CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

Sponsor: Zeneca Agrochemicals
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CTL Ref: Y04707/034
CTL Study No: SM0796

REPORT NO: CTL/P/4954

GLYPHOSATE ACID: MOUSE BONE MARROW MICRONUCLEUS TEST

by

[redacted]

Date of Issue: 21 March 1996

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STATEMENT OF DATA CONFIDENTIALITY CLAIM

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STATEMENT OF GLP COMPLIANCE

The study was conducted in compliance with the UK Principles of Good Laboratory Practice (The United Kingdom GLP Compliance Programme, Department of Health 1989) except for the deviations listed below. These Principles are in accordance with the OECD Principles of Good Laboratory Practice (1982) ISBN 9264 12367 9 (OECD Environment Monograph No 45, OCDE/GD(92)32) and are in conformity with, and implement, the requirements of the European Commission (Directives 87/18/EEC and 88/320/EEC).

These international standards are acceptable to the United States Environmental Protection Agency and this study, therefore, satisfies the requirements of 40 CFR Part 160.

The following GLP deviations are considered not to affect the integrity of the study or the validity of the conclusions drawn:

(i) the stability, homogeneity and achieved concentration of the test and control substances in the vehicles used were not determined by analysis

(vi) certified purity and stability of the control substances are not available.

[redacted] 21 March 1996

Study Director

This page may be required by some regulatory authorities.

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QUALITY ASSURANCE STATEMENT

In accordance with Zeneca policy and QA procedures for Good Laboratory Practice, this report has been audited and the conduct of this study has been inspected as follows:

Date	Date of QA Report
15 Mar 96	15 Mar 96
25 Mar 96	25 Mar 96

In addition, procedure inspections associated with this type of study were made as follows:

4 Oct 95	4 Oct 95
28 Nov 95	28 Nov 95
28 Nov 95	28 Nov 95
30 Nov 95	30 Nov 95
14 Dec 95	14 Dec 95
18 Dec 95	18 Dec 95

Facilities and process based procedures associated with this type of study were inspected in accordance with QA Standard Operating Procedures.

So far as can be reasonably established, the methods described and the results given in the final report accurately reflect the raw data produced during the study, SM0796

[redacted] (Unit Head, CTL Quality Assurance Unit) .. [redacted] ... 25 Mar 96

3166239

I, the undersigned declare that this report constitutes a true record of the actions undertaken and the results obtained in the above study.

[redacted] (Study Director) [redacted] 21 March 1996

[redacted] was Study Director from the start of this study until 4 January 1996. [redacted] was appointed as Study Director from 5 January 1996 to 7 January 1996 to cover [redacted] over this period. [redacted] was re-appointed as Study Director from 8 January 1996 until 13 March 1996 [redacted] assumed the responsibilities of Study Director from 14 March 1996.

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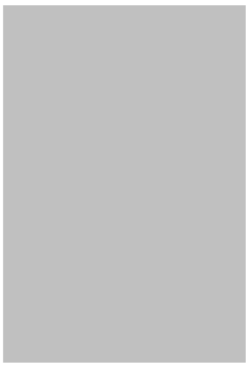
The following contributed to this report in the capacities indicated:



- Study Investigator
- Home Office Licensee
- Statistician
- Study Reviewer

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GLYPHOSATE ACID: MOUSE BONE MARROW MICRONUCLEUS TEST

SUMMARY

Glyphosate acid has been evaluated for its ability to induce micronucleated polychromatic erythrocytes in the bone marrow of CD-1 mice. A single oral dose was given to groups of 5 male and 5 female mice at a dose level of 5000mg/kg; this being the limit dose for this assay. Bone marrow samples were taken 24 and 48 hours after dosing.

No statistically or biologically significant increases in the incidence of micronucleated polychromatic erythrocytes, over the vehicle control values, were seen in either sex at either of the sampling times investigated.

Comparison of the percentage of polychromatic erythrocytes showed no statistically or biologically significant differences in either sex at either of the sampling times between the vehicle control animals and those treated with glyphosate acid.

The test system positive control, cyclophosphamide, induced statistically significant and biologically meaningful increases in micronucleated polychromatic erythrocytes, compared to the vehicle control values, thus demonstrating the sensitivity of the test system to a known clastogen.

