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Ciba Specialty Chemicals
USA
Water
Treatments

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FYI-98-001340

October 1, 1998



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TSCA Document Control Office (M/C 7407)
Room G-99, East Tower
Attention: FYI Docket
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
401 M Street, S.W. (M/C 7407)
Washington, D.C. 20460

Re: **FYI Submission**

Dear Sir/Madam:

We are submitting the enclosed studies on an FYI basis. On April 17, 1992, Allied Colloids, Inc. submitted a consolidated PMN (No. P-92-778) for an acrylic polymer, CAS No. 109292-17-3. Allied Colloids submitted three acute toxicity studies with the PMN in 1992.

Ciba Specialty Chemicals Corporation purchased Allied Colloids in 1998 and is in the process of integrating Allied Colloids into Ciba Specialty Chemicals as a new division called Water Treatments Division. Ciba Specialty Chemicals has identified several mutagenicity studies on a cosmetic ingredient containing the PMN substance which it appears should have been submitted with the PMN. (We are simultaneously advising the Office of Enforcement and Compliance Monitoring and the PMN docket of our findings.) These studies are enclosed with this letter. Ciba Specialty Chemicals has examined these mutagenicity studies in light of EPA's guidance for reporting of substantial risk information and has determined that they are not reportable under Section 8(e). A brief summary and analysis of the genotoxicity testing is attached. The test results taken as a whole indicate that the PMN substance does not appear to have genotoxic activity.

Allied Colloids Inc.
2301 Wilroy Rd.
P.O. Box 820
Suffolk, Virginia 23439-0820

Tel. 757 538 3700
Fax 757 538 3987

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October 1, 1998
Page 2

If you have questions regarding these studies, please contact me at (757) 538-3700.

Very truly yours,

A handwritten signature in cursive script that reads "Paul Whitwell".

Paul Whitwell
Technical Manager - Americas
Allied Colloids

Enclosures

SC 90 AND THE RESULTS OF GENOTOXICITY TESTING

Introduction

SC 90 (SAR 24221) is a formulation of an aqueous emulsion (30% concentration) of an acrylic copolymer that is used in personal care products. SC 90 was tested in several microbial genotoxicity assays and in an *in vitro* human lymphocyte metaphase analysis study. Components of SC 90 were also tested in microbial genotoxicity assays. The results of these assays are discussed below in the order in which they were conducted.

The present conclusions regarding a positive or negative outcome in each study herein reviewed are based upon the generally accepted criteria for evaluating the assay which are used in the scientific literature and used by the EPA. In the case of the Ames assays, the criteria are a doubling of the background incidence, the presence of a dose-response function, and the ability to replicate the observation. In some cases, application of these criteria resulted in a different interpretation of the data from that provided in the original study reports.

Study Results

Study #1 (September 1989). This Ames assay was conducted on a sample from a 200 Kg pilot batch of formulated material for research and development purposes for a new product. Five strains of *Salmonella typhimurium* were assayed (strains TA 1535, 1537, 1538, 98 and 100) both with and without metabolic activation. The results were strongly positive in all strains, with and without metabolic activation in the initial assay and in some cases exceeded the positive control values. In the replicate, the results were strongly positive only with activation. Conclusion – Strong positive.

Study #2 (July 1990). An *in vitro* chromosomal aberration study using human lymphocytes with and without metabolic activation was conducted from another sample from the same pilot batch used in Study #1. A positive response was observed only at relatively high concentrations in the presence of metabolic activation. Conclusion – Positive (with metabolic activation).

Study #3 (October 1990). This Ames assay was conducted with a 1% aqueous solution from the pilot batch used in Study #1 using only strains TA 98 and 100, with and without activation. Doubling of the number of revertants was seen only with TA 100 in the absence of metabolic activation. No dose-response was observed and the result could not be replicated. Conclusion – Negative.

Study #4-8 (October 1990). These Ames assays using strains TA 98 and 100 were conducted with various formulations of the product, including different monomer batches and emulsifiers. Study #8 involved a competitor's similar product. Conclusion – All negative.

Study #9 (October 1990). This Ames assay was conducted with a 0.1% concentration of precipitated polymer from the same pilot batch used in Study #1 using

strains TA 98 and 100. Doubling of the number of revertants was observed at the high concentration with Strain TA 98 both with and without metabolic activation. The result could not be replicated and a clear dose-response was not apparent. Conclusion – Negative.

Study #10 (October 1990). This Ames assay was conducted with a 1% concentration of the batch of the monomer used in the manufacture of the pilot batch used in Study #1. Strains TA 98 and 100 were exposed with and without activation. Conclusion – Negative.

Study #11-12 (December 1990). These Ames assays were conducted on samples from a typical production batch. (Study 12 was on a 1% concentration of the sodium salt.) Strains TA 98, 100, 1535, 1537, 1538, 98 and 100 were tested with and without metabolic activation. Conclusion – Negative.

Study #13 (May 1996). This study included both an Ames assay and an *E.coli* gene mutation assay and was conducted in a different laboratory than the other assays. The test material was from a typical production batch. Strains TA 1535, 1537, 98 and 100 and *E.coli* strain WP2uvrA were exposed with and without metabolic activation. A doubling or greater response was observed only with TA 1537 in the presence of metabolic activation. A dose-response was not observed. The response was replicated in TA 1537, other strains were negative. Conclusion – Negative/ Equivocal.

Summary and Discussion

Positive responses were observed only in Studies #1 and #2 from a pilot batch of the material formulated for R&D purposes. The genotoxicity of the pilot batch was indicated in these studies by the induction of gene mutations and chromosomal aberrations. Genotoxicity was not observed in the mutagenicity studies performed with subsequent batches (most notably in Studies #11 and 13). These studies demonstrate the absence of genotoxicity of SC 90 produced subsequent to the pilot batch. Studies #3-7, 9, 10, and 12 were also negative. These latter studies provide limited support for the absence of genotoxicity of SC 90.

The strong genotoxic activity observed with the pilot batch was only observed in *in vitro* studies and thus is not conclusive for the purpose of predicting human hazard. The inability to reproduce the clear positive genotoxicity findings associated with the pilot batch suggests that this R&D material was atypical. For this reason, the results of the testing of the pilot batch, although demonstrative of genotoxicity *in vitro*, do not seem relevant to the risk assessment of SC 90 produced subsequent to the preparation of the pilot batch in early 1989. Subsequent production batches of SC 90 do not show genotoxic activity in the microbial genotoxicity assays.

The test results taken as a whole indicate that SC 90, as formulated for personal care since 1990, does not appear to have genotoxic activity.

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**SALCARE SC90
GENOTOXICITY STUDIES**

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CONTENTS

GENOTOXICITY STUDIES:

- Study No. 1 (SALCARE SC90, July 1990)
- Study No. 2 (SALCARE SC90, July 1990)
- Study Nos. 3-10 (SALCARE SC90*, October 1990)
- Study No. 11 (SALCARE SC90, December 1990)
- Study No. 12 (SALCARE SC90, December 1990)
- Study No. 13 (SALCARE SC90, December 1990)

* Study No. 8 was performed on a competitor's similar product.

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STUDY NO. 1

A 10

Toxicol Study_No : M/AMES/19624

1st TEST.

PHARMAKOPIUS LTD
BACTERIAL REVERSE MUTATION ASSAY
SC90

September 1989

Sponsor

Pharmakopius Ltd.,
Alexander House,
Gatehampton Road,
Goring on Thames,
Reading.
RG8 0EN

Testing Facility

Toxicol Laboratories Limited,
Bromyard Road,
Ledbury,
Herefordshire.
HR8 1LH
England.

Tel : Ledbury (0531) 4121
Telex : 35644 Toxlab G
Fax : Ledbury (0531) 4753

SC90

BACTERIAL REVERSE MUTATION ASSAY

Toxicol Study No : M/AMES/19624

I certify that this study report provides a true and complete record of the data generated and that the study was conducted in accordance with the Principles of Good Laboratory Practice set forth in the following :-

1. The United Kingdom GLP Compliance Programme (DoH 1989).
2. The Principles of Good Laboratory Practice set forth in the OECD Guidelines for the Testing of Chemicals , ISBN 2-64-12367-9, Paris 1982.
3. The United States of America Food and Drug Administration (FDA) 21 CFR, Part 58 - Good Laboratory Regulations Federal Register (Vol. 52, No. 172 September 4, 1987).
4. Notification No. 313 of the Pharmaceuticals Affairs Bureau, Japanese Ministry of Health and Welfare (March 1982).

Signed: *J.C. Asquith* Study Director
(J. C. Asquith. B.Sc., M.Phil., Ph.D.)

Date: *12th February 1990*

SC22

BACTERIAL REVERSE MUTATION ASSAY

Toxicol Study No : M/AMES/19624

At the time of this study the Quality Assurance Unit of Toxicol Laboratories was inspecting one of each critical phase of every type of Mutagenicity study each month as part of a comprehensive evaluation of such studies.

This report has been audited in compliance with the Principles of Good Laboratory Practice. As far as can be reasonably established, the methods described and the results incorporated in the report accurately reflect the raw data produced during this study.

In addition, facilities associated with this study were inspected according to Quality Assurance Unit Standard Operating Procedures.

pp S. Trenchard-Morgan: ~~.....~~.....
(Head of Quality Assurance)

Date: FEBRUARY 12th 1990

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1.0 SUMMARY

SC90 was tested in vitro by the Ames plate incorporation method for its ability to induce mutations in five histidine dependent auxotrophic mutants of Salmonella typhimurium strains TA1535, TA1537, TA1538, TA98 and TA100.

Two independent mutation tests were performed, each in both the presence and absence of a metabolic activation system (S-9 mix) derived from the livers of Aroclor 1254 treated rats. The bacteria were exposed to the test substance dissolved in deionised water, which was also the solvent control. Based on the results of a preliminary toxicity rangefinder, doses used in the mutation experiments were :

0, 8, 40, 200, 1000 and 5000µg/plate both with and without S-9

All solvent (negative) controls gave counts of revertants within normal ranges.

All positive controls gave numbers of induced revertants within expected ranges demonstrating the sensitivity of the assay and the metabolising activity of the S-9 mix.

Dose related increases in revertants were found for TA1535 in the absence of S-9 and all five bacterial strains in the presence of S-9 in both experiments.

It is concluded that SC90 has been found to be a potent mutagen in the above test system when assayed up to 5000µg/plate in both the absence and presence of S-9.

2.0 INTRODUCTION

The purpose of this study was to assess the mutagenic potential of SC90 by testing reversing to prototrophy in five histidine auxotrophic mutants of Salmonella typhimurium. Two independent mutation tests were conducted, each in the presence and absence of a metabolic activation system derived from the livers of rats which had been treated with Aroclor 1254, an enzyme inducing agent. The test was performed in accordance with the plate incorporation method of Ames (references 1 and 2), and OECD Guideline 471.

3.0 TEST SUBSTANCE

The test substance SC90, an aqueous solution of acrylic copolymer, which was a colourless liquid was supplied in a white polythene bottle with a white polythene screw cap.

An aliquot of 2g of the test substance was taken for this study.

The test substance arrived at the testing facility on 28th July 1989.

The reference M/AMES/19624 was given to SC90 for this study. When not in use the substance was stored in a locked metal cupboard at room temperature.

The test substance is referred to as 'SC90' throughout this report.

4.0 MATERIALS4.1 Bacterial Strains

The following strains were used in the Ames test :

<u>S.</u>	<u>typhimurium</u>	TA1535	his G46	rfa ⁻	Δ	uvr B ⁻
<u>S.</u>	<u>typhimurium</u>	TA1537	his C3076	rfa ⁻	Δ	uvr B ⁻
<u>S.</u>	<u>typhimurium</u>	TA1538	his D3052	rfa ⁻	Δ	uvr B ⁻
<u>S.</u>	<u>typhimurium</u>	TA98	his D3052	rfa ⁻	Δ	uvr B ⁻ R ⁺
<u>S.</u>	<u>typhimurium</u>	TA100	his G46	rfa ⁻	Δ	uvr B ⁻ R ⁺

All five Salmonella strains are defective in DNA repair capacity (Δ uvrB⁻) and have a defective lipopolysaccharide barrier on the cell wall (rfa⁻). These two properties confer extra sensitivity to DNA damage and also greater permeability of large molecules into the cell. The strains TA98 and TA100 also contain a resistance transfer factor (plasmid pKM 101). This factor (R⁺), which confers resistance to ampicillin, enhances the operation of an error-prone repair system.

The strains are tested routinely for histidine dependence, cell membrane permeability and ampicillin resistance (reference 3) where appropriate.

TA1535 and TA100 are reverted to prototrophy by base substitution mutagens and TA1537, TA1538, TA98 and occasionally TA100 by frameshift mutagens.

Several days before the test, each bacterial strain was streaked from an overnight broth culture, prepared from freshly thawed frozen cells, onto nutrient agar plates. These plates were incubated at 37°C overnight to give isolated colonies and were then scored at 4°C until required.

On the day before the experiment, 50ml quantities of sterile nutrient broth were inoculated with a single colony of appropriate strain and incubated overnight at 37°C.

4.2 Sample Preparation

SC90 was dissolved in sterile deionised water at the following concentrations:-

Rangefinder : 50, 10, 2, 0.4, 0.08 and 0.016mg/ml
Mutation Experiments : 50, 10, 2, 0.4 and 0.08mg/ml

These concentrations give doses of

Rangefinder : 5000, 1000, 200, 40, 8, and 1.6 µg/plate
Mutation Experiment : 5000, 1000, 200, 40 and 8µg/plate

4.3 Metabolic Activation System (S-9 Mix)

The liver microsomal fraction (S-9) was prepared according to the method of Ames et al (reference 1) from Fischer 344 rats which had been induced with Aroclor 1254 (see Appendix 3).

The protein content, estimated by the method of Lowry et al (reference 4) was found to be 36.5mg/ml. For use, the S-9 protein content must not be less than 35mg/ml. The activity check and positive control results presented in this report showed normal activity.

The metabolic activation system (S-9 mix) used in this study was made as described in Appendix 3 and was kept on ice throughout the experiment.

4.4 Positive Controls**(a) Without S-9 mix**

<u>Strain</u>	<u>Positive Control</u>
TA1535) TA100)	Sodium azide (SA) at 1ug/plate
TA1537	9-Aminoacridine (9AA) at 100ug/plate
TA1538) TA98)	2-Nitrofluorene (2NF) at 0.5ug/plate

(b) With S-9 mix

<u>Strain</u>	<u>Positive Control</u>
TA1535) TA1537) TA1538) TA98) TA100)	2-Aminoanthracene (2AA) at 2ug/plate

5.0 METHODS**5.1 Toxicity Rangefinder**

In order to determine if SC90 was toxic to the test bacteria a preliminary rangefinder was carried out using Salmonella typhimurium strain T103. Only one Salmonella strain was used as in our experience all strains used show a similar toxic response. For this purpose, an Ames test was carried out with the above strain in the presence and absence of S-9 mix as described in 5.2. The concentrations used for the rangefinder were 0, 1.6, 8, 40, 200, 1000 and 5000µg/plate. After 24 hours incubation at 37°C the plates were examined for the appearance of a complete bacterial lawn as seen under a dissecting microscope.

