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TECHNICAL GLYPHOSATE:

REVERSE MUTATION ASSAY "AMES TEST"

USING *SALMONELLA TYPHIMURIUM*

AND *ESCHERICHIA COLI*

SPL PROJECT NUMBER: 434/014

SafePharm Laboratories

DERBY U.K.

SPL PROJECT NUMBER: 434/014

QUALITY ASSURANCE REPORT

The routine inspection of short term studies at Safepharm Laboratories is carried out as a continuous process designed to encompass all major phases of each study type once per month. Dates of relevant monthly inspections are given below.

Date(s) of Inspection and Reporting:

07, 20, 28 November 1995

This report has been audited by Safepharm Laboratories Quality Assurance Unit. It is considered to be an accurate account of the data generated and of the procedures followed.

Date of Report Audit:

02 January 1996

[redacted] DATE: .....

[redacted] C.Biol., M.I. Biol.

For Safepharm Quality Assurance Unit

GLP COMPLIANCE STATEMENT

I, the undersigned, hereby declare that the objectives laid down in the protocol were achieved and as nothing occurred to adversely affect the quality or integrity of the study, I consider the data generated to be valid. This report fully and accurately reflects the procedures used and data generated.

The work described was performed in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom Compliance Programme, Department of Health 1989). These Principles are in accordance with GLP standards published as OECD Environment Monograph No. 45 (OCDE/GD(92)32), and are in conformity with, and implement, the requirements of Directives 87/18/EEC and 88/320/EEC.

These international standards are acceptable to the United States Environmental Protection Agency and Food and Drug Administration, and fulfil the requirements of 40 CFR Part 160, 40 CFR Part 792 and 21 CFR Part 58 (as amended); and to the Japanese Ministry of Agriculture, Forestry and Fisheries (59 NohSan, Notification No. 3850, Agricultural Production Bureau) - confirmed by an Arrangement between the Ministry and UK Department of Health; the Japanese Ministry of Health and Welfare (Notification No. 313, Pharmaceutical Affairs Bureau - as amended, and Kanpogyo No. 39 Environmental Agency, Yakuhatsu No. 229); and the Japanese Ministry of International Trade and Industry (Chemical Substances Control Law, Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85).

.... DATE: 20 FEB 1996

Study Director
for Safepharm Laboratories

CONTENTS

SUMMARY

STUDY SPONSOR	:	MASTRA INDUSTRIES SDN. BHD.
CO-SPONSOR	:	MARUZEN KAKO CO., LTD.
STUDY TYPE	:	REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
TEST MATERIAL	:	TECHNICAL GLYPHOSATE

1. *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 and *Escherichia coli* strain WP2uvrA⁻ were treated with suspensions of the test material using the Ames plate incorporation method at five dose levels, in triplicate, both with and without the addition of a rat liver homogenate metabolising system (10% liver S9 in standard co-factors). This method conforms to the guidelines for bacterial mutagenicity testing published by the major Japanese Regulatory Authorities including MITI, MHW, MOL and MAFF. It also meets the requirements of the OECD, EC and USA, EPA (TSCA) guidelines. The dose range was determined in a preliminary toxicity assay and was 50 to 5000 μg/plate in the first experiment. The experiment was repeated on a separate day using the same dose range as experiment 1, fresh cultures of the bacterial strains and fresh test material formulations.

2. The vehicle (sterile distilled water) control plates produced counts of revertant colonies within the normal range.

3. All of the positive control chemicals used in the test produced marked increases in the frequency of revertant colonies, both with and without the metabolising system.

4. The test material caused no visible reduction in the growth of the bacterial lawn at any dose level either with or without metabolic activation, however a decrease in the frequency of revertant colonies was observed with some bacterial strains. The test material was tested up to the maximum recommended dose level of 5000 μg/plate.

5. No significant increase in the frequency of revertant colonies was recorded for any of the bacterial strains with any dose of the test material, either with or without metabolic activation. The test material was found to be non-mutagenic under the conditions of this test.

TECHNICAL GLYPHOSATE: REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

1. INTRODUCTION

This study was conducted according to Safepharm Standard Method Number JMOL 01 and was designed to assess the mutagenic potential of the test material using a bacterial/microsome test system. The study was based on the *in vitro* technique described by Ames and his co-workers (1, 2, 3) and Garner *et al* (4) in which mutagenic activity is assessed by exposing histidine auxotrophs of *Salmonella typhimurium* to various concentrations of the test material. This method conforms to the guidelines for bacterial mutagenicity testing published by the major Japanese Regulatory Authorities including MITI, MHW, MOL and MAFF. This method also conforms with the OECD Guidelines for the Testing of Chemicals, Protocol No. 471, Method B14 in EC Commission Directive 92/69/EEC and the USA, EPA (TSCA) guidelines. A copy of the Certificate of Compliance with GLP, issued by the UK Department of Health, is included as Appendix IV.

These mutant strains of *Salmonella* are incapable of synthesising histidine and are, therefore, dependent for growth on an external source of this particular amino acid. When exposed to a mutagenic agent these bacteria may undergo a reverse mutation to histidine independent forms which are detected by their ability to grow on a histidine deficient medium. Using various strains of this organism, revertants produced after exposure to a chemical mutagen may arise as a result of base-pair substitution in the genetic material (miscoding) or frame-shift mutation in which genetic material is either added or deleted. In order to make the bacteria more sensitive to mutation by chemical and physical agents, several additional traits have been introduced. These include a deletion through the excision repair gene (uvrB) which renders the organism incapable of DNA excision repair and deep rough mutation (rfa) which increases the permeability of the cell wall. In addition, a mutant strain of *Escherichia coli* (WP2uvrA$^-$), which requires tryptophan and which can be reverse mutated by base substitution to tryptophan independence, was used to complement the *Salmonella* strains. Since many compounds do not exert a mutagenic effect until they have been metabolised by enzyme systems not available

in the bacterial cell, the test material and the bacteria are also incubated in the presence of a liver microsomal fraction (S9) prepared from rats pre-treated with a compound known to induce an elevated level of these enzymes.

The study was performed between 19 August 1995 and 13 November 1995.

2. TEST MATERIAL

Sponsor's identification	:	TECHNICAL GLYPHOSATE
Chemical name	:	N-(phosphonomethyl)glycine
Lot number	:	H95D 161 A
Purity	:	95.3% w/w
Date received	:	4 August 1995
Description	:	white powder
Sponsor's description	:	white to off-white crystals
Storage conditions	:	room temperature

Data relating to the identity, purity and stability of the test material are the responsibility of the sponsor.

3. METHODS

3.1 Tester Strains

Salmonella typhimurium	TA1535, TA1537, TA98 and TA100
Escherichia coli	WP2uvrA$^-$

The *Salmonella typhimurium* strains were obtained from the University of California at Berkeley on culture discs on 4 August 1995 whilst the *Escherichia coli* strain WP2uvrA$^-$ was obtained from the British Industrial Biological Research Association on 14 August 1987. All of the strains were stored at -196°C in a Statebourne liquid nitrogen freezer, model SXR 34. Prior to the master strains being used, characterisation checks were carried out to determine the amino-acid requirement, presence of rfa, R factors, uvrB mutation and the spontaneous reversion rate.

In this assay, overnight sub-cultures of the appropriate coded stock cultures were prepared in nutrient broth and incubated at 37°C for approximately 10 hours.

