scantox

TEST REPORT

STUDY TITLE:	Mutagenicity test: In vitro Mammalian Cell Gene Mutation Test with Glyphosate, batch 206-JaK-25-1.
DATA REQUIREMENT:	FIFRA 84-2 OECD 476
AUTHOR:	[redacted] M.Sc.
REPORT DATE:	10.09.1991
PERFORMING LABORATORY:	Scantox A/S
LABORATORY NO.:	12325
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] DVM

Date: 13.09.1991

Signature: [redacted]

Lab. No. 12325

AUTHENTICATION

The investigation described in this report (In vitro Mammalian Cell Gene Mutation Test 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 April 4, 1991 and May 13, 1991.

10.09.1991

[redacted]

Study Director

Lab. No. 12325

QUALITY ASSURANCE STATEMENT

Study number: 12325

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: 19.07.1991

Date of report to study director: 19.07.1991

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

Lab. No. 12325

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 in vitro mammalian cell gene mutation test. The test was conducted in accordance with the guideline recommended by OECD "In Vitro Mammalian Cell Gene Mutation Test" No. 476, 1983 (1), as well as US CFR part 700 (F) § 798.5265 (1987).

The compound was tested in Mouse Lymphoma cells (L5178Y) at the dose levels of 5.0 mg/ml, 2.5 mg/ml, 1.3 mg/ml and 0.63 mg/ml without metabolic activation, and 4.2 mg/ml, 2.1 mg/ml, 1.0 mg/ml and 0.52 mg/ml with metabolic activation (S-9 mix).

Addition of Glyphosate in the above stated doses induced no increase in the mutation frequency as compared to the controls. Thus, the Glyphosate was found to be non-mutagenic in the In Vitro Mammalian Cell Gene Mutation Test.

TABLE OF CONTENTS

1. INTRODUCTION

The in vitro mammalian cell gene mutation test is a short term mutagenicity test for the evaluation of possible mutagenic effects of chemicals.

2. TEST ARTICLE

Scantox received on 08.04.1991 the test article "Glyphosate technical, batch 206-JaK-25-1, purity 98.6%". The sample was further labelled with Scantox Lab. No. 12325 and stored at room temperature in the dark until use.

The Glyphosate was a white powder, soluble in cell culture medium up to at least 10 mg/ml.

No chemical analysis on the test article has been performed by Scantox A/S.

3. TEST SYSTEM

The test substance was added to cell cultures of mouse lymphoma cells (L5178Y). After an exposure period of 4 hours without metabolic activation and 3 hours with metabolic activation the cells were sedimented by centrifugation and resuspended in fresh medium. Four dose levels were used with and without metabolic activation.

The dose range was selected on the basis of a preliminary toxicity test in which the cell growth was measured for a period of 2 days after exposure. The principles for dose selection can be summarized as follows: If possible the highest dose is chosen so the survival (cloning efficiency) is approximately 20% of the control level, and so the cells grow at a normal rate before plating. If the test article is of limited solubility and causes no toxicity, the maximum dose may be chosen as the highest dose which can be added without precipitation of the test article or without toxic effects of the solvent. In any case 5 mg/ml or 10 mM is generally considered the highest dose relevant to include in the assay.

The present study was performed with 5.0 mg/ml as the highest dose without S-9 mix and 4.2 mg/ml with S-9 mix.

Before the mutagenicity assay the cells were grown for 1 day in medium containing thymidine (9 µg/ml), hypoxanthine (15 µg/ml), methotrexate (0.3 µg/ml) and glycine (22.5 µg/ml) (THMG-medium). After a recovery period of 2 - 3 days in medium containing the above mentioned chemicals except methotrexate (THG-medium), all the cells should have the genotype $TK^{+}/^{-}$ with respect to the heterozygous thymidine kinease gene.

