79
Steve R. Haworth, Ph.D. Date
Study Director

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John Cameron 6/29/79
John Cameron Date
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Introduction

U. S. Fish and Wildlife Service Retenone Crystalline Code #864200, Lot #735-RAP-1502 (MRI #165) was received on November 3, 1978, for testing in the Salmonella/ mammalian-microsome mutagenicity assay at six dose levels using five tester strains, TA98, TA100, TA1535, TA1537 and TA1538, both with and without metabolic activation by Aroclor induced rat liver microsomes. The test article was assayed twice with a minimum of two weeks elapsing between assays.

Materials and Methods

The experimental protocol (see Appendix) is a modification of that described by Ames, B. N., et al. Methods for detecting carcinogens and mutagens with the Salmonella/ mammalian-microsome mutagenicity test. Mutation Research 31:347-364, 1975.

RESULTS

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Preliminary Toxicity Determination
of Test Article

Table 1

019-563-165-1
Study Number

Lot #735-RAP-1502
Code #864200 Retrorse Crysta
Test Article Identification

Test Compound Concentration µg/Plate	TA100 Viable Count/ Plate	TA100 Spontaneous Revertants/Plate	TA100 Background Bacterial Lawn
50 ul DMSO	353	103	normal
100 ul DMSO	186	104	normal
2.0	355	106	normal
5.0	371	93	normal
15.0	373	102	normal
48.0	317	93	normal
153.0	243	95	normal w/precip
489.0	291	104	normal w/precip
1,563.0	* 339	* 81	normal w/precip
5,000.0	* 348	* 97	normal w/heavy precip
10,000.0/100 ul	* 246	* 85	normal w/heavy prec

* hand count due to precipitate

Date Plated 5/1/79

machine Counted By: G. Smith 5/7/79

Eight serial half-log dilutions of the test compound are plated with TA100 on minimal agar plus 10XSA and on minimal agar plus 1XSA. Equal numbers of cells are seeded on each plate in the presence of the test compound. The percent survival of an appropriately diluted TA100 culture on the 10XSA supplemented plates is determined by comparing numbers of colonies on the solvent control with those on the plates containing test compound. Toxicity on the 1XSA supplemented plates is detectable by a decrease in the number of revertant colonies occurring per plate and by a thinning or disappearance of the background bacterial lawn. The highest concentration of test compound used in the broad range mutagenesis assay is that which gives a detectable reduction of spontaneous revertants on the 1XSA supplemented plates.

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TABLE 2

SALMONELLA MUTAGENESIS ASSAY

019-563-165-1	Retenone Crystalline Lot# 735-RAP-1502 Code# 864200	30-10,000 ug
Study Number	Test Article Identification	Dose Range

		Solvent DMSO 100 ml	Concentration 30	100	330	1,000	3,300	10,000	
Strain: T.# 98 Date Plated: 5-15-79 No. of Cells Seeded: 1.3 x10^8 Metabolic Activation: Rat Liver Hand Counted by: P.T. Burke 5-21-79 Calculations by: G. Rickard 5/22/79	Revertants per Plate	20	30	25	26	29	25	26	
		25	25	38	19	29	24	20	
		26	28	27	26	16	23	24	
	Averaged Revertants	24	28	30	24	26	24	23	
	Standard Deviation	3	3	7	4	6	1	3	
Strain: TA98 Date Plated: 5-15-79 No. of Cells Seeded: 1.3 x10^8 Metabolic Activation: None Hand Counted by: P.T. Burke 5-21-79 Calculations by: G. Rickard 5/22/79	Revertants per Plate	19	17	16	17	14	17	8	
		18	18	17	12	17	19	10	
		25	15	16	17	16	11	6	
	Averaged Revertants	21	17	16	15	16	16	8	
	Standard Deviation	4	2	1	3	2	4	2	

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TABLE 3

SALMONELLA MUTAGENESIS ASSAY

019-563-16.5-1	Retenone Crystalline Lot #735-RAF-1502 Code #864200	30-10,000 ug
Study Number	Test Article Identification	Dose Range