3.2 Preparation of Test and Control Materials

The test material was accurately weighed and approximate half-log suspensions in sterile distilled water prepared on the day of each experiment. An allowance for purity (95%) was made prior to test material formulation. Analysis for concentration, homogeneity and stability of the test material formulations is not a requirement of the test guidelines and was, therefore, not determined.

Vehicle and positive controls were used in parallel with the test material. A solvent treatment group was used as the vehicle control and the positive control materials were as follows:

N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) 2 μg/plate for WP2uvrA$^-$, 3 μg/plate for TA100 and 5 μg/plate for TA1535

9-Aminoacridine (9AA) 80 μg/plate for TA1537

4-Nitroquinoline-1-oxide (4NQO) 0.2 μg/plate for TA98

In addition, the material 2-Aminoanthracene (2AA), which is non-mutagenic in the absence of metabolising enzymes was used in the S9 series of plates at the following concentrations:

1 μg/plate for TA100

2 μg/plate for TA1535 and TA1537

10 μg/plate for WP2uvrA$^-$

0.5 μg/plate for TA98

3.3 Microsomal Enzyme Fraction

S9 was prepared in-house on 9 August 1995 and 11 October 1995. It was prepared from the livers of male Sprague-Dawley rats weighing ~ 200g. These had each received a single i.p. injection of Aroclor 1254 at 500 mg/kg, 5 days before S9 preparation. Prior to use, all batches of S9 were checked for suitability using a recognised mutagenic compound (2AA).

The S9 was stored at -196°C in a Statebourne liquid nitrogen freezer, model SXR 34.

3.4 S9-Mix and Agar

The S9-mix was prepared at 4°C as follows:

S9	5.0 ml
1.65 M KCl/0.4 M $MgCl_2$	1.0 ml
0.1 M Glucose-6-phosphate	2.5 ml
0.1 M NADP	2.0 ml
0.2 M Sodium phosphate buffer (pH 7.4)	25.0 ml
Sterile distilled water	14.5 ml

A known aliquot (0.5 ml) of S9-mix and 2 ml of molten, trace histidine/tryptophan supplemented media were overlaid onto a sterile vogel-bonner agar plate in order to assess the sterility of the S9-mix. This procedure was repeated, in triplicate, on the day of each experiment.

Top agar was prepared using 0.6% Difco Bacto agar and 0.5% sodium chloride with 5 ml of 1.0 mM histidine/1.0 mM biotin and 1.0 mM tryptophan solution added to each 100 ml of top agar. Base agar plates were prepared using 1.2% Oxoid Agar Technical No.3 with Vogel-Bonner Medium E and 20 mg/ml D-glucose.

3.5 Test Procedure

3.5.1 Preliminary Toxicity Study

In order to select appropriate dose levels for use in the main study, a preliminary test was carried out to determine the toxicity of the test material to the tester organisms. A mixture of 0.1 ml of bacterial suspension (TA100 or WP2uvrA$^-$), 0.1 ml of test solution, 0.5 ml phosphate buffer and 2 ml of molten, trace histidine/tryptophan supplemented media was overlaid onto sterile plates of Vogel-Bonner Minimal agar (30 ml/plate). Five doses of the test material and a vehicle control (sterile distilled water) were tested in duplicate. In addition, 0.1 ml of the maximum concentration of test solution and 2 ml of molten, trace histidine/tryptophan supplemented media were overlaid onto a sterile vogel-bonner agar plate in order to

assess the sterility of the test material.After approximately 48 hours incubation at 37°C the plates were scored for revertant colonies using a colony counter and examined for a thinning of the background lawn.

3.5.2 Mutation Study - Experiment 1 (Range-finding Study)

Five concentrations of the test material were assayed in triplicate against each tester strain, using the direct plate incorporation method in accordance with the standard methods for mutagenicity tests using bacteria.

3.5.2.1 Test Material and Vehicle Controls

Known aliquots (0.1 ml) of one of the bacterial suspensions were dispensed into sets of sterile test tubes followed by 2.0 ml of molten trace histidine/tryptophan supplemented top agar at 45°C, 0.1 ml of the appropriately diluted test material or vehicle control and either 0.5 ml of the S9 liver microsome mix or 0.5 ml of pH 7.4 buffer. The contents of each test tube were mixed and equally distributed onto the surface of Vogel-Bonner agar plates (one tube per plate). This procedure was repeated, in triplicate, for each bacterial strain and for each concentration of test material with and without S9-mix.

3.5.2.2 Positive Controls

Without Activation: A known aliquot (0.1 ml) of one of the positive control solutions (ENNG, 9AA or 4NQO) was added to a test tube containing 2.0 ml of molten, trace histidine/tryptophan supplemented top agar and 0.1 ml of the appropriate bacterial suspension. Finally, 0.5 ml of pH 7.4 buffer was added to the tube, the contents mixed and poured onto an agar plate. This procedure was then repeated, in triplicate, for each tester strain.

With Activation: A known aliquot (0.1 ml) of 2AA solution was added to a test tube containing 2.0 ml of molten, trace histidine/tryptophan supplemented top agar and 0.1 ml of the appropriate bacterial suspension. Finally, 0.5 ml of S9-mix was added to the tube, the contents mixed and poured onto an agar plate. This procedure was then repeated, in triplicate, for each tester strain.

The plates were incubated at 37°C for approximately 48 hours and the number of revertant colonies counted using a colony counter.

3.5.3 Mutation Study - Experiment 2 (Main Study)

The second experiment was performed using methodology as described for experiment 1, using fresh bacterial cultures, test material and control solutions in triplicate.

3.6 Interpretation of Results

For a substance to be considered positive in this test system, it should have induced a dose-related and statistically(5) significant increase in mutation rate (of at least twice the spontaneous reversion rate) in one or more strains of bacteria in the presence and/or absence of the S9 microsomal enzymes in both experiments at sub-toxic dose levels. If the two experiments give conflicting results or equivocal results are obtained, then a third experiment may be used to confirm the correct response. To be considered negative the number of induced revertants compared to spontaneous revertants should be less than twofold at each dose level employed, the intervals of which should be between 2 and 5 fold and extend to the limits imposed by toxicity, solubility or up to the maximum recommended dose of 5000 μg/plate. In this case the limiting factor was the maximum recommended dose.

4. ARCHIVES

Unless instructed otherwise by the sponsor, all original data and a copy of the final report will be retained in the archives of Safepharm Laboratories Limited for a period of 10 years. After this period, the sponsor's instructions will be sought.

5. RESULTS

5.1 Preliminary Toxicity Study

The dose range of the test material used in the preliminary toxicity study was 0, 50, 150, 500, 1500 and 5000 µg/plate. The test material exhibited toxicity to the revertant colonies in bacterial strain TA100 and was non-toxic in bacterial strain WP2uvrA$^-$.

The mean numbers of revertant colonies for the toxicity assay were:

Strain	Dose (µg/plate)					
	0	50	150	500	1500	5000
TA100	69	65	69	76	54	12
WP2uvrA$^-$	16	11	19	16	10	17

5.2 Mutation Study

Prior to use the master strains were checked for characteristics, viability and spontaneous reversion rate and were all found to be satisfactory.

Results for the negative controls (spontaneous mutation rates) are presented in Table 1, Appendix I.

The individual plate counts, the mean number of revertant colonies and the standard deviations for the test material, vehicle and positive controls both with and without metabolic activation, are presented in Appendix I with the results also expressed graphically in Appendix III.

Information regarding the equipment and methods used in these experiments and the details of the personnel involved, as required by the Japanese Ministry of Labour, Japanese Ministry of International Trade and Industry and Japanese Ministry of Health and Welfare are presented as Appendix II.

