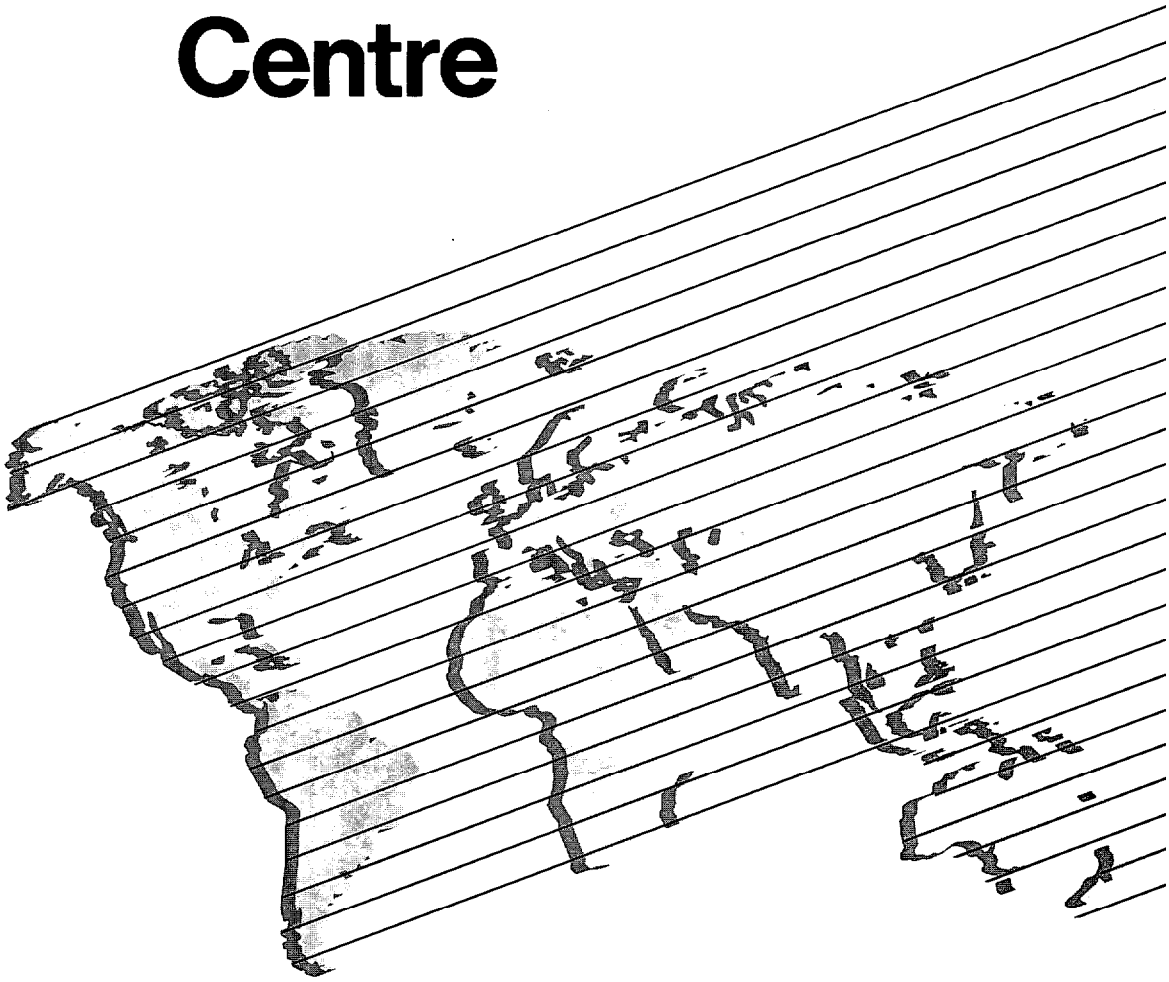
HRC Report

CHLORODIBROMOMETHANE

IN VIVO RAT LIVER
DNA REPAIR TEST

Huntingdon Research Centre



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CHLORODIBROMOMETHANE

IN VIVO RAT LIVER DNA REPAIR TEST

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Report issued 12 September 1995

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health & Social Security 1986 and subsequent revision, Department of Health 1989.

EC Council Directive, 87/18 EEC of 18 December 1986, (No. L 15/29).

Good Laboratory Practice in the testing of Chemicals OECD, ISBN 92-64-12367-9, Paris 1982, subsequently republished OECD Environment Monograph No. 45, 1992.

United States Environmental Protection Agency, (FIFRA), Title 40 Code of Federal Regulations Part 160, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

United States Environmental Protection Agency, (TSCA), Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Japan Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Federal Register, 22 December 1978, and subsequent amendments.

Japan Ministry of Health and Welfare, Notification No. Yakuhatsu 313 Pharmaceutical Affairs Bureau, 31 March 1982 and subsequent amendment Notification No. Yakuhatsu 870, Pharmaceutical Affairs Bureau, 5 October 1988.

R.J. Proudlock

12 Sept 1995

Raymond J. Proudlock, B.Sc. (Hons.), M.I.Biol.,
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QUALITY ASSURANCE STATEMENT

This report has been audited by the Huntingdon Research Centre Quality Assurance Department. The methods, practices and procedures reported herein are an accurate description of those employed at HRC during the course of the study. Observations and results presented in this final report form a true and accurate representation of the raw data generated during the conduct of the study at HRC.

Certain studies such as that described in this report are conducted at HRC in a setting which involves frequent repetition of similar or identical procedures. At or about the time the study described in this report was in progress, 'process-based' inspections were made by the Quality Assurance Department of critical procedures relevant to this study type. The findings of these inspections were reported promptly to the Study Director and to HRC Management.

Date(s) of inspection	8 - 28 March 95
Date(s) of reporting inspection findings to the Study Director and HRC Management	29 March 95
Date of reporting audit findings to the Study Director and HRC Management	30 August 95

Rod Scammell,
Audit Team Supervisor,
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11.9.95
Date

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SUMMARY

Chlorodibromomethane was assessed for induction of DNA repair in hepatocytes following acute oral administration to Specific Pathogen Free outbred albino Hsd/Ola Sprague-Dawley male rats at dosages of 600 and 2000 mg/kg bodyweight. The high dose of 2000 mg/kg chosen for the DNA repair test is the maximum level recommended by the UKEMS and by OECD guidelines and is also the OECD and EEC limit dose for acute oral toxicity testing; we consider it to be an appropriate limit for use in this system.

A negative control group was treated with the vehicle, aqueous 1% methylcellulose, and a positive control group was treated with dimethylnitrosamine at 4 mg/kg (for the 2 hour expression) or 2-acetylaminofluorene at 50 mg/kg (for the 14 hour expression). Hepatocytes were isolated by enzymatic dissociation at 2 or 14 hours after exposure of the animals to the test substance. Four animals were assessed at each experimental point, with the exception that only two animals from the positive control group were assessed at each expression time.