It is therefore concluded that glyphosate acid, under the conditions of test, is not clastogenic in the mouse micronucleus test.

1. INTRODUCTION

Glyphosate acid was tested for its ability to induce clastogenic effects using the mouse bone marrow micronucleus test.

The micronucleus test is capable of detecting the clastogenic effect of a chemical. After chromosomal damage has been induced by a test compound or its metabolites, acentric fragments of chromosomal material lag behind at anaphase. At telophase a large proportion of these fragments is not included in the main daughter nuclei. This can result in the formation of small secondary nuclei or micronuclei.

Micronuclei can be formed in a wide variety of cell types, but in this test system bone marrow erythrocytes are observed because micronuclei can easily be detected in this cell type, since the nucleus proper is extruded during maturation.

A few hours after their last division is completed, erythroblasts expel their nuclei and become polychromatic erythrocytes. The term polychromatic is derived from the reaction of the cell with Romanovsky stains; residues of nucleic acids remain for a short time after the expulsion of the nucleus causing the cell to stain a blue-grey colour, whereas the mature erythrocyte appears pink.

Polychromatic erythrocytes are useful for the detection of clastogenic chemicals because they persist for only 24 hours before maturing into normochromatic erythrocytes. Consequently, any micronuclei in these cells will have been produced at the last mitotic division and their formation will be due to the effects of the chemical in the preceding 48 hours.

The clastogenic potential of glyphosate acid was assessed in the micronucleus assay, following its administration as a single oral dose, as recommended in OECD 474 (1983), US EPA (1991) and EEC Annex V B12 (1992). The established clastogen, cyclophosphamide, was used as a positive control in order to demonstrate the sensitivity of the test system.

All data pertaining to this study are stored in the Archives at Central Toxicology Laboratory (CTL), Alderley Park, Macclesfield, Cheshire, UK. A copy of this report is held by the Report Centre at the same address.

The experimental phase of this study was carried out between 11 December 1995 and 11 January 1996. The slides were analysed between 16 January and 30 January 1996.

2. EXPERIMENTAL PROCEDURES

2.1 Test Sample

The test sample of glyphosate acid (Batch Ref: P24) was obtained from Zeneca Agrochemicals, Jealott's Hill, and was submitted for testing by Zeneca Agrochemicals, Fernhurst. It had Sponsor reference 17984 and was given CTL reference number Y04707/034. The test sample was supplied as a white solid with a certified purity of 95.6% w/w (Analytical reference M0118, dated 2 February 1995).

The test substance was stored in anti-static bags at ambient temperature in the dark until required. From the information supplied by the Sponsor, the test substance was used within the stated expiry date. The test substance was tested with no correction for purity.

2.2 Control Chemicals

The positive control, cyclophosphamide, was supplied by Sigma Chemical Company Ltd, Fancy Road, Poole, UK, and was given the CTL reference number Y01259/007.

The vehicle control was physiological saline, supplied by Central Dispensary, CTL, and was given the CTL reference number Y06538/003.

2.3 Preparation of Dosing Solutions/Suspensions

Dosing suspensions of the test material and a dosing solution of cyclophosphamide were prepared in physiological saline immediately prior to use. All dosing preparations were administered at a volume of 20ml/kg bodyweight (0.2ml/10g).

2.4 Animals and Husbandry

Male and female CD-1 mice in the age range of 4-12 weeks were used for Phase I and mice in the age range 6-7 weeks were used for Phase II of the study. The animals were supplied by Charles River Breeding Laboratories, Margate, UK.

On arrival the mice were housed by sex with up to 5 per cage on mobile mouse cage racks and given food, CT1 (supplied by Special Diets Services, Stepfield, Witham, Essex, UK; Appendix A) and water (via an automatic water system) ad libitum.

The animal rooms used for Phases I and II are designed to be maintained within a temperature range of 19-23°C, and within a relative humidity range of 40-70%. Temperature and relative humidity were monitored and the data were recorded in the animal husbandry diaries. Lighting was controlled to provide 12 hours artificial light followed by 12 hours darkness. The animal room was under positive pressure with respect to the access corridor and had at least 15 air changes per hour.