5.2 Ames Test

All procedures were carried out under low intensity yellow lighting in a class II cabinet.

SC90 was tested against the above (4.1) bacterial strains at five dose levels, both with and without S-9 mix, in triplicate in two separate experiments. The test substance was assayed in both experiments at the dose levels given in Section 4.2.

5.2.1 Without metabolic activation

The following components were added sequentially to 2ml of histidine and biotin supplemented molten top agar :-

- 0.1ml of a dilution of SC90
- or 0.1ml of appropriate positive control
- or 0.1ml of deionised water (negative control)
- 0.1ml of appropriate overnight bacterial culture (approximately 10^8 organisms)
- 0.5ml of sterile 0.2M phosphate buffer (pH 7.4)

The components were rapidly mixed on a Whirlimixer and poured onto Vogel Bonner E minimal agar plates. Three plates were prepared for each dose point or control. When the agar had set, the plates were inverted and incubated at 37°C for 48 hours. Numbers of revertants per plate were counted using a Biotran III automatic colony counter and the results recorded on form HU20F1.

5.2.2 With metabolic activation

Methodology was as described in 5.2.1 except that 0.5ml of S-9 mix (see Appendix 5) was added to test tubes instead of sterile phosphate buffer.

5.3 Evaluation of Results

Mean and standard deviations for each treatment group are calculated and recorded on form MU20F1.

An analysis of variance is performed on each set of data to obtain the F-statistic. If this statistic is significant ($p < 0.05$) and dose related increases are apparent, the correlation coefficient is calculated for the response range and the significance of the result is determined from standard tables. These are recorded on form MUSF.1.

A statistically significant increase in the mean number of revertants that exceeds twice the concurrent negative control, plus evidence of a dose response relationship with SC90 is considered necessary to indicate a positive result.

6.0 RESULTS

6.1 Toxicity Rangefinder

After 24 hours incubation, growth assessment of the bacterial background lawn following treatment with SC90 was as follows :-

Strain	% S-9	Concentration of Test Substance ($\mu\text{g}/\text{plate}$)*						
		0	1.6	8	40	200	1000	5000
TA98	0	+	+	+	+	+	+	+
TA98	10	+	+	+	+	+	+	+

* These are actual amounts of test substance added and correspond to solutions of test compound with concentrations from 0.016 to 50mg/ml.

+ = normal growth

SC90 was not toxic to the bacteria in the rangefinder study. According to OECD Guideline 471, a compound should be tested to a solubility or toxicity limit within a maximum concentration of 5000 $\mu\text{g}/\text{plate}$. SC90 was therefore tested at

5000, 1000, 200, 40 and 8 $\mu\text{g}/\text{plate}$ with and without S-9.

6.2 Mutation Experiment 1

6.2.1 Raw Data

The responses of the tester strains to the negative control, SC90 and the positive control mutagens are given as individual plate counts in Appendix 1. The data are summarised in Table 1 (page 14).

All solvent controls gave counts of spontaneous revertants within normal ranges.

All positive controls gave counts of induced revertants within expected ranges.

Dose related increases in revertants which were greatly in excess of a doubling of the spontaneous revertant ratio for all strains except TA100 in the absence of S-9. In all cases, the increases in the presence of S-9 were larger than those seen in the absence of S-9.

6.2.2 Statistical Analysis

An analysis of variance was carried out on the data from Experiment 1.

All five strains, both with and without S-9, gave significant F-values. With the exception of TA100 without S-9, these increases were associated with large dose related increases in revertants. The significant F-value for TA100 in the absence of S-9 does not reflect mutagenic activity of SC90.

Correlation coefficients, taken by least squares regression analysis, were all found to be significant.

The slopes of the regression lines, which give the value of revertants induced/ μg of test compound were also calculated and are given (column b, p14) with the other results of statistical analysis.

TABLE 1

MEAN NUMBER OF REVERTANTS PER PLATEFOR SC90EXPERIMENT 1

Strain	% S-9	Concentration of test substance ($\mu\text{g}/\text{plate}$)						
		0	8	40	200	1000	5000	PC
TA1535	0	4.0	8.0	8.7	14.7	55.3	151.0	258.0
TA1537	0	7.0	3.3	8.3	7.3	15.3	29.0	970.7
TA1538	0	8.7	8.0	12.3	37.0	122.0	374.7	132.7
TA98	0	27.6	24.3	25.7	33.0	70.0	299.7	164.0
TA100	0	94.7	86.7	91.3	90.0	116.7	117.3	236.3
TA1535	10	6.0	8.3	16.0	65.7	238.0	851.0	170.0
TA1537	10	6.7	7.3	13.0	33.0	119.7	524.0	56.3
TA1538	10	14.3	28.5	43.3	147.7	511.7	1031.0	326.7
TA98	10	16.3	19.3	17.0	60.7	188.0	627.0	728.3
TA100	10	83.3	89.0	115.7	103.0	198.7	570.3	1082.3

PC = positive control

ANALYSIS OF VARIANCE

MSP. 1

Test Substance :- SC90

Ref. No. M/AMES/19624

EXPERIMENT 1

Strain	% S-9	F Statistic	Degrees of freedom		Sig of F (p)	R	p	b
			N	D				
TA1535	0	18.47	5	10	<0.01	0.99	<0.01	0.03
TA1537	0	7.27	5	12	<0.01	0.98	<0.01	4.17×10^{-3}
TA1538	0	2659.24	5	12	<0.01	0.99	<0.01	0.07
TA98	0	140.37	5	12	<0.01	1.00	<0.001	0.05
TA100	0	5.72	5	12	*			
TA1535	10	89.73	5	12	<0.01	1.00	<0.001	0.17
TA1537	10	84.93	5	12	<0.01	1.00	<0.001	0.10
TA1538	10	237.01	5	11	<0.01	0.96	<0.01	0.20
TA98	10	170.46	5	12	<0.01	1.00	<0.001	0.12
TA100	10	47.10	5	12	<0.01	1.00	<0.001	0.10

R = Correlation coefficients
b = Slope (revertants/ μ g)
NS = Not significant ($p > 0.05$)
* = See section 6.2.2

6.3 Mutation Experiment 2

6.3.1 Raw Data

The responses of the tester strains to the negative control, SC90 and the positive control mutagens are given as individual plate counts in Appendix 2. The data are summarised in Table 2 (page 16).

All solvent controls gave counts of spontaneous revertants within normal ranges.

All positive controls gave counts of induced revertants within expected ranges.

Dose related increases in revertants exceeding a doubling of the spontaneous revertant rates were seen for TA1535 and TA100 in the absence of S-9 and for all five bacterial strains in the presence of S-9. The increases for TA1535 and TA100 were larger in the presence of S-9 than in the absence of S-9.

All the increases, except for TA100 without S-9, were smaller than found in Experiment 1.

6.3.2 Statistical Analysis

As in Experiment 1, an analysis of variance was carried out on the data from Experiment 2.

Significant F-values, all of which were associated with dose related increases in revertants, were found for all five strains in the presence of S-9 and for TA1535 and TA100 in the absence of S-9.

Regression analysis was carried out in the results for strains which gave significant F-values. The correlation coefficients obtained were all statistically significant, when calculated over the range of concentration levels of SC90 giving increased revertant counts.

The slopes of the regression lines (induced revertants/ μ g) were again calculated.

TABLE 4MEAN NUMBER OF REVERTANTS PER PLATEFOR SC90EXPERIMENT 2

Strain	% S-9	Concentration of test substance (µg/plate)						
		0	3	40	200	1000	5000	PC
TA1535	0	11.7	6.3	8.7	10.7	21.3	30.0	289.3
TA1537	0	6.0	9.3	9.3	6.3	7.0	8.3	988.3
TA1538	0	9.0	8.7	9.3	12.0	12.0	9.5	146.7
TA98	0	16.0	16.0	20.7	16.0	28.7	16.0	118.7
TA100	0	76.3	96.3	110.0	165.7	138.7	125.7	245.7
TA1535	10	10.7	11.0	17.7	60.3	246.7	635.0	87.7
TA1537	10	13.3	10.3	15.3	28.3	95.0	305.7	51.3
TA1538	10	17.5	16.3	19.0	23.3	47.0	236.7	480.7
TA98	10	24.0	26.3	26.7	34.0	51.3	98.0	330.5
TA100	10	92.0	73.3	78.3	94.3	125.0	219.7	480.3

PC = positive control

ANALYSIS OF VARIANCE

MUSF.1

Test Substance :- SC90

Ref. No. M/AMES/19624

EXPERIMENT 2

Strain	% S-9	F Statistic	Degrees of freedom		Sig of F (p)	R	p	b
			N	D				
TA1535	0	5.20	5	12	<0.01	0.91	<0.05	3.43 x 10 ⁻³
TA1537	0	0.79	5	12	NS			
TA1538	0	0.38	5	10	NS			
TA98	0	3.96	5	11	NS			
TA100	0	5.77	5	12	<0.01	0.98	<0.01	0.4
TA1535	10	166.07	5	12	<0.01	0.98	<0.01	0.12
TA1537	10	8.16	5	12	<0.01	1.00	<0.001	0.06
TA1538	10	9.46	5	11	<0.01	1.00	<0.001	0.04
TA98	10	7.79	5	12	<0.01	0.99	<0.01	0.01
TA100	10	34.38	5	12	<0.01	0.99	<0.01	0.03

NS = Not significant (p>0.05)

ANALYSIS OF VARIANCE

MUSF.1

Test Substance :- , SC90

Ref. No. H/AMES/19624

EXPERIMENT 2

Strain	% S-9	F Statistic	Degrees of freedom		Sig of F (p)	R	p	b
			N	D				
TA1535	0	5.20	5	12	<0.01	0.91	<0.05	3.43×10^{-3}
TA1537	0	0.79	5	12	NS			
TA1538	0	0.38	5	10	NS			
TA98	0	3.96	5	11	NS			
TA100	0	5.77	5	12	<0.01	0.98	<0.01	0.4
TA1535	10	166.07	5	12	<0.01	0.98	<0.01	0.12
TA1537	10	8.16	5	12	<0.01	1.00	<0.001	0.06
TA1538	10	9.46	5	11	<0.01	1.00	<0.001	0.04
TA98	10	7.79	5	12	<0.01	0.99	<0.01	0.01
TA100	10	34.38	5	12	<0.01	0.99	<0.01	0.03

NS = Not significant (p>0.05)

APPENDIX 1

Individual Plate Counts for Experiment 1

BACTERIAL MUTATION TESTS

Date of Experiment :- 08/09/89

Ref. No. :- M/AMES/19624

Study Investigators :- CW/DMW

Test Substance :- SC90

Bacterial Strain :- TA1535

Positive Control :- SA -S-9
2AA +S-9

Solvent :- Deionised Water

S-9 0%	Concentration ($\mu\text{g}/\text{plate}$)						
	Solvent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	1
No. of revertants per plate	6 PL 2	8 8 PL	6 10 10	11 13 15	59 55 52	200 153 100	299 239 236
Mean	4.0	8.0	8.7	14.7	55.3	151.0	258.0
S.D.	2.83	0.00	2.31	3.51	3.51	50.03	35.54

S-9 10%	Concentration ($\mu\text{g}/\text{plate}$)						
	Solvent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	2
No. of revertants per plate	6 6 6	6 8 ii	22 14 12	94 61 42	274 247 193	936 928 689	179 PL 161
Mean	6.0	8.3	16.0	65.7	238.0	851.0	170.0
S.D.	0.00	2.52	5.29	26.31	41.24	140.35	10.73

PL = plate lost, fungal contaminant

BACTERIAL MUTATION TESTS

Date of Experiment :- 08/09/89

Ref. No. :- M/AMES/19624

Study Investigators :- CW/DMW

Test Substance :- SC90

Bacterial Strain :- TA1537

Positive Control :- 9AA -S-9
2AA +S-9

Solvent :- Deionised Water

S-9 0%	Concentration ($\mu\text{g}/\text{plate}$)						
	Solvent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	100
No. of revertants per plate	9 7 5	2 5 3	10 7 8	9 7 6	6 24 16	27 19 41	932 998 982
Mean	7.00	3.3	8.3	7.3	15.3	29.0	970.7
S.D.	2.00	1.53	1.53	1.53	9.02	11.14	34.43

S-9 10%	Concentration ($\mu\text{g}/\text{plate}$)						
	Solvent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	2
No. of revertants per plate	5 11 4	8 3 11	18 11 10	27 37 35	46 159 154	518 595 459	57 49 63
Mean	6.7	7.3	13.0	33.0	119.7	524.0	56.3
S.D.	3.79	4.04	4.36	5.29	63.85	68.20	7.02

BACTERIAL MUTATION TESTS

Date of Experiment :- 11/09/89

Ref. No. :- M/AMES/19624

Study Investigators :- CW/DMW

Test Substance :- SC90

Bacterial Strain :- TA1538

Positive Control :- 2NF -S-9
2AA +S-9

Solvent :- Deionised Water

S-9 0%	Concentration ($\mu\text{g}/\text{plate}$)						
	Solvent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	0.5
No. of revertants per plate	10 6 10	12 6 6	8 18 11	30 40 41	124 121 121	380 378 366	147 148 103
Mean	8.7	8.0	12.3	37.0	122.0	374.7	132.7
S.D.	2.31	3.46	5.13	6.08	1.73	7.57	25.70

S-9 10%	Concentration ($\mu\text{g}/\text{plate}$)						
	Solvent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	2
No. of revertants per plate	13 12 18	PL 31 26	39 55 36	140 146 157	521 511 503	1123 1052 918	310 356 314
Mean	14.3	28.5	43.3	147.7	511.7	1031.0	326.7
S.D.	3.21	3.54	10.21	8.62	9.02	104.10	25.48

PL = plate lost, fungal contaminant

BACTERIAL MUTATION TESTS

Date of Experiment :- 08/09/89

Ref. No. :- M/AMES/19624

Study Investigators :- CW/DMW

Test Substance :- SC90

Bacterial Strain :- TA98

Positive Control :- 2NF -S-9

2AA +S-9

Solvent :- Deionised Water

S-9 0%	Concentration ($\mu\text{g}/\text{plate}$)						Positive Control
	Solvent Control	Test Substance					
	0	8	40	200	1000	5000	
No. of revertants per plate	30 26 25	22 19 32	26 22 29	33 35 31	74 57 79	311 329 259	162 201 129
Mean	27.0	24.3	25.7	33.0	70.0	299.7	164.0
S.D.	2.65	6.81	3.51	2.00	11.53	36.35	36.04

