scantox

TEST REPORT

STUDY TITLE:	Mutagenicity test: Ames Salmonella Assay with Glyphosate, batch 206-JaK-25-1.
DATA REQUIREMENT:	FIFRA 84-2 OECD 471
AUTHOR:	[redacted]
REPORT DATE:	10.09.1991
PERFORMING LABORATORY:	Scantox A/S
LABORATORY NO.:	12323
CLIENT:	Cheminova Agro A/S P.O. Box 9 DK-7620 Lemvig Denmark

The testing is subjected to the conditions stated overleaf.

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These conditions shall apply to the performance of the testing and to the preparation of this present test report:

1. In respect of tests made and reports thereon, the laboratory shall as against the party ordering be liable in damages under the Danish general rules on liability in tort, subject to the limitations set out in paras. 2-6 of these present conditions.

2. The test and report have been made by the laboratory on the basis of the knowledge and facilities available to the laboratory at the time of execution of the test. The laboratory shall not be held liable where later developments may indicate that laboratory knowledge or facilities are insufficient or incorrect.

3. The laboratory shall not be held liable for damage caused by a product manufactured by the party ordering the test where

   - the tort is referable to the party ordering prior to the laboratory report having been submitted by the laboratory,

   -the specific product causing the damage has not been tested by the laboratory, save where the party ordering proves that the product causing the damage is identical with a specific product tested by the laboratory, and

   - the damage is due to a characteristic of the product, or an application of the product, that either has not been tested and described in the test report or that varies from the laboratory test report description of product characteristics and possible applications of the product.

4. The laboratory shall not be held liable for damage referable to use made of any opinion given by the laboratory where such opinion is stated to be based on estimated judgment or assessment.

5. In instances other than those set out in paras. 2-4 above, the laboratory may be held liable where damage is proved to be caused by default or negligence on the part of the laboratory. However, laboratory liability in respect of material damage shall not, unless otherwise expressly agreed, exceed DKK 1.500.000 for each individual claim. The laboratory shall not be liable for loss of production, consequential loss, loss of profit, or any other indirect loss. The laboratory shall be liable to pay damages only where the claim is made in writing within three years from the date of this present test report.

6. Where in a legal action against the laboratory, the claim set up goes beyond the limitations to laboratory liability stated in paras. 2-5 above, the client shall at the request of the laboratory take on himself the conduct of such action.

   The client shall indemnify the laboratory to the extend that the laboratory may be held liable - or may otherwise have defrayed any expenses - in excess of the limitations to laboratory liability stated in paras. 2-5 above.

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claims 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).

Company: Cheminova Agro A/S

Company Agent: [redacted]

Date: 13.09.1991

Signature: [redacted]

AUTHENTICATION

The investigation described in this report (Ames Salmonella Assay with Glyphosate, batch 206-JaK-25-1) was carried out under my supervision and in accordance with the principles of Good Laboratory Practice (GLP) according to OECD codes of GLP, May 1981, Doc C (81) 30 (Final) Annex 2. The study was conducted according to the procedures herein described and the report is a complete and accurate account of the methods employed and the data obtained. The experimental work was carried out between February 5, 1991 and March 8, 1991.

10.09.1991

Study Director

QUALITY ASSURANCE STATEMENT

Study number: 12323

The quality of non-clinical safety studies performed at Scantox A/S is secured by an independent Quality Assurance Unit.

Short term routine studies such as are reported here, are inspected by the Quality Assurance Unit in a process based manner, i.e. critical phases in the actual study type are inspected regularly. In addition, the test system facilities are inspected approximately once a week. Documented inspection reports are communicated to the study director and to the facility management.

All reports are audited before release.

Date of audit:	13.06.1991
Date of report to study director:	13.06.1991

This report accurately describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

The Quality Assurance of this study complies with OECD codes of GLP and, in general, also with the FIFRA Good Laboratory Practice Standards (40 CFR part 160) with the exception that the above mentioned inspections are conducted regularly and not on an individual basis.

10.09.1991

[redacted]

Quality Assurance Manager

PERSONNEL RESPONSIBLE FOR THE STUDY

Study Director	[redacted]
Quality Assurance	[redacted]
Sponsor Monitor	[redacted]

SUMMARY

"Glyphosate, batch 206-JaK-25-1" was tested in the Ames test.

The test was performed in accordance with the guideline recommended by OECD "Salmonella typhimurium, Reverse Mutation Assay" No. 471, 1983, (3) as well as US CFR part 700 (F) § 798.5265 (1987).

The compound was tested in strains TA 100, TA 98, TA 1537 and TA 1535 at the dose levels of 5.0 mg, 2.5 mg, 1.3 mg, 0.63 mg and 0.31 mg per plate with S-9 mix and at the dose levels of 2.5 mg, 1.3 mg, 0.63 mg, 0.31 mg and 0.16 mg per plate without S-9 mix.

It is concluded from the results that the addition of Glyphosate in the above stated doses induced no increase in the number of revertants as compared to the controls. Thus, the Glyphosate was found non-mutagenic in the Ames Test.

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1. PURPOSE

The Ames Mutagenicity Assay is designed to provide an evaluation of the mutagenic potential of chemicals using mutant strains of Salmonella typhimurium as indicator organisms.