		Solvent DMSO 100 ul	Concentration 30	100	330	1,000	3300	10,000	
Strain: TA 100 Date Plated: 5-15-79 No. of Cells Seeded: 1.5 x10^8 Metabolic Activation: RAT LIVER 5-21-79 Hand Counted by: P.T. Burke Calculations by: P. Rockland 5/22/79	Revertants per Plate	126	141	122	155	131	114	133	
		108	133	114	110	97	132	127	
		124	130	123	121	128	129	107	
	Averaged Revertants	119	131	120	135	119	125	122	
	Standard Deviation	10	9	5	23	19	10	14	
Strain: TA 100 Date Plated: 5-15-79 No. of Cells Seeded: 1.5 x10^8 Metabolic Activation: NONE 5-21-79 Hand Counted by: P.T. Burke Calculations by: P. Rockland 5/22/79	Revertants per Plate	138	157	140	133	140	119	116	
		141	135	157	154	170	132	115	
		171	128	162	158	147	138	114	
	Averaged Revertants	150	140	153	148	152	130	115	
	Standard Deviation	18	15	12	13	16	10	1	

Form No. NL-105

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TABLE 4

SALMONELLA MUTAGENESIS ASSAY

019-563-165-1	Retenene Crystalline Lot#735-RAP-1502 Cole#864200	30-10,000 ug
Study Number	Test Article Identification	Dose Range

		Solvent DMSO 100 ul	Concentration 30	100	330	1,000	3,300	10,000	
Strain: TA1535 Date Plated: 5-15-79 No. of Cells Seeded: 1.1 x10^8 Metabolic Activation: Rat Liver Hand Counted by: P.J. Burke 5-21-79 Calculations by: H Rickard 5/22/79	Revertants per Plate	11	11	10	14	14	9	5	
		16	7	12	12	10	7	7	
		10	16	15	8	7	10	16	
	Averaged Revertants	12	9	12	11	10	9	9	
	Standard Deviation	3	2	3	3	4	2	6	
Strain: TA1535 Date Plated: 5-15-79 No. of Cells Seeded: 1.1 x10^8 Metabolic Activation: None Hand Counted by: P.J. Burke 5-21-79 Calculations by: H Rickard 5/23/79	Revertants per Plate	19	28	24	31	23	33	13	
		35	24	32	24	24	21	13	
		32	21	16	36	24	20	17	
	Averaged Revertants	29	25	24	30	24	25	14	
	Standard Deviation	9	3	8	6	1	7	2	

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TABLE 5

SALMONELLA MUTAGENESIS ASSAY

619-563-165-1 (Study Number)	Retenone Crystalline Lot#735-RAP-1502 Calc#864200 (Test Article Identification)	30 - 10,000 ug (Dose Range)

		Solvent DMSO 100 ml	Concentration 30	100	330	1,000	3,300	10,000	
Strain: TA 1537	Revertants per Plate	6	9	8	9	10	7	6	
Date Plated: 5-15-79		8	7	10	13	6	9	4	
No. of Cells Seeded: 0.7 x10^8		4	6	8	8	8	4	5	
Metabolic Activation: Rat Liver	Averaged Revertants	6	7	9	10	8	7	5	
Hand Counted by: P.J. Burke 5-21-79	Standard Deviation	2	2	1	3	2	3	1	
Calculations by: G Reichard 5/22/79									
Strain: TA 1537	Revertants per Plate	6	8	7	8	9	7	7	
Date Plated: 5-15-79		10	9	6	10	7	6	6	
No. of Cells Seeded: 0.7 x10^8		8	7	10	10	7	8	2	
Metabolic Activation: None	Averaged Revertants	8	8	8	9	8	7	5	
Hand Counted by: P.J. Burke 5-21-79	Standard Deviation	2	1	2	1	1	1	3	
Calculations by: G Reichard 5/22/79									

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TABLE 6

SALMONELLA MUTAGENESIS ASSAY

019-563-165-1 (Study Number)	Retenene Crystalline Lot#735-RAP-1502 Code#864200 (Test Article Identification)	30-10,000 ug (Dose Range)