The test material caused no visible reduction in the growth of the bacterial lawn at any dose level either with or without metabolic activation, however a decrease in the frequency of revertant colonies was observed with some bacterial strains.

No significant increase in the frequency of revertant colonies was recorded for any of the bacterial strains with any dose of the test material either with or without metabolic activation.

All of the positive control chemicals used in the test produced marked increases in the frequency of revertant colonies and the activity of the S9 fraction was found to be satisfactory.

6. CONCLUSION

The test material was found to be non-mutagenic under the conditions of this test.

7. REFERENCES

1. Ames, B.N., Durston, W.E., Yamasaki, E., and Lee, F.D. Proc. Nat. Acad. Sci. USA (1970), 70, 2285.

2. Ames, B.N., McCann, J. and Yamasaki, E., Mutation Research (1975), 31, 347.

3. McCann, J., Coi, E., Yamasaki, E., and Ames, B.N. Proc. Nat. Acad. Sci. USA (1975) 75, 5135.

4. Garner, R.C., Miller, E.C., and Miller, J.A. Cancer Res. (1972), 33, 2058.

5. Kirkland, D.J., (Ed). Statistical Evaluation of Mutagenicity Test Data. UKEMS Sub-committee on Guidelines for Mutagenicity Testing. Report - P.. III (1989) - Cambridge University Press.

APPENDICES

REVERSE MUTATION ASSAY "AMES TEST" REPORT

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

APPENDIX I

KEY TO TABLES OF TEST RESULTS

NOTES:

1. When bacterial growth inhibition is found, the applicable value is marked with an asterisk.

2. The average number of colonies for each concentration is recorded in parentheses.

3. Figures immediately below the average values refer to standard deviation.

4. "Number of revertants" - The observed values and average value are shown in order, beginning with the lowest concentration of the test substance.

5. The following postfixes are used when required:

   C = contaminated
   P = precipitate
   X = plate unscorable

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX I (continued)
TABLES OF TEST RESULTS

TABLE 1

SPONTANEOUS MUTATION RATES

RANGE-FINDING STUDY

Number of Revertants (Number of Colonies per plate)									
Base-pair Substitution Type						Frameshift Type			
TA100		TA1535		WP2uvrA⁻		TA98		TA1537	
91		7		12		12		6	
62	(74)	7	(8)	11	(11)	10	(11)	7	(6)
68		9		11		12		4	

MAIN STUDY

Number of Revertants (Number of Colonies per plate)									
Base-pair Substitution Type						Frameshift Type			
TA100		TA1535		WP2uvrA⁻		TA98		TA1537	
108		18		19		28		16	
121	(123)	12	(16)	20	(20)	22	(26)	5	(11)
141		17		20		29		12	

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

APPENDIX I (continued)

TABLES OF TEST RESULTS

TABLE 2

EXPERIMENT 1 - WITHOUT METABOLIC ACTIVATION

With or Without S9-Mix	Test Substance concentration (µg/plate)	Number of revertants (Number of colonies per plate)									
		Base-pair substitution type						Frameshift type			
		TA 100		TA 1535		WP2uvrA-		TA 98		TA 1537	
-	0	117 140 127	(128) 11.5	15 13 10	(13) 2.5	20 11 24	(18) 6.7	26 15 18	(20) 5.7	9 9 5	(8) 2.3
-	50	143 115 113	(124) 16.8	15 16 20	(17) 2.6	15 13 10	(13) 2.5	16 32 27	(25) 8.2	10 16 6	(11) 5.0
-	150	119 115 84	(106) 19.2	10 10 18	(13) 4.6	19 24 18	(20) 3.2	19 13 20	(17) 3.8	9 3 7	(6) 3.1
-	500	118 119 125	(121) 3.8	14 11 19	(15) 4.0	16 19 14	(16) 2.5	22 20 16	(19) 3.1	11 10 14	(12) 2.1
-	1500	97 101 121	(106) 12.9	19 15 6	(13) 6.7	22 23 11	(19) 6.7	32 14 16	(21) 9.9	13 7 9	(10) 3.1
-	5000	101 94 132	(109) 20.2	13 15 11	(13) 2.0	26 18 23	(22) 4.0	19 26 7	(17) 9.6	8 12 7	(9) 2.6
Positive controls	Name	ENNG		ENNG		ENNG		4NQO		9AA	
S9-Mix	Concentration (µg/plate)	3		5		2		0.2		80	
-	No. colonies per plate	313 306 301	(307) 6.0	755 672 335	(587) 222.4	1961 1817 1999	(1926) 96.0	176 174 143	(164) 18.5	170 341 278	(263) 86.5

ENNG N-ethyl-N'-nitro-N-nitrosoguanidine

4NQO 4-nitroquinoline-1-oxide

9AA 9-aminoacridine

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX I (continued)
TABLES OF TEST RESULTS

TABLE 3

EXPERIMENT 1 - WITH METABOLIC ACTIVATION

With or Without S9-Mix	Test Substance concentration (µg/plate)	Number of revertants (Number of colonies per plate)									
		Base-pair substitution type						Frameshift type			
		TA 100			TA 1535			WP2uvrA		TA 98			TA 1537		
+	0	109	(116)		9	(12)		14	(18)	27	(36)		11	(8)	
		125		8.2	10		3.8	15		48		10.8	10		4.4
		114			16			26	6.7	33			3		
+	50	94	(105)		10	(10)		14	(15)	29	(32)		10	(9)	
		119		12.9	12		2.0	12		35		3.1	5		3.6
		101			8			18	3.1	33			12		
+	150	117	(101)		9	(10)		14	(18)	32	(29)		14	(12)	
		86		15.6	7		4.2	18		28		2.6	9		2.9
		99			15			22	4.0	27			14		
+	500	105	(118)		11	(11)		15	(18)	19	(29)		10	(11)	
		117		13.5	12		1.0	19		34		9.0	7		4.0
		132			10			21	3.1	35			15		
+	1500	97	(93)		8	(11)		17	(20)	27	(36)		7	(10)	
		100		10.2	14		3.1	19		40		8.1	11		2.6
		81			10			23	3.1	42			12		
+	5000	122	(116)		10	(10)		28	(22)	27	(36)		12	(11)	
		131		18.7	10		0.6	17		38		8.6	8		2.3
		95			11			22	5.5	44			12		
Positive controls	Name	2AA			2AA			2AA		2AA			2AA		
S9-Mix	Concentration (µg/plate)	1			2			10		0.5			2		
+	No. colonies per plate	388	(406)		107	(110)		528	(616)	873	(981)		127	(148)	
		482		69.2	111		2.3	741		1043		93.9	146		21.5
		347			111			579	111.2	1027			170		