2. TEST ARTICLE

Scantox A/S received on 24.01.1991 from Sponsor a container with the test article marked "Glyphosate, batch 206-JaK-25-1". The sample was labelled Scantox Lab. No. 12323 and stored at room temperature in the dark until use.

The test article was a white powder of 98.6% purity, according to Sponsor. The powder was soluble in distilled water at concentrations up to at least 16.7 mg/ml which was the highest concentration needed in the assay.

No analyses on the test article were performed by Scantox.

3. TEST SYSTEM

3.1 Principle

The Ames Mutagenicity Assay, originally described by Ames et al. (1), is an in vitro short term test used to detect gene mutations.

Mutagenic effect is measured by exposing the following four strains of Salmonella typhimurium to the test substance:

Salmonella typhimurium TA 100
Salmonella typhimurium TA 98
Salmonella typhimurium TA 1537
Salmonella typhimurium TA 1535

Unlike the original Salmonella typhimurium these mutant strains have lost the ability to synthesize the amino-acid histidine. Without histidine in the culture medium, the strains are unable to grow and form colonies. If the histidine-requiring mutants are exposed to a mutagen, they may revert to the original histidine-independent form and can thus be counted as colonies. Mutations detected by this assay are of the types which can cause base-pair substitutions or frameshift mutations.

A measure of the mutagenic properties of the test article is obtained by comparing the number of induced revertants with the number of spontaneously occurring revertants.

3.2 Bacterial strains

The strains listed below were received from National Collection of Type Cultures, Central Public Health Laboratory, London NW9 5HT, UK.

Strain	His Mutations	Mutation Type	Excision Repair	LpS	Plasmid PKM 101
TA 100	His G 46	Basepair substitution	uvrB	rfa	R^+
TA 98	His D 3052	Frameshift	uvrB	rfa	R^+
TA 1537	His C 3076	Frameshift	uvrB	rfa	R^-
TA 1535	His G 46	Basepair substitution	uvrB	rfa	R^-

Beside the mutations in the histidine operon the strains have a deletion through the uvrB region of the chromosome resulting in the lack of excision repair. The lack of excision repair makes the organisms more sensitive to DNA damage which otherwise might be corrected by the excision repair process.

The rfa mutation results in deletion of the polysaccharide side chain of the liposaccharide of the bacterial cell surface, making the cells more permeable to compounds, especially large molecules.

The strains TA 100 and TA 98 have in addition the R-factor plasmid, pKM 101, which increases their sensitivity to mutagens by making use of an error prone repair process. The plasmid also carries a gene for resistance to ampicillin.

The genotypes of the tester strains were checked for:

his mutation (histidine requirement)
uvrB mutation (sensitivity to uv-radiation)
rfa mutation (crystal violet sensitivity)
pKM 101 plasmid (ampicillin resistance)

3.3 Growth and storage of bacterial strains

Cultures for mutagenicity testing

Cultures were set up from frozen stock cultures in culture tubes containing 7 ml bouillon (Oxoid nutrient broth, No. 2), and incubated in a water bath with a shaking device at 37°C. The growth period was planned so that a density of 10^8 to 10^9 bact./ml was reached by the time of the performance of the test.

Frozen stock cultures

Stock cultures were prepared from fresh broth cultures (10^8 - 10^9 bact./ml) to which 0.09 ml dimethylsulfoxide was added per ml broth culture. The cultures were frozen in a freezer and stored at -196°C.

3.4 Metabolic activation

Some chemicals do not exert a mutagenic effect in this system unless they are activated by mammalian enzymes. The activation was accomplished by the addition of rat liver postmitochondrial fraction supplemented with salts and co-factors (S-9 mix). The microsome fraction was obtained from rats pre-treated with the PCB mixture Aroclor(R) 1254.

The test was carried out in the presence and absence of the metabolic activation system.

4. TEST MATERIALS

4.1 Media

Vogel-Bonner agar

$MgSO_4 \cdot 7H_2O$	0.2 g
Citric acid $\cdot H_2O$	2.0 g
$NaNH_4HPO_4 \cdot 7 H_2O$	3.5 g
K_2HPO_4	10.0 g
Agar	15.0 g
Glucose	20.0 g
Distilled water ad	1000.0 ml

The solutions of salts, glucose and agar were autoclaved separately and mixed afterwards.

20 ml of Vogel-Bonner agar was used per plate.

Top Agar

Agar	0.6 g
NaCl	0.5 g
Distilled water ad	100.0 ml

The autoclaved top agar was supplemented with 10.0 ml of a sterile solution of 0.5 mM L-histidine and 0.5 mM biotin before use.

4.2 S-9 mix

	1. Test series (Plate incorporation)	2. Test series (Pre-incubation)
S-9 fraction (rat liver homogenate)	2.4 ml	2.4 ml
Phosphate buffer (0.2 M, pH 7.4)	30.0 ml	30.0 ml
Salt solution (0.4 M $MgCl_2$, 1.65 M KCl)	1.2 ml	1.2 ml
Glucose-6-phosphate solution (0.1 M)	0.3 ml	0.3 ml
NADP solution (0.1 M)	2.4 ml	2.4 ml
Distilled water	24.0 ml	

The S-9 mix was kept on ice until addition.