The isolated hepatocytes were allowed to attach to glass coverslips and were cultured *in vitro* with (methyl-^{3}H)thymidine at 10 μCi/ml for four hours to 'radiolabel' replicating DNA. The hepatocytes were 'chased' for 24 hours with unlabelled thymidine then they were fixed and processed for autoradiography.

DNA repair was assessed by comparing the labelling levels of hepatocyte nuclei from treated animals with control values and with the accompanying cytoplasmic labelling levels (usually a total of 150 cells per animal were examined).

Chlorodibromomethane did not cause any significant increases in either the gross nuclear grain count or the net nuclear grain count (*ie* the gross nuclear grain count minus the cytoplasmic grain count) at any dose level at either sampling time.

Positive control group animals showed a large and highly significant increase ($P < 0.001$) in the net nuclear grain count which was accompanied by a large increase in the gross nuclear grain count.

It is concluded that Chlorodibromomethane did not elicit any evidence of DNA-damage in the rat liver in this *in vivo* test system.

INTRODUCTION

The Drinking Water Inspectorate of the Department of the Environment has sought advice on the genotoxicity of the trihalomethanes (THMs) from the Department of Health's Committee on Mutagenicity (CoM). The CoM concluded that the *in vitro* data indicated that chlorodibromomethane had mutagenic potential. The *in vivo* data was reviewed: negative results were obtained in a bone marrow micronucleus test after intraperitoneal administration; although positive results were reported for a bone marrow metaphase study, conclusions could not be drawn because the data were regarded as questionable; a very small increase in sister chromatid exchanges was not regarded as significant. The bone marrow data was therefore considered essentially negative. The CoM concluded that results were needed from an assay using a second tissue *in vivo*, before the mutagenic potential seen *in vitro* could be discounted. The CoM recommended that an *in vivo* rat liver DNA repair test be performed.

In view of this recommendation, chlorodibromomethane was tested for potential DNA-damaging activity in the *in vivo* rat hepatocyte DNA repair system using techniques recommended by the American Society for Testing and Materials, the United Kingdom Environmental Mutagen Society and appropriate procedures from the OECD guideline for the *in vitro* version of this test:

> BUTTERWORTH, B.E. *et al* ASTM task group (1987) A protocol and guide for the *in vivo* rat hepatocyte DNA-repair assay. *Mutation Research*, **189**, 123-133.
>
> KENNELLY, J.C. *et al* (1993) *In vivo* rat liver UDS assay. In: KIRKLAND, D.J. and FOX, M. (Eds) Supplementary Mutagenicity Tests: UKEMS recommended procedures Part II revised, p. 52. Cambridge University Press, England.
>
> OECD (1986) Guideline for testing of chemicals No. 482 Genetic Toxicology: DNA damage and repair/unscheduled DNA synthesis in mammalian cells *in vitro*.

The experimental design also takes into account important modifications of the standard procedure as recommended in proposed OECD guidelines:

> OECD (Draft) Proposal for a new guideline. DNA damage and repair/unscheduled DNA synthesis in mammalian cells *in vivo*.

Chemicals which damage DNA (so called 'genotoxins') are also capable of causing mutations and, frequently, cancer. DNA damage normally results in either cell death or removal of the damaged region and replacement by newly synthesised DNA. The latter process is called 'excision repair' and can be quantified autoradiographically by measuring the amount of radioactive thymidine incorporated into the nuclear DNA of the cells not in the S-phase of cell cycle; the process is frequently referred to as "unscheduled DNA synthesis" or UDS.

Many carcinogens/mutagens are not themselves directly genotoxic but become chemically transformed to the ultimate active species in the exposed animal. *In vitro* cultured cell systems cannot be expected to accurately reproduce the complex chemical transformations which can occur in the intact animal - in the *in vivo* system described here many of the factors involved in carcinogen/mutagen activation process are intrinsically taken into account. Thus, not only is the rat liver capable of performing

many of these transformations (*ie* it is metabolically competent) but it is also directly exposed to the test substance after absorption through the gut.

To measure DNA repair induction in the liver, animals are normally treated orally with the test substance. After a few hours, during which time DNA-damage may accumulate, the liver cells are isolated by enzymatic dissociation then cultured with radioactive thymidine *in vitro* to optimise labelling of newly synthesised DNA. The incorporated radioactive thymidine results in the deposition of silver grains in the overlying autoradiographic emulsion. The optimum time between treatment and harvesting is thought to vary between compounds so that two 'expression periods' are normally used for each test substance. The cells are washed, fixed and stained, then incorporation of thymidine into nuclear DNA is quantified autoradiographically. Increases in the nuclear grain density of cells from treated animals are indicative of DNA repair and hence DNA damage.

The protocol for the proposed test was approved by the Study Director and HRC Management on 1 May 1995 and by the Sponsor on 9 May 1995.

The experimental phase of the study was conducted between 22 May and 30 July 1995.

TEST SUBSTANCE

Identity:	Chlorodibromomethane
CAS registry number:	124-48-1
Supplier:	Aldrich Chemical Co., UK.
Product number:	206326
Batch number:	41028
Expiry:	9 May 1997 - taken as two years from the date of arrival at HRC
Purity:	98%
Appearance:	Pale yellow liquid
Storage conditions:	Approximately 4°C in dry conditions, in a tightly closed container
Date received:	9 May 1995

The above information, with regard to the physical characterisation of the test substance, was supplied by Aldrich Chemical Co., UK.

EXPERIMENTAL PROCEDURE

ANIMAL MANAGEMENT

All animals in this study were Specific Pathogen Free outbred albino Hsd/Ola Sprague-Dawley male rats specified as being approximately five weeks old and weighing 150 - 159 grams on despatch from Harlan Olac UK Ltd, Bicester, Oxon, England.

On arrival, the weight of the animals was checked and found to be acceptable. The animals were randomly assigned to groups and tail marked. Each group of rats was kept in a plastic disposable cage with a stainless steel grid top and maintained in a controlled environment with 20 changes of air per hour, the thermostat set at 22°C and relative humidity set at 55%. Relative humidity and temperature were monitored continuously throughout the acclimatisation and experimental phase of the study and were found to vary between 45 - 58% and 20 - 24°C respectively. The room was illuminated by artificial light for 12 hours per day. All animals were allowed free access to pelleted Biosure LAD 1 rodent diet and tap water. Food and water are routinely analysed for quality at source. Dietary contaminants are not suspected of having any significant effect on previous *in vivo* rat liver UDS tests at any time in this laboratory and are not expected to have had any effect on the present study. Animals were acclimatised for four to six days, examined daily and weighed prior to dosing and cell isolation.