2.5 Test Method

2.5.1 Study Design: Phase I involved the determination of a maximum tolerated dose (MTD), based on patterns of lethalities or severe toxicity observed over a four-day observation period following a single oral dose as shown in Appendix B.

After acclimatisation, the mice for Phase II were randomly distributed on to racks according to the rack plan in Appendix C. The animals were identified by cage cards and by ear punching.

In Phase II, male and female animals were weighed and given a single oral dose of physiological saline, cyclophosphamide (65mg/kg) or glyphosate acid at a dose level of 5000mg/kg bodyweight as detailed in Appendix D. The bodyweights were recorded prior to dosing and are detailed in Appendix H.

2.5.2 Summary of Methodology: Bone marrow smears were prepared 24 and 48 hours after dosing for the vehicle control and glyphosate acid treated animals and 24 hours after dosing for the cyclophosphamide treated animals. The preparations were stained with polychrome methylene blue and eosin to visualise the various cell types. Two thousand polychromatic erythrocytes per slide were evaluated for the presence of micronuclei. In addition 1000 erythrocytes were counted to determine the percentage of polychromatic erythrocytes in the total erythrocyte population. This provides an indication of any cytotoxicity in the bone marrow. Detailed methodology is shown in Appendix E.

2.6 Statistical Analyses

The incidence of micronucleated polychromatic erythrocytes and percentage polychromatic erythrocytes in the erythrocyte sample, were considered by analysis of variance at 24 and 48 hours, separately for males and females.

The data for the incidence of micronucleated polychromatic erythrocytes were transformed using a square root transformation, prior to analysis. The data for the percentages of polychromatic erythrocytes were transformed using the double arcsine transformation of Freeman and Tukey (1950), prior to analysis.

Analyses were carried out using the GLM procedure in SAS (1989). Each treatment group mean was compared with the control group mean at the corresponding sampling time using a one-sided Student's t-test, based on the error mean square in the analysis.

3. RESULTS

3.1 Phase I - Determination of the Maximum Tolerated Dose

A group of 5 males and 5 females was dosed with glyphosate acid at 5000mg/kg (Appendix B). As no clinical signs or lethalities were observed over a four day observation period, at the limit dose level of 5000mg/kg, this was selected to represent the maximum tolerated dose for both males and females.

3.2 Phase II - Micronucleus Test

The data for individual animals are shown in Appendices F and G and the group data are summarised in Tables 1-4.

No adverse reactions to treatment were observed for either males or females dosed with glyphosate acid at the limit dose of 5000mg/kg.

No statistically or biologically significant increases in the incidence of micronucleated polychromatic erythrocytes, over the vehicle control values, were observed in either males or females at either sampling time investigated.

No statistically significant differences in the percentage of polychromatic erythrocytes, between the vehicle control and glyphosate acid treated animals, were observed in either males or females at either sampling time investigated.

The test system positive control, cyclophosphamide, induced statistically and biologically significant increases in the frequency of micronucleated polychromatic erythrocytes in both male and female mice at the 24 hour sampling time.

4. DISCUSSION

The criteria for a valid test system as laid down by OECD Guideline 474 (1983), US EPA (1991) and EEC Annex V B12 (1992) for the conduct of micronucleus studies, are that the positive control substance should induce a significant elevation in micronucleated polychromatic erythrocytes compared to the vehicle control values, and that the test material should be tested at a level that causes a decrease in the percentage of polychromatic erythrocytes (indicating a cytotoxic effect on the bone marrow) or at the maximum tolerated dose level.

The study satisfies these criteria in that glyphosate acid was tested at the limit dose level of 5000mg/kg in both males and females. The positive control substance, cyclophosphamide, gave a statistically significant and biologically meaningful increase in micronucleated polychromatic erythrocytes, compared to vehicle control values.

No statistically or biologically significant increases in the incidence of micronucleated polychromatic erythrocytes, compared to the vehicle control values, were seen in any glyphosate acid treated mice at either of the sampling times investigated.

The data from the study do not indicate any clastogenic activity of glyphosate acid in the mouse bone marrow when tested up to the limit dose of 5000mg/kg in both male and female mice.

5. CONCLUSION

Under the conditions of test, glyphosate acid is not clastogenic in the mouse bone marrow micronucleus test.