In the mutagenicity test a series of cell cultures was exposed to the test substance as described above and subsequently grown for a period of 2 days which is necessary for the phenotypic expression of mutations induced in the TK-gene (2). The cell cultures were then seeded (cloned) in 96-well microtiter plates in medium containing trifluorothymidine (TFT) (2000 cells/well) as well as in normal medium (2 cells/well). Because only cells that have mutated from $TK^{+}/^{-}$ to $TK^{-/-}$ can grow in medium containing TFT, the mutation frequency can be determined on the basis of the number of cell clones in each type of medium after 8 - 10 days incubation.

4. CELLS AND CULTURE METHODS

The assay was performed with the mouse lymphoma cell line L5178Y received from [redacted] University of Sussex, BN1 9RR, UK. The cell line has been used in a large number of gene mutation assays.

The cells were grown as suspension cultures under gentle mixing in RPMI 1640 medium supplemented with 10% horse serum (Gibco), 200 µg/ml sodium pyruvate and 50 µg/ml gentamycin. The medium for plating and the THG-medium was supplemented with 50% conditioned medium.

Sterile NUNC plastic flasks and microtiter plates were used and incubation was carried out in a CO_2-incubator (5% CO_2) at 37°C. During normal maintenance and during the phenotypic expression period (see section 3) the cells were diluted daily and kept at a density of 2×10^5 to 1.5×10^6 cells/ml.

4.1 Frozen stock cultures

Frozen stock cultures were kept in a liquid nitrogen tank at -196°C. New stock cultures were made periodically. To cell suspensions of approx. 10^6 cells/ml was added 1 ml of DMSO per 10 ml of cell suspension. Freezing was accomplished by use of freezer (-20°C), so the temperature decreased approximately 1°C per min until -20°C was reached, whereafter the vials were transferred to the liquid nitrogen tank. Thawing was performed in a 37°C water bath.

Before new stock cultures were used in assays, the cells were checked for general morphology, growth characteristics and absence of mycoplasma.

5. 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 incubating the cells for 3 hours under gentle shaking together with the test compound and rat liver post mitochondrial fraction supplemented with salts and co-factors (S-9 mix). The microsome fraction was obtained from rats pretreated with Aroclor® 1254.

The test was carried out with and without metabolic activation.

5.1 S-9 fraction (rat liver homogenate)

SPF Wistar rats of the strain Mol:WIST were obtained from 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® 1254 at a dose of 500 mg/kg body weight was given to each rat. The animals were killed 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, rinsed briefly in 0.15 M KCl, and minced in 0.15 M KCl solution (3.0 ml per gram wet liver). After homogenization, the homogenate was centrifugated 9000 x g for 15 minutes. The supernatant (S-9 fraction) was decanted, frozen and stored at -196°C until use.

5.2 S-9 mix (20 ml)

S-9 fraction (rat liver homogenate)	6.0	ml
Hepes buffer (1 M, pH 7.2)	0.4	ml
$MgCl_2$ (50 mM)	2.0	ml
KCl (330 mM)	2.0	ml
Glucose-6-phosphate (0.1 M)	0.6	ml
NADP (0.1 M)	0.8	ml
Distilled H_2O	8.0	ml
Phenol red, sodium salt (0.5 mg/ml)	0.2	ml

The S-9 fraction was added after adjustment of pH to 7.2 with NaOH or HCl. The S-9 mix was kept on ice, and warmed to 37°C immediately before addition.

6. TEST PROCEDURES

6.1 Test solutions

The Glyphosate was dissolved in culture medium at the concentration of 10 mg/ml. Two-fold dilutions were prepared in cell culture medium to obtain the additional concentrations of 5.0, 2.5, 1.3 and 0.63 mg/ml.

6.2 Dosing of cultures

Without S-9 mix

Cell suspensions of about 6 x 10^5 cells/ml were mixed 1:1 with selected test solutions (see 6.1) in order to obtain the appropriate final concentrations (see 7). All cultures were set up in final volumes of 15 ml. The cultures were then incubated under gentle shaking for 4 hours at 37°C. After the incubation period the cells were sedimented by centrifugation for 10 min at 800 rpm, the supernatant discarded and the cells resuspended in 15 ml fresh medium.