S-9 10%	Concentration ($\mu\text{g}/\text{plate}$)						Positive Control
	Solvent Control	Test Substance					
	0	8	40	200	1000	5000	
No. of revertants per plate	18 17 14	19 18 21	16 17 18	66 50 66	196 191 177	696 641 544	770 754 661
Mean	16.3	19.3	17.0	60.7	188.0	627.0	728.3
S.D.	2.08	1.53	1.00	9.24	9.85	76.96	58.86

BACTERIAL MUTATION TESTS

Date of Experiment :- 09/08/89

Ref. No. :- M/AMES/19624

Study Investigators :- G/D/MW

Test Substance :- SC90

Bacterial Strain :- TA100

Positive Control :- SA -S-9
2AA +S-9

Solvent :- Deionised Water

S-9 0%	Concentration (µg/plate)						Positive Control
	Solvent Control	Test Substance					
	0	8	40	200	1000	5000	
No. of revertants per plate	97	80	96	93	105	119	200
	97	79	85	89	134	129	212
	90	101	93	88	111	104	297
Mean	94.7	86.7	91.3	90.0	116.7	117.3	236.3
S.D.	4.04	12.42	5.69	2.65	15.31	12.58	52.88

S-9 10%	Concentration (µg/plate)						Positive Control
	Solvent Control	Test Substance					
	0	8	40	200	1000	5000	
No. of revertants per plate	75	79	153	110	225	612	1083
	85	97	76	107	207	648	1097
	90	91	118	92	164	451	1067
Mean	83.3	89.0	115.7	103.0	198.7	570.3	1082.3
S.D.	7.64	9.17	38.55	9.64	31.34	104.90	15.01

APPENDIX 2

Individual Plate Counts for Experiment 2

D 05



STUDY NO. 2

Toxicol Study No : M/HL/21719

Pharmakopius Limited
Metaphase Analysis of
Human Lymphocytes
Treated with SC 90

July 1990

Sponsor

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SC 90

METAPLEASE ANALYSIS OF HUMAN LYMPHOCYTES

TOXICOL REPORT REFERENCE M/HL/21719

I certify that this study report provides a true and complete record of the data generated and that the study was conducted in accordance with the Principles of Good Laboratory Practice set forth in the following :-

1. The United Kingdom GLP Compliance Programme (D.o.H. 1989).
2. The Principles of Good Laboratory Practice set forth in the OECD Guidelines for the Testing of Chemicals . ISBN 92-64-12367-9. Paris 1982.
3. The United States of America Code of Federal Regulations (FDA) Title 21 Part 56 (1978) as amended by CFR 21 Part 58 Final Rule (Volume 52 No. 172 September 4th 1987).
4. Notification No. 313 of the Pharmaceuticals Affairs Bureau, Japanese Ministry of Health and Welfare (March 1982).

Signed:  Study Director
 (J. C. Asquith, B.Sc., M.Phil., Ph.D.)

Date: ... 20th July 1990

SC 90

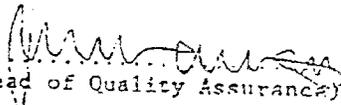
METAPHASE ANALYSIS OF HUMAN LYMPHOCYTES

Toxicol Study No : M/HL/21719

At the time of this study the Quality Assurance Unit of Toxicol Laboratories was inspecting one of each critical phase of every type of Mutagenicity study each month as part of a comprehensive evaluation of such studies.

This report has been audited in compliance with the Principles of Good Laboratory Practice. As far as can be reasonably established, the methods described and the results incorporated in the report accurately reflect the raw data produced during this study.

In addition, facilities associated with this study were inspected according to Quality Assurance Unit Standard Operating Procedures.

S. Trenchard-Morgan: 
(Head of Quality Assurance)

Date: July 2nd 1980

C O N T E N T S

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1.0 SUMMARY

1.1

A dose rangefinder was carried out on SC 90 using concentrations up to 5000µg/ml. Based on the results obtained, concentrations of 500, 1667 and 5000µg/ml in the absence and presence of S-9 were selected for the cytogenetic study.

1.2

Whole blood cultures from two donors were stimulated with phytohaemagglutinin (PHA) for 45 hours before treatment with SC 90 in the presence of S-9 for 3 hours, and 48 hours before treatment with SC 90 in the absence of S-9 for 24 hours. Positive control cultures without S-9 were treated with 0.5µg/ml Mitomycin C (MMC) while positive control cultures with S-9 were treated with 20µg/ml cyclophosphamide (CPA).

All cultures received 0.15µg/ml demecolcine 2 hours before harvesting at 72 hours. Metaphase spreads were made from fixed cells and stained by conventional methods. Slides were coded by an independent observer and 100 cells scored from each culture.

1.3

Solvent control cultures gave means of 1 and 3 aberrations (excluding gaps) per 100 cells with and without S-9, which are acceptable values for the controls.

1.4

Both positive control chemicals gave significantly more structural aberrations than the relevant negative controls, showing the cells to be sensitive to the effects of known clastogenic (chromosome breaking) agents.

1.5

SC 90 caused statistically significant increases in chromosome aberrations at 1667 and 5000µg/ml with a dose response relationship in the presence of S-9 only. In the absence of S-9, SC 90 caused no statistically significant increases in chromosome aberrations, nor did the aberrations scored form a dose response relationship.

1.6

SC 90 has been found to be a clastogen to human lymphocytes under the conditions of this study in the presence of S-9 only.

2.0 INTRODUCTION

The sponsor requested that SC 90 be tested for its clastogenic (chromosome-breaking) potential, using human lymphocytes in whole blood cultures.

The study was designed to meet the requirements of Method B10 of Annex V of the EEC.

3.0 TEST SUBSTANCE

The test substance, a clear viscous liquid at room temperature, was received at Toxicol Laboratories Limited on the 8th September 1989. It was supplied in an opaque polythene bottle with a white polythene screw cap, and labelled :

Salcare SC 90

When not in use the test substance was stored in the dark at room temperature, was assigned Toxicol Reference Number M/HL/21719 for this study and is referred to as SC 90 throughout this report.

4.0 METHODS

4.1 Cells

Peripheral blood was drawn from two healthy volunteers with no history of chromosome fragility, no recent exposure to hazardous chemicals or ionizing radiation and no recent virus infection.

The blood was taken fresh on the day of the experiment, into sterile lithium heparin tubes and 0.8ml aliquots were added to sterile plastic universal bottles to give 10ml cultures in bicarbonate buffered RPMI 1640 medium (Imperial Laboratories Limited) containing 15% Foetal bovine serum (Flow Laboratories Limited) 0.1ml of phytohaemagglutinin (Sigma Chemicals Limited) and 100 units/ml penicillin and streptomycin.

4.2 Sample Preparation

SC 90 was soluble in the cultura medium. This solubility was facilitated by continually mixing the sample on addition of the cultura medium solvent.

Solutions of SC 90 were prepared in cultura medium without foetal bovine serum at ten times the final top dose. Foetal bovine serum was omitted at this stage to avoid problems of frothing when dissolving SC 90. This ten times solution was then diluted to the test doses of 5000, 1667 and 500 μ g/ml to give two sets of dosing solutions. One was used for dosing cultures in the presence of S-9, with the other being used for dosing cultures in the absence of S-9. The latter dosing solutions contained foetal bovine serum at 20% final volume. The plus S-9 cultures did not contain foetal bovine serum during the dosing period. Positive control chemicals were mitomycin C (MMC) at 0.5 μ g/ml in the absence of S-9 and cyclophosphamide (CPA) at 20 μ g/ml in the presence of S-9. Both positive controls were dissolved in water at 100 x the required concentration and diluted in medium.

All solutions were prepared immediately before use. High concentrations of SC 90 tended to increase the viscosity of the cultura medium, which could alter the osmolarity of the medium and thus cause non-compound related aberrations, hence a concentration limit of 5000 μ g/ml was used in this chromosome aberration study.

4.3 Liver Microsomal Fraction S-9

S-9 was made according to the method of Ames *et al* (Mutation Research 1975, 31, 347) from Fischer 344 rats which had been induced with Aroclor-1254 at 500mg/kg over a 5 day period. The protein content of the S-9 as determined by the Lowry method (J. Biol. Chem. 1951, 193, 265-275) must not be less than 35mg/ml to be acceptable for use. The batch of S-9 used in this study had a protein content of 54.4mg/ml

4.4 Lymphocyte Culture and Treatment

Blood from each donor was established in 10 cultures as described in 4.1 and incubated at 37°C. Cells for treatment in the presence of S-9 and absence of S-9 were centrifuged at 1200 rpm for 6 minutes and the medium removed at 45 hours and 48 hours after cultures were established, respectively. Treatment in the presence of S-9, which was for 3 hours, was carried out in serum-free medium. Treatment in the absence of S-9, which was for 24 hours, was carried out in medium containing 20% serum. Positive control treatments were for the same lengths of time as treatments with SC 90.

After 3 hours treatment in the presence of S-9 the cells were again pelleted by centrifugation at 1200 rpm for 6 minutes, the treatment medium removed, the cells washed with sterile phosphate buffered saline, centrifuged again and resuspended in fresh medium containing 20% serum. In the absence of S-9, treatment continued until the cells were harvested.

4.5 Metaphase Arrest and Harvest

Two hours before harvesting, cells were arrested in mitosis by adding demecolcine to each culture at a final concentration of 0.15µg/ml and incubating at 37°C. After metaphase arrest each culture was centrifuged at 1200 rpm for 5 minutes.

The supernatants were removed and cells were swollen in 0.075M KCl for 7 minutes at 37°C. The cells were then fixed. After centrifugation, the KCl solution was removed and replaced by an equal volume of fresh methanol/glacial acetic acid (3:1), followed by several changes of fixative, using centrifugation and resuspension, until the supernatants were clear.

4.6 Slide Preparation

Fixed cells which had been left at room temperature for 30 minutes were pelleted and resuspended in approximately 0.2ml of fresh fixative. This suspension was then dropped onto scrupulously clean, dry microscope slides. 2 or 3 drops of cell suspension were put on each slide and 2 slides were made from each culture. The slides were air dried before staining with 5% Gurr's R66 Giemsa in pH 6.8 buffer for 5 minutes. The slides were rinsed, air dried and mounted in Gurr's Neutral Mounting Medium.

4.7 Coding of Slides

The mounted, dried slides were coded by a responsible person not connected with the scoring of the slides. This person devised 20 separate, unambiguous codes, one for each experimental culture, and used adhesive labels to cover the existing information so that the cytogeneticists could only see the study reference number, the donor and the code. The slide code was kept in the files of another department while the slides were scored.

4.8 Scoring of Chromosome Damage

100 cells were scored from each set of 2 slides with one code, thus resulting in 200 cells scored for each level of treatment either with or without S-9. Only well-spread metaphases with a chromosome count of 44 or more were scored.

Chromatid and chromosome gaps and breaks as well as fragments and re-arrangements were all recorded on raw data sheets MU29F.1 with the stage co-ordinates (vernier readings) of every cell containing aberrations.

The mitotic index for each culture was determined, based on a total of 500 cells per culture, in order to check whether mitosis was being inhibited at the higher doses.

4.9 Statistical Analysis

All SC 90 treated and positive control results were compared with solvent controls using the formula:

$$\text{Chi squared} = \sum \frac{(O - E - 0.5)^2}{E}$$

where O = observed total number of aberrations

E = expected total number of aberrations

All points were compared as the total aberrations (excluding gaps) scored in 200 cells (100 from each of 2 donors) or 50 cells for positive controls. not as the mean aberrations per 100 cells scored.

5.0 RESULTS

5.1 Dose-rangefinder

A dose rangefinder was carried out, over a wide range of concentrations. The concentrations used were spaced at half-log intervals with 5000µg/ml as the top dose plus and minus S-9. The results (Table 1 below and Graph A, Appendix 1) of the dose rangefinder showed greater toxicity of SC 90 in the absence of S-9 than in the presence of S-9.

In Table 1 results are given as percentage mitotic cells (Mitotic index, MI) and as relative mitotic index (MI for control = 100%)

Table 1

Point		MI	relative MI	Point		MI	relative MI
Control	-S-9	7.03	100.0	Control	+S-9	9.55	100.0
8	µg/ml -S-9	6.35	90.3	8	µg/ml +S-9	10.21	106.9
40	µg/ml -S-9	5.63	80.1	40	µg/ml +S-9	4.74	49.6
200	µg/ml -S-9	8.86	126.0	200	µg/ml +S-9	9.09	95.2
1000	µg/ml -S-9	6.13	87.2	1000	µg/ml +S-9	8.97	93.9
5000	µg/ml -S-9	3.37	47.9	5000	µg/ml +S-9	7.43	77.8

Doses of 500, 1667 and 5000µg/ml in the presence and absence of S-9 were selected for the cytogenetic study. These doses are logarithmically equally spaced over one decade of concentrations to meet the requirements of Method B10 of Annex V.

5.2 Chromosome Aberration Assay

5.2.1 Raw Data

The raw data forms MU29P.1. are not included in this report, but are available to the sponsor on request.

The data for SC 90 in the absence of S-9 are summarised in Table 2, data for SC 90 in the presence of S-9 are in Table 3. Results for positive control chemicals are summarised in Table 4.

5.2.2 Analysis of Raw Data

Mitotic index figures for cultures treated with SC 90 showed again that SC 90 was more toxic in the absence of S-9 than in the presence of S-9, compared to the controls. The top dose in the absence of S-9 gave a relative mitotic index of 81.6% which was not as marked as in the range-finder results. In the presence of S-9 no toxicity was seen as all points were around the control mitotic index value with a slight increase in mitotic index seen in donor A in the presence of S-9. It is also clear to see that the mitotic indices produced by donor A are much smaller than those seen in donor B. This is purely a donor effect with donor B responding to culture much better than donor A, for some reason not ascertainable in this study. Donor B was also used for the range-finder experiment.

Negative control cultures gave a mean total of 3 and 1 aberrations (excluding gaps) in the absence and presence S-9 respectively. These are within the acceptable backgrounds for chromosome aberrations in human lymphocytes.

Both positive control chemicals have given large numbers of aberrations compared to negative controls ($p < 0.001$, see page 15, summary of statistical analysis) showing that the cells were sensitive to the effects of known clastogens (chromosome breaking agents) and that the S-9 was able to metabolise an inactive precursor (CPA) to an actively genotoxic intermediate.