6. REFERENCES

Annex V to Council Directive 67/548/EEC on the approximation of law, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances, published in the Seventeenth Adaptation, Commission Directive 92/69/EEC of 31st July 1992, OJ L383A 29.12.92 : B12 (Mutagenicity Micronucleus Test)

Freeman M F and Tukey J W (1950). Transformations related to the angular and the square root. Annals of Maths Stats 21, 607

OECD Guidelines for Testing of Chemicals (1983). Genetic Toxicology: Micronucleus Test - No 474.

SAS Institute Inc. SAS/STAT User's Guide (1989). Version 6, Fourth Edition, Volume 2, Cary, NC: SAS Institute Inc.

US Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation Human and Domestic Animals, Series 84, Mutagenicity, Addendum 9. Publication No. EPA-540/09-91-122 NTIS Report No. PB91-158394 Feb 1991 (as specified in US Environmental Protection Agency Code of Federal Regulations 40CFR Part 798 Health Effects Testing Guidelines, Subpart F - Genetic Toxicology).

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TABLE 1

MEAN INCIDENCE OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES/1000 POLYCHROMATIC ERYTHROCYTES ± STANDARD DEVIATION (SD) AT TWO SAMPLING TIMES

GROUP MEAN ANIMAL DATA - MALES

Group	Compound	Dose	Mean Incidence of MPE/1000 PE ± SD	
			24 hours	48 hours
11	Vehicle Control	20ml/kg	1.6 ± 0.8	1.7 ± 1.3
12	Cyclophosphamide	65mg/kg	22.2 ± 6.1**	
13	Glyphosate acid	5000mg/kg	2.1 ± 1.6	2.1 ± 1.9

PE = polychromatic erythrocytes.
MPE = micronucleated polychromatic erythrocytes.
SD = standard deviation.

** Statistically significant increase in micronucleated polychromatic erythrocytes at $p<0.01$ in the Student's 't' test (one-sided) on transformed data.

All means based on 10 observations (2 counts of 1000 PE per animal).

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TABLE 2

MEAN INCIDENCE OF MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES/1000 POLYCHROMATIC ERYTHROCYTES ± STANDARD DEVIATION (SD) AT TWO SAMPLING TIMES

GROUP MEAN ANIMAL DATA - FEMALES

Group	Compound	Dose	Mean Incidence of MPE/1000 PE ± SD	
			24 hours	48 hours
11	Vehicle Control	20ml/kg	1.4 ± 0.7	0.7 ± 0.6
12	Cyclophosphamide	65mg/kg	23.3 ± 4.9**	
13	Glyphosate acid	5000mg/kg	2.1 ± 2.5	0.8 ± 0.8

PE = polychromatic erythrocytes.
MPE = micronucleated polychromatic erythrocytes.
SD = standard deviation.

** Statistically significant increase in micronucleated polychromatic erythrocytes at $p<0.01$ in the Student's 't' test (one-sided) on transformed data.

All means based on 10 observations (2 counts of 1000 PE per animal).

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TABLE 3

MEAN PERCENTAGE OF POLYCHROMATIC ERYTHROCYTES
± STANDARD DEVIATION (SD) AT TWO SAMPLING TIMES

GROUP MEAN ANIMAL DATA - MALES

Group	Compound	Dose	Mean % Polychromatic Erythrocytes ± SD	
			24 hours	48 hours
11	Vehicle Control	20ml/kg	46.0 ± 4.1	49.8 ± 4.8
12	Cyclophosphamide	65mg/kg	46.5 ± 2.0	
13	Glyphosate acid	5000mg/kg	46.4 ± 4.3	48.4 ± 4.0

SD = standard deviation.

All means based on 5 observations (1 count of 1000 erythrocytes per animal).

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TABLE 4

MEAN PERCENTAGE OF POLYCHROMATIC ERYTHROCYTES
± STANDARD DEVIATION (SD) AT TWO SAMPLING TIMES

GROUP MEAN ANIMAL DATA - FEMALES

Group	Compound	Dose	Mean % Polychromatic Erythrocytes ± SD	
			24 hours	48 hours
11	Vehicle Control	20ml/kg	46.6 ± 1.9	46.4 ± 4.4
12	Cyclophosphamide	65mg/kg	46.0 ± 4.1	
13	Glyphosate acid	5000mg/kg	42.3 ± 6.2	45.8 ± 2.9

SD = standard deviation.