With S-9 mix

Normally growing cell cultures were sedimented by centrifugation and for each culture 1.8 x 10^6 cells were resuspended in 2.5 ml medium containing the test substance (see 6.1) in order to obtain the appropriate final concentrations (see 7). To each culture was then added 0.5 ml S-9 mix. The cultures were incubated at 37°C under gentle shaking for 3 hours, whereafter the cells were sedimented by centrifugation for 10 min at 800 rpm, the supernatant discarded and the cells resuspended in 15 ml fresh medium to obtain a density of 3 x 10^5 cells/ml.

6.3 Plating

After the exposure period a small sample of cells from each culture was diluted and seeded in a microtiter plate for determination of the cloning efficiency (2 cells/well). During the phenotypic expression period of 2 days each culture was diluted daily and the growth rate was recorded. At the end of the expression period, the cultures were diluted and a sample of cells was seeded in two microtiter plates

(2000 cells/well) in medium containing 4 µg/ml trifluorothymidine, while another sample was seeded in two microtiter plates in normal medium (2 cells/well) for determination of the cloning efficiency.

After 10 days incubation (5% CO_2, 37°C) the number of cell clones were counted for each dose as well as for negative and positive controls. The clones were differentiated into large clones (L) and small dense clones (S) because these two types of clones have been shown to represent gene mutations and chromosomal deletions, respectively.

7. MUTAGENICITY ASSAY

7.1 Preliminary toxicity test

The dose range for the mutagenicity assay was determined on the basis of a preliminary toxicity test. A series of duplicate cultures were dosed as described in 6.2 using the doses of 5.0, 2.5, 1.3 and 0.63 mg/ml without S-9 mix and 4.2, 2.1, 1.0 and 0.52 mg/ml with S-9 mix (final concentrations) in medium and incubation mixture, respectively.

After treatment the cultures were diluted and a sample of cells from each culture seeded in a 96-well microtiter plate (2 cells/well) for determination of the cloning efficiency. In addition the growth rates of the cultures were monitored for a period of 2 days after treatment.

After 8 - 10 days incubation (5% CO_2, 37°C) the number of cell clones were counted and the cloning efficiencies determined.

The growth rates after dosing with and without S-9 mix were similar for the control and test cultures and there was no difference in cloning efficiency between control and test cultures.

7.2 Mutagenicity test

The Glyphosate was tested in two independent assays each performed using 4 dose levels with and without metabolic activation (S-9 mix). Duplicate cultures were used for each dose level. Solvent controls and positive controls were included in both assays. As positive control without S-9 mix 100 µg/ml ethylnitrosourea (ENU) was used and as positive control with S-9 mix 5 µg/ml dimethylbenzanthracene (DMBA) was used in the first test series and 10 µg/ml DMBA in the second test series.

The doses were chosen on the basis of the results of the preliminary toxicity test. Since no toxicity was observed at the doses of 5.0/4.2 mg/ml, these doses were chosen as highest doses (see section 3).

The four dose levels included in both assays were 5.0, 2.5, 1.3 and 0.63 mg/ml without S-9 mix and 4.2, 2.1, 1.0 and 0.52 mg/ml with S-9 mix (final concentrations) in medium and incubation mixture, respectively.

The cloning efficiency and the mutation frequency were calculated using the formulae:
$CE = -1/2 \ln X/N$ and $MF = -1/2000 \ln X/N$.
(X = number of empty wells, N = number of wells seeded).

The mutation frequency was further corrected by division with the corresponding CE.

8. RESULTS

The results are presented in tables 1 - 2.

The control mutation frequency was in the expected range in all test series and all positive control cultures were clearly positive. Since a relatively moderate effect was seen at 5.0 µg/ml DMBA (with S-9 mix) in the first test series, it was decided to increase the dose to 10 µg/ml in the second test series. The higher dose of DMBA gave a much stronger response although the survival was corresponding lower. In any case the positive controls with and without S-9 mix clearly demonstrated the efficiency of the test system in both test series.