S-9 fraction (rat liver homogenate)

SPF Wistar rats of the strain Mol:WIST were obtained from the Møllegaard Breeding Center Ltd, Ejby, DK-4623 Lille Skensved. Rats weighing approximately 200 g were used for induction of liver enzymes. A single intraperitoneal injection of Aroclor(R) 1254 at a dose of 500 mg/kg body weight was given to each rat. The animals were killed by gassing with CO_2 5 days after being injected and following a 16 hour period of fasting.

All steps in preparation of the liver homogenate were performed on ice using aseptic techniques and cold sterile solutions. The livers were removed and minced in 0.15 M KCl solution (3.0 ml KCl solution per gram wet liver). After homogenization, the homogenate was centrifuged at 9000 g for 15 minutes. The supernatant (S-9 fraction) was decanted, frozen and stored at -196°C until use.

5. TEST PROCEDURES

5.1 Preliminary toxicity test

A preliminary toxicity test was carried out using TA 98 in the following way:

Test tubes containing 2 ml of top agar with extra histidine (0.5 mM) and normal biotine (0.05 mM) were kept at 46°C in a water bath. To each test tube was added 0.3 ml test solution, 0.5 ml S-9 mix when required and 0.1 ml bacterial suspension diluted to about 10^3 bact./ml in phosphate buffer. After mixing, each top agar solution was spread on a Vogel-Bonner plate and incubated at 37°C for 2 days.

A three-fold dilution series from 5.0 mg/plate to 0.56 mg/plate was tested with and without S-9 mix.

At the dose of 5.0 mg/plate without S-9 mix only 1 colony was found on the three plates compared to 164 colonies on the three control plates. At the dose of 1.67 mg/plate without S-9 mix a total of 74

colonies were found and at the dose of 0.56 mg/plate without S-9 mix a total of 184 colonies was observed. For all doses with S-9 mix the number of colonies were similar to the control, i.e. close to 200 on three plates.

On this basis it was decided to use the following doses in the mutagenicity assay: 5.0 mg, 2.5 mg, 1.3 mg, 0.63 mg and 0.31 mg per plate with S-9 mix, and 2.5 mg, 1.3 mg, 0.63 mg, 0.31 mg and 0.16 mg per plate without S-9 mix.

5.2 Mutagenicity test

The mutagenicity test included two independent test series each performed with and without S-9 mix using the four strains described in section 3. Three plates were used per dose point.

In order to obtain the most reliable assessment of the mutagenic potential of the test chemical two different dosing procedures were followed.

The first test series was performed as a socalled plate incorporation assay while the second test series was performed using a preincubation procedure (see below).

Positive and negative controls were included in both test series. As positive controls without S-9 mix 0.5 µg/plate Na-azide was used for TA 100, 1.0 µg/plate Na-azide for TA 1535 and 0.6 µg/plate 2-nitrofluorene for TA 98 and TA 1537. With S-9 mix 1.25 µg/plate 2-aminoanthracene was used for all four strains.

5.3 Dosing procedures

Plate incorporation assay

To each test tube was added 0.3 ml test solution, 0.5 ml S-9 mix when required, 0.1 ml bacterial suspension (10^8 - 10^9 bact./ml) and 2.0 ml top agar. After whirl mixing the mixture was spread on a Vogel-Bonner agar plate. After incubation for 48 to 72 hours at 37°C the number of colonies was counted.

Preincubation assay

To each test tube was added 0.3 ml test solution, 0.3 ml S-9 mix or phosphate buffer pH 7.4 and 0.1 ml bacterial suspension (10^8 - 10^9 bact./ml). The test tube was incubated for 30 min at 37°C under gentle shaking whereafter 2.0 ml top agar was added and after whirl mixing the mixture was spread on a Vogel-Bonner agar plate. After incubation for 48 to 72 hours at 37°C the number of colonies was counted.

5.4 Statistical analysis

Statistical analysis of the negative control versus test data was performed using the Analysis of Variance method (general linear model, least square mean) (4). Statistical analysis of the negative versus positive control data was performed using the Student's t-test.

6. RESULTS

The results are presented in tables 1 to 4.

The negative and positive controls were all in the expected range. No clear depression of the background growth was observed at any dose with or without S-9 mix in the main test. However, the generally lower numbers of revertants in the highest dose groups indicate that some toxicity occurred.

The test article induced no statistically significant increases in the number of revertants in any strains with or without S-9 mix.

7. CONCLUSION

The test article, Glyphosate, batch 206-JaK-25-1, was found non-mutagenic in the Ames Test.

8. REFERENCES

1. Ames, B.N., McCann, I and Yamasaki, E. (1975). Methods for detection of carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31, 347 - 364.

2. Maron, D.M. and Ames, B.N. (1983). Revised methods for the Salmonella mutagenicity test. Mutat. Res. 113, 173 - 215.

3. OECD Guidelines for testing of chemicals, (No. 471), OECD Publication Office, 2 rue André-Pascal, F-75775 Paris Cedex 16, France (1983).