FORMULATION OF THE TEST SUBSTANCE AND CHEMICAL ANALYSIS

Suspensions of the test substance were freshly prepared on the day of use (separately using identical methods for each phase of the test) and were diluted to the required concentration in aqueous 1% methylcellulose obtained from Courtaulds, batch number T32398.

Chemical analysis of achieved concentration and stability of test substance formulation were not performed in this study.

POSITIVE CONTROL COMPOUNDS

Dimethylnitrosamine (DMN) >99% pure, obtained from Aldrich Chemical Co, batch number 03736LY, was used as the positive control compound for the 2 hour expression. It was prepared as a solution in sterile distilled water just prior to use at a concentration of 0.4 mg/ml.

2-Acetylaminofluorene (2AAF) 95 - 97% pure, obtained from Sigma Chemical Co Ltd, batch number 45F3657 was used as the positive control compound for the 14 hour expression. It was prepared as a suspension in aqueous 1% methylcellulose obtained from Courtaulds, batch number T32398 at a concentration of 5 mg/ml.

TREATMENT PROCEDURE

All animals in all groups were dosed orally by gastric intubation with the standard dose volume of 10 ml/kg bodyweight. Animals in the negative control group were treated with the vehicle, aqueous 1% methylcellulose. Animals in the positive control group were treated with dimethylnitrosamine at 4 mg/kg for the 2 hour expression or 2-acetylaminofluorene at 50 mg/kg for the 14 hour expression.

Animals in the preliminary toxicity test were treated on 22 May 1995 (Phase I) and 5 June 1995 (Phase II). Animals in the DNA repair test were treated on 21 June 1995.

PRELIMINARY TOXICITY TEST

This test was performed in order to determine the maximum dose level for the DNA repair test. This test was performed in two phases. The dose levels used for the Phase I test were selected in view of the expected high toxicity of the test substance. The doses selected for the Phase II test were based on the results of the Phase I test. Thirty-two male rats were used, and the experimental design is shown below.

Experimental design

Phase	Group	Material	Concentration (mg/ml)	Dosage (mg/kg)	Number of animals
I	1	Chlorodibromomethane	17.6	176	4
	2		26.4	264	4
	3		39.6	396	4
	4		59.4	594	4
II	5	Chlorodibromomethane	68.6	686	4
	6		98	980	4
	7		140	1400	4
	8		200	2000	4

Following dosing, the animals were observed regularly during the working day for a period of 4 days and any mortalities or signs of malreaction during the experiment were recorded.

DNA REPAIR TEST

From the results of the preliminary toxicity test (see Page 15), dosages of 600 and 2000 mg/kg bodyweight were chosen for use in the DNA repair test. More than the required minimum of four animals (2 for positive controls) were treated at each experimental point to allow for possible mortalities or problems encountered during perfusion. Two expression times were utilised in order

to allow for variations in the rate of absorption, metabolism and accumulation of DNA damage. The experimental design is shown below.

Experimental design

Group	Material	Concentration (mg/ml)	Dosage (mg/kg)	Number of animals used	
				2 hour*	14 hour*
1	Vehicle	-	-	4(5)	4(5)
2	Chlorodibromomethane	60	600	4(5)	4(5)
3	Chlorodibromomethane	200	2000	4(5)	4(5)
4a	Dimethylnitrosamine	0.4	4	2(3)	not done
4b	2-Acetylaminofluorene	5	50	not done	2(3)

* Expression time. The numbers below indicate the number of animals used for hepatocyte culture, the numbers in parentheses indicate the number of animals treated. Cells isolated from animals not used for hepatocyte culture were discarded.

HEPATOCYTE ISOLATION AND CULTURE

Hepatocytes were isolated from each rat by enzymatic dissociation of the liver using the perfusion procedure detailed in Appendix 4. Formulation details for perfusing solutions and culture media are given in Appendix 5.

The isolated cells were suspended in Williams' medium E supplemented with 10% foetal calf serum (WEC) at a density of approximately 0.2×10^6 cells per ml. This cell suspension was dispensed in 2 ml aliquots into the 35 mm diameter wells of multi-well tissue culture plates, each well containing a sterile 22 mm diameter No. 1½ glass coverslip. Twelve replicate cultures were initiated per animal. The cultures were incubated at 37°C in a humid atmosphere containing 5% carbon dioxide for 90 minutes to allow hepatocytes to attach to the coverslips. After this attachment period the supernatant medium was removed and the cells were gently rinsed with one wash of Williams' medium E without serum (WEI).

The medium was then replaced with WEI containing high specific activity (methyl-^{3}H)thymidine (Amersham International, batch numbers 143 and 144; specific activities 80 and 83 Ci/mmol respectively) at a final activity of 10 μCi/ml. The cultures were incubated in this medium for a period of 4 hours. After this labelling period, the supernatant medium was removed and replaced by WEI containing 250 μM cold (*ie* unlabelled) thymidine. The cultures were then incubated for a 'chase' period of 24 hours. This additional culture period helps to wash out excess radiolabel and improves cell morphology thus facilitating subsequent grain count analysis of autoradiographs.

CELL HARVEST

After the 24 hour cold chase with thymidine, coverslips with attached cells were removed from the culture medium, given three 5 minute washes in Hanks' balanced salts solution then fixed in 2.5% v/v acetic acid in ethanol and allowed to dry. They were mounted on glass microscope slides, with the cell layer uppermost, using DPX mountant. The mountant was allowed to harden at approximately 37°C.

AUTORADIOGRAPHY

Autoradiographs were prepared from six cultures per animal; slides from the remaining six cultures per animal were held in reserve in case of any technical problems with the first set of autoradiographs.

Ilford K2 emulsion was applied to the slides in the dark room working under a 25 watt Kodak Number 1 red safelight. The emulsion was melted then diluted with an equal volume of water containing 4% v/v glycerol. The melted emulsion was placed in a dipping chamber and held at approximately 43°C. Each slide was in turn dipped into the emulsion, withdrawn and held vertically for a few seconds, then excess emulsion was wiped off the back of the slide and it was placed on a chilled metal plate for a few minutes to allow the emulsion to gel. The slides were partially dried in a gentle stream of air for approximately one hour then they were sealed in a light-tight box containing desiccated silica gel and allowed to dry overnight at room temperature. The silica gel was renewed and the autoradiographs exposed for a further 13 days at approximately 4°C.