The mutation frequencies of the test cultures were generally similar to the control cultures in both test series with as well as without S-9 mix. Statistical analysis using the Analysis of Variances method on the corresponding test and control cultures revealed no statistically significant differences (5% level) in any of the test series with or without S-9 mix.

9. CONCLUSION

The test article, Glyphosate, batch 206-JaK-25-1, was found non-mutagenic in the In Vitro Mammalian Cell Gene Mutation Test.

10. REFERENCES

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

(2) Handbook of Mutagenicity Test Procedures, chapter 11: Procedures for the L5178Y/TK$^{+/-}$ → TK^{-} / mouse lymphoma cell mutagenicity assay, N.T. Turner et. al, Edt. B.J. Kilbey et.al., Elsevier (1984).

(3) D. Clive et. al., Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity, Mutation Research 189, pp. 143 - 156 (1987).

(4) W.J. Caspary et. al., The mutagenic activity of selected compounds at the TK locus: rodent vs. human cells, Mutation Research 196 pp. 61 - 81 (1988).

(5) J. Cole, et al., Comparative induction of gene mutations and chromosome damage by 1-methoxy-1,3,5-cycloheptatriene (MCHT), 2. Results using L5178Y mouse lymphoma cells to detect both gene and chromosome damage; validation with ionizing radiation, methyl methanesulphonate, ethyl methanesulphonate and benzo(a)pyrene, Mutation Research, 230, pp. 81 - 91 (1990).

TABLE 1. EXPERIMENT 1, WITHOUT S-9 MIX :

DOSE MG/ML	CLONING EFFICIENCY DAY 0 PLATING	CLONING EFFICIENCY DAY 2 PLATING	CLONES IN TFT (192 WELLS) SMALL	LARGE	MUTATION FREQUENCY (PER 10000 CELLS) SMALL	LARGE	TOTAL
0.0	0.84	0.87	13	13	0.40	0.40	0.84
0.0	0.90	0.69	15	18	0.59	0.71	1.4
5.0	0.90	0.69	8	16	0.31	0.63	0.97
5.0	0.87	0.90	11	20	0.33	0.61	0.98
2.5	0.78	0.84	14	20	0.45	0.66	1.2
2.5	0.84	0.69	14	18	0.55	0.71	1.3
1.3	0.96	0.67	14	18	0.57	0.74	1.4
1.3	0.90	0.71	13	20	0.49	0.78	1.3
0.61	1.00	0.87	17	12	0.53	0.37	0.9
0.61	0.96	0.87	15	15	0.47	0.47	1.0
ENU (1)	0.71	0.67	30	98	1.3	5.3	8.2
ENU	0.76	0.76	29	97	1.1	4.6	7.0

TABLE 2. EXPERIMENT 1, WITH S-9 MIX :

DOSE MG/ML	CLONING EFFICIENCY DAY 0 PLATING	CLONING EFFICIENCY DAY 2 PLATING	CLONES IN TFT (192 WELLS) SMALL	LARGE	MUTATION FREQUENCY (PER 10000 CELLS) SMALL	LARGE	TOTAL
0.0	0.87	0.81	19	20	0.64	0.68	1.4
0.0	0.76	0.71	19	22	0.73	0.86	1.7
4.2	0.69	0.81	8	15	0.26	0.50	1.1
4.2	0.87	0.71	26	24	1.0	0.94	2.1
2.1	1.00	0.81	15	15	0.50	0.50	1.0
2.1	0.87	0.71	19	21	0.73	0.82	1.6
1.0	0.93	0.67	15	16	0.61	0.65	1.3
1.0	0.84	0.71	15	24	0.57	0.94	1.6
0.52	0.93	0.74	15	23	0.55	0.86	1.5
0.52	0.87	0.74	14	22	0.51	0.82	1.4
DMBA(2)	0.55	0.52	31	47	1.7	2.7	5.0
DMBA	0.37	0.43	30	39	2.0	2.6	5.2

(1) POSITIVE CONTROL, 100 uG/ML ENU.
(2) POSITIVE CONTROL, 5.0 uG/ML DMBA.