2AA 2-aminoanthracene

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

APPENDIX I (continued)
TABLES OF TEST RESULTS

TABLE 4
EXPERIMENT 2 - WITHOUT METABOLIC ACTIVATION

With or Without S9-Mix	Test Substance concentration (μg/plate)	Number of revertants (Number of colonies per plate)				
		Base-pair substitution type			Frameshift type	
		TA 100	TA 1535	WP2uvrA-	TA 98	TA 1537
-	0	163 144 162 (156) 10.7	33 46 35 (38) 7.0	21 28 37 (29) 8.0	27 33 37 (32) 5.0	17 16 15 (16) 1.0
-	50	164 167 166 (166) 1.5	41 43 41 (42) 1.2	24 24 27 (25) 1.7	30 41 32 (34) 5.9	17 12 13 (14) 2.6
-	150	165 167 162 (165) 2.5	30 37 30 (32) 4.0	17 22 16 (18) 3.2	31 37 24 (31) 6.5	11 16 7 (11) 4.5
-	500	151 154 152 (152) 1.5	36 26 36 (33) 5.8	85 32 13 (43) 37.3	20 33 31 (28) 7.0	9 16 6 (10) 5.1
-	1500	154 143 145 (147) 5.9	20 26 34 (27) 7.0	11 14 14 (13) 1.7	28 42 28 (33) 8.1	10 10 16 (12) 3.5
-	5000	68 87 78 (78) 9.5	30 30 29 (30) 0.6	16 21 21 (19) 2.9	5 18 17 (13) 7.2	6 8 9 (8) 1.5
Positive controls	Name	ENNG	ENNG	ENNG	4NQO	9AA
S9-Mix	Concentration (μg/plate)	3	5	2	0.2	80
-	No. colonies per plate	722 693 761 (725) 34.1	493 466 487 (482) 14.2	995 974 1003 (991) 15.0	145 164 241 (183) 50.8	846 738 1011 (865) 137.5

ENNG N-Ethyl-N'-nitro-N-nitrosoguanidine

4NQO 4-nitroquinoline-1-oxide

9AA 9-Aminoacridine

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

APPENDIX I (continued)

TABLES OF TEST RESULTS

TABLE 5

EXPERIMENT 2 - WITH METABOLIC ACTIVATION

With or Without S9-Mix	Test Substance concentration (μg/plate)	Number of revertants (Number of colonies per plate)				
		Base-pair substitution type			Frameshift type	
		TA 100	TA 1535	WP2uvrA-	TA 98	TA 1537
+	0	132 166 150 (149) 17.0	19 21 11 (17) 5.3	31 25 26 (27) 3.2	27 49 46 (41) 11.9	11 9 14 (11) 2.5
+	50	153 127 145 (142) 13.3	18 10 10 (13) 4.6	24 28 23 (25) 2.6	38 25 29 (31) 6.7	7 8 6 (7) 1.0
+	150	146 146 131 (141) 8.7	9 17 12 (13) 4.0	24 24 28 (25) 2.3	34 36 28 (33) 4.2	12 8 19 (13) 5.6
+	500	133 127 104 (121) 15.3	14 10 16 (13) 3.1	28 22 18 (23) 5.0	35 31 40 (35) 4.5	9 10 13 (11) 2.1
+	1500	108 105 115 (109) 5.1	20 13 10 (14) 5.1	25 21 21 (22) 2.3	30 29 25 (28) 2.6	13 10 10 (11) 1.7
+	5000	80 121 121 (107) 23.7	7 8 10 (8) 1.5	14 16 15 (15) 1.0	33 25 39 (32) 7.0	4 7 11 (7) 3.5
Positive controls	Name	2AA	2AA	2AA	2AA	2AA
S9-Mix	Concentration (μg/plate)	1	2	10	0.5	2
+	No. colonies per plate	594 608 554 (585) 28.0	109 112 110 (110) 1.5	273 226 244 (248) 23.7	269 256 337 (287) 43.5	189 239 237 (222) 28.3

2AA 2-Aminoanthracene

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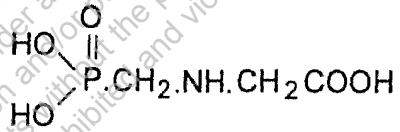
TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II

REPORT OF RESULTS IN MUTAGENICITY TEST USING MICRO-ORGANISMS (INDUSTRIAL SAFETY AND HEALTH LAW)

1. GENERAL ITEMS (to be completed by the sponsor)

Name of the new chemical substance (IUPAC nomenclature)	N-(phosphonomethyl)glycine			
Other name	TECHNICAL GLYPHOSATE			
Structural formula or rational formula (or outline of manufacturing method, in case both are unknown)				
Purity of the new chemical substance tested	95%	Lot no. of the new chemical substance tested		
Name and concentration of impurities				
CAS No.		Vapour pressure	1.94×10^{-7} mm Hg at 45°C	
Molecular weight		Partition coefficient		
Melting point	230°C	Appearance at ordinary temperature	White to off-white crystals	
Boiling point				
Stability	Stable for >2 years under normal storage conditions			
Degree of solubility in solvent	Solvent	Degree of solubility	Solvent	Degree of solubility
	Water	Doseable suspension at 50 mg/ml	D M S O	
	Acetone		Others ( )	

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

2. TESTER STRAINS

(1) Procurement

Test strain	Obtained from	Date obtained	Strain check date for coded stock cultures used for this study
TA100	UCB**	4 August 1995	05.08.95 (range-finding study) 17.10.95 (main study)
TA1535	UCB**	4 August 1995	05.08.95 (range-finding study) 17.10.95 (main study)
WP2uvrA	BIBRA*	14 August 1987	05.08.95 (range-finding study) 17.10.95 (main study)
TA98	UCB**	4 August 1995	05.08.95 (range-finding study) 17.10.95 (main study)
TA1537	UCB**	4 August 1995	05.08.95 (range-finding study) 17.10.95 (main study)

*BIBRA: British Industrial Biological Research Association
**UCB: University of California at Berkeley

(2) Storage

(Encircle the applicable number and fill in the relevant entries)

Storage method	(1.) Freezing in small aliquot 2. Freezing in large aliquot 3. Others ( )	
Storage temperature	-196°C	
Composition	Bacterial suspension: 8.0 ml	DMSO: 0.7 ml
	Others ( ):	

3. S9-MIX

(1) Source of S9

(Encircle the applicable number and fill in the relevant entries)

Made in-house or purchase	(1.) Made in-house	2. Purchase (Supplier BIBRA*)
Prepared on	09.08.95 (range-finding study) and 11.10.95 (main study)	
Lot No. (in case of purchase)		
Storage temperature	-196°C	

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

3. S9-MIX (continued)

(2) Preparation of S9

Animal used		Inducing substance	
Species strain	Rat Sprague-Dawley	Name	AROCLOR 1254
Sex	Male	Administration method	single i.p. injection
Age (in weeks)	7 weeks	Administration period and amount (g/kg body weight)	5 days 0.5 g/kg
Weight	~ 200g		

(3) Composition of S9-Mix

Constituents	Amount in 1 ml S9-Mix	Constituents	Amount in 1 ml S9-Mix
S9	0.1 ml	NADPH	μmol
$MgCl_2$	8.0 μmol	NADH	μmol
KCl	33.0 μmol	Na-phosphate buffer	100.0 μmol
Glucose-6-phosphate	5.0 μmol	Others (NADP)	4.0 μmol
Glucose-6-phosphate dehydrogenase			

4. POSITIVE CONTROLS AND SOLVENT FOR POSITIVE CONTROLS

(Encircle the applicable number and fill in the relevant entries)

Substance name		Supplier	Lot No.	Grade	Purity (%)	Solvent
Positive control	ENNG	SIGMA	67F-3700	Technical	97	DMSO
	4NQO	SIGMA	33H 2517	Technical	99	DMSO
	9AA	SIGMA	96F-05641	Technical	98	DMSO
	2AA	SIGMA	121H3475	Technical	97.5	DMSO
Solvent	DMSO	SIGMA	103H0433		99.5	SIGMA
Preparation and storage of positive control		1. Freshly prepared /		(2.) Storage in small aliquot (Storage temperature -20°C)		
		3. Others ( )				

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

5. PREPARATION OF THE TEST MATERIAL IN SOLUTION

(Encircle the applicable response regarding purity conversion)