After the total exposure period of 14 days the autoradiographs were allowed to warm for several hours and then they were developed using the following method:

Kodak D-19 developer	5 minutes at 15°C
0.5% v/v acetic acid	1 minute at 15 - 20°C
Kodak Unifix	2 minutes at 15 - 20°C
Running tap water	20 minutes at 15 - 20°C
Distilled water	5 minutes at 15 - 20°C

The slides were stained in Mayer's Haemalum (BDH 35060) for 1 minute, rinsed in distilled water, washed in running tap water then allowed to air dry.

EXAMINATION OF SLIDES

The slides were randomised, encoded and grain count analysis performed using a Zeiss Photomicroscope II connected to an AMS 40-10 image analyser via a high resolution camera fitted with a 1" Chalnicon tube. A Tulip AT compact 3 microcomputer (IBM compatible PC) linked to the image analyser was used to provide a direct data capture system to record grain counts. Three slides per animal were examined using high magnification, oil immersion optics; the remaining autoradiographs prepared from each animal were held as reserves in case of any technical problems with the three slides initially examined.

The image analyser was used in the area count mode and the count obtained was automatically converted to an equivalent grain count using a conversion factor of 0.15 grains per pixel, which remained constant throughout the experiment. This method is believed to give the most accurate assessment of labelling levels because actual grain counting methods do not take into account variation in grain size or overlapping of grains at the high density seen in the hepatocyte UDS system (Kennelly *et al* 1993).

Usually, fifty hepatocytes over several widely-separated, randomly chosen fields of view, from each of three cultures per animal were analysed. Only results from hepatocytes not in S-phase with a normal morphology (*ie* not pyknotic or lysed) without staining artifacts or debris were recorded. For each cell the number of silver grains overlying the nucleus was estimated using the image analysis system, then the number of silver grains in an equivalent and most heavily-grained, adjacent area of cytoplasm was estimated. The cytoplasmic grain count was subtracted from the gross nuclear grain count to give the net nuclear grain count. Mean grain counts were calculated for each slide examined. For slides showing a strong response, *ie* where the mean net grain count was in excess of 10, only 25 cells were examined. The number of cells with a net grain count of greater than or equal to five was recorded in the raw data as an indication of the proportion of cells undergoing repair.

EVALUATION OF RESULTS

Both gross and net nuclear grain counts for treated animals were compared with vehicle control counts using classical one-way analysis of variance followed by a Student's *t* test with an appropriate transformation of values if indicated by excessive variance (Snedecor and Cochran 1967).

A positive response is normally indicated by a substantial dose-associated statistically significant increase in the net nuclear grain count which is accompanied by a substantial increase in the gross nuclear grain count over concurrent control values.

ARCHIVES

All specimens, raw data and study related documents generated during the course of this study, together with a copy of the final report, have been lodged in the Huntingdon Research Centre Archive.

Such specimens and records will be retained for a minimum period of five years from the date of issue of the final report. At the end of the five year retention period the Sponsor will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the Sponsor's knowledge.

RESULTS

PRELIMINARY TOXICITY TEST

The details of clinical signs and mortalities obtained in the Phase I and II preliminary toxicity tests are given in Appendices 1 and 2 respectively.

As no mortalities occurred in the Phase I test, it was necessary to conduct a Phase II test. In the Phase II test at approximately 29 hours after treatment, one animal from the 2000 mg/kg dose group and one animal from the 1400 mg/kg dose group were showing severe clinical signs. These two animals were therefore sacrificed *in extremis*. Since the longest exposure time to be used in the DNA repair test was 14 hours and this critical time point had passed with no mortalities occurring at the high dose level of 2000 mg/kg bodyweight, this dose level was selected as an appropriate maximum for use in the DNA repair test. This is the maximum dose level recommended by the UKEMS and by proposed OECD guidelines and is the OECD and EEC limit dose for acute oral toxicity testing; we consider it to be an appropriate limit for use in this system.

DNA REPAIR TEST

The details of any clinical signs and mortalities obtained in the DNA repair test are presented in Appendix 3. Details of individual animal bodyweights, cell yields and viabilities are given in Appendix 6.

The results of the DNA repair test using the 2 hour and 14 hour expression periods are presented in Tables 1 and 2 respectively.

Animals treated with chlorodibromomethane did not show any statistically significant increase ($P > 0.01$) in the gross or net nuclear grain count at any dosage level at either the 2 or 14 hour expression time. Grain counts were similar to vehicle control values and were within the range of historical control values - see Appendix 7.

Animals treated with dimethylnitrosamine or 2-acetylaminofluorene showed a statistically significant increase ($P < 0.001$) in the net nuclear grain count which was accompanied by a substantial increase in the gross nuclear grain count.

CONCLUSION

Chlorodibromomethane did not elicit any evidence of causing DNA-damage in the rat liver in this *in vivo* test system.

REFERENCES

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MIRSALIS, J.C. and BUTTERWORTH, B.E. (1980) Detection of unscheduled DNA synthesis in hepatocytes isolated from rats treated with genotoxic agents: an *in vivo - in vitro* assay for potential carcinogens and mutagens. *Carcinogenesis*, 1, 621.

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WILLIAMS, G.M. (1976) Carcinogen-induced DNA repair in primary rat liver cell cultures; a possible screen for chemical carcinogens. *Cancer Letters*, 1, 231.

TABLE 1

Results for the 2 hour expression time

Test substance	Dosage (mg/kg)	Gross nuclear grain count					Cytoplasmic grain count			Net nuclear grain count				
		x^1	x^2	x^3	$\bar{x}$	$\bar{\bar{x}}$†	x^1	x^2	x^3	x^1	x^2	x^3	$\bar{x}$	$\bar{\bar{x}}$†
Vehicle	–	11.0	12.1	12.4	11.8	**10.9**	13.0	13.1	14.4	-2.0	-0.9	-2.0	-1.6	**-2.3**
		13.3	10.2	10.0	11.2		14.8	12.3	11.9	-1.5	-2.1	-1.8	-1.8	
		10.3	10.6	8.9	9.9		12.3	12.7	11.8	-2.0	-2.1	-2.9	-2.3	
		9.6	11.4	10.4	10.5		11.6	16.7	13.6	-2.1	-5.3	-3.3	-3.6	
Clorodibromomethane	600	10.5	11.0	9.9	10.5	**9.8**	15.3	16.1	14.8	-4.8	-5.0	-4.9	-4.9	**-3.7**
		11.0	7.6	8.8	9.1		13.1	10.9	14.1	-2.2	-3.4	-5.3	-3.6	
		9.0	11.3	8.7	9.7		13.4	14.8	12.1	-4.4	-3.6	-3.4	-3.8	
		9.7	8.0	11.5	9.7		12.6	9.8	14.8	-2.9	-1.8	-3.3	-2.7	
	2000	16.2	9.5	9.5	11.7	**10.5**	17.8	11.6	15.2	-1.5	-2.1	-5.7	-3.1	**-4.1**
		16.3	8.1	9.0	11.1		19.7	12.1	12.0	-3.4	-4.0	-3.0	-3.5	
		6.2	10.0	10.8	9.0		8.9	14.7	18.4	-2.7	-4.7	-7.7	-5.0	
		7.6	10.1	12.2	10.0		12.4	16.1	16.1	-4.9	-6.0	-3.9	-4.9	
Dimethylnitrosamine	4	23.6	24.6	40.8	29.7	**27.4****	10.0	11.1	11.8	13.5	13.5	28.9	18.6	**15.7****
		20.3	31.7	23.0	25.0		9.2	16.4	11.1	11.1	15.3	11.9	12.8	

x^1, x^2, x^3 Mean results for each replicate culture

$\bar{x}$ Mean for each animal

$\bar{\bar{x}}$ Group mean

† Results of statistical analysis (one-way analysis of variance followed by a Student's *t* test with critical one-sided probability levels) :