TABLE 3. EXPERIMENT 2, WITHOUT S-9 MIX :

DOSE MG/ML	CLONING EFFICIENCY DAY 0 PLATING	CLONING EFFICIENCY DAY 2 PLATING	CLONES IN TFT (192 WELLS) SMALL	LARGE	MUTATION FREQUENCY (PER 10000 CELLS) SMALL	LARGE	TOTAL
0.0	0.65	0.62	16	26	0.70	1.2	2.0
0.0	0.63	0.63	25	27	1.1	1.2	2.5
5.0	0.60	0.53	17	26	0.88	1.4	2.4
5.0	0.63	0.53	23	35	1.2	1.9	3.4
2.5	0.62	0.50	21	29	1.2	1.6	3.0
2.5	0.63	0.50	17	24	0.93	1.3	2.4
1.3	0.65	0.53	23	32	1.2	1.7	3.2
1.3	0.65	0.57	20	28	0.97	1.4	2.5
0.61	0.63	0.50	25	24	1.4	1.3	2.6
0.61	0.60	0.55	30	17	0.86	0.86	2.6
ENU (1)	0.53	0.49	30	103	1.7	7.8	12
ENU	0.52	0.37	40	79	3.2	7.2	13

TABLE 4. EXPERIMENT 2, WITH S-9 MIX :

DOSE MG/ML	CLONING EFFICIENCY DAY 0 PLATING	CLONING EFFICIENCY DAY 2 PLATING	CLONES IN TFT (192 WELLS) SMALL	LARGE	MUTATION FREQUENCY (PER 10000 CELLS) SMALL	LARGE	TOTAL
0.0	0.63	0.53	24	36	1.3	2.0	3.5
0.0	0.60	0.55	17	38	0.86	2.0	3.1
4.2	0.62	0.58	23	43	1.1	2.2	3.6
4.2	0.62	0.62	24	32	1.1	1.5	2.8
2.1	0.57	0.50	24	44	1.3	2.6	4.2
2.1	0.50	0.54	29	39	1.5	2.1	4.0
1.0	0.57	0.50	26	30	1.5	1.7	3.4
1.0	0.58	0.49	17	32	0.95	1.9	3.0
0.52	0.57	0.53	20	31	1.0	1.7	2.9
0.52	0.52	0.60	18	29	0.82	1.4	2.3
DMBA(2)	0.09	0.04	15	31	10	22	34
DMBA	0.07	0.02	13	20	45	28	47

(1) POSITIVE CONTROL, 100 uG/ML ENU.
(2) POSITIVE CONTROL, 10.0 uG/ML DMBA.

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IN VITRO MAMMALIAN CELL GENE MUTATION TEST PERFORMED WITH MOUSE LYMPHOMA CELLS (L5178Y)

(FIFRA guidelines, subdivision F, § 84-2)

Protocol

Test article: Glyphosate technical
Batch 206-JaK-25-1

Sponsor: Cheminova Agro A/S
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Date: 07.02.1991

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IN VITRO MAMMALIAN CELL GENE MUTATION TEST PERFORMED WITH MOUSE LYMPHOMA CELLS (L5178Y)

Test article: Glyphosate technical
Batch 206-JaK-25-1

Sponsor: Cheminova Agro A/S

Protocol approval

Scantox A/S:

Issued by: [redacted] Date: 08.02.1991
(Head of Genetic Toxicology Unit)

Sponsor:

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

Approved by: [redacted] Date 11/2-1991

Details concerning the experiment are described in the following protocol:

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Authorization no. 145

Lab. No. 12325

IN VITRO MAMMALIAN CELL GENE MUTATION TEST PERFORMED WITH MOUSE LYMPHOMA CELLS (L5178Y)

Protocol

1. INTRODUCTION

The in vitro mammalian cell gene mutation 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 "In Vitro Mammalian Cell Gene Mutation Test" No. 476, 1983 (1), as well as US CFR part 700 (F) § 798.5265 (1987).