			Concentration						
		Solvent DMSO 100 ul	30	100	330	1,000	3,300	10,000	
Strain: TA1538 Date Plated: 5-15-79 No. of Cells Seeded: 1.4 x10^8 Metabolic Activation: Rat Liver Hand Counted by: P.J. Burke 5-21-79 Calculations by: G. Reinhard 5/22/79	Revertants per Plate	17	23	23	23	27	24	11	
		20	19	19	24	28	18	19	
		36	21	27	22	21	10	20	
	Averaged Revertants	24	21	23	23	25	17	17	
	Standard Deviation	10	2	4	1	4	7	5	
Strain: TA1538 Date Plated: 5-15-79 No. of Cells Seeded: 1.4 x10^8 Metabolic Activation: None Hand Counted by: P.J. Burke 5-21-79 Calculations by: G. Reinhard 5/22/79	Revertants per Plate	19	13	16	16	16	6	16	
		10	22	16	15	18	10	8	
		13	22	10	14	16	15	9	
	Averaged Revertants	14	19	14	15	17	10	11	
	Standard Deviation	5	5	3	1	1	5	4	

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5/79

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SALMONELLA MUTAGENESIS ASSAY

Positive Controls

TABLE 7

019-563-165-1
Study Number

Retenone Crystalline
Code # 864200 Lot # 735-RAP-1502
Test Article Identification

Date	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per Plate	S.D.
5-15-79	TA98	2-Aminoanthracene	1.0 ug	Rat Liver	1345	1412	1682	1480	178
5-15-79	TA98	2-Nitrofluorene	10.0 ug	None	1033	1049	1117	1066	45
5-15-79	TA100	2-Aminoanthracene	1.0 ug	Rat Liver	2322	2952	2276	2517	378
5-15-79	TA100	1,3-Propane Sultone	0.04 µl	None	998	1140	1163	1100	89
5-15-79	TA1535	1,3-Propane Sultone	0.04 µl	None	1086	953	977	1005	71
5-15-79	TA1537	9-Aminoacridine	75 ug	None	811	733	621	722	96
5-15-79	TA1538	2-Nitrofluorene	10.0 ug	None	953	956	1209	1039	147

MACHINE Counted by: M Williams 5/21/79
Calculations by: H. Reichard 5/29/79

Form No. WL-106
5/79

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TABLE 8

SALMONELLA MUTAGENESIS ASSAY

Lot # 735-RNP-1502

019-563-165-1	Reference Crystalline Cycle # 364200	30.0 → 10,000.0 u.g.
Study Number	Test Article Identification	Dose Range

		Solvent 100 µl. Dmso	Concentration 30.0	100.0	333.0	1,000.0	3,333.0	10,000.0	
Strain: TA98	Revertants per Plate	18	20	33	22	21	28	8	
Date Plated: 6/5/79		20	22	18	17	21	19	15	
No. of Cells Seeded: 1.2 x10^8		31	24	16	25	32	23	14	
Metabolic Activation: Rat liver									
Hand Counted by: Smith 6/11/79	Averaged Revertants	23	22	22	21	25	23	13	
Calculations by: H. Rickard 6/12/79	Standard Deviation	7	2	9	4	6	5	5	
Strain: TA98	Revertants per Plate	22	12	17	18	26	20	19	
Date Plated: 6/5/79		20	8	24	21	22	13	14	
No. of Cells Seeded: 1.2 x10^8		14	14	17	25	16	21	14	
Metabolic Activation: None									
Hand Counted by: Smith 6/11/79	Averaged Revertants	19	11	19	21	21	18	16	
Calculations by: H. Rickard 6/12/79	Standard Deviation	4	3	4	4	5	4	3	

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TABLE 9

SALMONELLA MUTAGENESIS ASSAY

019-563-165-1 (Study Number)	Lot # 735-AAP-1562 Reference Coprisulfone Code # 864200 (Test Article Identification)	30.0 → 10,000.0 ug. (Dose Range)

			Concentration						
		Solvent 100 µl DMSO	30.0	100.0	330.0	1,000.0	3,300.0	10,000.0	
Strain: TA100 Date Plated: 6/5/79 No. of Cells Seeded: 1.3 x10^8 Metabolic Activation: Rat liver	Revertants per Plate	111	95	88	102	107	103	102	
		96	94	103	113	103	115	89	
		112	109	89	118	107	90	97	
Hand Counted by: C. Smith 6/11/79 Calculations by: H. Reichard 6/12/79	Averaged Revertants	106	99	93	111	106	103	83	
	Standard Deviation	9	8	8	8	2	13	18	
Strain: TA100 Date Plated: 6/5/79 No. of Cells Seeded: 1.3 x10^8 Metabolic Activation: None	Revertants per Plate	101	116	114	126	129	100	106	
		117	118	107	105	128	112	108	
		110	129	128	116	131	109	107	
Hand Counted by: C. Smith 6/11/79 Calculations by: H. Reichard 6/12/79	Averaged Revertants	109	121	116	116	129	107	107	
	Standard Deviation	8	7	11	11	2	6	1	