4. SAS users Guide, Statistics, 1982 Edt. SAS Institute Inc. Box 8000, Cary, North Carolina, 27511, USA.

TABLE 1. TA 100

STRAIN=TA 100, ENZYME= -S9, TEST= PLATE INCORPORATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	184	184	159	175.7	14.4	
2.5 mg	56	82	92	76.7	18.6	0.44
1.3 mg	104	89	100	97.7	7.8	0.56
0.63 mg	158	146	140	151.3	14.7	0.86
0.31 mg	189	189	169	182.3	11.5	1.04
0.16 mg	161	159	169	163.0	5.3	0.93
Na-azide 0.5 ug	990	800	850	880.0	98.5	5.01 **

STRAIN=TA 100, ENZYME= -S9, TEST= PREINCUBATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	179	175	193	182.3	9.5	
2.5 mg	85	87	111	94.3	14.5	0.52
1.3 mg	110	96	124	110.0	14.0	0.60
0.63 mg	149	125	141	138.3	10.6	0.76
0.31 mg	197	163	155	185.0	13.1	1.01
0.16 mg	201	193	188	193.7	6.8	1.07
Na-azide 0.5 ug	670	540	640	616.7	68.1	3.38 **

STRAIN=TA 100, ENZYME= +S9, TEST= PLATE INCORPORATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	190	195	210	198.3	10.4	
5.0 mg	78	63	88	76.3	12.6	0.38
2.5 mg	108	94	110	104.0	8.7	0.52
1.3 mg	135	134	114	124.3	10.0	0.63
0.63 mg	210	170	183	187.7	20.4	0.95
0.31 mg	209	154	184	182.3	27.5	0.92
2-Aminoanthr. 1.25 ug	1250	1130	980	1120.0	158.2	5.65 **

STRAIN=TA 100, ENZYME= +S9, TEST= PREINCUBATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	218	188	185	197.0	18.2	
5.0 mg	64	86	80	76.7	11.4	0.39
2.5 mg	100	94	88	94.0	6.0	0.48
1.3 mg	126	122	118	122.0	4.0	0.62
0.63 mg	169	178	162	169.7	8.0	0.86
0.31 mg	195	181	192	182.7	18.8	0.93
2-Aminoanthr. 1.25 ug	670	670	700	680.0	17.3	3.45 **

. = PLATE NOT READABLE
STD = STANDARD DEVIATION
RATIO = MEAN NUMBER OF REVERTANTS/MEAN NUMBER OF SPONTANEOUS REVERTANTS
* = STATISTICALLY SIGNIFICANT AT 5 % LEVEL (ANALYSIS OF VARIANCE)
** = STATISTICALLY SIGNIFICANT AT 1 % LEVEL (ANALYSIS OF VARIANCE)

TABLE 2. TA 98.

STRAIN=TA 98, ENZYME= -S9, TEST= PLATE INCORPORATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	45	34	20	33.0	12.5	
2.5 mg	22	21	26	23.0	2.6	0.70
1.3 mg	32	29	21	27.3	5.7	0.83
0.63 mg	35	30	26	30.3	4.5	0.92
0.31 mg	39	32	28	33.0	5.6	1.00
0.16 mg	30	31	30	30.3	0.6	0.92
2-Nitrofluor. 0.6 ug	330	316	306	317.3	12.1	9.62 **

STRAIN=TA 98, ENZYME= -S9, TEST= PREINCUBATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	52	44	37	44.3	7.5	
2.5 mg	42	27	26	31.7	9.0	0.71
1.3 mg	32	36	37	35.0	2.6	0.79
0.63 mg	42	54	36	44.0	9.2	0.99
0.31 mg	63	41	44	49.3	11.9	1.11
0.16 mg	48	56	52	52.0	4.0	1.17
2-Nitrofluor. 0.6 ug	316	272	310	299.3	23.9	6.75 **

STRAIN=TA 98, ENZYME= +S9, TEST= PLATE INCORPORATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	41	34	33	36.0	4.4	
5.0 mg	27	29	22	26.0	3.6	0.72
2.5 mg	42	31	34	35.7	5.7	0.99
1.3 mg	36	28	41	35.0	6.6	0.97
0.63 mg	39	40	49	42.7	5.5	1.19
0.31 mg	40	45	31	38.7	7.1	1.07
2-Aminoanthr. 1.25 ug	1250	1280	700	1076.7	325.5	29.91 **

STRAIN=TA 98, ENZYME= +S9, TEST= PREINCUBATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	22	19	20	20.3	1.5	
5.0 mg	20	21	18	19.7	1.5	0.97
2.5 mg	19	20	20	19.7	0.6	0.97
1.3 mg	24	23	28	25.0	2.6	1.23
0.63 mg	23	22	22	22.3	0.6	1.10
0.31 mg	19	19	32	23.3	7.5	1.15
2-Aminoanthr. 1.25 ug	1200	1000	760	986.7	220.3	48.52 **

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TABLE 3. TA 1537.