- ** $P < 0.001$ (highly significant)
- * $P < 0.01$ (significant)
- Otherwise $P > 0.01$ (not significant)

NB For each cell examined, Net nuclear grain count = Gross nuclear grain count - Cytoplasmic grain count. An occasional apparent discrepancy of 0.1 net grains may occur due to rounding of mean values for presentation in the table

TABLE 2

Results for the 14 hour expression time

Test substance	Dosage (mg/kg)	Gross nuclear grain count					Cytoplasmic grain count			Net nuclear grain count				
		x^1	x^2	x^3	$\bar{x}$	$\bar{\bar{x}}$ †	x^1	x^2	x^3	x^1	x^2	x^3	$\bar{x}$	$\bar{\bar{x}}$ †
Vehicle	–	8.5	9.7	7.2	8.5	**8.7**	10.3	11.1	9.3	-1.9	-1.5	-2.0	-1.8	**-2.3**
		8.5	11.0	9.1	9.5		10.5	14.4	11.2	-2.1	-3.4	-2.1	-2.5	
		9.4	7.8	9.5	8.9		11.7	10.1	11.5	-2.3	-2.3	-2.0	-2.2	
		8.5	7.7	7.7	8.0		11.4	10.0	10.3	-2.9	-2.3	-2.6	-2.6	
Chlorodibromomethane	600	10.0	8.9	10.3	9.7	**11.2**	12.5	11.1	11.9	-2.4	-2.2	-1.6	-2.1	**-2.6**
		14.7	10.7	14.2	13.2		15.4	14.0	17.6	-0.7	-3.3	-3.5	-2.5	
		9.2	12.6	11.6	11.1		12.3	16.1	14.3	-3.1	-3.5	-2.8	-3.1	
		13.3	10.1	8.5	10.6		16.8	12.6	11.3	-3.4	-2.6	-2.8	-2.9	
	2000	11.9	10.4	13.4	11.9	**9.9**	13.7	12.9	15.9	-1.8	-2.5	-2.5	-2.3	**-2.6**
		8.9	8.3	9.9	9.0		12.0	11.6	12.6	-3.2	-3.3	-2.8	-3.1	
		13.7	9.6	9.3	10.9		14.9	12.5	11.8	-1.2	-2.9	-2.6	-2.2	
		7.1	8.3	7.9	7.8		10.0	11.2	10.2	-2.9	-2.9	-2.3	-2.7	
2-Acetylaminofluorene	50	20.3	21.0	20.4	20.6	**22.8****	10.1	10.3	9.5	10.2	10.7	10.8	10.6	**11.8****
		24.3	24.4	26.4	25.0		11.5	11.3	13.5	12.8	13.0	12.9	12.9	

x^1, x^2, x^3 Mean results for each replicate culture

$\bar{x}$ Mean for each animal

$\bar{\bar{x}}$ Group mean

† Results of statistical analysis (one-way analysis of variance followed by a Student's *t* test with critical one-sided probability levels) :

- ** $P < 0.001$ (highly significant)
- * $P < 0.01$ (significant)
- Otherwise $P > 0.01$ (not significant)

NB For each cell examined, Net nuclear grain count = Gross nuclear grain count - Cytoplasmic grain count. An occasional apparent discrepancy of 0.1 net grains may occur due to rounding of mean values for presentation in the table

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APPENDIX 1

Clinical signs and mortalities

Preliminary toxicity test - Phase I

Approximate time after treatment (hr : min)	Treatment and dosage (mg/kg)			
	Chlorodibromomethane			
	176	264	396	594
0 : 30	P^{++} IR^{+}	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{+}L^{++}$
1 : 00	$P^{++}IR^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{+}L^{+}$	$2(P^{++}IR^{+}L^{+++}PT^{+++})$ $P^{++}IR^{+}L^{++}$
2 : 00	$P^{++}IR^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{++}L^{+++}PT^{+++}$
3 : 00	$P^{++}IR^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{++}L^{+++}PT^{+++}$ SG^{++}
4 : 00	$P^{++}IR^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{++}PT^{+++}L^{+++}$ SG^{++}
5 : 00	$P^{++}IR^{+}$	$P^{++}IR^{+}L^{+}$	$P^{++}IR^{+}L^{+}$	$PT^{+++}P^{++}IR^{+}L^{+++}$ SG^{++}
21 : 15	$P^{+}IR^{+}$	$P^{+}IR^{+}$	$P^{+}IR^{+}$	$P^{+}IR^{+}SG^{+}$
24 : 00	$P^{+}IR^{+}$	$P^{+}IR^{+}$	$P^{+}IR^{+}$	$P^{+}IR^{+}SG^{+}$
29 : 00	$P^{+}IR^{+}$	$P^{+}IR^{+}$	$P^{+}IR^{+}$	$P^{+}IR^{+}$
45 : 30	P^{+}	P^{+}	$P^{++}IR^{+}$	$P^{++}H^{++}L^{++}RS^{+}SC^{+}$ UG^{++}
48 : 00	P^{+}	P^{+}	$P^{++}IR^{+}$	$1(P^{++}H^{+++}L^{++}RS^{+}$ $PT^{++}IR^{+}T^{++})$ $P^{++}H^{++}$ $L^{++}RS^{+}SC^{+}UG^{++}$
53 : 00	P^{+}	P^{+}	$P^{+}IR^{+}$	$1(P^{++}T^{++}RS^{+}SG^{+}UG^{++}$ $IR^{++})P^{++}L^{+}H^{+}$
69 : 30	P^{+}	P^{+}	P^{+}	$1(P^{++}T^{++}RS^{++}SH^{+}$ $UG^{++})$ $P^{+}L^{+}H^{+}$
72 : 00	P^{+}	P^{+}	P^{+}	$1(P^{++}RS^{++}SH^{+}UG^{++})$ $P^{+}L^{+}H^{+}$
77 : 00	P^{+}	P^{+}	P^{+}	$1(P^{++}RS^{++}SH^{+}UG^{++})$ $P^{+}L^{+}H^{+}$
93 : 30	P^{+}	P^{+}	P^{+}	$1(P^{++}UG^{+}RS^{+}L^{+})$ $P^{+}L^{+}$
96 : 00	P^{+}	P^{+}	P^{+}	$1(P^{++}L^{+}UG^{+})$ $P^{+}L^{+}$
Mortality ratio	0/4	0/4	0/4	0/4