All means based on 5 observations (1 count of 1000 erythrocytes per animal).

APPENDIX A

COMPOSITION OF CT1 DIET

Manufacturer - Special Diets Services Ltd, Stepfield, Witham, Essex, UK.

Dietary constituents and a proximate analysis are given below. The diet is prepared to a constant formula, details of which are available on request.

Dietary Constituents	Proximate Analysis	%
Wheat	Crude protein	20.0
Wheat feed	Crude oil	3.4
Wheat bran	Crude fibre	3.0
Maize	Moisture	9.0
Cornflour	Ash	6.0
Soya bean meal extract	Calcium	0.96
British white fish meal	Phosphorus	0.93
Skim milk powder (spray dried)		
PCD vitamin and mineral premix		

All batches of CT1 diet complied with the following contaminants specification:

Chemical Contaminant	Maximum Permitted Concentration (ppm)	Microbiological Contaminant	Maximum Permitted
Arsenic	1.0	Total viable organisms	2×10^4/ g
Cadmium	0.5		
Lead	3.0		
Mercury	0.1	Mesophilic spores	2×10^4/ g
Selenium	0.5		
DDT (total)	0.1	Salmonella sp	None / g
Dieldrin	0.02		
Heptachlor	0.01	Faecal E coli (Type 1)	None / g
Lindane	0.1		
PCB's (total)	0.05		
		Coliforms	None / g
Fluorine	40		
Nitrite	5.0	Fungal units	200 / g
Nitrate	100		
		Antibiotic activity	None / g
Aflatoxins (total)	0.001		
Malathion	0.5		

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APPENDIX B

COMPOUND ADMINISTRATION : MTD DETERMINATION

Glyphosate acid was administered as a single oral dose to a group of 5 male and 5 female mice at dose level of 5000mg/kg. The results are shown below:-

Group	Compound	Dose (mg/kg)	Sex	Animal Number	No. of deaths /No. dosed
1	Glyphosate acid	5000	Male Female	1-5 6-10	0/5 0/5

As no clinical signs or lethalities were observed at the limit dose level of 5000mg/kg, this was taken to represent the maximum tolerated dose for both males and females. This dose level was administered in Phase II of the study.

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APPENDIX C

RACK PLAN - PHASE II

Males 24h Kill	81-85 (12)	91-95 (13)	71-75 (11)
Females 24h Kill	96-100 (13)	76-80 (11)	86-90 (12)
Males 48h Kill	101-105 (11)	111-115 (13)	
Females 48h Kill	116-120 (13)	106-110 (11)	

Group numbers are shown in parentheses.

h = hour

Group 11 = Vehicle control - 20ml/kg
Group 12 = Cyclophosphamide - 65mg/kg
Group 13 = Glyphosate acid - 5000mg/kg

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APPENDIX D

ANIMAL ALLOCATION TO DOSING GROUPS - PHASE II

Group	Compound	Dose	Sex	Animal Numbers/Time of Kill	
				24 hours	48 hours
11	Vehicle Control	20ml/kg	M	71-75	101-105
			F	76-80	106-110
12	Cyclophosphamide	65mg/kg	M	81-85	
			F	86-90	
13	Glyphosate acid	5000mg/kg	M	91-95	111-115
			F	96-100	116-120

M = male
F = female

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APPENDIX E

PROCESSING OF BONE MARROW AND CRITERIA FOR IDENTIFICATION OF MICRONUCLEI

The animals were killed by asphyxiation in halothane Ph. Eur. (FLUOTHANE, Zeneca Pharmaceuticals) followed by cervical dislocation 24 and 48 hours after receiving a single oral dose of the test material.

a) Femurs were removed and stripped clean of muscle.

b) The iliac end of the femur was removed and a fine paint brush was rinsed in saline, wiped to remove the excess and wetted with a solution of albumin (6% w/v in physiological saline). This was then dipped into the marrow canal and two smears were painted on an appropriately labelled clean, dry microscope slide. This procedure was repeated to give four smears of marrow per slide. The brush was rinsed in physiological saline between animals of the same group, and a separate brush and pot of physiological saline were used between groups to avoid cross contamination.

c) The slides were allowed to air dry.

d) The slides were then stained with polychrome methylene blue and eosin using an automatic staining machine.

e) Slides were coded and scored blind, in numerical slide code order.