STRAIN=TA 1537, ENZYME= -S9, TEST= PLATE INCORPORATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	10	11	12	11.0	1.0	
2.5 mg	9	10	9	9.3	0.6	0.85
1.3 mg	9	9	13	10.3	2.3	0.94
0.63 mg	10	9	11	10.0	1.0	0.91
0.31 mg	12	12	9	11.0	1.7	1.00
0.16 mg	12	7	13	10.7	3.2	0.97
2-Nitrofluor. 0.6 ug	112	118	153	127.7	22.1	11.61 **

STRAIN=TA 1537, ENZYME= -S9, TEST= PREINCUBATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	12	15	13	13.7	2.1	
2.5 mg	13	9	12	11.3	2.1	0.83
1.3 mg	18	13	18	16.3	2.9	1.20
0.63 mg	11	9	5	8.3	3.1	0.61
0.31 mg	11	10	21	14.0	6.1	1.02
0.16 mg	15	14	17	15.7	1.5	1.15
2-Nitrofluor. 0.6 ug	149	141	141	143.7	4.6	10.51 **

STRAIN=TA 1537, ENZYME= +S9, TEST= PLATE INCORPORATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	12	14	13	13.0	1.0	
5.0 mg	9	9	8	8.7	0.6	0.67
2.5 mg	13	8	18	13.0	5.0	1.00
1.3 mg	10	14	11	11.7	2.1	0.90
0.63 mg	10	13	14	12.3	2.1	0.95
0.31 mg	8	14	9	10.3	3.2	0.79
2-Aminoanthr. 1.25 ug	140	150	152	147.3	6.4	11.33 **

STRAIN=TA 1537, ENZYME= +S9, TEST= PREINCUBATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	5	6	5	5.3	0.6	
5.0 mg	4	5	5	4.7	0.6	0.88
2.5 mg	7	6	5	6.0	1.0	1.12
1.3 mg	7	6	5	6.0	1.0	1.12
0.63 mg	7	5	6	6.0	1.0	1.12
0.31 mg	9	8	5	7.3	2.1	1.37
2-Aminoanthr. 1.25 ug	134	150	153	145.7	10.2	27.31 **

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STD = STANDARD DEVIATION
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** = STATISTICALLY SIGNIFICANT AT 1 % LEVEL (ANALYSIS OF VARIANCE)

TABLE 4. TA 1535.

STRAIN=TA 1535, ENZYME= -S9, TEST= PLATE INCORPORATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	20	19	21	20.0	1.0	
2.5 mg	14	12	13	13.0	1.0	0.65
1.3 mg	12	15	13	13.3	1.5	0.67
0.63 mg	18	25	15	19.3	5.1	0.97
0.31 mg	25	19	15	19.7	5.0	0.98
0.16 mg	23	23	23	23.0	0.0	1.15
Na-azide 1.0 ug	1280	1050	830	1053.3	225.0	52.67 **

STRAIN=TA 1535, ENZYME= -S9, TEST= PREINCUBATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	23	32	24	26.3	4.9	
2.5 mg	22	19	21	20.7	1.5	0.78
1.3 mg	32	30	26	29.3	3.1	1.11
0.63 mg	25	33	40	32.7	7.5	1.24
0.31 mg	32	33	26	30.3	3.8	1.15
0.16 mg	29	33	24	28.7	4.5	1.09
Na-azide 1.0 ug	850	950	1220	1020.0	166.8	38.73 **

STRAIN=TA 1535, ENZYME= +S9, TEST= PLATE INCORPORATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	24	19	19	20.7	2.9	
5.0 mg	13	17	10	13.3	3.5	0.65
2.5 mg	16	17	16	16.3	0.6	0.79
1.3 mg	18	27	19	21.3	4.9	1.03
0.63 mg	17	23	20	20.0	3.0	0.97
0.31 mg	25	30	22	25.7	4.0	1.24
2-Aminoanthr. 1.25 ug	200	224	232	218.7	16.7	10.58 **

STRAIN=TA 1535, ENZYME= +S9, TEST= PREINCUBATION ASSAY

DOSE	PLATE 1	PLATE 2	PLATE 3	MEAN	STD	RATIO
Control	15	14	15	14.7	0.6	.
5.0 mg	16	14	14	14.7	1.2	1.00
2.5 mg	14	16	15	15.0	1.0	1.02
1.3 mg	14	15	14	14.3	0.6	0.98
0.63 mg	16	14	15	15.0	1.0	1.02
0.31 mg	14	18	15	15.7	2.1	1.07
2-Aminoanthr. 1.25 ug	195	110	190	165.0	47.7	11.25 **

. = PLATE NOT READABLE
STD = STANDARD DEVIATION
RATIO = MEAN NUMBER OF REVERTANTS/MEAN NUMBER OF SPONTANEOUS REVERTANTS
* = STATISTICALLY SIGNIFICANT AT 5 % LEVEL (ANALYSIS OF VARIANCE)
** = STATISTICALLY SIGNIFICANT AT 1 % LEVEL (ANALYSIS OF VARIANCE)

scantox

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Authorization no. 145

Lab. No. 12323

AMES TEST

Protocol

Test article: Glyphosate

Sponsor: Cheminova Agro A/S
P.O. Box 9
DK-7620 Lemvig
Denmark

Study performance: Scantox A/S
40, Tornbjergvej, Ejby
DK-4623 Lille Skensved
Denmark

Date: 08.01.1991

Management of study:

Study director:

Head, QAU:

Sponsor contact:

AMES TEST

Test article: Glyphosate

Sponsor: Cheminova Agro A/S

Protocol approval

Scantox A/S:

Issued by: [redacted] Date: 10.01.1991

Sponsor:

This protocol is accepted without revision and my signature authorises the study to proceed as described in this document.