2. TEST SYSTEM

The test substance is added in varying concentrations to cell cultures of mouse lymphoma cells (L5178Y). After an exposure period of 4 hours without metabolic activation and 3 hours with metabolic activation the cells are spun down and resuspended in fresh medium. Four dose levels are used with and without metabolic activation.

The dose range is selected on the basis of a preliminary toxicity test in which the cell-growth is measured for a period of up to 3 days after exposure. If possible the maximum dose is chosen so the survial (cloning efficiency) is approximately 20%, and so the cells grow at a normal rate before the end of the 3 day period. If the test article is of limited solubility and causes no toxicity, the maximum dose may be chosen as the highest dose which can be added without precipitation of the test article or without toxic effects of the solvent. In any case 5 mg/ml or 10 mM is generally considered the highest dose relevant to include in the assay.

Before the mutagenicity assay the cells are grown for 1 day in medium containing thymidine (9 µg/ml), hypoxanthine (15 µg/ml), methotrexate (0.3 µg/ml) and glycine (22.5 µg/ml) (THMG-medium). After a recovery period of 1 - 3 days in medium containing the above mentioned chemicals except methotrexate (THG-medium), all the cells will have the genotype $TK^{+}/^{-}$ with respect to the heterozygous thymidine kinase gene.

In the mutagenicity test a series of cell cultures are exposed to the test substance as described above and subsequently grown for a period of 2 - 3 days. The period of 2 - 3 days is nescesary for the phenotypic expression of mutations induced in the TK-gene (2). The cell cultures are then seeded (cloned) in 96-well microtiter plates in medium containing trifluorothymidine (TFT) (2000 cells/well) as well as in normal medium (2 cells/well). Because only cells that have mutated from $TK^{+}/^{-}$ to $TK^{-/-}$ can grow in medium containing TFT the mutation frequency can be determined on the basis of the number of cell clones in each type of medium after 8 - 10 days incubation.

3. CELLS AND CULTURE METHODS

The assay is performed with the mouse lymphoma cell line L5178Y received from [redacted] University of Sussex, BN1 9RR, UK. The cell line has been used in a large number of gene mutation assays.

The cells are grown as suspension cultures in RPMI 1640 medium supplemented with 10% horse serum (Flow or Gibco), 200 µg/ml sodium pyruvate and 50 µg/ml gentamycin.

The doubling time during optimal growth is approximately 12 hours. Sterile NUNC plastic flasks and microtiter plates are used and incubation is carried out in a humidified CO_2-incubator (5% CO_2) at 37°C. During normal maintenance and during the phenotypic expression period (see section 2) the cells are diluted daily and and kept at a density of 2 x 10^5 to 1.5 x 10^6 cells/ml.

4.1 Frozen stock cultures

Frozen stock cultures are kept in a liquid nitrogen tank at -196°C. New stock cultures are made periodically. To cell suspensions of approx. 10^6 cells/ml is added 1 ml of DMSO per 10 ml of cell suspension. Freezing is accomplished by use of freezer (-20°C), so the temperature decreases approximately 1°C per min until -20°C is reached, whereafter the vials are transferred to the liquid nitrogen tank. Thawing is performed in a 37°C water bath.

Before new stock cultures are used in assays, the cells are checked for general morphology, growth characteristics and absence of mycoplasma.

5. METABOLIC ACTIVATION

Some chemicals do not exert a mutagenic effect in this system, unless they are activated by mammalian enzymes. The activation is accomplished by incubating the cells for 3 hours under gentle shaking together with the test compound and rat liver post mitochondrial fraction supplemented with salts and co-factors (S-9 mix). The microsome fraction is obtained from rats pretreated with Aroclor® 1254.

The test is carried out with and without metabolic activation.