PROCESSING OF BONE MARROW AND CRITERIA FOR IDENTIFICATION OF MICRONUCLEI

f) Two thousand (2 x 1000) polychromatic erythrocytes were examined for the presence of micronuclei using x10 or x12.5 eye pieces and a x100 oil immersion objective lens for each animal. The slides were also examined for evidence of cytotoxicity, which may be manifest by alterations in the ratio of different cell types in the bone marrow. This was assessed by counting the ratio of polychromatic to normochromatic erythrocytes in a sample of 1000 erythrocytes.

Criteria for identification of micronuclei are as described by Schmid (1976):

(i) Spherical (or rounded) with well-defined edges.
(ii) Diameters of not less than approximately 1/20 of a polychromatic erythrocyte diameter.
(iii) Dark purple/dark blue staining.
(iv) Lie in the same plane as the polychromatic erythrocyte in which it is contained (determined by focusing).

Reference:

Schmid W (1976). The Micronucleus Test for Cytogenetic Analysis. In: A Hollaender (Ed). Chemical Mutagens : Principles and Methods For Their Detection. Vol 4, Plenum, New York 31-43.

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APPENDIX F

INDIVIDUAL ANIMAL DATA
MICRONUCLEATED POLYCHROMATIC ERYTHROCYTES/2000 POLYCHROMATIC ERYTHROCYTES

PHASE II

Group	Treatment	Dose	Sex	24 Hour Sampling Time					48 Hour Sampling Time				
11	Vehicle control	20ml/kg	Male	4	5	4	2	1	1	2	5	2	7
12	Cyclophosphamide	65mg/kg	Male	33	45	45	35	64					
13	Glyphosate acid	5000mg/kg	Male	6	2	3	1	9	1	1	4	10	5
11	Vehicle control	20ml/kg	Female	5	3	1	2	3	3	2	1	0	1
12	Cyclophosphamide	65mg/kg	Female	36	49	41	62	45					
13	Glyphosate acid	5000mg/kg	Female	1	13	3	3	1	0	0	3	3	2

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APPENDIX G

INDIVIDUAL ANIMAL DATA - % POLYCHROMATIC ERYTHROCYTES

PHASE II

Group	Treatment	Dose	Sex	24 Hour Sampling Time	48 Hour Sampling Time
11	Vehicle control	20ml/kg	Male	47.7 44.0 49.5 39.8 49.0	42.7 49.2 56.2 51.2 49.5
12	Cyclophosphamide	65mg/kg	Male	48.2 45.0 44.8 45.5 49.0	
13	Glyphosate acid	5000mg/kg	Male	44.8 51.0 41.5 51.0 43.9	43.1 47.3 54.2 47.7 49.5
11	Vehicle control	20ml/kg	Female	46.2 44.5 45.4 49.1 47.8	47.4 49.1 38.8 49.6 46.9
12	Cyclophosphamide	65mg/kg	Female	42.5 51.0 42.5 49.9 44.3	
13	Glyphosate acid	5000mg/kg	Female	38.9 40.3 47.3 50.0 35.0	42.6 44.8 49.6 48.1 43.9

APPENDIX H

INDIVIDUAL BODYWEIGHTS (g) - PHASE II

Animal Number	Bodyweight (g)	Animal Number	Bodyweight (g)
71	33.4	96	27.2
72	32.1	97	26.3
73	33.2	98	28.4
74	32.6	99	25.5
75	37.6	100	27.1
76	31.0	101	34.0
77	26.5	102	33.7
78	27.4	103	35.2
79	28.0	104	34.1
80	25.9	105	28.0
81	32.1	106	26.8
82	36.8	107	24.2
83	36.2	108	29.8
84	35.4	109	27.6
85	34.3	110	28.0
86	22.8	111	34.6
87	30.2	112	35.9
88	28.4	113	35.2
89	27.4	114	35.3
90	24.2	115	37.9
91	31.6	116	25.9
92	32.1	117	25.3
93	33.7	118	26.1
94	31.1	119	24.8
95	33.1	120	24.2