Approved by: [redacted] Date 23/1-1991

Details concerning the experiment are described in the following protocol:

AMES TEST

SALMOMELLA TYPHIMURIUM, REVERSE MUTATION ASSAY

Protocol

1. INTRODUCTION

The Ames Test is a short term mutagenicity test for the evaluation of possible mutagenic effects of chemicals.

The test is conducted in accordance with the guideline recommended by OECD *"Salmonella typhimurium, Reverse Mutation Assay"*, No. 471, 1983 (3) as well as US CFR part 700 (F) § 798.5265 (1987).

2. PRINCIPLE

Mutagenic effects are measured by exposing the following four bacterial tester strains to the test chemical:

Salmonella typhimurium	TA	100
Salmonella typhimurium	TA	98
Salmonella typhimurium	TA	1537
Salmonella typhimurium	TA	1535

Unlike the original Salmonella typhimurium LT2, the above tester strains all carry a mutation in the operon (gene) for synthesis of the amino acid histidine. Therefore, the tester strains are not able to grow in substrate without histidine.

The mutations are either of the type basepair-substitution or frameshift mutations. A basepair-substitution occurs when one basepair in a DNA sequence is exchanged with a different basepair, while a frameshift mutation occurs, when one or more basepairs are added to or deleted from a DNA sequence.

When the tester strains are exposed to a test compound, mutations may be induced in the defect histidine genes, whereby some of the bacteria can revert to the wild-type. The revertants will be able to grow and form colonies on agar plate substrate without histidine.

A measure of the mutagenic properties of the test article is obtained by comparing the number of induced revertants with the number of spontaneously occurring revertants.

3. TESTER STRAINS

The bacterial tester strains listed below were received from National Collection of Type Cultures, Central Public health Laboratory, London NW9 5HT, UK.

In addition to the already described mutation in the histidine operon, all the strains carry two more mutations, uvrB and rfa, which increase the sensitivity of the strains. Furthermore two of the strains also carry the R-factor plasmid pKM 101, see below.

Strain	His Mutations	Mutation Type	Excision Repair	LpS	Plasmid PKM 101
TA 100	His G 46	Basepair substitution	uvrB	rfa	R^+
TA 98	His D 3052	Frameshift	uvrB	rfa	R^+
TA 1537	His C 3076	Frameshift	uvrB	rfa	R^-
TA 1535	His G 46	Basepair substitution	uvrB	rfa	R^-

3.1. His mutation

As described in section 2, the tester strains carry mutations in the histidine operon. The presence of the His mutation is checked by plating bacterial suspension on Vogel-Bonner plates with biotin, but without histidine. No background growth and only very few colonies must be seen.

3.2. uvrB mutation

This mutation includes the genetic information for the excision repair process and results in a reduced capacity for repair of damage to the DNA. The presence of the uvrB mutation is checked by testing the sensitivity of the strains to ultraviolet light.

3.3. rfa mutation

This mutation causes partial loss of the lipopolysaccharide barrier that coates the surface of the bacteria, thereby increasing the permeability of the cell wall to large molecules. The presence of the rfa mutation is checked by testing the sensitivity of the strains to the toxic effect of crystal violet.

3.4. R-factor plasmid (pKM 101)

The strains TA 100 and TA 98 carry the R-factor plasmid pKM 101, which further increases the sensitivity of these strains by enhancing an error-prone DNA repair process. The plasmid also carries a gene for ampicillin resistance. The presence of the plasmid is checked by testing the resistance of the strains to ampicillin.

4. PROCEDURES FOR GROWING AND MAINTAINING THE TESTER STRAINS

4.1. Stock cultures

Frozen stock cultures are kept in a liquid nitrogen tank at -196°C. New stock cultures are reisolated when appropriate and frozen.

Reisolation is accomplished by streaking out with a platinum wire a small amount of bacterial suspension from a grown up broth culture on a Vogel-Bonner agar plate with biotin and extra histidine (0.5 mM). For plasmid strains 25 μg/ml ampicillin is added to the agar.

After incubation for two days, single colonies are inoculated in broth cultures and grown to a density of 10^8-10^9 bct/ml. Parts of these cultures are used for control testing as described in Section 3, while the rest are frozen. In addition the spontaneous reversion level and the response to positive controls (with and without S-9 mix) are checked for each broth culture. On the basis of these control testing data the best cultures which carry all the characteristic mutations are selected and used as new stock cultures.

4.2. Freezing of bacterial cultures

Freshly grown up broth cultures are supplemented with 9% DMSO and frozen in a -20°C freezer for one day, whereafter the cryo tubes are transferred to a liquid nitrogen tank (-196°C).

4.3. Cultures for mutagenicity testing

Broth cultures for mutagenicity testing are inoculated directly from frozen stock cultures and incubated at 37°C in a water bath under shaking until a density of 10^8-10^9 bact/ml is reached (photometrical measurements). Only freshly grown up cultures are used for mutagenicity testing.