Degree of reaction: 0 no reaction, $^{+}$ slight reaction, $^{++}$ moderate reaction, $^{+++}$ severe reaction

Type of reaction: H hunched posture, IR increased respiratory rate, L lethargy, P piloerection, PT ptosis, RS red staining, SC spasmodic contractions of the abdomen, SG staggering gait, SH shivering, T twitching, UG ungroomed

Clinical signs refer to all animals within the dose group, except where $x(....)$; x denoting the number of animals displaying the clinical signs within the bracket

APPENDIX 2

Clinical signs and mortalities

Preliminary toxicity test - Phase II

Approximate time after treatment (hr : min)	Treatment and dosage (mg/kg)			
	Chlorodibromomethane			
	686	980	1400	2000
0 : 00	$P^{+}L^{+}$	$P^{+}L^{+}$	$P^{+}L^{++}$	$P^{+}L^{++}$
0 : 30	$2(P^{+}L^{+}G^{+}PT^{+})$ $P^{+}L^{+}$ PT^{+}	$P^{+}L^{+}$	$3(P^{+}L^{++}PT^{+})$ $P^{+}L^{++}$	$P^{+}L^{++}PT^{+}$
1 : 00	$2(P^{+}L^{+}G^{+}RS^{++}PT^{+})$ P^{+} L^{+}	$P^{+}L^{+}$	$1(SL^{+++}L^{+++}P^{+}PT^{++}$ $SG^{+++})$ $W^{+}P^{+}PT^{+}L^{++}$	$P^{+}L^{++}PT^{+}W^{+}IR^{+}$
1 : 30	$2(P^{+}L^{++}G^{+}RS^{++}PT^{+})$ $P^{++}L^{++}PT^{+}$	$P^{++}L^{+++}$	$1(SL^{+++}L^{+++}P^{+}PT^{++}$ $SG^{+++}IR^{+})$ $W^{+}P^{+}PT^{+}L^{++}$	$P^{++}L^{+++}PT^{+}W^{+}IR^{++}$
2 : 00	$2(P^{+}L^{++}G^{+}RS^{++}PT^{+})$ $P^{++}L^{++}PT^{+}$	$P^{++}L^{+++}$	$1(SL^{+++}L^{+++}P^{+}PT^{++}$ $SG^{+++})$ $W^{+}P^{+}PT^{++}L^{+}$	$P^{++}L^{+++}PT^{+}W^{+}IR^{++}$
3 : 00	$1(P^{+}L^{++}G^{+}RS^{++})$ $1(P^{+}$ $L^{++}RS^{++})$ $P^{++}L^{++}PT^{+}$	$P^{++}L^{+++}$	$1(SL^{+++}L^{+++}P^{+}PT^{++}$ $IR^{++})$ $W^{+}P^{+}PT^{++}L^{+}$	$P^{++}L^{+++}PT^{++}W^{+}IR^{++}$
4 : 00	$2(P^{+}L^{++}RS^{++})$ $P^{++}L^{++}$ PT^{+}	$P^{++}L^{+++}$	$1(SL^{+++}L^{+++}P^{+}PT^{++}$ $IR^{++})$ $W^{+}P^{+}PT^{++}L^{+}$	$1(NR^{+}P^{++}L^{+++}PT^{++}$ $W^{+}IR^{++})$ $P^{++}L^{+++}$ $PT^{++}W^{+}IR^{++}$
5 : 00	$2(P^{+}L^{++}RS^{++})$ $P^{++}L^{++}$ PT^{+}	$P^{++}L^{+++}$	$1(SL^{+++}L^{+++}P^{+}PT^{++}$ $IR^{++})$ $W^{+}P^{+}PT^{++}L^{+}$	$1(NR^{+}IL^{+}P^{++}L^{+++}$ $PT^{++}W^{+}IR^{++})$ P^{++} $L^{+++}PT^{++}W^{+}IR^{++}$
6 : 00	$2(P^{+}L^{++}RS^{+})P^{++}L^{+}RS^{+}$	$1(P^{++}L^{++})$ $P^{+}L^{++}$	$1(SL^{+++}L^{+++}P^{++}PT^{+++}$ $IR^{++})$ $W^{+}P^{++}PT^{++}L^{+}$	$1(NR^{+}S^{+}IL^{+}P^{++}L^{+++}$ $PT^{++}W^{+}IR^{++})$ P^{++} $L^{+++}PT^{++}W^{+}IR^{++}$
22 : 30	P^{+}	P^{+}	$1(C)$ $1(RS^{+}P^{+})$ P^{+}	$1(L^{+++}RS^{+}P^{+}SG^{++}$ $G^{++})$ $P^{+}L^{++}SG^{++}$
24 : 00	P^{+}	P^{+}	$1(C)$ $1(RS^{+}P^{+}PT^{+})$ P^{+}	$1(NR^{+}S^{+}IL^{+}P^{++}L^{+}$ $PT^{++}W^{+}IR^{++})$ $P^{+}L^{++}$
28 : 00	P^{+}	P^{+}	$1(C)$ $1(RS^{+}P^{+}PT^{+})$ P^{+}	$1(NR^{+})$ $1(NR^{+}S^{+}IL^{+}$ $P^{++}L^{++}PT^{+}W^{+})$ P^{+} L^{++}
29 : 00	P^{+}	P^{+}	$1(C)$ $1(RS^{+}P^{+}PT^{+})$ P^{+}	$1(NR^{+}S^{+}IL^{+}P^{++}L^{++}$ $PT^{+}W^{+})$ $P^{+}L^{+}$

Degree of reaction: 0 no reaction, $^{+}$ slight reaction, $^{++}$ moderate reaction, $^{+++}$ severe reaction

Type of reaction: C coma, G gasping, IL increased lacrimation, IR increased respiratory rate, L lethargy, NR noisy respiration, P piloerection, PT ptosis, RS red staining around eyes, S squeaking, SG staggering gait, SL splayed limbs

Clinical signs refer to all animals within the dose group, except where $x(....)$; x denoting the number of animals displaying the clinical signs within the bracket