Form No. NL-105

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TABLE 10

SALMONELLA MUTAGENESIS ASSAY

LT# 735-RAF-1562

019-563-105-1	Reference Crystalline Sdx # 864200	30.0 → 10,000.0 ug.
Study Number	Test Article Identification	Dose Range

		Solvent Control DMSO	Concentration 30.0	100.0	370.0	1,000.0	3300.0	10,000.0	
Strain: TA1535 Date Plated: 6/5/79 No. of Cells Seeded: 1.7 x10^8 Metabolic Activation: Rat Liver Hand Counted by: J. Smith 6/11/79 Calculations by: G. Richard 6/12/79	Revertants per Plate	11	14	9	12	5	9	8	
		6	11	4	16	11	11	4	
		15	9	20	10	10	5	10	
	Averaged Revertants	11	11	11	13	9	8	7	
	Standard Deviation	5	3	8	3	3	3	3	
Strain: TA1535 Date Plated: 6/5/79 No. of Cells Seeded: 1.7 x10^8 Metabolic Activation: None Hand Counted by: J. Smith 6/11/79 Calculations by: G. Richard 6/12/79	Revertants per Plate	27	21	25	21	18	21	12	
		23	20	11	18	27	22	17	
		20	19	18	21	19	20	9	
	Averaged Revertants	23	20	18	20	21	21	13	
	Standard Deviation	4	1	7	2	5	1	4	

Form No. WL-105

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TABLE 11

SALMONELLA MUTAGENESIS ASSAY

019-563-165-1 (Study Number)	Lot # 735-KNR 152 Retrorone Crystalline Code # 81.42cc (Test Article Identification)	30.0 → 10,000.0 ug. (Dose Range)

			Concentration						
		Solvent 100ul DMSO	30.0	100.0	330.0	1,000.0	3,300.0	10,000.0	
Strain: TA1537 Date Plated: 1.15.79 No. of Cells Seeded: 1.5 x10^8 Metabolic Activation: Rat Liver Hand Counted by: C. Smith 1.17.79 Calculations by: H Reichard 1/12/79	Revertants per Plate	6	10	7	6	8	5	4	
		10	9	7	7	7	5	6	
		8	6	4	5	4	12	3	
	Averaged Revertants	8	8	6	6	6	7	4	
	Standard Deviation	2	2	2	1	2	4	2	
Strain: TA1537 Date Plated: 1.15.79 No. of Cells Seeded: 1.5 x10^8 Metabolic Activation: None Hand Counted by: C. Smith 1.17.79 Calculations by: H. Reichard 6/12/79	Revertants per Plate	2	4	6	7	7	5	2	
		7	8	6	8	3	5	6	
		8	4	6	5	9	5	9	
	Averaged Revertants	6	5	6	7	6	5	6	
	Standard Deviation	3	2	0	2	3	0	4	

Form No. WL-105

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SALMONELLA MUTAGENESIS ASSAY

Positive Controls

TABLE 12

019-563-165-1
Study Number

Lot# 735-RAP-1502 Code# SCY200
RETENONE CRYSTALLINE
Test Article Identification

Date	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per Plate	S.D.
6.5.79	TA92	2-Aminoanthracene	1.0 µg	Rat Liver	1574	1387	1565	1509	105
6.5.79	TA98	2-Nitrofluorene	10.0 µg	None	1163	1375	1515	1351	177
6.5.79	TA100	2-Aminoanthracene	1.0 µg	Rat Liver	1307	1115	1479	1300	182
6.5.79	TA100	1,3-Propane Sultone	0.04 µl	None	1194	1354	1163	1247	120
6.5.79	TA1535	1,3-Propane Sultone	0.04 µl	None	1370	1437	1239	1349	101
6.5.79	TA1537	9-Aminoacridine	75 µg	None	1246	1754	1630	1543	265
6.5.79	TA1538	2-Nitrofluorene	10.0 µg	None					