SC 90 produced significant increases in chromosome aberrations at 1667 and 5000 $\mu\text{g/ml}$ in the presence of S-9 only. There was also a dose response relationship in the presence of S-9 (see graph B Appendix 1). In the absence of S-9 no point produced a significant increase in chromosome aberrations, although two fragmented cells with a minimum of ten aberrations each were scored in donor B.

There was no significant increase in numerical aberrations scored either plus or minus S-9 indicating that SC 90 is not a spindle poison.

0029.F2

Test Compound :- SC 90

Ref. No. :- M/10/21719

Treatment	Replicate No.	No. cells scored	Aberrations per 100 cells						Total Aberrations + gaps - gaps	% cells with aberrations + gaps - gaps	Miotic Index			
			Gaps	Chromosome Del. Exch.	Chromatid Del. Exch Del.	iso-locus Del.	+ gaps	- gaps						
CONTROL -S-9	A	100	4	0	0	0	0	0	5	1	5	1	0	8.59
	B	100	1	0	0	0	0	0	6	3	5	4	1	11.99
Mean/100 cells based on 200 cells scored														
	A	100	4	0	0	0	0	0	5.5	3.0	5.0	2.5	0.5	10.29
	B	100	0	1	0	0	1	0	8	4	6	4	0	5.62
									1	1	1	1	1	14.54
Mean/100 cells based on 200 cells scored														
	A	100	5	0	0	0	0	0	4.5	2.5	3.5	2.5	0.5	10.08
	B	100	3	0	0	0	0	0	9	4	9	4	3	6.62
									3	0	3	0	1	13.16
Mean/100 cells based on 200 cells scored														
	A	100	5	0	0	0	0	0	6.0	2.0	6.0	2.0	2.0	9.89
	B	100	0	2	0	0	0	1	10	5	9	5	0	7.13
									5	5	3	3	2	9.66
Mean/100 cells based on 200 cells scored														
	A	100	2.5	1.0	0	0	0	2.0	7.5	5.0	6.0	4.0	1.0	8.40
	B	100	4.0	0	0	0	0	0	6.0	2.0	6.0	2.0	2.0	9.89
									10	5	9	5	0	7.13
									5	5	3	3	2	9.66

* Cells which show fragmentation are excluded from the calculation of aberrations per 100 cells but are included in cells with aberrations.

Test Compound :- SC 90

Ref. No. :- M/HL/21719

Treatment	Repl- cate No.	No. scored	Aberrations per 100 cells					Total Aberrations + gaps - gaps	% cells with aber- rations + gaps - gaps	Mitotic Index				
			Gaps	Chromosome Del. Exch.	Chromatid Del. Exch	iso- locus Del.	iso- locus Del.				Oth- ers*			
CONTROL	A	100	6	0	0	1	0	0	2	8	2	0	4.30	
	B	100	0	0	0	0	0	0	0	0	0	3	11.31	
Mean/100 cells														
based on 200			3.0	0	0	0.5	0	0.5	1.0	4.0	1.0	1.0	1.5	7.81
cells scored														
500	A	100	5	1	0	1	0	1	0	3	3	0	4.96	
	B	100	4	0	0	2	0	0	2	2	2	0	10.45	
Mean/100 cells														
based on 200			4.5	0.5	0	1.5	0	0.5	2.5	7.0	2.5	0	7.71	
cells scored														
1667	A	100	6	0	0	7	0	0	13	7	12	7	5.61	
	B	100	1	0	0	3	0	0	4	3	4	3	12.18	
Mean/100 cells														
based on 200			3.5	0	0	5.0	0	0	8.5	5.0	5.0	1.0	9.21	
cells scored														
5000	A	100	6	0	0	5	0	3	14	8	13	8	5.62	
	B	100	1	0	0	3	0	0	4	3	3	2	11.29	
Mean/100 cells														
based on 200			3.5	0	0	4.0	0	1.5	9.0	5.5	8.0	5.0	2.5	8.46
cells scored														

* Cells which show fragmentation are excluded from the calculation of aberrations per 100 cells but are included in cells with aberrations.

MD29.F2

TABLE 4

Test Compound :- Positive controls

Ref. No. :- H/NL/21719

Treat- ment	Repli- cate No.	No. cells scored	Aberrations per 100 cells					Iso- locus Del.	Total Aberrations + gaps - gaps	% cells with aber- rations + gaps - gaps	Oth- ers*		
			Gaps	Chromosome Del. Exch.	Chromatid Del. Exch.	Del. Exch.	Del. Exch.						
HMC	A	25	4	8	0	28	16	16	72	68	48	0	
	B	25	8	0	0	36	20	8	72	64	44	4	
Mean/100 cells based on 50 cells scored			6.0	4.0	0	32.0	18.0	12.0	72.0	66.0	46.0	46.0	2.0
CPA	A	25	12	4	4	12	0	12	44	32	32	28	0
	B	25	8	0	0	28	0	12	48	40	44	36	4
Mean/100 cells based on 50 cells scored			10.0	2.0	2.0	20.0	0	12.0	46.0	36.0	38.0	32.0	2.0

* Cells which show fragmentation are excluded from the calculation of aberrations per 100 cells but are included in cells with aberrations.

Summary of Statistical Analysis

All points were tested against the relevant negative controls as the results for two donors combined, using the chi-squared test.

Point	Chi squared
500µg/ml -S-9	0.50 NS
1667µg/ml -S-9	0.10 NS
5000µg/ml -S-9	0.56 NS
MHC -S-9	97.77 p<0.001
500µg/ml +S-9	0.57 NS
1667µg/ml +S-9	4.08 p<0.05
5000µg/ml +S-9	4.92 p<0.05
CPA +S-9	56.95 p<0.001

NS = Not significant

6.0 DISCUSSION AND CONCLUSION

Positive control chemicals are included in all in vitro genetic toxicology tests in order to demonstrate :

- a) Sensitivity of the cells to the effects of known genotoxins
- b) The ability of the metabolic activation system used to metabolize an active precursor (CPA) to an actively genotoxic intermediate.

Both these points have been satisfactorily demonstrated in the current study.

Two criteria were preset (Protocol Section 10.2) as the requirements for a positive result. These are:-

- a) A dose - response relationship.
- b) At least one point with statistically significantly more chromosome aberrations than the negative controls.

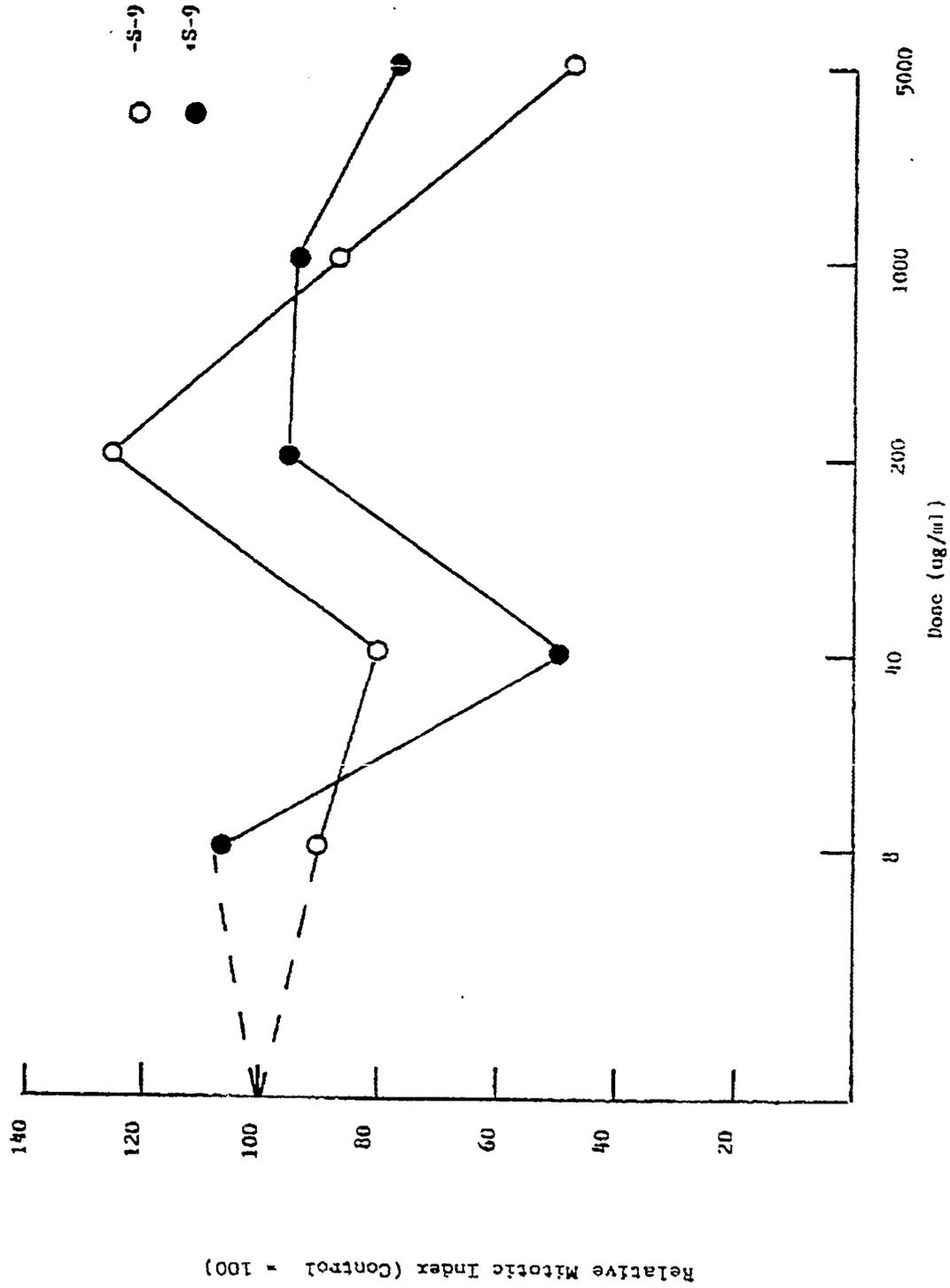
In the presence of S-9 the above two criteria have been met with a dose response relationship (see graphs B and C in Appendix 1) and two points which show statistical significance against the negative control. Neither of these criteria have been met in the absence of S-9.

Having met these criteria SC 90 has been found to be a clastogen to human lymphocytes in the presence of S-9 when tested up to a limit of 5000µg/ml (5mg/ml).