APPENDIX 2

(Continued)

Approximate time after treatment (hr : min)	Treatment and dosage (mg/kg)			
	Chlorodibromomethane			
	686	980	1400	2000
30 : 00	P^+	P^+	1SE(C) 1($RS^+P^+PT^+$) P^+	1SE($NR^+S^+IL^+P^{++}L^{++}$ PT^+W^+) P^+L^+
47 : 00	P^+	P^+	1(RS^+P^+) P^+	$P^{++}H^+W^+$
48 : 00	P^+	P^+	1(RS^+P^+) P^+	$P^{++}H^+W^+$
54 : 00	P^+	P^+	1($P^+PT^{+++}LB^{++}L^+$) P^+	1($RS^+LB^{++}P^+T^{++}$ L^+) 1($RS^+PT^{+++}T^+$) P^+H^+
71 : 00	P^+	P^+	1($P^+RS^+LB^{++}L^+$) P^+	1($RS^+PT^+T^{++}LB^+P^+L^+$) 1($RS^+PT^+T^+$) P^+H^+
72 : 00	P^+	P^+	1($P^+RS^+LB^{++}L^+RE^+$) P^+	1($RS^+PT^+T^+LB^+P^+$ L^+) 1(RS^+PT^+) P^+H^+
78 : 00	P^+	P^+	P^+RS^+	1($RS^+P^+T^+LB^+$) P^+H^+
95 : 00	P^+	P^+	P^+	2(RS^+P^+) P^+
96 : 00	P^+	P^+	P^+	2(RS^+P^+) P^+
Mortalities	0/4	0/4	0/4 (**SE** 1/4)	0/4 (**SE** 1/4)

Degree of reaction: 0 no reaction, $^+$ slight reaction, $^{++}$ moderate reaction, $^{+++}$ severe reaction

Type of reaction: C coma, H hunched posture, L lethargy, LB laboured breathing, IL increased lacrimation, NR noisy respiration, P piloerection, PT ptosis, RE red eyes, RS red staining around eyes, S squeaking, T twitching, W waddling

SE Animal sacrificed *in extremis* immediately after recording clinical signs

Clinical signs refer to all animals within the dose group, except where x(....); x denoting the number of animals displaying the clinical signs within the bracket

APPENDIX 3

Clinical signs and mortalities

DNA repair test

Expression time	Approximate time after treatment (hr : min)	Treatment and dosage (mg/kg)			
		Chlorodibromomethane		DMN	2AAF
		600	2000	4	50
2 Hour	0 : 00	P^{+}	$1(P^{+}L^{+}PT^{+}SC^{+})$ $P^{+}L^{+}PT^{+}$ DR^{+}	P^{+}	-
	2 : 00	$P^{+}L^{+}W^{+}$ PT^{+}	$1(P^{++}L^{++}PT^{+++}W^{+}$ $SC^{++})$ $P^{++}L^{++}PT^{+++}DR^{++}W^{+}$	P^{+}	-
Mortality ratio		0/5	0/5	0/3	-
14 Hour	0 : 32 - 1: 44	$PT^{+}L^{+}$	$PT^{+}L^{++}DR^{+}$	-	0
	13 : 00	$P^{+}L^{+}$	$P^{+}L^{++}SG^{+}$	-	0
	14 : 00	NR	$1(P^{++}L^{++}PT^{+}SG^{+}W^{+})$ P^{++} $L^{++}SG^{+}$	-	0
Mortality ratio		0/5	0/5	-	0/3

DMN Dimethylnitrosamine
2AAF 2-Acetylaminofluorene
NR clinical signs not recorded for this group at this time point
Degree of reaction: 0 no reaction, $^{+}$ slight reaction, $^{++}$ moderate reaction, $^{+++}$ severe reaction
Type of reaction: DR decreased respiratory rate, L lethargy, NR clinical signs not recorded, P piloerection, PT ptosis, SC spasmodic contractions of the abdomen, SG staggering gait, W waddling
No clinical signs were noted for the vehicle control animals
Clinical signs refer to all animals within the dose group, except where $x(....)$; x denoting the number of animals displaying the clinical signs within the bracket

APPENDIX 4

Procedural details for hepatocyte isolation

The hepatocytes were isolated by enzymatic digestion as detailed below:

The animal was killed by exposure to an increasing concentration of carbon dioxide.

The liver was exposed and the hepatic portal vein cannulated using a 18 gauge 1¼" Angiocath intravenous catheter placement unit (BD 028721).

The liver was perfused with EGTA solution* (at approximately 37°C) for 5 minutes at a flow rate of 10 ml per minute.

Pressure on the liver was avoided by cutting the subhepatic vena cava just below the right renal vein.

The liver was allowed to drain freely throughout the perfusion.

The liver was then perfused with collagenase solution* (at approximately 37°C) for 10 minutes at a flow rate of 10 ml per minute.

After perfusion the liver was excised and placed in a petri dish with a further aliquot of collagenase solution. The liver cells were combed into suspension using forceps and scissors then filtered through nylon bolting cloth (200 μm mesh).

The hepatocytes were partially purified by differential centrifugation and finally resuspended in WEC*.

A viable cell count was performed after diluting an aliquot of the cells with an equal volume of trypan blue solution. Normally, mean viability values of about 75% are routinely obtained in this laboratory. The viability of the cultures is not an absolute determinant of the validity of the experiment, subsequent attachment and washing stages tend to remove non-viable cells. Results are largely independent of the initial viability of the cultures but cells from vehicle control animals should have a minimum viability of 50%. None of the animals gave a cell viability of less than 50% - see Appendix 6. The cell yield was also calculated.

* Formulation of media/stock solutions - see Appendix 5.