RETENE Counted by: [signature] 6/11/79
Calculations by: [signature] 6/12/79

Form No. ML-106
5/79

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TABLE 13

SALMONELLA MUTAGENESIS ASSAY

Lot # 735-RAP-1502

019-563-165-1	Retinoic Crystalline Code # 864200	30.0 → 10,000.0 ug.
Study Number	Test Article Identification	Dose Range

			Concentration						
		Solvent 100 µ. Dmso	30.0	100.0	270.0	1,000.0	3,000.0	10,000.0	
Strain: TA1535 Date Plated: 6/19/79 No. of Cells Seeded: 1.1 x10^8 Metabolic Activation: Rat Liver Hand Counted by: [signature] 6/20/79 Calculations by: J. Cimons 6/26/79	Revertants per Plate	21	22	21	23	16	26	20	
		25	17	27	28	25	29	18	
		21	23	32	26	20	26	9	
	Averaged Revertants	22	21	27	25	20	27	16	
	Standard Deviation	2	3	6	3	5	2	6	
Strain: TA1535 Date Plated: 6/19/79 No. of Cells Seeded: 1.1 x10^8 Metabolic Activation: None Hand Counted by: [signature] 6/20/79 Calculations by: J. Cimons 6/26/79	Revertants per Plate	18	16	17	19	20	17	14	
		13	15	8	15	14	14	17	
		15	15	13	16	15	20	13	
	Averaged Revertants	15	15	13	17	16	17	15	
	Standard Deviation	3	1	5	2	3	3	2	

Form No. WL-105

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SALMONELLA MUTAGENESIS ASSAY

Positive Controls

TABLE 14

Lt* 735-RAP-15c2

019-563-165-1 — Study Number

Reference Crystalline Code # 864200 — Test Article Identification

Date	Strain	Chemical	Concentration per plate	Metabolic Activation	Revertants/Plate			Averaged Revertants per Plate	S.D.
	TA98	2-Aminoanthracene	1.0 ug	Rat Liver					
	TA98	2-Nitrofluorene	10.0 ug	None					
6/19/79	TA100	2-Aminoanthracene	1.0 ug	Rat Liver	3678	3500	3900	3793	111
	TA100	1,3-Propane Sultone	0.04 µl	None					
	TA1535	1,3-Propane Sultone	0.04 µl	None					
	TA1537	9-Aminoacridine	75 ug	None					
6/19/79	TA1538	2-Nitrofluorene	10.0 ug	None	995	875	923	931	60

Machine Counted by: Smith 6/24/79

Calculations by: J. Cannon 6/24/79

Form No. ML-106
5/79

Conclusions

The results of the Salmonella/mammalian-microsome plate incorporation mutagenicity assays indicate that FWS test article Retenone Crystalline Code #864200 (MRI #165) did not cause a significant increase in the number of revertants per plate of any of the tester strains neither with nor without metabolic activation by Aroclor 1254 induced rat liver microsomes.

In the experiment of 6/5/79, the TA1538 culture was grossly contaminated. The strain was retested on 6/19/79.

APPENDIX

Media

Top agar is initially prepared with 8 g/liter Difco Bacto Agar and 5 g/liter NaCl. After autoclaving, the molten agar is distributed in 100 ml aliquots into sterile bottles where it is stored at room temperature. Immediately before its use in the mutagenesis assay, the top agar is melted and supplemented with 10 ml/100 ml agar of a sterile solution containing 0.5mM L-histidine and 0.5mM biotin (1XSA). Twenty-five ml of sterile deionized water is added per 100 ml top agar when it is used in assays without metabolic activation. This insures that final top agar and amino acid supplement concentrations are the same on plates with or without metabolic activation.

10XSA supplement contains 5.0mM L-histidine and 0.5mM biotin.

Bottom agar is the Vogel-Bonner minimal medium E described by Ames.

Nutrient broth used for growing overnight cultures of the tester strains contains 25g per liter of Nutrient Broth No. 2 (Oxoid).