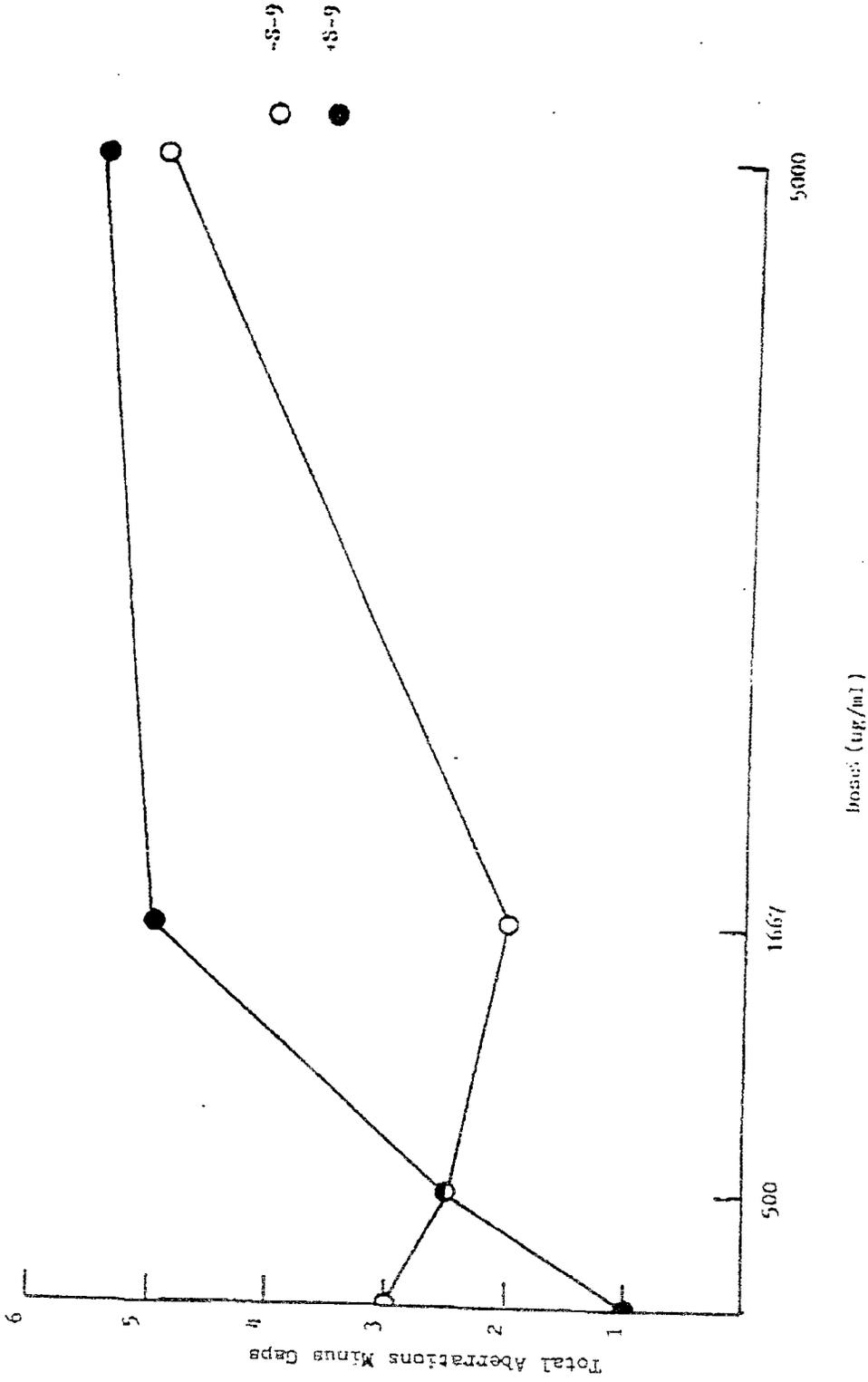
APPENDIX 1

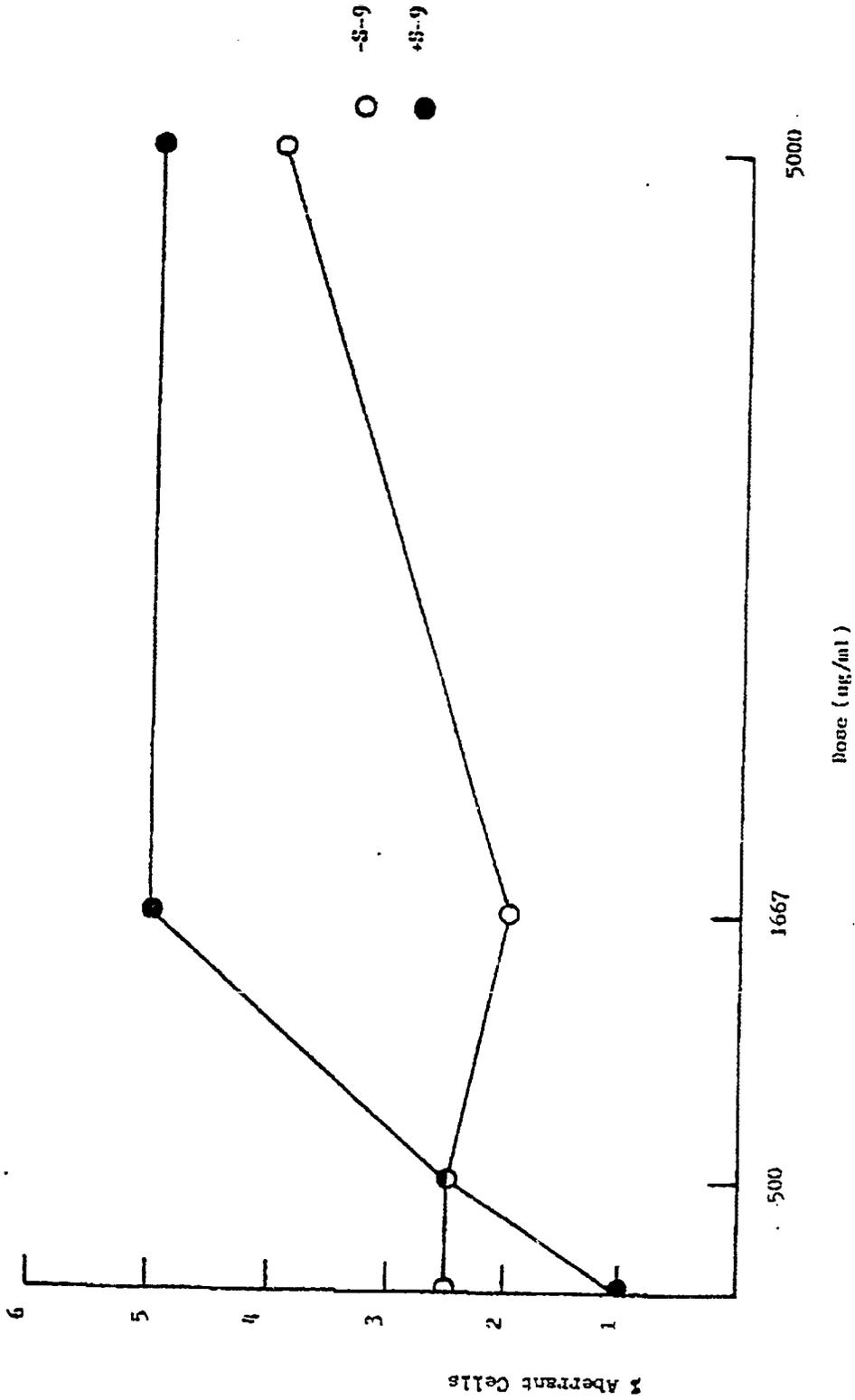
- Graph A : Dose Rangefinder Relative Mitotic Indices
- Graph B : Total aberrations (excluding gaps) against dose of SC 90
- Graph C : Percentage cells with aberrations (excluding gaps, including fragmented cells) against dose of SC 90)

GRAPH A
Range-finder Experiment on SC 90



CHAPI B Total Aberrations (Excluding Caps) Against Dose of SC 90







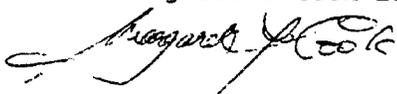
STUDY NOS. 3-10

SALCARE SC90

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Report No: BKPS/TOX/05
Project No: 0004/91

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Summary

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As Salcare SC90 had been shown to have mutagenic potential both in the Ames test and in the metaphase analysis of human lymphocytes a series of 2-strain Ames tests was undertaken on various SC90-derived products. Three of the tests were positive, one of them being on SC90 itself.

There is no clear cut explanation for the findings in this study. Two factors could be involved: one is the possible interaction of the preservative with the constituents but this is considered unlikely. The duration of the addition time in the process could also be involved.

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It is recommended that further Ames tests should be conducted on:

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- SC90 before neutralisation and dilution

- PRP 5329A and PRP 5330A with the addition of a preservative.

If one of these products is non-mutagenic and has the required properties this could be considered as a replacement for SC90.

J /

MJC/lg/TOX-03

Method

The original eight samples (labelled PRP5327 to 5334 consecutively) were received from the General Industries Division, Allied Colloids on 14 March 1990.

Samples were transferred from the stock bottles to amber/yellow clear plastic tubes with white snap-on caps and labelled PKPS/PRP5327-5334. They were delivered to Toxicol Laboratories, Ledbury on 3 April 1990.

The original samples were found to be contaminated with fungal growth so replacement samples in 1 litre plastic bottles were received from the General Industries Division, Allied Colloids on 22 June 1990. These were labelled PRP 5327A to 5334A consecutively by Allied Colloids to distinguish them from the original samples. On the same day ie 22 June 1990 the same bottles were relabelled PKPS/PRP 5327A-5334A and delivered to the Toxicol Laboratories.

PKPS/PRP 5327A-5334A were tested in vitro by the Ames plate incorporation method for its ability to induce mutations in two histidine dependent auxotrophic mutants of *Salmonella typhimurium*: strains TA98 and TA100.

The methods employed in the Ames tests are presented in Appendices 1-8.

All of the studies were carried out in accordance with the principles of Good Laboratory Practice.

Results

The results of the six individual tests are also presented in Appendices 1-8.

The results can be summarised as follows:

Sample number	TA 98		TA 100	
	without S-9	with S-9	without S-9	with S-9
PKPS/PRP 5327A	+	+	+	+
PKPS/PRP 5328A	+	-	+	+
PKPS/PRP 5329A	-	-	-	-
PKPS/PRP 5330A	-	-	-	-
PKPS/PRP 5331A	-	-	-	-
PKPS/PRP 5332A	-	-	-	-
PKPS/PRP 5333A	+	-	+	-
PKPS/PRP 5334A	-	-	-	-

Three of the samples therefore were shown to be mutagenic in the Ames plate incorporation assay, namely PKPS/PRP 5327A, PKPS/PRP 5328A and PKPS/PRP 5333A.

Discussion

The eight Ames tests were carried out 'blind' ie neither Pharmakopius (Services) nor Toxicol Laboratories staff were aware of the identity of the samples. However when the results of the studies had been reported, details of the constitution and preparation of the eight samples were made available to Pharmakopius (Services) Ltd and are presented in Appendix 9.

It was revealed that PKPS/PRP 5327A was, in fact, Salcare SC90 which had been shown to be mutagenic in both the 5-strain Ames test¹ and the human chromosome study². The polymer component of PKPS/PRP 5333A consisted of the powder extracted from PKPS/PRP 5327A as the active polymer ingredient. This too was shown to be mutagenic. The third mutagenic compound was PKPS/PRP 5328A which had the same polymer component as the original product PKPS/PRP 5327A (ie Salcare SC90). However the addition time was shorter (2 hour vs 5 hour), the free toluene concentration was lower (30 ppm vs 5000 ppm), Perlankrol RN75 was used instead of Perlankrol FN65 and was at a lower level (2% vs 4%).

Compound PKPS/PRP 5330A was identical to PKPS/PRP 5327A in Perlankrol use (ie 4% total Perlankrol FN65), and in the amount of free toluene in the RS10AE patch but the addition time was shorter (4 hours vs 5 hours). This compound was not mutagenic in the Ames test. It could therefore be that, for some unknown reason, the lengthening of the addition time renders the compound mutagenic.

Compound PKPS/PRP 5329A was similar to PKPS/PRP 5328A in that the same amount of the same batch of surfactant (2% Perlankrol RN75) was used. The amount of toluene however, was greater in 5329A (5000 ppm vs 30 ppm) and the addition time was longer (4 hr vs 2 hr). This latter factor would seem to indicate that the addition time may not be relevant:

Addition time 5327
(mutagenic)
5328
(mutagenic)

Another factor should be considered
(PKPS/PRP 53327A) contained a polymer
this was not present in the other
another difference between the
non-mutagenic PKPS/PRP 5330A. C
in some way with the compound
PKPS/PRP 53328A, a laboratory pre
preservative, but was shown to
This would suggest, therefore,
to be implicated.

An Ames test is already planned
This liquid is at pH 4 and it
neutralisation and dilution pro
conferred mutagenic potential on

From the foregoing it is evident
indication as to why some of the
negative. It is considered unlikely
a preservative or vaccination in
been implicated.

In the absence of any explanation
on both PKPS/PRP 53328A and PKPS/PRP
recommended that further 5-strain
after the addition of preservative
these tests, one of these products
replacement for the original SC90

Addition time	5327	>	5330
	(mutagenic)		(non-mutagenic)
	5328	<	5329
	(mutagenic)		(non-mutagenic)

Another factor should be considered. The works prepared sample (PKPS/PRP 5327A) contained a preservative, propyl paraben, but this was not present in the other samples. This was therefore another difference between the mutagenic PKPS/PRP 5327A and the non-mutagenic PKPS/PRP 5330A. Could the preservative be reacting in some way with the compound to render it mutagenic? However PKPS/PRP 5328A, a laboratory prepared samples, did not contain a preservative, but was shown to be positive in the Ames test. This would suggest, therefore, that the preservative is unlikely to be implicated.

An Ames test is already planned on Salcare SC90 'as supplied'. This liquid is at pH4 and it has been postulated that the neutralisation and dilution procedure may have, in some way, conferred mutagenic potential on this product.

From the foregoing it is evident that there is no clear indication as to why some of the tests were positive and other negative. It is considered unlikely that either the addition of a preservative or variation in addition time are likely to have been implicated.

In the absence of any explanation for these findings and as tests on both PKPS/PRP 5329A and PKPS/PRP 5330A were negative it is recommended that further 5-strain Ames tests should be conducted, after the addition of preservative. Depending on the results of these tests, one of these products could be considered as a replacement for the original SC90.

Recommendations

The following 5-strain Ames tests should be conducted:

- i) PKPS/PRP 5329A + preservative
- ii) PKPS/PRP 5330A + preservative
- iii) Un-neutralised, undiluted Salcare SC90.

References

1. Toxicol Report M/Ames/19624
2. Toxicol Report M/Ames/21719
3. Pharmakopius Report PKPS/TOX/01

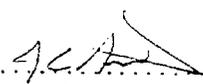
FKPS/PRP 5327

BACTERIAL REVERSE MUTATION ASSAY

Toxicol Study No : M/AMES/24667

I certify that this study report provides a true and complete record of the data generated and that the study was conducted in accordance with the Principles of Good Laboratory Practice as set forth in the following :-

1. The United Kingdom GLP Compliance Programme (DoH 1989).
2. The Principles of Good Laboratory Practice as set forth in the OECD Guidelines for the Testing of Chemicals, ISBN 92-64-12367-9, Paris 1982.
3. The United States of America Code of Federal Regulations (EPA-TSCA) Toxic Substances Control Act CFR 40 Part 792 (Volume 48 No. 230 November 29th 1983).

Signed:  Study Director
(J. C. Asquith, B.Sc., M.Phil., Ph.D.)

Date: 31st October 1990

FKPS/PRP 5327

BACTERIAL REVERSE MUTATION ASSAY

Toxicol Study No : M/AMES/24667

At the time of this study the Quality Assurance Unit of Toxicol Laboratories was inspecting one of each critical phase of every type of Mutagenicity study each month as part of a comprehensive evaluation of such studies.

This report has been audited in compliance with the Principles of Good Laboratory Practice. As far as can be reasonably established, the methods described and the results incorporated in the report accurately reflect the raw data produced during this study.

In addition, facilities associated with this study were inspected according to Quality Assurance Unit Standard Operating Procedures.

S. Trenchard-Morgan: *[Signature]*.....
(Head of Quality Assurance)

Date: *November 5, 1970*

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1.0 SUMMARY

FKPS/PRP 5327 was tested in vitro by the Ames plate incorporation method for its ability to induce mutations in two histidine dependent auxotrophic mutants of Salmonella typhimurium : strains TA98 and TA100.

Two independent mutation tests were performed, each in both the presence and absence of a metabolic activation system (S-9 mix) derived from the livers of Aroclor 1254 treated rats. The bacteria were exposed to the test substance dissolved in deionised water, which was also the solvent control. Based on the results of a preliminary toxicity rangefinder, doses used in the mutation experiments were :

5000, 1000, 200, 40 and 8 µg/plate

All solvent (negative) controls gave counts of revertants within normal ranges.

All positive controls gave numbers of induced revertants within expected ranges demonstrating the sensitivity of the assay and the metabolising activity of the S-9 mix.

FKPS/PRP 5327 gave increased numbers of revertants with statistical significance, at all dose levels tested in both experiments, in TA100 in the absence of S-9. Increased numbers of revertants were scored for TA98 in both the absence and presence of S-9 and TA100 in the presence of S-9 in both experiments. These increases were, however, only statistically significant in the second experiment.

FKPS/PRP 5327 has been found to be a direct acting mutagen in the Ames plate incorporation assay.

2.0 INTRODUCTION

The purpose of this study was to assess the mutagenic potential of PKPS/PRP 5327 by testing reversion to prototrophy in two histidine auxotrophic mutants of Salmonella typhimurium. Two independent mutation tests were conducted, each in the presence and absence of a metabolic activation system derived from the livers of rats which had been treated with Aroclor 1254, an enzyme inducing agent. The test was performed in accordance with the plate incorporation method of Ames (references 1 and 2).

This study was part of an initial screen of eight test substances.

3.0 TEST SUBSTANCE

The test substance PKPS/PRP 5327 was a clear viscous liquid in an amber/yellow, clear plastic tube with a white plastic snap-on cap. The bottle was labelled 'PKPS/PRP 5327'.

The test substance arrived at the testing facility on 3rd April 1990.

The reference M/AMES 24667 was given to PKPS/PRP 5327 for this study. When not in use the substance was stored in a locked metal cupboard at room temperature.

The test substance is referred to as 'PKPS/PRP 5327' throughout this report.

As the initial sample was found to be contaminated with fungal growth, a second sample, in a white plastic bottle with a white plastic screw cap, was delivered to the testing facility on 22nd June 1990.