APPENDIX 5

Formulation details of perfusing solutions and culture media

0.5 mM EGTA solution

500 ml Hanks' balanced salt solution without Ca^+ or Mg^+ (Gibco 14170-088)
0.5 ml 190 mg/ml solution of EGTA (Sigma E4378) in 2M NaOH (BDH AnalaR 19138) 5 ml 1M HEPES buffer (Imperial Laboratories 4-760-07)

Collagenase perfusion solution

500 ml WEI (see below)
5 ml 1M HEPES buffer (Imperial Laboratories 4-760-07)
1.5 ml 1M $CaCl_2$ solution (BDH AVS volumetric solution 19046)
2.45 ml 1M NaOH (BDH AnalaR 19139)
50000 units lyophilised collagenase (Gibco 17018-037)

Williams' medium E, incomplete (WEI)

500 ml Williams' medium E 1 × liquid (Flow Laboratories 12-502-54)
5 ml 200 mM L-glutamine (Gibco 043-05030H)
0.5 ml 50 mg/ml solution of gentamicin (Biological Industries 03-035-18)

Williams' medium E, complete (WEC)

500 ml WEI (see above)
50 ml foetal calf serum (PAA 0150HQ)

APPENDIX 6

Details of animal weights, cell viabilities and yields

Expression time	Test substance	Dosage (mg/kg)	Animal number	Animal bodyweight (grams)*	Cell viability (%)	Cell yield (x 10^6 cells)
2 Hour	Vehicle	-	11	199	93	556
			12	199	89	472
			13	209	94	568
			14	207	94	560
	Chlorodibromomethane	600	21	208	91	496
			22	201	95	400
			23	200	95	436
			24	205	87	440
		2000	31	213	95	584
			32	206	93	400
			33	199	92	328
			34	213	94	376
	DMN	4	41	209	95	408
			42	204	94	468
14 Hour	Vehicle	-	111	208	95	444
			112	210	97	588
			113	205	98	332
			114	209	97	484
	Chlorodibromomethane	600	121	198	95	456
			122	203	93	384
			123	194	96	288
			124	209	93	388
		2000	131	195	98	380
			132	189	95	284
			133	202	97	448
			134	193	94	624
	2AAF	50	141	219	93	568
			142	217	96	304

* At sacrifice
DMN Dimethylnitrosamine
2AAF 2-Acetylaminofluorene

APPENDIX 7

Historical control values

The frequency distributions shown below present results for 302 individual vehicle control animals used in 88 previous unrelated experiments during the period June 1990 to June 1994. The mean animal gross nuclear grain count is 12.0 (standard deviation = 3.6). Group means range from 4.5 to 20.5 gross nuclear grains for experiments with three, four or five animals in each control group. The mean animal net nuclear grain count is -2.4 (standard deviation = 0.9). Group means range from -4.3 to -0.7 net nuclear grains for experiments with three, four or five animals per control group.

Gross nuclear grain count	Incidence	Net grain count	Incidence
< 4.1	0	< -6.0	0
4.1 - 5.0	9	-5.9 - -5.0	3
5.1 - 6.0	8	-4.9 - -4.0	13
6.1 - 7.0	11	-3.9 - -3.0	62
7.1 - 8.0	14	-2.9 - -2.0	138
8.1 - 9.0	19	-1.9 - -1.0	73
9.1 - 10.0	23	-0.9 - 0.0	12
10.1 - 11.0	37	0.1 - 1.0	1
11.1 - 12.0	26	> 1.0	0
12.1 - 13.0	46		
13.1 - 14.0	30		
14.1 - 15.0	29		
15.1 - 16.0	16		
16.1 - 17.0	9		
17.1 - 18.0	6		
18.1 - 19.0	9		
19.1 - 20.0	5		
20.1 - 21.0	4		
21.1 - 22.0	0		
22.1 - 23.0	0		
23.1 - 24.0	1		
> 24.0	0		

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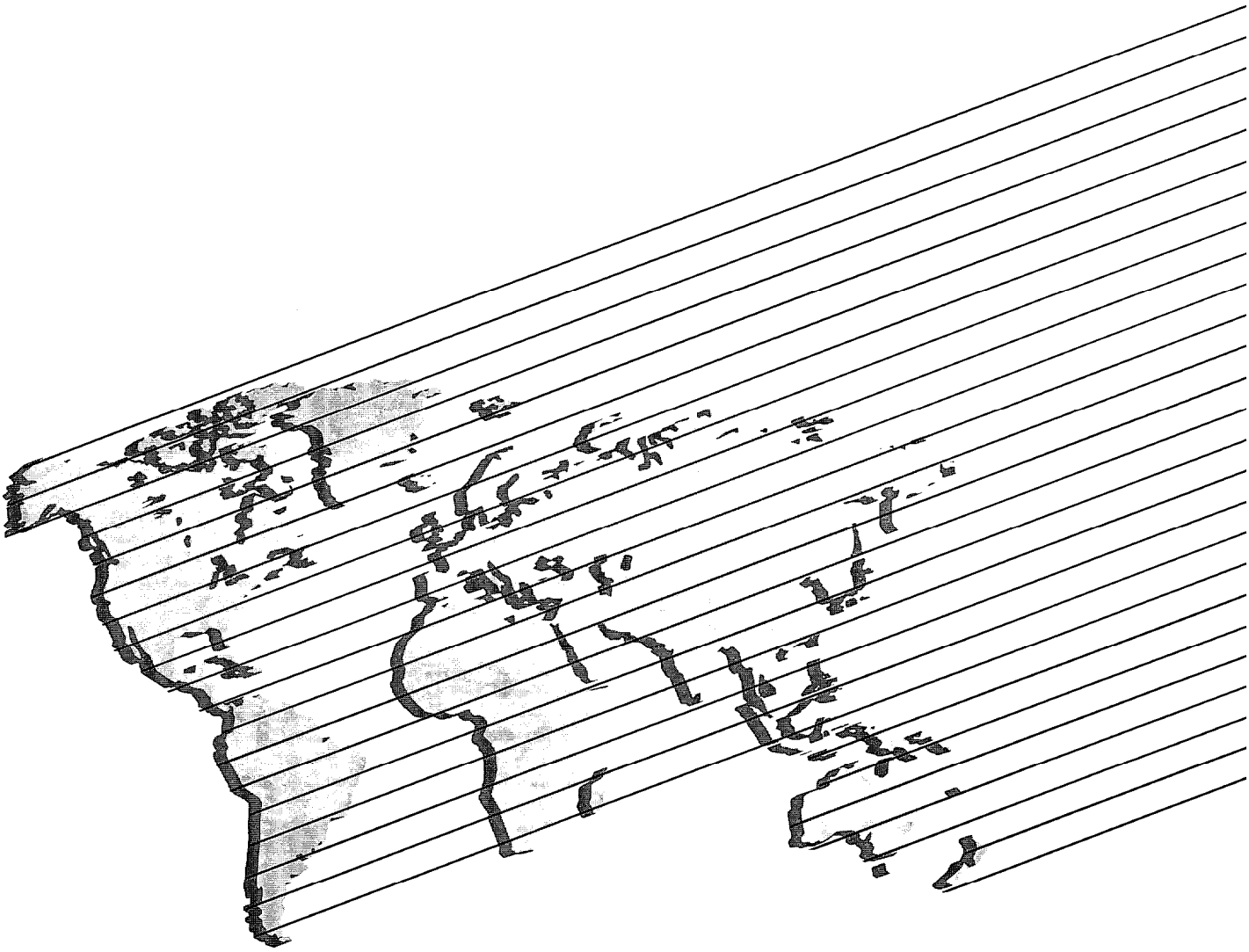
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