	Name	Supplier	Lot No.	Grade	Purity (%)
Solvent used	Sterile distilled water	Steripak	401038 401019		
Stability of test substance in solvent					
Reason for selection of solvent	Doseable suspension at 50 mg/ml				
Suspension and other methods when test substance difficult to dissolve					
Storage time and temperature of solution from preparation until use	1½ hrs room temperature (range-finding study) 25 mins room temperature (main study)				
Conversion of purity	(Yes) No				

6. CONDITIONS ETC. OF PRE-CULTURE

(1) Conditions

Nutrient Broth	Name	Manufacturer	Lot No.
	Oxoid	Oxoid Ltd.	130 54491 4/99
Period of pre-culture	10 hr		
Storage time and temperature of cultures from inoculation until shaking	Approx 6½ hours at room temperature		
Storage time and temperature of cultures from shaking to harvest	Up to 10 hours at room temperature		
Shaking method (shaking type and frequency)	Orbital shaker 130 revs/min, 37°C		
Culture flask (form, size)	Plastic universal container, 50 ml		
Amount of culture medium	5 ml	Amount of strain inoculated	20 µl

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

6. CONDITIONS ETC. OF PRE-CULTURE (continued)

(2) Cell viability at the end of pre-culture

		Base-pair substitution type				Frameshift type		
		TA100	TA1535	WP2uvrA		TA98	TA1537	
Cell number ($\times 10^9$/ml)	Range-finding study	9.0	7.6	2.4		3.8	6.3	
	Main study	6.2	6.2	5.3		4.9	4.0	
Count method (encircle the applicable number)		1. Conversion from O.D. value (2.) Stepwise dilution method 3. Others ( )						

7. AGAR PLATE MEDIUM

(1) Top Agar

Top Agar	Name	Difco-Bacto agar
	Manufacturer	Difco
	Lot No.	71889 AJB 5/00 and 55284 AJA 8/99

(2) Minimum Glucose Agar Plate Medium

(Encircle the applicable number and fill in the relevant entries)

Made in-house or purchase	(1.) Made in-house 2. Purchase (supplier)
Prepared on	15.08.95 (range-finding study) 24.10.95 (main study)
Lot No. in case purchase	
Name of supplier and lot no. of the used agar	Oxoid Ltd. Oxoid Technical No.3, Lot Nos. B061 90268 1/00 (range-finding study) 214 94981 7/00 (main study)

8. STERILITY TEST (Encircle the applicable response in the right hand column)

	Bacterial growth other than expected	
Test substance solution	Yes	(No)
S9-Mix	Yes	(No)

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

9. TEST METHOD

		Pre-incubation	Plate Method
Composition	Bacterial suspension	ml	0.1 ml
	Test substance solution	ml	0.1 ml
	Na-phosphate buffer	ml	0.5 ml
	S9-mix (in case of metabolic activation method)	ml	0.5 ml
	Top agar solution	ml	2.0 ml
	Others ( )		
Pre-incubation	Temperature	°C	
	Time	min	
Incubation	Temperature	°C	37°C
	Time	hours	48 hours

10. COUNT METHOD OF COLONIES (Encircle 1 and 2 if both methods are used)

Count method	1. By hand (2.) Colony counter
Reason why both methods were used	
Name, type and manufacture of colony counter	SEESCAN COLONY METER
Correction method	1. No correction 2. Area-correction 3. Miscount-correction (4.) Area and miscount-correction

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

11. TEST RESULTS

(1) The results should be reported on the attached form

(2) Judgement of the results

Judgement (encircle one)	Positive	(Negative)

Reason for judgement and referential matters: No significant increase in the frequency of revertant colonies was recorded for any bacterial strain used with any dose of the test material in two separate experiments either with or without metabolic activation. A statistical analysis of the data was not required to determine the result of the test. An allowance for purity (95%) was made prior to test material formulation. A reduction in the frequency of revertant colonies was observed with some bacterial strains.

NADPH was not added to the S9 co-factors, however, NADPH is generated in-situ by the addition of the NADP and excess G-6-P and this is considered to be equivalent to direct addition of NADPH. Aroclor 1254 was used to induce higher enzyme levels in the rat liver S9. Aroclor 1254 is considered to be equally effective as the use of the equivalent combination of phenobarbital,5,6-benzoflavone and methylcholanthrene.

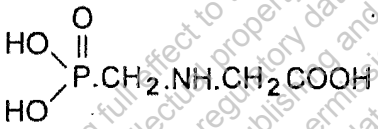
12. OTHERS

	Name	Safepharm Laboratories Ltd.		
	Address	P.O. Box 45, Derby, United Kingdom		
Administrator	Title	Head of Genetic Toxicology	Name	[redacted]
Archives Director	Title	Head of Quality Assurance	Name	
Study Director	Title	Study Director - Genetic Toxicology	Name	
	Years of experience: 8			
Personnel engaged in study	Title	Genetic Toxicologist	Name	
	Years of experience: 7			
	Title	Leading Technican - Genetic Toxicology	Name	
	Years of experience: 1			
Test dates	From: 19.08.95		To: 13.11.95	
	Protocol authorised: 15.05.95		Final report authorised: 20. FEB. 1996	
Study number	434/014			

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

REPORT OF RESULTS OF REVERSE-MUTATION ASSAY IN BACTERIA

[1] GENERAL ITEMS

<table>
<tr><td>Name of the new chemical substance (IUPAC nomenclature)</td><td colspan="4">N-(phosphonomethyl)glycine</td></tr>
<tr><td>Other name</td><td>TECHNICAL GLYPHOSATE</td><td colspan="2">Molecular weight</td><td></td></tr>
<tr><td rowspan="5">Structural formula or rational formula (or outline of manufacturing method, in case both are unknown)</td><td rowspan="5">  </td><td colspan="2">Appearance at ordinary temperature</td><td>white to off-white crystals</td></tr>
<tr><td colspan="2">Stability</td><td>Stable for >2 years under normal storage conditions</td></tr>
<tr><td colspan="2">Melting point</td><td>230°C</td></tr>
<tr><td colspan="2">Boiling point</td><td></td></tr>
<tr><td colspan="2">Vapour pressure</td><td>1.94×10^{-7} mmHg at 45°C</td></tr>
<tr><td rowspan="2">Purity of the new chemical substance tested</td><td rowspan="2">95%</td><td rowspan="5">Physicochemical properties of the new chemical substance</td><td colspan="2">Partition coefficient</td><td></td></tr>
<tr><td colspan="2">Solubility</td><td></td></tr>
<tr><td rowspan="4">Name and concentration of impurities</td><td rowspan="4"></td><td rowspan="4">Degree of solubility</td><td>Water</td><td>Doseable suspension at 50 mg/ml</td></tr>
<tr><td>DMSO</td><td></td></tr>
<tr><td>Acetone</td><td></td></tr>
<tr><td>Others ( )</td><td></td></tr>
</table>

1. **"STABILITY"** - Fill in the stability for water, other solvents, heat, light, etc.
2. **"VAPOUR PRESSURE"** - Fill in the vapour pressure of the test substance at 25°C
3. **"PARTITION COEFFICIENT"** - Fill in the value, the temperature used and the name of solvent used for the measurement.
4. **"SOLUBILITY"** - Fill in such information as water soluble, soluble in oil.
5. **"DEGREE OF SOLUBILITY"** - Fill in the solubility at 25°C for each solvent.