5. NEGATIVE AND POSITIVE CONTROLS

5.1. Negative controls

In all mutagenicity tests are included negative solvent controls, which reflect the number of spontaneous revertants. The normal spontaneous revertant levels for the four strains are:

TA 100 :	100 - 220	per plate
TA 98 :	20 - 70	per plate
TA 1537:	5 - 15	per plate
TA 1535:	10 - 30	per plate

5.2. Positive controls

Positive controls are included in all test series. The sensitivity of the strains without S-9 mix is checked using the following mutagens:

TA 100 :	0.5 µg Sodium-azide per plate
TA 98 :	0.6 µg 2-nitrofluorene per plate
TA 1537:	0.6 µg 2-nitrofluorene per plate
TA 1535:	1.0 µg Sodium-azide per plate

When S-9 mix is applied, 1.25 µg 2-aminoanthracene per plate is used for all four strains.

6. METABOLIC ACTIVATION

Some chemicals do not exert a mutagenic effect in this system unless they are activated by mammalian enzymes. The activation is normally accomplished by the addition of rat liver postmitochondrial fraction supplemented with salts and co-factors (S-9 mix). The microsome fraction is obtained from rats pre-treated with Aroclor® 1254. The test is carried out in the presence and absence of metabolic activation system.

6.1. S-9 fraction (rat liver homogenate)

F wistar rats of the strain Mol:WIST are obtained from the Møllegaard Breeding Center Ltd, Ejby, DK-4623 Lille Skensved. Rats weighing approximately 200 g are used for induction of liver enzymes. A single intra-peritoneal injection of Aroclor® 1254 at a dose of 500 mg/kg body weight is given to each rat. The animals are killed by gassing with CO_2 5 days after being injected and following a 16 hour period of fasting.

All steps in preparation of the liver homogenate are performed on ice using aseptic techniques and cold sterile solutions. The livers are removed and minced in 0.15 M KCl solution (3.0 ml KCl solution per gram wet liver). After homogenization, the homogenate is centrifuged at 9000 g for 15 minutes. The supernatant (S-9 fraction) is decanted, frozen and stored at -196°C until use.

6.2. S-9 mix

S-9 m fraction (rat liver homogenate)	2.4 ml
Sodium phosphate buffer (0.2 M, pH 7.4)	30.0 ml
Salt solution (0.4 M $MgCl_2$, 1.65 M KCl)	1.2 ml
Glucose-6-phosphate (0.1 M)	0.3 ml
NADP (0.1 M)	2.4 ml
Distilled water	24.0 ml

The S-9 mix is kept on ice until addition.

7. SUBSTRATES AND SOLUTIONS

7.1. Liquid growth medium (broth)

12.5 g Oxoid Nutrient Broth No. 2 per 500 ml H_2O

7.2. Vogel-Bonner agar plates

15 g Bacto agar, 20 g glucose, 0.2 g $MgSO_4 \cdot 7H_2O$, 2 g citric acid, 10 g K_2HPO_4, 3.5 g $NaNH_4HPO_4 \cdot 4\ H_2O$ and distilled water ad 1 litre.

20 ml of agar is used per 9 cm petri disk plate.

7.3. Top-agar

6 g Bacto agar, 5 g NaCl and distilled water ad 1 litre. The top agar is supplemented with 0,05 mM histidine and 0.05 mM biotin before use.

8. TEST METHODS

8.1. Test article

The test article is dissolved in a solvent which has no effect on the system in the concentration applied. Distilled water, DMSO or ethanol are examples of usable solvents.

Sterile dilutions of the test article with logarithmic concentration intervals are prepared fresh for each test, unless other storage conditions are specified.

Preliminary toxicity test

The general bactericidal effect of the test compound is tested in a preliminary toxicity test using TA 98. The test is performed in the following way:

Test tubes containing 2 ml top agar with extra histidine (0.5 mM) and normal biotin (0,05 mM) are kept at 45°C in a water bath. To each test tube is added 0.1 ml test solution, 0.5 ml S-9 mix when required and 0.1 ml bacterial suspension diluted to about 10^8 bact./ml in phosphate buffer. Solvent controls are included with and without S-9 mix. After mixing, each top agar solution is spread on a Vogel-Bonner plate and incubated at 37°C for 2 days.

On the basis of the toxicity, measured as a reduction in number of colonies on the test plates as compared to the control plates, the dose levels are selected for the mutagenicity test.

Toxic effects, as measured in the above toxicity test, are further investigated in the mutagenicity test by inspection of the level of background growth (micro-colonies) on the test plates.

8.3. Mutagenicity test

On the basis of the preliminary toxicity test, five dose levels are selected for the mutagenicity test. If possible, the dose range is selected so the highest dose is clearly toxic, while the lower doses should show no signs of toxicity.

If the test compound is found to be atoxic, the highest dose level is set to 5 mg/plate, or to the highest amount of the test compound which is practically applicable due to the solubility or other characteristics of the test compound.