5.1 S-9 fraction (rat liver homogenate)

SPF Wistar rats of the strain Mol:WIST are obtained from Møllegaard Breeding Center Ltd, Ejby, DK-4623 Lille Skensved. Rats weighing approximately 200 g are used for induction of liver enzymes. A single intraperitonal injection of Aroclor® 1254 at a dose of 500 mg/kg body weight is given to each rat. The animals are killed 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, rinsed briefly in 0.15 M KCl, and minced in 0.15 M KCl solution (3.0 ml per gram wet liver). After homogenization, the homogenate is centrifugated 9000 x g for 15 minutes. The supernatant (S-9 fraction) is decanted, frozen and stored at -196°C until use.

5.2 S-9 mix (20 ml)

S-9 fraction (rat liver homogenate)	6.0	ml
Hepes buffer (1 M, pH 7.2)	0.4	ml
$MgCl_2$ (50 mM)	2.0	ml
KCl (330 mM)	2.0	ml
Glucose-6-phosphate (0.1 M)	0.6	ml
NADP (0.1 M)	0.8	ml
Distilled H_2O	8.0	ml
Phenol red, sodium salt (0.5 mg/ml)	0.2	ml

The S-9 fraction is added after adjustment of pH to 7.2 with NaOH or HCl. The S-9 mix is kept on ice, and warmed to 37°C immediately before addition.

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6. TEST PROCEDURES

6.1 Test article

The test article is: Glyphosate technical
Batch 206-JaK-25-1
Purity 98.6%

The test substance is a white powder.

The powder will be stored at room temperature protected from light until use.

6.2 Dosing of cultures

The test substance is dissolved in culture medium, water, DMSO, ethanol or another suitable solvent.

Without S-9 mix

For each dose duplicate cultures containing 3 x 10^5 cells/ml are set up in normal medium. For weakly or non-toxic dose levels as well as for negative and positive controls are used 15 ml cultures, while 45 ml cultures are used for strongly toxic doses. The test solution is added in a volume of maximum 1% (2% if the solvent is water)* and the cultures are incubated under gentle shaking for 4 hours at 37°C. After the incubation period the cells are spun down by centrifugation for 10 min at 800 rpm, the supernatant discarded and the cells resuspended in the original volume of fresh medium (15 or 45 ml).

With S-9 mix

For each dose duplicate cell suspensions of 2 x 10^6 cells/ml are set up in normal medium. For weakly or non-toxic dose levels and negative and positive controls are used 2.5 ml cell suspensions while 7.5 ml cell suspension are used for strongly toxic doses. To each culture is added 0.5 ml S-9 mix and the test solution in a volume of maximum 1% (2% if water)*. The cultures are incubated at 37°C under gentle shaking for 3 hours, whereafter the cells are spun down by centrifugation for 10 min at 800 rpm, the supernatant discarded and the cells resuspended in fresh medium at a density of 3 x 10^5 cells/ml.

* If the test article is dissolved in culture medium larger volumes may be used because the test solution can replace part of the cell suspension medium.

6.3 Preliminary toxicity test

The dose range to be used in the mutagenicity assay is determined in a preliminary toxicity test. A series of duplicate cultures are dosed with and without S-9 mix as described in 6.2. When convenient cultures smaller than 15 ml cells may be used since the mutant number is of no importance in this connection.

After treatment the cultures are diluted and a sample of cells from each culture is seeded in a 96-well microtiter plate (2 cells/well) for determination of the cloning efficiency. In addition the growth rates of the cultures are monitored for a period of 1 -3 days after treatment.

After 8 - 10 days incubation (5% CO_2, 37°C) the number of cell clones are counted and the cloning efficiencies d[illegible]nined.

6.4 Mutagenicity test

The test article is tested in two independent assays each performed using 4 dose levels with and without

metabolic activation (S-9 mix). For each dose level duplicate cultures are used. Solvent conctrols and positive controls are included in both assays. As positive control without S-9 mix is used 100 µg/ml ethylnitrosourea (ENU) and as positive control with S-9 mix is used 5 µg/ml dimethylbenzanthracene (DMBA).