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

APPENDIX II (continued)

[2] TESTER STRAINS

(1) Procurement

Obtained from	Date obtained
British Industrial Biological Research Association (*E. coli* strain WP2uvrA⁻)	14 August 1987
University of California at Berkeley (*Salmonella* strains)	4 August 1995

(2) Storage

Storage temperature	Composition	
-196°C	Bacterial suspension	8.0 ml
	DMSO	0.7 ml
	Others ( )	ml

[3] S9-MIX

(1) Source of S9 (Encircle the applicable number and fill in the entries)

(1.) Made-in house	Prepared on 09.08.95 and 11.10.95	(date)
2. Purchase	Supplier	
	Prepared on	(date)
	Purchased on	(date)
	Lot number	

(2) Storage Temperature, etc. of S9

Storage Temperature	-196°C	Name and model of storage apparatus	Statebourne SXR 34

(3) Preparation of S9 (If purchased material, fill in spaces to extent possible)

Animal used		Inducing substance	
Species, strain	Rat, Sprague-Dawley	Name	Aroclor 1254
Sex	Male	Administration method	Single i.p. injection
Age (in weeks)	7 weeks	Administration period and amount (g/kg weight)	5 days 0.5 g/kg
Weight	~ 200g		

(4) Composition of S9-Mix

Constituents	Amount in 1 ml S9-Mix	Constituents	Amount in 1 ml S9-Mix
S9	0.1 ml	NADPH	μmol
$MgCl_2$	8.0 μmol	NADP	4.0 μmol
Glucose-6-phosphate dehydrogenase		Na-phosphate buffer pH 7.4	100.0 μmol
KCl	33.0 μmol		
Glucose-6-phosphate	5.0 μmol	Others ( )	-

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST"
USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

[4] PREPARATION OF THE SOLUTION OF THE TEST SUBSTANCE

Solvent used	Method of suspension etc. when test substance is difficult to dissolve
Sterile distilled water	

[5] CONDITIONS OF PRE-CULTURE

Name of nutrient broth and manufacturer	Oxoid Ltd., UK. - Nutrient Broth
Period of pre-culture	10 hours

[6] MINIMUM GLUCOSE AGAR PLATE MEDIUM (Encircle the applicable number and fill in the relevant entries)

(1.) Made in-house	Agar	Name	Oxoid Technical No. 3
		Manufacturer	Oxoid Ltd., U.K.
		Lot Numbers	B061 90268 1/00 (range-finding study) 214 94981 7/00 (main study)
	Volume of agar plate medium		30 ml
2. Purchase	Manufacturer		
	Prepared on		
	Purchased on		
	Lot number		

[7] STERILITY TEST (Encircle the applicable response in the right hand column)

	Bacterial growth other than those used for test	
Test substance solution	Yes	(No)
S9-Mix	Yes	(No)

[8] TEST METHOD

(1) Test Method (Encircle the applicable number)

1. Pre-incubation method

(2.) Plate method

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*
APPENDIX II (continued)

[8] TEST METHOD (continued)

(2) Test Condition

		Pre-incubation method	Plate method
Composition	Bacterial suspension	ml	0.1 ml
	Test substance solution	ml	0.1 ml
	Na-phosphate buffer	ml	0.5 ml
	S9-Mix (in case of metabolic activation method)	ml	0.5 ml
	Top agar solution	ml	2.0 ml
	Others ( )		-
Pre-incubation	Temperature	°C	
	Time	min.	
Incubation	Temperature	°C	37°C
	Time	hours	48 hours

[9] TEST RESULTS

(1) Test results should be reported on the attached form

(2) Judgement of the results

Judgement (Encircle one)	Positive	(Negative)

Reason for judgement: No significant increase in the frequency of revertant colonies was recorded for any bacterial strain used with any dose of the test material in two separate experiments either with or without metabolic activation.

NADPH was not added to the S9 co-factors, however, NADPH is generated in-situ by the addition of NADP and excess G-6-P and this is considered to be equivalent to direct addition of NADPH. Aroclor 1254 was used to induce higher enzyme levels in the rat liver S9. Aroclor 1254 is considered to be equally effective as the use of the equivalent combination of phenobarbital,5,6-benzoflavone and methylcholanthrene.

(3) Referential matters

An allowance for purity (95%) was made prior to test material formulation. A reduction in the frequency of revertant colonies was observed with some bacterial strains.

A statistical analysis of the data was not required to determine the result of the test.

[REMARK] **"Referential matters"** - (fill in the view etc. of the Study Director on the test results)

[10] OTHERS

Testing Institution	Name	Safepharm Laboratories Ltd.	
	Address	P.O. Box 45, DERBY, UK	Telephon
Study Director	Name		Signature
Test Dates	From: 19.08.95	To: 13.11.95	
	Protocol authorised: 15.05.95	Final report authorised:	20. FEB. 1996

TECHNICAL GLYPHOSATE : REVERSE MUTATION ASSAY "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

APPENDIX II (continued)

TABLE OF TEST RESULTS (RELATIVE ACTIVITY)

TEST SUBSTANCE: TECHNICAL GLYPHOSATE

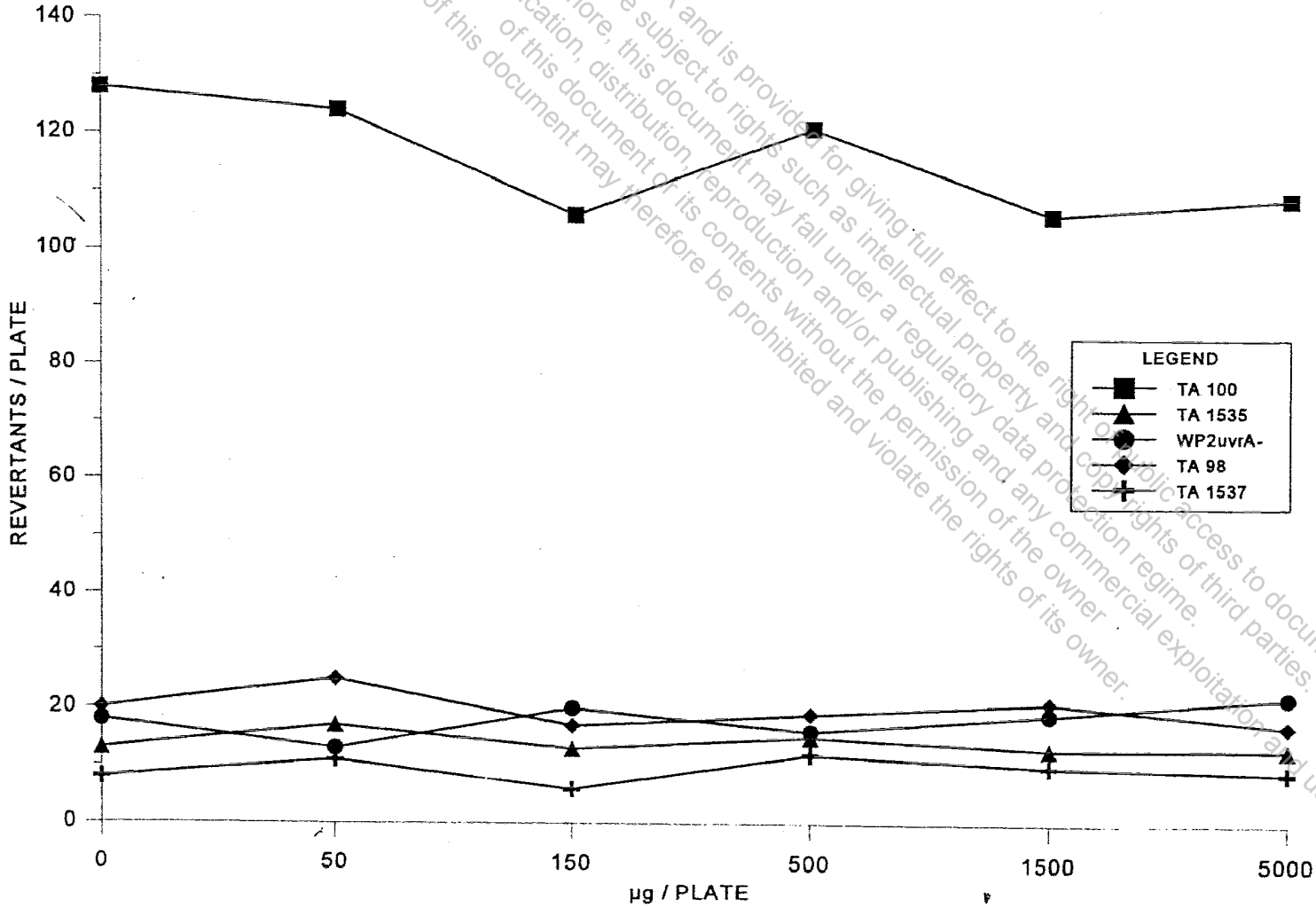
		- S9-Mix		+ S9-Mix	
		Relative activity	Dose Levels used to calculate	Relative activity	Dose levels used to calculate
Range-finding Study	TA100	N/A		N/A	
	TA1535	N/A		N/A	
	WP2uvrA⁻	N/A		N/A	
	TA98	N/A		N/A	
	TA1537	N/A		N/A	
Main Study	TA100	N/A		N/A	
	TA1535	N/A		N/A	
	WP2uvrA⁻	N/A		N/A	
	TA98	N/A		N/A	
	TA1537	N/A		N/A	