4.0 MATERIALS

4.1 Bacterial Strains

The following strains were used in the Ames test :

S. typhimurium TA98 his D3052 rfa⁻ Δ uvr B⁻R⁺

S. typhimurium TA100 his G46 rfa⁻ Δ uvr B⁻R⁺

Both Salmonella strains are defective in DNA repair capacity (Δ uvrB⁻) and have a defective lipopolysaccharide barrier on the cell wall (rfa⁻). These two properties confer extra sensitivity to DNA damage and also greater permeability of large molecules into the cell. They also contain a resistance transfer factor (plasmid pKM 101). This factor (R⁺), which confers resistance to ampicillin, enhances the operation of an error-prone repair system.

The strains are tested routinely for histidine dependence, cell membrane permeability and ampicillin resistance (reference 1) where appropriate.

TA100 is reverted to prototrophy by base substitution mutagens and TA98 and occasionally TA100 by frameshift mutagens.

Several days before the test, each bacterial strain was streaked from an overnight broth culture, prepared from freshly thawed frozen cells, onto nutrient agar plates. These plates were incubated at 37°C overnight to give isolated colonies and were then stored at 4°C until required.

On the day before the experiment, 50ml quantities of sterile nutrient broth were inoculated with a single colony of appropriate strain and incubated overnight at 37°C.

4.2 Sample Preparation

PXPS PRP 5327 was miscible with water. It was mixed with sterile deionized water by addition of the water to a sterile plastic universal bottle containing the test substance, while the bottle was being agitated on a rotary mixer. The highest concentration solution was prepared in this way and lower concentrations were then prepared by serial dilution.

PXPS, PRP 5327 was tested at the following concentrations:-

Rangefinder: 50, 10, 2, 0.4 0.05 and 0.015 mg/ml

Mutation Experiment: 50, 10, 2, 0.4 and 0.08 mg/ml

These concentrations gave dose levels of:-

Rangefinder: 5000, 1000, 200, 40, 8 and 1.6 µg/plate

Mutation Experiment: 5000, 1000, 200, 40 and 8 µg/plate

4.3 Metabolic Activation System (S-9 Mix)

The liver microsomal fraction (S-9) was prepared according to the method of Ames et al (reference 1) from Fischer 344 rats which had been induced with Aroclor 1254 (see Appendix 3).

The protein content, estimated by the method of Lowry et al (reference 4) was found to be 51.6 mg/ml. For use, the S-9 protein content must not be less than 35mg/ml.

The metabolic activation system (S-9 mix) used in this study was made as described in Appendix 3 and was kept on ice throughout the experiment.

4.4 Positive Controls

(a) Without S-9 mix

<u>Strain</u>	<u>Positive Control</u>
TA100	Sodium azide (SA) at 1ug/plate
TA98	2-Nitrofluorene (2NF) at 0.5ug/plate

(b) With S-9 mix

<u>Strain</u>	<u>Positive Control</u>
TA98)	2-Aminoanthracene (2AA) at 2ug/plate
TA100)	

5.0 METHODS5.1 Toxicity Rangefinder

In order to determine if PKPS/PRP 5327 was toxic to the tester bacteria a preliminary rangefinder was carried out using Salmonella typhimurium strain TA98. Only one Salmonella strain was used as in our experience both strains used show a similar toxic response. For this purpose, an Ames test was carried out with the above strain in the presence and absence of S-9 mix as described in 5.2. The concentrations used for the rangefinder were 5000, 1000, 200, 40, 8 and 1.6 µg/plate. After 24 hours incubation at 37°C the plates were examined for the appearance of a complete bacterial lawn as seen under a dissecting microscope.

5.2 Ames Test

All procedures were carried out under low intensity yellow lighting in a class II cabinet.

PKPS/PRP 5327 was tested against the above (4.1) bacterial strains at five dose levels, both with and without S-9 mix, in triplicate in two separate experiments. The test substance was assayed in both experiments at the dose levels given in Section 4.2.

5.2.1 Without metabolic activation

The following components were added sequentially to 2ml of histidine and biotin supplemented molten top agar :-

- 0.1ml of a dilution of PKPS/PRP 5327
- 0.1ml of appropriate positive control
- 0.1ml of sterile distilled water (negative control)
- 0.1ml of appropriate overnight bacterial culture (approximately 10^8 organisms)
- 0.5ml of sterile 0.2M phosphate buffer (pH 7.4)

The components were rapidly mixed on a Whirlimixer and poured onto Vogel Bonner E minimal agar plates. Three plates were prepared for each dose point or control. When the agar had set, the plates were inverted and incubated at 37°C for 48 hours. Numbers of revertants per plate were counted using a Biotran III automatic colony counter and the results recorded on form MU20F1.

5.2.2 With metabolic activation

Methodology was as described in 5.2.1 except that 0.5ml of S-9 mix (see Appendix 3) was added to test tubes instead of sterile phosphate buffer.

5.3 Evaluation of Results

Mean and standard deviations for each treatment group are calculated and recorded on form MU2CF1.

Dunnnett's test is used to determine the statistical significance of the data for each strain with and without S-9 and for each experiment, by obtaining the t - statistic for combined treatments with the control (reference 5). This analysis is used as being recommended by UKEMS (reference 6).

These are recorded on Form MUSF.2.

A statistically significant increase in the mean number of revertants plus a dose response relationship in two experiments with PKPS/BRP 3327 is considered necessary to indicate a positive result.

5.4 Archiving

Copies of the final report, study protocol and protocol amendments and all raw data relating to the study will be archived at Toxicol Laboratories Ltd.

This documentation will be retained in the archive for 7 years.

At the end of the archive period, after due consultation, the documentation will be returned to the sponsor or destroyed by Toxicol Laboratories.

6.0 RESULTS6.1 Toxicity Rangefinder

After 24 hours incubation, growth assessment of the bacterial background lawn following treatment with PKPS/PRP 5327 was as follows :-

Strain	Concentration of Test Substance ($\mu\text{g}/\text{plate}$)*							
	5-9	0	1.6	8	40	200	1000	5000
TA98	0	+	+	+	+	+	+	+
TA98	10	+		+	+		+	+

* These are actual amounts of test substance added and correspond to solutions of test compound with concentrations from 0.016 to 50 mg/ml.

+ = normal growth

PKPS/PRP 5327 was not toxic to the bacteria at any concentration tested, as shown by normal growth of the background lawns.

PKPS/PRP 5327 was therefore tested in the mutagenicity experiments at dose levels of:-

5000, 1000, 200, 40 and 8 $\mu\text{g}/\text{plate}$ in both the absence and presence of S-9.

6.2 Mutation Experiment 1

6.2.1 Raw Data

The responses of the tester strains to the negative control, PKPS/PRP 5327 and the positive control mutagens are given as individual plate counts in Appendix 1. The data are summarised in Table 1 (page 13).

All solvent controls gave counts of spontaneous revertants within normal ranges.

All positive controls gave counts of induced revertants within expected ranges.

The revertant numbers scored on plates treated with PKPS/PRP 5327 were consistently higher than the spontaneous revertant numbers scored on solvent control plates, for both bacterial strains in both the absence and the presence of S-9.

6.2.2 Statistical Analysis

Dunnnett's test was carried out on the data from experiment 1.

Significant values of the t - statistic were obtained only for CA100 in the absence of S-9. All the dose points for this strain gave significant t - statistic values.

As no dose - relation of the increased revertant numbers scored could be seen over the dose-range tested, no further analysis was carried out.

TABLE 1

MEAN NUMBER OF REVERTANTS PER PLATEFOR PKPS/PRP 5327EXPERIMENT 1

Strain	± S-9	Concentration of test substance (µg/plate)						
		0	8	40	200	1000	5000	PC
TA98	0	25.0	31.3	28.3	37.3	23.3	29.7	222.7
TA100	0	96.3	160.0	186.7	171.7	181.0	179.3	513.3
TA98	10	30.7	41.7	38.7	36.3	35.3	38.7	432.3
TA100	10	145.3	166.3	172.7	167.3	181.7	156.3	678.3

PC = positive control

t - Statistic from Dunnett's test for each dose in Experiment 1

TEST SUBSTANCE :- PKPS/PRP 5327

Ref. No. : M/AMES/24667

Strain	Z	S-9	Concentration of test substance (µg/plate)					Degrees of Freedom	
			8	40	200	1000	5000	N	D
TA 98	0	0	1.36	0.77	2.65	-0.45	1.09	5	12
TA 100	0	0	5.21*	7.13*	6.07*	6.75*	6.63*	5	12
TA 98	10	10	3.07	2.27	1.61	1.32	2.27	5	12
TA 100	10	10	1.49	1.99	1.58	2.59	0.81	5	12

* = p < 0.01

6.3 Mutation Experiment 2

6.3.1 Raw Data

The responses of the tester strains to the negative control, PKPS/PRP 5327 and the positive control mutagens are given as individual plate counts in Appendix 2. The data are summarised in Table 2 (page 16).

All solvent controls gave counts of spontaneous revertants within normal ranges.

All positive controls gave counts of induced revertants within expected ranges.

As in Experiment 1, the numbers of revertants scored on dosed plates was consistently higher than the spontaneous revertant numbers for both bacterial strains, in both the absence and presence of S-9, although there was no obvious dose-response relationship over the dose range used in this experiment.

6.3.2 Statistical Analysis

Dunnnett's test was carried out on the data from Experiment 2.

Significant values of the t - statistic were obtained at all dose levels for both strains of bacteria in the absence of S-9. In the presence of S-9 significant values of the t - statistic were obtained for TA98 at 8, 1000 and 5000 µg/plate and for TA100 at 1000 µg/plate.

TABLE 2
MEAN NUMBER OF REVERTANTS PER PLATE
FOR PKPS/PRP 5327
EXPERIMENT 2

Strain	Z	Concentration of test substance ($\mu\text{g}/\text{plate}$)						
	S-9	0	8	40	200	1000	5000	PC
TA98	0	35.0	60.0	64.7	55.3	57.0	63.0	273.7
TA100	0	78.0	153.3	158.0	163.7	169.7	153.0	587.7
TA98	10	33.0	52.0	44.7	48.3	56.0	49.3	1004.7
TA100	10	128.3	154.3	161.0	159.7	165.3	159.0	737.0

PC = positive control

t - Statistic from Dunnett's test for each dose in Experiment 2

TEST SUBSTANCE :- PKPS/PRP 5327

Ref. No. : M/AMES/24667

Strain	Z	S-9	Concentration of test substance ($\mu\text{g}/\text{plate}$)					Degrees of Freedom	
			8	40	200	1000	5000	N	D
TA 98	0	0	5.98*	6.95*	4.94*	5.36*	6.61*	5	12
TA 100	0	0	8.17*	8.61*	9.10*	9.66*	8.14*	5	12
TA 98	10	10	4.18*	2.63	3.38	4.92*	3.63*	5	12
TA 100	10	10	2.71	3.35	3.22	3.79*	3.13	5	12

* = $p < 0.01$

7.0 DISCUSSION AND CONCLUSION

Responses to the solvent controls were within the normal ranges obtained in this laboratory.

All positive controls gave counts of induced revertants within expected ranges.

Satisfactory responses to the positive control compounds show :-

1. That the bacteria are sensitive, as expected, to known mutagens.
2. That the S-9 used in this study is capable of metabolising an inactive precursor (2AA) to a genotoxic intermediate.

It is necessary to demonstrate both of these points for the study to be acceptable.

In the experiments reported here, there were consistent increases in the numbers of revertants scored on PKPS/PRP 5327 treated plates. At first sight, these increases do not appear to be dose related. However, examination of the graphically presented results in Appendix 3 shows that increases in revertants occur over a range of concentrations to a maximum concentration of 40 µg/plate. At higher concentrations, a plateau has been reached and the results show scatter about this plateau level, except for TA100 in the absence of S-9 in Experiment 2 and in TA100 in the presence of S-9 in Experiment 1, where a decrease in revertants scored at 5000 µg/plate occurred. This is thought to be an early indication of toxicity, as this effect is frequently seen at a dose level lower than that giving a reduction of the background lawn.

In this study, it was considered impractical to assign a value of revertants per µg to the increases seen, as there were insufficient points in the dose range over which the increases occurred to be able to define this accurately.

Statistical analysis showed some difference in the results between the two experiments in this study. While significant t - values have been found for all dose levels tested against TA100 in the absence of S-9 in both experiments, significant t - values have only been found in Experiment 2 for TA98 in both the absence and presence of S-9 and TA100 in the presence of S-9.

The criteria for a positive result with PKPS, PRP 5327 have been met for TA100 in the absence of S-9, and the test substance has been found to be a direct acting mutagen in the Ames plate incorporation assay.

8.0 REFERENCES

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BACTERIAL MUTATION TESTS

MU20.F1

Date of Experiment :- 26/6/90

Ref. No. :- M/AMES/24667

Study Investigators :- JA/VM

Test Substance :- PKPS/PRP 5327

Bacterial Strain :- TA100

Positive Control :- SA -S-9
2AA +S-9Solvent :- H₂O

S-9 0%	Concentration (µg/plate)						Positive Control
	Solvent Control	Test Substance					
							1
No. of revertants, per plate	73 77 84	138 158 164	155 170 149	154 150 187	169 150 180	165 137 137	526 590 587
Mean	78.0	153.3	158.0	163.7	169.7	153.0	587.7
S.D.	5.57	13.61	10.82	20.31	10.02	14.42	2.08

S-9 10%	Concentration (µg/plate)						Positive Control
	Solvent Control	Test Substance					
							2
No. of revertants per plate	135 125 125	151 157 155	163 174 146	149 153 177	171 168 157	178 161 138	728 763 370
Mean	128.3	154.3	161.0	159.7	165.3	159.3	787.0
S.D.	5.77	3.06	14.11	15.14	7.37	20.07	73.98