Storage and Preparation of Tester Strains

All tester strains are stored in liquid nitrogen, and fresh cultures are inoculated directly from these frozen stocks. Broth cultures are grown overnight at 37°C with shaking. If necessary, the cultures are then centrifuged and resuspended in their culture medium to give an appropriate final cell concentration. Each culture is routinely checked for crystal violet sensitivity and ampicillin resistance before use in the mutagenesis assay.

Toxicity Determination of Test Articles

Each test article is checked for toxicity to the tester strains up to a concentration of 10 mg/plate for solids and up to 10 µl/plate in the case of liquids. Eight serial half-log dilutions of the test article are plated with TA100 on minimal agar plus 10XSA and on minimal agar plus 1XSA. Equal numbers of cells are seeded on each plate in the presence of the test article. The percent survival of an appropriately diluted TA100 culture on the 10XSA supplemented plates is determined by comparing numbers of colonies on the solvent control with those on the plates containing test article. Toxicity on the 1XSA supplemented plates is detectable by a decrease in the number of revertant colonies occurring per plate and by a thinning or disappearance of the background bacterial lawn. The highest concentration of test article used in the broad range mutagenesis assay is that which gives a detectable reduction of spontaneous revertants on the 1XSA supplemented plates. The results of the preliminary toxicity study are recorded on Form No. WL-107.

Plating Procedures for Mutagenesis Plate Incorporation Assay

Routinely, the test article is prepared immediately before its use in the mutagenesis assay. Six doses of the test article are first plated with all five tester strains (TA98, TA100, TA1535, TA1537, TA1538) with metabolic activation, after which they are immediately plated on all tester strains without metabolic activation. All positive controls, solvent controls, and test article dilutions are plated in triplicate. Without metabolic activation, 50 µl of tester strain and 50 µl of solvent or test article are added to 2.5 ml of molten top agar at 45°C. With metabolic activation, 50 µl of tester strain, 50 µl of solvent or test article, and 0.5 ml of S-9 mix are added to 2.0 of molten top agar at 45°C. To achieve the desired maximum concentration per plate of the test article it is sometimes necessary to plate 200 µl or 100 µl aliquots

instead of the usual 50 µl aliquots. Appropriate solvent controls are included in these instances. After vortexing, the mixture is poured onto the surface of 25 ml of bottom agar contained in a 15x100 mm plastic petri dish.

Positive Controls

Positive controls are run with each tester strain. All combinations of positive controls and tester strains are listed on Form No. WL-106.

Tester Strain Titers

Tester strain titers are determined by viable count assays on 10XSA supplemented minimal agar plates.. The number of cells plated per plate is reported on Form No. WL-105.

Preparation and Storage of Liver Microsomal Enzymes

Liver microsomal enzymes are routinely prepared from male Sprague-Dawley rats that have been injected with Aroclor 1254 at 500 mg/kg. The Aroclor is diluted in corn oil to a concentration of 200 mg/ml. Five days after their i.p. injection with the Aroclor, the rats are sacrificed by decapitation, and their livers are excised. The rats are denied access to food for 12 hours immediately preceding sacrifice.

The preparation of the microsomal enzyme fraction is carried out with sterile glassware and solutions at 0-4°C. The liver from each rat is excised and placed in 20 ml of 0.15M KCl contained in a pre-weighed beaker. After weighing the liver, it is transferred to another beaker containing 3 volumes of 0.15M KCl (3ml/g of wet liver) where it is minced with sterile scissors. The minced liver is homogenized in a Potter-Elvehjen apparatus with a teflon pestle. The homogenate is centrifuged at 9000 x g for 10 minutes in the SS-34 rotor of a Sorvall SS-3 centrifuge. The supernatant (referred to by Ames as the S-9 fraction) is decanted, and small aliquots are distributed into freezing ampules which are stored in liquid nitrogen.

One ml of the microsomal enzyme reaction mixture (S-9 mix) which is added to the soft agar overlay contains the following components:

S-9	0.05 ml
0.4M $MgCl_2$	0.02 ml
1.65M KCl	0.02 ml
0.04M NADP	0.10 ml
0.05M Glucose-6-phosphate	0.10 ml
1.00M NaH_2PO_4, pH 7.4	0.10 ml
H_2O	0.61 ml
	1.00 ml

Evaluation of Mutagenesis Assay Data

For a test article to be considered positive, it must cause at least a doubling in the observed revertants per plate of at least one tester strain. This increase in revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.