N/A = not applicable as no evidence of mutagenic activity was observed

TECHNICAL GLYPHOSATE : REVERSE MUTATION "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND ESCHERICHIA COLI

APPENDIX III

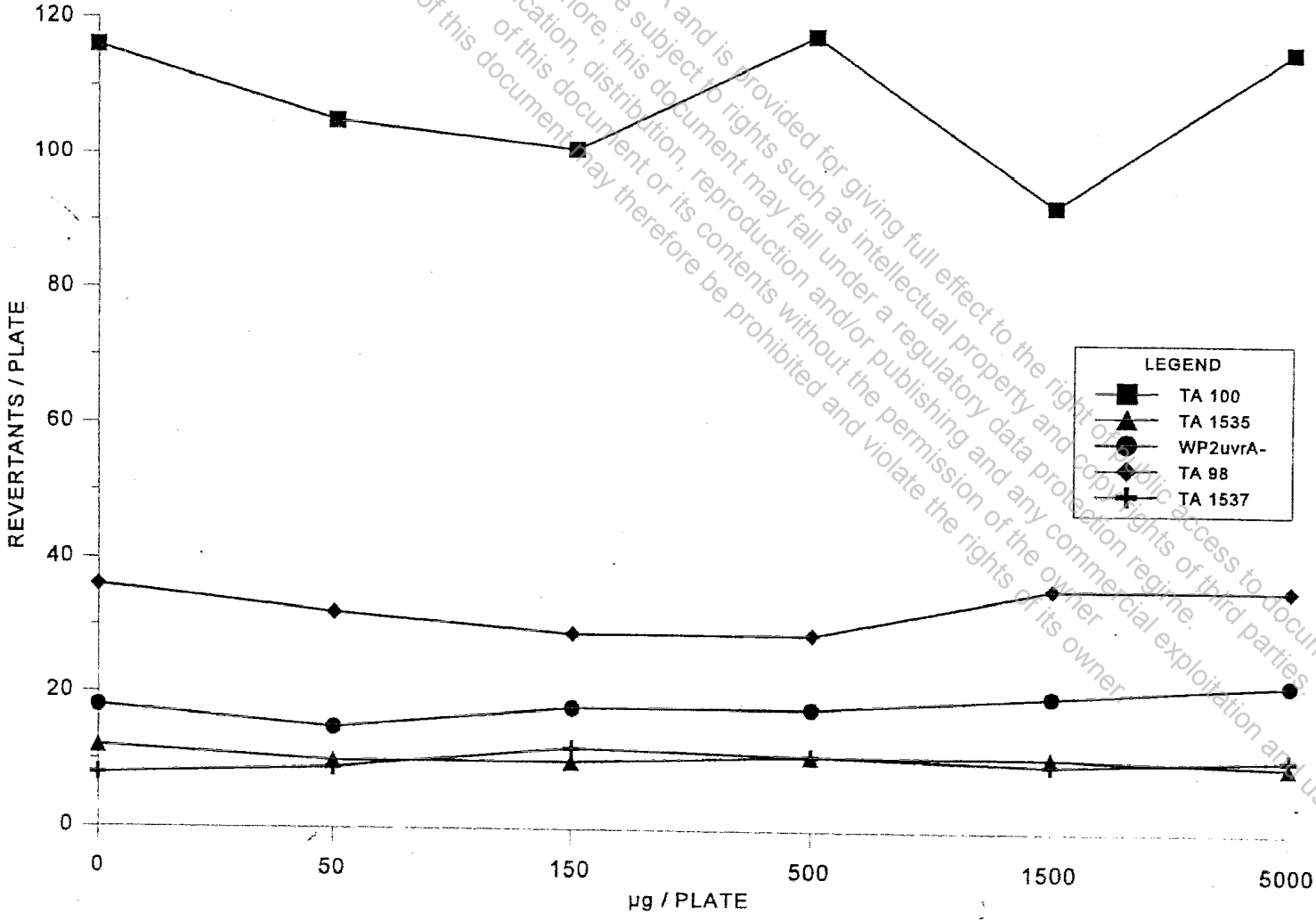
DOSE RESPONSE CURVE: EXPERIMENT 1 - WITHOUT METABOLIC ACTIVATION



TECHNICAL GLYPHOSATE : REVERSE MUTATION "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND ESCHERICHIA COLI

APPENDIX III (continued)