APPENDIX 3

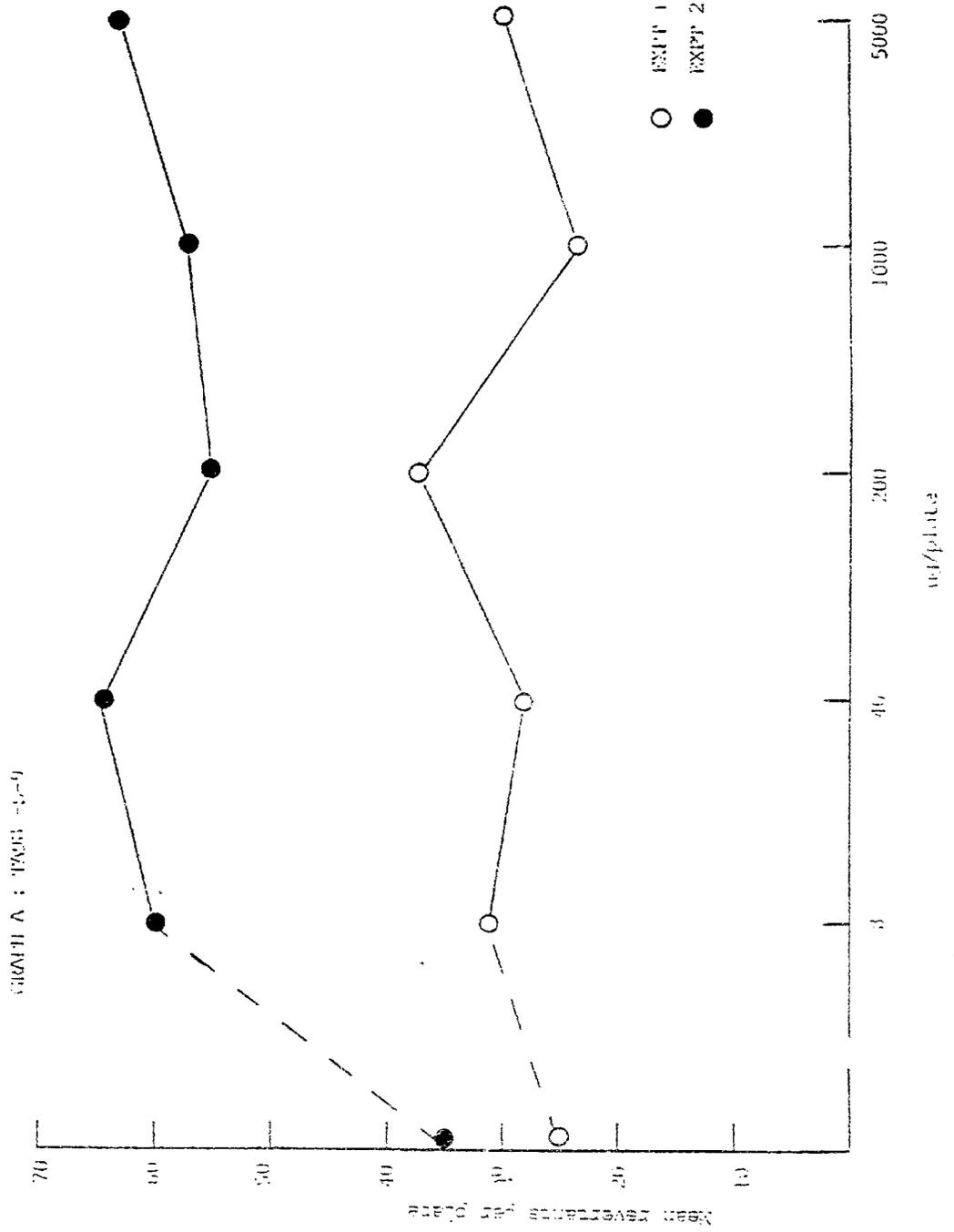
GRAPHS OF RESULTS

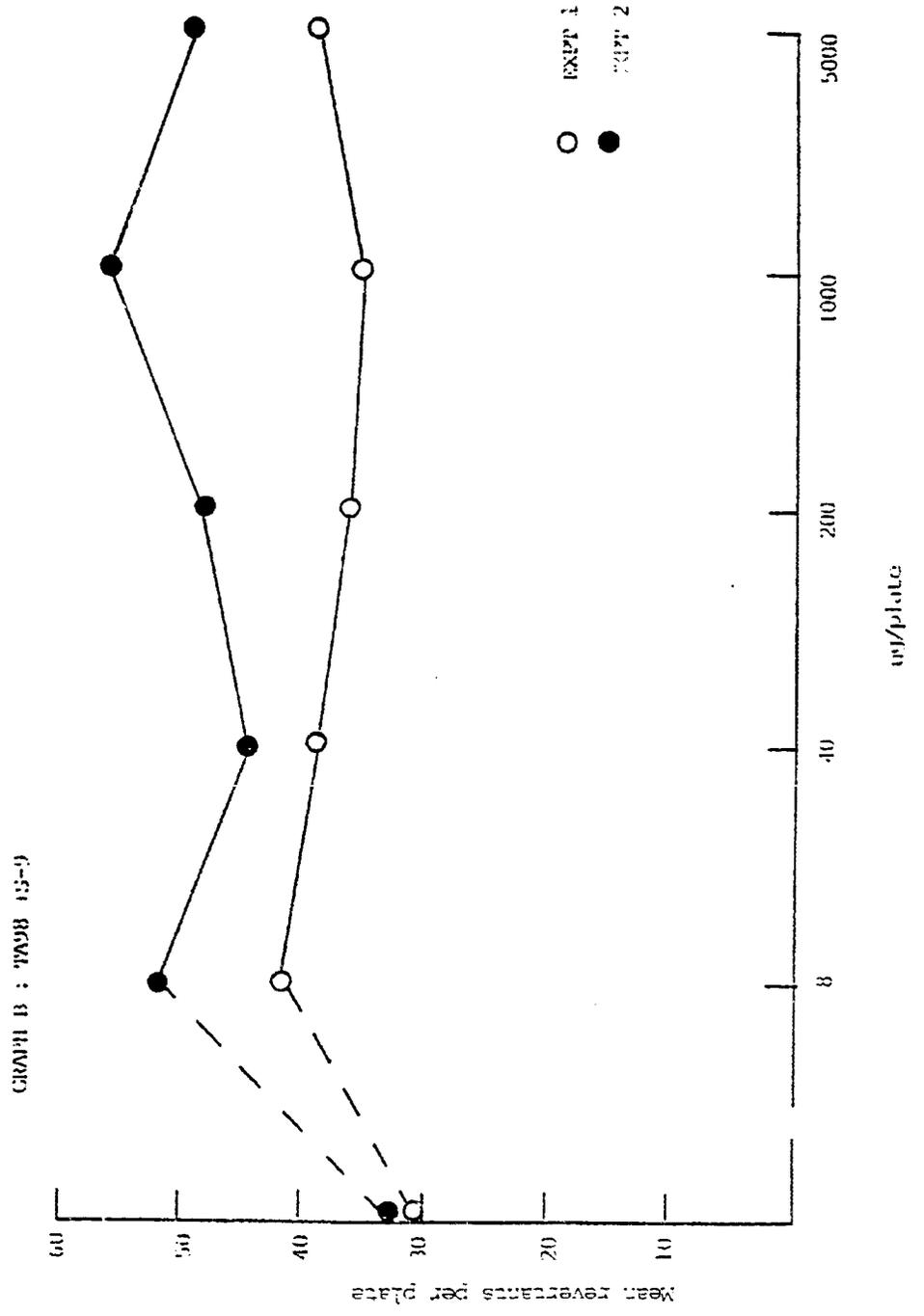
GRAPH A . TA98 - S-9

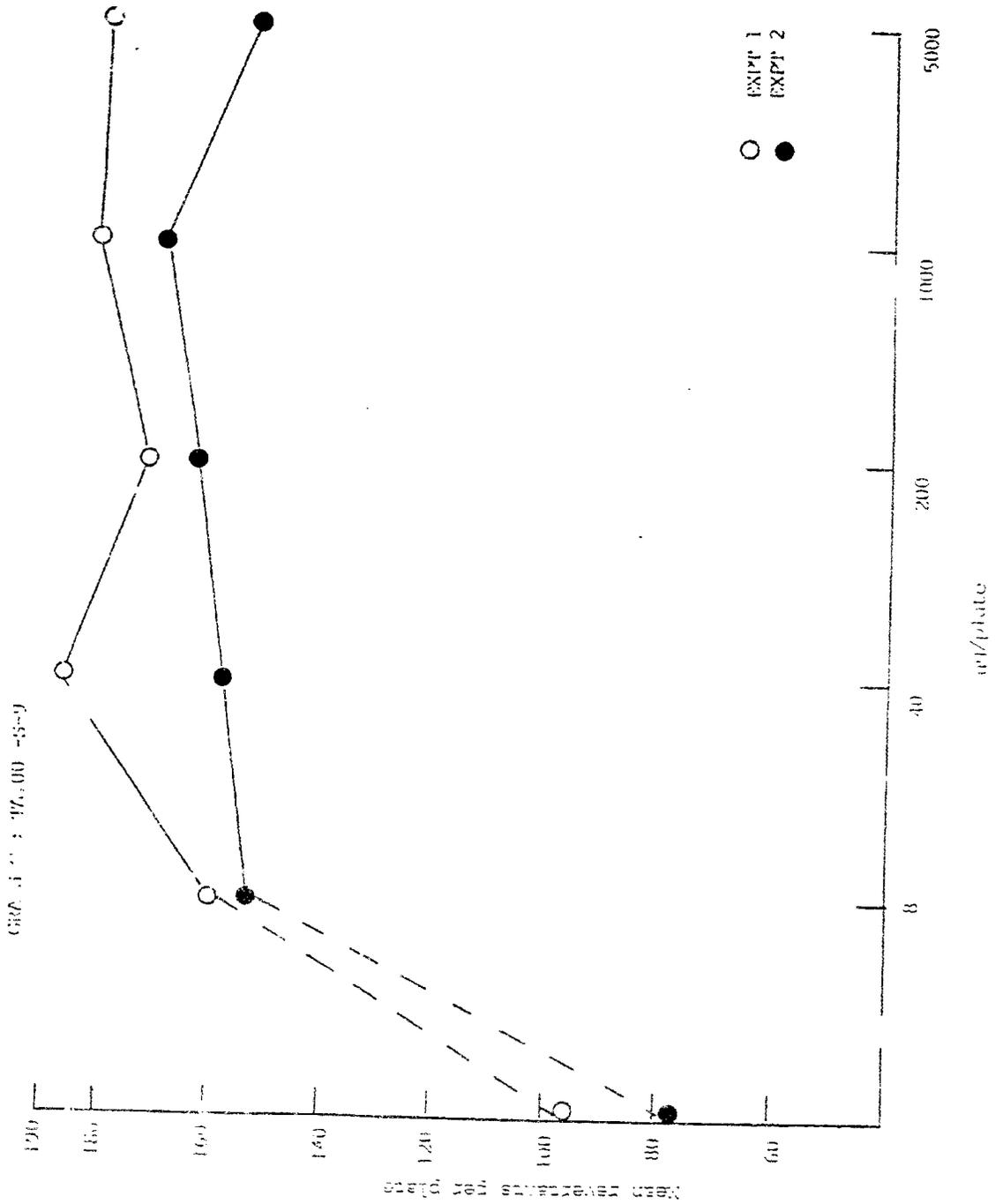
GRAPH B : TA98 + S-9

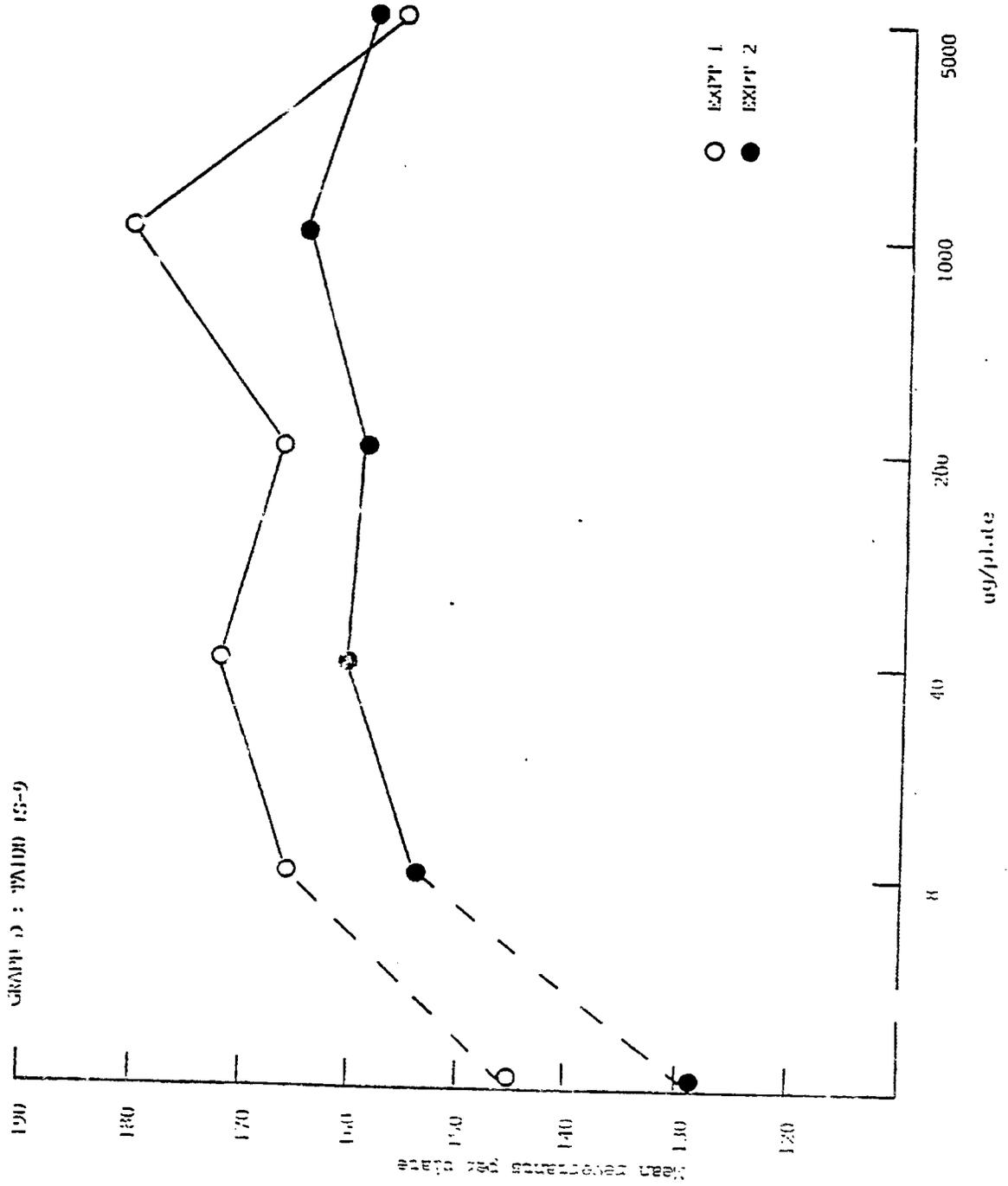
GRAPH C : TA100 - S-9

GRAPH D : TA100 + S-9









Toxicol Study No : M/AMES/24669

PHARMAKOPIUS LIMITED
BACTERIAL REVERSE MUTATION ASSAY
PKPS/PRP 5329

October 1990

Sponsor

Pharmakopius (Services) Limited.
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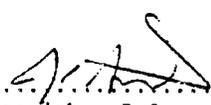
PKPS/PRP 5329

BACTERIAL REVERSE MUTATION ASSAY

TOXICOL REPORT REFERENCE : M/AMES/24669

I certify that this study report provides a true and complete record of the data generated and that the study was conducted in accordance with the Principles of Good Laboratory Practice as set forth in the following :-

1. The United Kingdom GLP Compliance Programme (DoH 1989).
2. The Principles of Good Laboratory Practice as set forth in the OECD Guidelines for the Testing of Chemicals, ISBN 92-64-12367-9, Paris 1982.
3. The United States of America Code of Federal Regulations (EPA-TSCA) Toxic Substances Control Act CFR 40 Part 792 (Volume 48 No. 230 November 29th 1983).