The four dose levels are chosen on the basis of the preliminary toxicity test using the criteria described in section 2, i.e. the dose interval ranges from a strongly cytotoxic level to a non-toxic level, if possible.

After the exposure period the cultures are diluted and a sample of cells is seeded in a microtiter plate for determination of the cloning efficiency (2 cells/well). During the phenotypic expression period each culture is diluted daily and the growth rate is recorded. At the end of the expression period the cultures are diluted and a sample of cells is seeded in two microtiter plates (2000 cells/well) in medium containing 4 µg/ml triflourothymidine, while another sample is seeded in two mictotiter plates in normal medium (2 cells/well) for determination of the cloning efficiency.

After 8 - 10 days incubation (5% CO_2, 37°C) the number of cell clones are counted and the mutation frequencies determined for each dose and for the negative and positive controls.

7. EVALUATION OF RESULTS

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

a. Statistically significant and reproducible increase in the mutation frequency of test cultures as compared to the negative control cultures, (Chi-square tests).

b. Dose-response is seen.

c. The mutation frequency at the dose level where the highest effect is found should be more than twice the concurrent spontaneous mutation frequency.

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. If a is not met the test article is considered to be non mutagenic.

8. 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.

9. REPORT

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

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10. ARCHIVES

All original data, including copies of correspondance, 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.

11. LITERATURE

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

(2) Handbook of Mutagenicity Test Procedures, chapter 11: Procedures for the L51784/TK$^{+/-}$ -> TK$^{-/-}$ mouse lymphoma cell mutagenicity assay, N.T. Turner et. al, Edt. B.J. Kilbey et.al., Elsevier (1984).

(3) D. Clive et. al., Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity, Mutation Research 189, pp. 143 - 156 (1987).

(4) W.J. Caspary et. al., The mutagenic activity of selected compounds at the TK locus: rodent vs. human cells, Mutation Research 196 pp. 61 - 81 (1988).

(5) J. Cole, et al., Comparative induction of gene mutations and chromosome damage by 1-methoxy-1,3,5-cycloheptatriene (MCHT), 2. Results using L5178Y mouse lymphoma cells to detect both gene and chromosome damage; validation with ionizing radiation, methyl methanesulphonate, ethyl methanesulphonate and benzo(a)pyrene, Mutation Research, 230, pp. 81 - 91 (1990).

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Lab. No. 12325

IN VITRO MAMMALIAN CELL GENE MUTATION TEST

Test article: Glyphosate, batch 206-JaK-25-1

Sponsor: Cheminova Agro A/S

Amendment No. 1

to Protocol

1. Protocol page 5, section 6.2: with S-9 mix, 1. line: "...cell suspensions of 2 x 10^5 cells/ml are set up" is changed to "cell suspensions of 1.8 x 10^5 cells/ml are set up".

   The change is made in order to clearly indicate that the same number of cells are treated with and without S-9 mix.

2. Protocol page 6, section 6.4: In the end of the section the following amendment is added:

   During the counting a distinction is made between "large" (L) and "small" (S) clones in order to make possible a separate analysis for supposed gene mutations (large clones) and chromosomal deletions (small clones).

3. Protocol page 3, section 2, line 8-9: concerning the statement: "In any case 5 mg/ml or 10 mM..."

   At the dosing with S-9 mix in the preliminary toxicity test and in both test series 0.5 ml S-9 mix was added to test tubes with 2.5 ml cell suspension containing 5.0 mg/ml Glyphosate.

   Thus, the actual concentration in the incubation mixture was 4.2 mg/ml and not as originally intended 5.0 mg/ml.

   Since 4.2 mg/ml is in any case an extremely high dose and since the intended dose of 5.0 mg/ml was more or less arbitrarily chosen as the maximum dose, the slightly lower dose used should make no difference for the interpretation of the results.

Approved by:

St[redacted]

02.07.1991
Date

[redacted]
Sponsor

4/7-91
Date