The mutagenicity test includes two independent tests separated by at least one day. In each test is used all four strains with and without S-9 mix, 3 plates per dose level. Positive and negative controls with and without S-9 mix are included in both tests.

In order to obtain the most reliable assessment of the mutagenic potential of a chemical two different dosing procedures are usually followed in the two test series.

The first test series is performed as a socalled plate incorporation assay while the second test series is performed using a preincubation procedure (see below).

8.4 Dosing Procedures

Plate incorporation assay

To each test tube is added the test solution (0.1 ml), 0.5 ml S-9 mix when required, 0.1 ml bacterial suspension (10^8 - 10^9 bact./ml) and 2.0 ml top agar. After whirl mixing the mixture is spread on a Vogel-Bonner agar plate. After incubation for 48 to 72 hours at 37°C the number of colonies is counted.

Preincubation assay

To each test tube is added the test solution (0.1 ml), 0.5 ml S-9 mix or phosphate buffer pH 7.4 and 0.1 ml bacterial suspension (10^8 - 10^9 bact./ml). The test tube is incubated for 30 min at 37°C under gentle shaking whereafter 2.0 ml top agar is added and after whirl mixing the mixture is spread on a Vogel-Bonner agar plate. After incubation for 48 to 72 hours at 37°C the number of colonies is counted.

EVALUATION OF DATA

The data obtained are evaluated with respect to the following criteria:

a. Statistically significant increase in the level of revertants on the test plates as compared to the control plates. Analysis of variance test is used to compare the test and negative control groups.

b. Dose-response is seen.

c. The number of revertants at the dose level where the highest effect is found should be more than twice the concurrent spontaneous level.

If a, b and c are all met the test article is considered to be mutagenic. If only a and b are met the test article is considered to be weakly mutagenic. A weak effect should preferably be confirmed either in the parallel test or in a supplementary assay using the same dosing procedure (note: Supplementary testing is not included in the standard protocol). If a is not met the test article is considered to be non mutagenic.

10. GOOD LABORATORY PRACTICE (GLP)

This study will be conducted in accordance with the principles of Good Laboratory Practice (GLP) according to OECD codes of GLP, May 1981, Doc C(81)30 (Final) Annex 2 and Scantox Standard Operation Procedures.

The Quality Assurance Unit (QAU) will carry out periodic and independent routine inspections on critical phases and repeated processes in this type of study, but not necessarily on activities of this study. The report on the study will be audited.

11. REPORT

The report is made in English language covering description of the test procedures, tables with the relevant observations, statistical analysis, evaluation and interpretation of results.

12. ARCHIVES

All original data, including copies of correspondence, all reports issued and a sample of the test article will be stored at ambient temperature in the GLP Archives of Scantox Biological Laboratory Ltd. for a period of 5 years. Samples that are unstable may be disposed of before that time or stored under specified conditions after consultation with the Sponsor. At the end of the period Sponsor will be consulted regarding requirements for disposal or further storage.

13. LITERATURE

(1) B.N. Ames, McCann, I. and Yamasaki, E.: Mutation Res. 31, 347-364 (1975).

(2) D.M. Maron and Ames B.N.: Mutation Res. 113, 173-215 (1983).

(3) OECD Guidelines for testing of chemicals, (1983), OECD Publication office, 2 rue André-Pascal, D-75775 Paris Cedex 16, France.

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Lab. No. 12323

AMES TEST

Test article: Glyphosate

Sponsor: Cheminova Agro A/S

Ammendment No. 1

to Protocol

1. Regarding the test article "Glyphosate".
The sample was labelled:
Glyphosate technical
Batch 206-JaK-25-1
Purity: 98.6%

The same identification appeared in the letter following the test article.

2. The test article is stored at Scantox at room temperature protected from light until use.

Approved by:

[redacted] 08.02.1991
Date

[redacted]
Sponsor

4/2-91
Date

scantox

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Lab. No. 12323

AMES TEST

Test article: Glyphosate

Sponsor: Cheminova Agro A/S

Ammendment No. 2

to Protocol

Since the Glyphosate was soluble in distilled water only up to approx. 20 mg/ml it was necessary to add the test substance in a volume of 0.3 ml (16.7 mg/ml) - instead of 0.1 ml as usually used - in order to obtain the dose of 5.0 mg/plate.

In the plate incorporation assay small changes in the dose volume is of very little importance since the mixture of test solution, S-9 mix (0.5 ml), bacterial suspension (0.1 ml) and top agar (2.0 ml) is spread on the test agar plate (20 ml) immediately after mixing.

However, in the preincubation assay the total volume of incubation mixture changes from 0.7 ml (0.1 ml test solution, 0.1 ml bacterial suspension and 0.5 ml S-9 mix) to 0.9 ml (0.3 ml test solution, 0.1 ml bacterial suspension and 0.5 ml S-9 mix or buffer).

Since the usual S-9 mix formulation contains 0.2 ml distilled water per 0.5 ml S-9 mix it was decided to omit the distilled water in the S-9 mix formulation for the preincubation assay and instead add only 0.3 ml (more concentrated) S-9 mix in order to maintain the usual enzyme/co-factor concentration.

Approved by:

[redacted] 29.07.1991
Date

[redacted]
Sponsor 30/7-91
Date