DOSE RESPONSE CURVE: EXPERIMENT 1 - WITH METABOLIC ACTIVATION

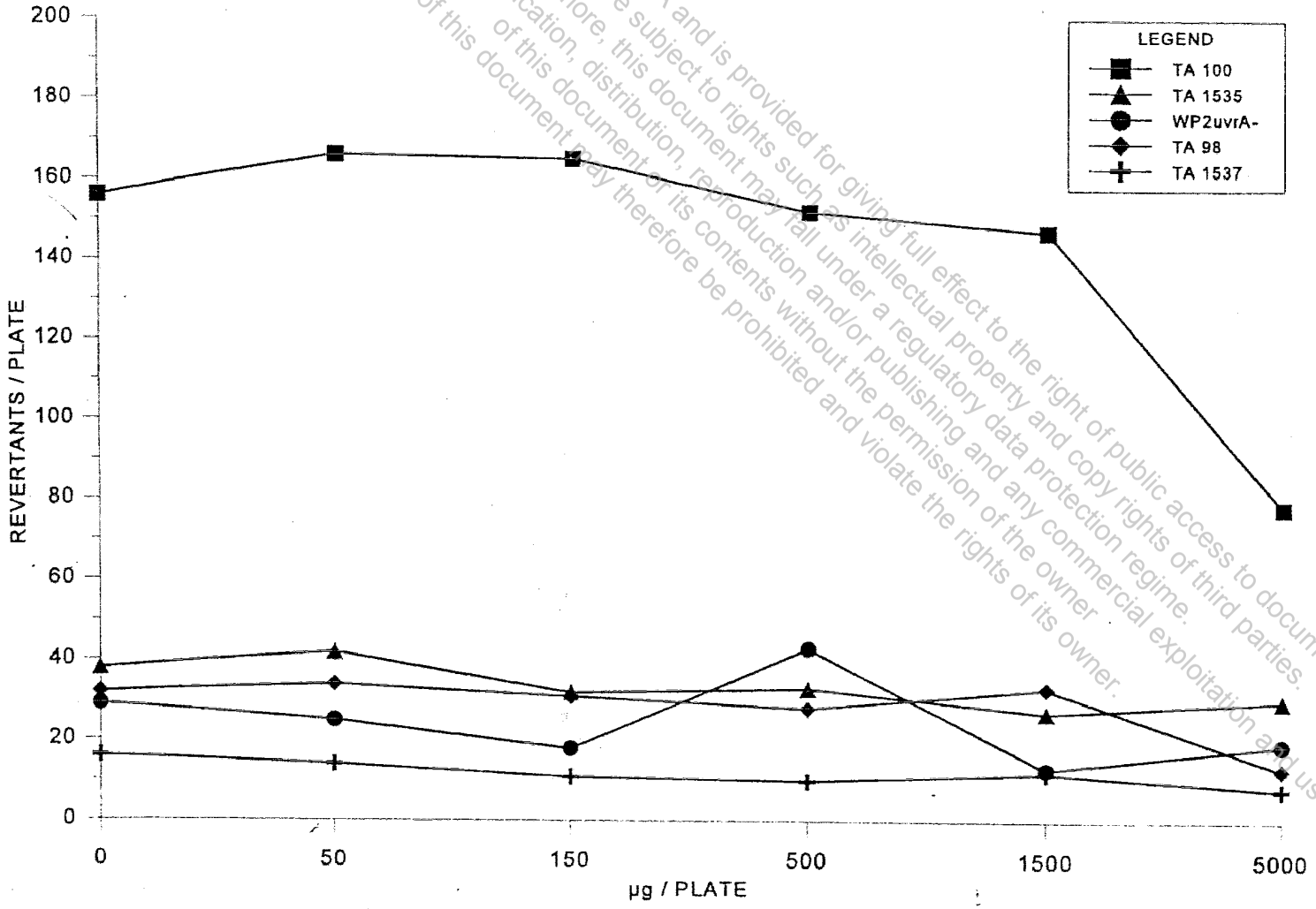


SPL PROJECT NUMBER: 434/014

TECHNICAL GLYPHOSATE : REVERSE MUTATION "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND ESCHERICHIA COLI

APPENDIX III (continued)

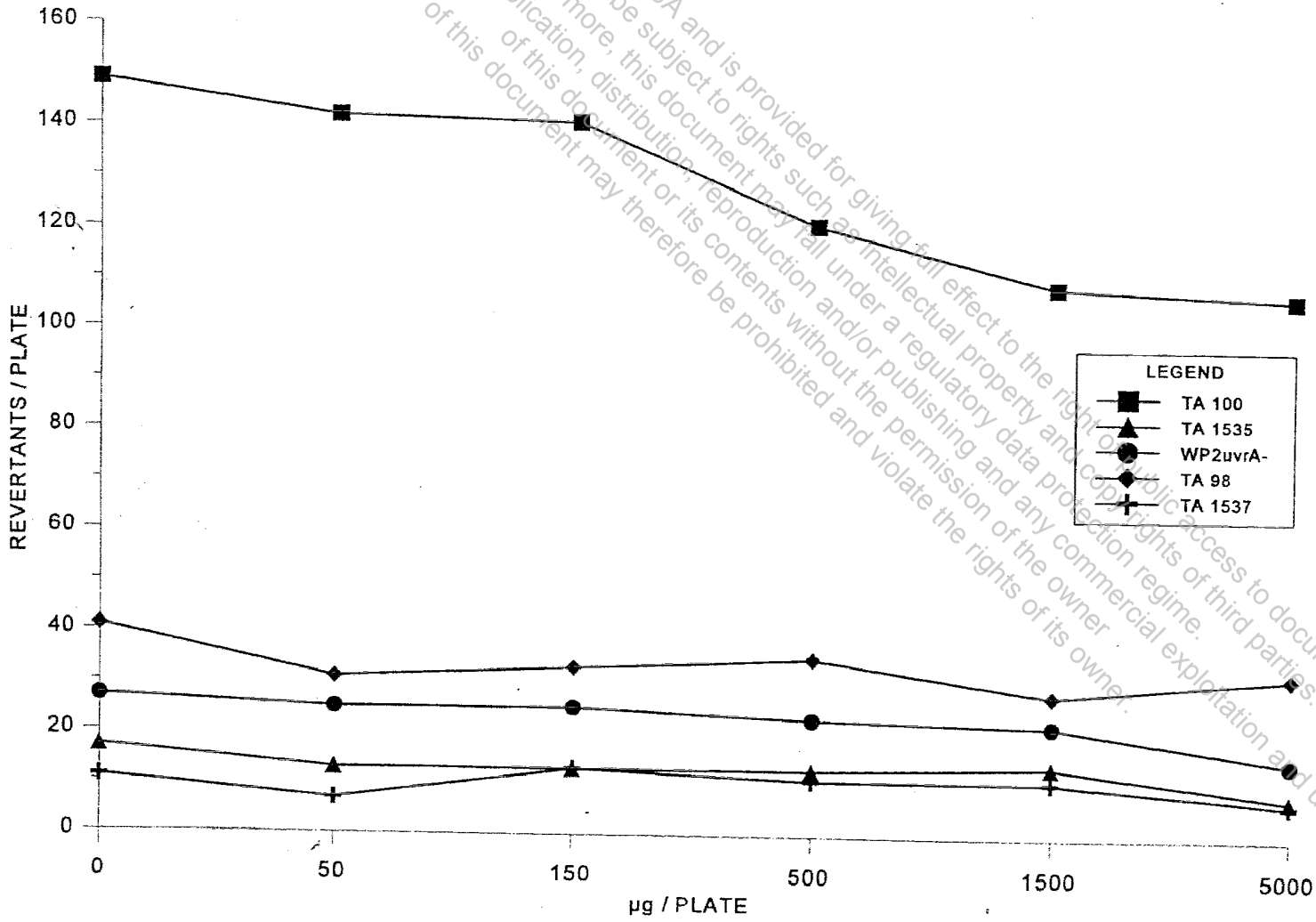
DOSE RESPONSE CURVE: EXPERIMENT 2 - WITHOUT METABOLIC ACTIVATION



TECHNICAL GLYPHOSATE : REVERSE MUTATION "AMES TEST" USING *SALMONELLA TYPHIMURIUM* AND ESCHERICHIA COLI

APPENDIX III (continued)

DOSE RESPONSE CURVE: EXPERIMENT 2 - WITH METABOLIC ACTIVATION



SPL PROJECT NUMBER: 434/014

APPENDIX IV

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE

IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY

SafePharm Laboratories Limited
P O Box No 45
Derby
DE1 2BT

DATE OF INSPECTION

31 January 1994

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of studies performed at these facilities.

[redacted]

16/3/94. [redacted]

Director
UK GLP Monitoring Unit