Signed:  Study Director
 (J. C. Asquith. B.Sc., M.Phil., Ph.D.)

Date: 31st October 1990

PKPS/PRP 5329

BACTERIAL REVERSE MUTATION ASSAY

TOXICOL REPORT REFERENCE . M/AMES/24669

At the time of this study the Quality Assurance Unit of Toxicol Laboratories was inspecting one of each critical phase of every type of Mutagenicity study each month as part of a comprehensive evaluation of such studies.

This report has been audited in compliance with the Principles of Good Laboratory Practice. As far as can be reasonably established, the methods described and the results incorporated in the report accurately reflect the raw data produced during this study.

In addition, facilities associated with this study were inspected according to Quality Assurance Unit Standard Operating Procedures.

S. Trenchard-Morgan: *[Signature]*
(Head of Quality Assurance)

Date: *November 5, 1970*

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1.0 SUMMARY

PKPS/PRP 5329 was tested in vitro by the Ames plate incorporation method for its ability to induce mutations in two histidine dependent auxotrophic mutants of Salmonella typhimurium strains TA98 and TA100.

Two independent mutation tests were performed, each in both the presence and absence of a metabolic activation system (S-9 mix) derived from the livers of Aroclor 1254 treated rats. The bacteria were exposed to the test substance dissolved in deionized water, which was also the solvent control. Based on the results of a preliminary toxicity rangefinder, doses used in the mutation experiments were :

5000, 1000, 200, 40 and 8µg/plate

All solvent (negative) controls gave counts of revertants within normal ranges.

All positive controls gave numbers of induced revertants within expected ranges demonstrating the sensitivity of the assay and the metabolising activity of the S-9 mix.

Significant increase in revertants were scored for both strains in the absence of S-9 in one experiment only. These increases were not seen to be dose related. The criteria for a positive result have not been met.

PKPS/PRP 5329 has not been found to be mutagenic in the Ames plate incorporation assay.

4.3 Metabolic Activation System (S-9 Mix)

The liver S-9 fraction (S-9) was prepared according to the method of Ames et al (reference 1) from Fischer 344 rats which had been induced with Aroclor 1254 (Appendix 3).

The protein content, estimated by the method of Lowry et al (reference 4) was found to be 51.6mg/ml. For use, the S-9 protein content must not be less than 35mg/ml.

The metabolic activation system (S-9 mix) used in this study was made as described in Appendix 3 and was kept on ice throughout the experiment.

4.4 Positive Controls

(a) Without S-9 mix

<u>Strain</u>	<u>Positive Control</u>
TA100	Sodium azide (SA) at 1µg/plate
TA98	2-Nitrofluorene (2NF) at 0.5µg/plate

(b) With S-9 mix

<u>Strain</u>	<u>Positive Control</u>
TA98)	2-Aminonaphthalene (2AA) at 2µg/plate
TA100)	

5.0 METHODS

5.1 Toxicity Rangefinder

In order to determine if PKPS/PRP 5329 was toxic to the tester bacteria a preliminary rangefinder was carried out using Salmonella typhimurium strain TA98. Only one Salmonella strain was used as in our experience all strains used show a similar toxic response. For this purpose, an Ames test was carried out with the above strain in the presence and absence of S-9 mix as described in 5.2. The concentrations used for the rangefinder were 5000, 1000, 200, 40, 8 and 1.6µg/plate. After 24 hours incubation at 37°C the plates were examined for the appearance of a complete bacterial lawn as seen under a dissecting microscope.

5.2 Ames Test

All procedures were carried out under low intensity yellow lighting in a class II cabinet.

PKPS/PRP 5329 was tested against the above (4.1) bacterial strains at five dose levels, both with and without S-9 mix, in triplicate in two separate experiments. The test substance was assayed in both experiments at the dose levels given in Section 4.2.

5.2.1 Without metabolic activation

The following components were added sequentially to 2ml of histidine and biotin supplemented molten top agar :-

- 0.1ml of a dilution of PKPS/PRP 5329
- or 0.1ml of appropriate positive control
- or 0.1ml of sterile deionized water (negative control)
- 0.1ml of appropriate overnight bacterial culture (approximately 10^8 organisms)
- 0.5ml of sterile 0.2M phosphate buffer (pH 7.4)

The components were rapidly mixed on a Whirlmixer and poured onto Vogel Bonner E minimal agar plates. Three plates were prepared for each dose point or control. When the agar had set, the plates were inverted and incubated at 37°C for 48 hours. Numbers of revertants per plate were counted using a Biotran III automatic colony counter and the results recorded on form MU20F1.

5.2.2 With metabolic activation

Methodology was as described in 5.2.1 except that 0.5ml of S-9 mix (see Appendix 3) was added to test tubes instead of sterile phosphate buffer.

5.3 Evaluation of Results

Mean and standard deviations for each treatment group are calculated and recorded on form MU20F1.

Dunnett's test is used to determine the statistical significance of the data for each strain with and without S-9 and for each experiment, by obtaining the t statistic for combined treatments with the control (reference 5). This analysis is used as recommended by UKEMS (reference 5).

These are recorded on Form MUSE.2.

A statistically significant increase in the mean number of revertants plus a dose response relationship in two experiments with PKPS/PRP 5329 is considered necessary to indicate a positive result.

5.4 Archiving

Copies of the final report, study protocol and protocol amendments and all raw data relating to the study will be archived at Toxicol Laboratories Ltd.

This documentation will be retained in the archive for 7 years.

At the end of the archive period, after due consultation, the documentation will be returned to the sponsor or destroyed by Toxicol Laboratories.

6.0 RESULTS6.1 Toxicity Rangefinder

After 24 hours incubation, growth assessment of the bacterial background lawn following treatment with PKPS/PRP 5329 was as follows :-

Strain	Z S-9	Concentration of Test Substance ($\mu\text{g}/\text{plate}$)*						
		0	1.6	8	40	200	1000	5000
TA98	0	+	+	+	+	+	+	+
TA98	10	+	+	+	+	+	+	+

* These are actual amounts of test substance added and correspond to solutions of test compound with concentrations from 0.016 to 50mg/ml.

+ = normal growth

PKPS/PRP 5329 was not toxic to the bacteria at any concentration tested, as shown by normal growth of the background lawns.

PKPS/PRP 5329 was therefore tested in the mutagenicity experiments at dose levels of :-

5000, 1000, 200, 40 and 8 $\mu\text{g}/\text{plate}$ in both the absence and presence of S-9.

5.2 Mutation Experiment 1

6.2.1 Raw Data

The responses of the tester strains to the negative control, PKPS/PRP 5329 and the positive control mutagens are given as individual plate counts in Appendix 1. The data are summarised in Table 1 (page 13).

All solvent controls gave counts of spontaneous revertants within normal ranges.

All positive controls gave counts of induced revertants within expected ranges.

In the presence of S-9, the revertants scored on PKPS/PRP 5329 treated plates showed only normal variation around the spontaneous revertant numbers scored on solvent control plates. In the absence of S-9, the numbers of revertants scored was higher on PKPS/PRP 5329 treated plates than on the solvent control plates.

6.2.2 Statistical Analysis

Dunnett's test was carried out on the data from experiment 1.

Significant values of the t-statistic were obtained in the absence of S-9 only, for TA98 at 6, 200 and 5000 μ g/plate and for TA100 at 8 μ g/plate.

There was no evidence of a dose-response relationship for either strain.

No further analysis was carried out

TABLE 1
MEAN NUMBER OF REVERTANTS PER PLATE
FOR PKPS/PRP 5329
EXPERIMENT 1

Strain	I S-9	Concentration of test substance (µg/plate)						
		0	8	40	200	1000	5000	PC
TA98	0	17.7	26.0	24.7	31.3	17.0	30.3	178.7
TA100	0	116.7	151.3	140.7	136.0	123.7	141.0	285.0
TA98	10	26.0	28.0	26.3	24.7	24.7	22.0	746.0
TA100	10	136.0	157.3	144.0	145.3	127.0	130.3	875.0

negative control

t - Statistic from Dunnett's test for each dose in Experiment 1

Test Substance : FKPS/PRP 5329

Ref. No. M/AMES/24669

Strain	Z S-9	Concentration of test substance ($\mu\text{g}/\text{plate}$)						Degrees of Freedom	
		8	40	200	1000	5000	N	D	
TA 98	0	3.56*	3.05	5.49*	-0.27	5.17*	5	12	
TA 100	0	4.59*	2.34	1.89	0.69	2.35	5	12	
TA 98	10	0.47	0.12	-0.33	-0.32	-1.09	5	12	
TA 100	10	2.41	0.68	0.84	-1.44	-0.51	5	12	

* = $P < 0.01$

6.3 Mutagen Experiment 2

6.3.1 Raw Data

The responses of the test strains to the negative controls, TESTSAR 52.9 and the positive control mutagens are given as individual plate counts in Appendix 2. The data are summarized in Table 2 (page 16).

All solvent controls gave counts of spontaneous revertants within normal ranges.

All positive controls gave counts of induced revertants within expected ranges.

The number of revertants scored on 200/475 5.0 treated plate were normal variation around the spontaneous revertant number. For TA100 in the presence of S-9, the revertant counts on 10 treated plates were higher than the spontaneous revertant numbers but these increases were all very small and would be considered to be within normal variation around the solvent control revertant numbers.

6.3.2 Statistical Analysis

Dunnnett's test was carried out on the data from Experiment 2.

No significant values of the t-statistic were found. No further analysis was carried out.

TABLE 1

MEAN NUMBER OF REVERTANTS PER PLATE

FOR 200/475 5.0

EXPERIMENT 2

Strain	Concentration of test substance (ug/plate)								
	0	5-9	0	8	14	200	1000	5000	PC
TA98	0	14.3	13.7	17.7	11.7	14.7	10.0	158.7	
TA100	0	88.3	81.3	81.3	88.3	93.7	88.0	547.3	
TA98	10	15.3	14.3	11.7	15.3	13.3	11.7	487.3	
TA100	10	113.3	113.0	111.3	123.7	127.7	124.0	1046.7	

PC = positive control

TABLE 2

MEAN NUMBER OF REVERTANTS PER PLATE

FOR PKPS/PRP 5329

EXPERIMENT 2

Strain	Z		Concentration of test substance (µg/plate)					PC
	S-9	0	8	40	200	1000	5000	
TA98	0	14.3	13.7	17.7	11.7	14.7	10.0	168.7
TA100	0	89.3	83.3	82.3	88.0	93.7	88.0	547.3
TA98	10	15.3	14.3	14.7	15.3	13.3	11.7	487.3
TA100	10	112.3	119.0	121.3	123.7	127.7	124.0	1046.7

PC = positive control

B 06

t - Statistic from Dunnett's test for each dose in Experiment 2

Test Substance : PKPS/PRP 5329

Ref. No. M/AMES/24669

Strain	Z S-9	Concentration of test substance ($\mu\text{g}/\text{plate}$)					Degrees of Freedom	
		8	40	200	1000	5000	N	D
TA 98	0	-0.30	1.41	-1.30	0.11	-2.12	5	12
TA 100	0	-1.11	-1.27	-0.21	0.82	-0.21	5	12
		8	40	200	1000	5000		
TA 98	10	-0.47	-0.44	-0.04	-0.96	-1.81	5	12
TA 100	10	1.42	1.96	2.46	3.30	2.53	5	12

7.0 DISCUSSION AND CONCLUSION

Responses to the solvent controls were within the normal ranges obtained in this laboratory.

All positive controls gave counts of induced revertants within expected ranges.

Satisfactory responses to the positive control compounds show :-

1. That the bacteria are sensitive, as expected, to known mutagens.
2. That the S-9 used in this study is capable of metabolising an inactive precursor (2AA) to a genotoxic intermediate.

It is necessary to demonstrate both of these points for the study to be acceptable.

Although significant values of the t-statistic were obtained for both strains in the absence of S-9 in Experiment 1, these were not repeated in Experiment 2 and no evidence of a dose-response relationship was seen for either strain. The required criteria for a positive result have not been satisfied. It is concluded that PKPS/PRP 5329 has been found not to be mutagenic in the Ames plate incorporation assay.

8.0 REFERENCES

1. Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D., Proc. Nat. Acad. Sci. USA (1973) 70, 2281
2. Ames, B.N., McCann, J. and Yamasaki, E., Mutation Res. (1975) 31, 347-364
3. de Serres, F.J. and Shelby, M.D., Mutation Res. (1979) 61, 159.
4. Lowry et al., J. Biol. Chem. (1951) 193, 265-275
5. Dunnet, C.W., Journal of the American Statistical Association (1955) 50, 1096-1121.
6. Mahon G.A.T. et al in 'Statistical Analysis of Mutagenicity Test Data' (1989) ed. D. J. Kirkland. Cambridge University Press.

APPENDIX 1

Individual Plate Counts for Experiment 1

PHARMAKOPIUS LIMITED
BACTERIAL REVERSE MUTATION ASSAY
PKPS/PRP 5328

October 1990

Sponsor

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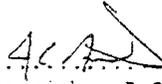
PKPS/PRP 5328

BACTERIAL REVERSE MUTATION ASSAY

Toxicol Study No : M/AMES/24668

I certify that this study report provides a true and complete record of the data generated and that the study was conducted in accordance with the Principles of Good Laboratory Practice as set forth in the following :-

1. The United Kingdom GLP Compliance Programme (DoH 1989).
2. The Principles of Good Laboratory Practice as set forth in the OECD Guidelines for the Testing of Chemicals, ISBN 92-64-12367-9, Paris 1982.
3. The United States of America Code of Federal Regulations (EPA-TSCA) Toxic Substances Control Act CFR 40 Part 792 (Volume 48 No. 230 November 29th 1983).

Signed:  Study Director
(J. C. Asquith. B.Sc., M.Phil., Ph.D.)

Date: 31st October 1990

PKFS/PR^o 5328

BACTERIAL REVERSE MUTATION ASSAY

Toxicol Study No : M/AMES/24668

At the time of this study the Quality Assurance Unit of Toxicol Laboratories was inspecting one of each critical phase of every type of Mutagenicity study each month as part of a comprehensive evaluation of such studies.

This report has been audited in compliance with the Principles of Good Laboratory Practice. As far as can be reasonably established, the methods described and the results incorporated in the report accurately reflect the raw data produced during this study.

In addition, facilities associated with this study were inspected according to Quality Assurance Unit Standard Operating Procedures.

S. Trenchard-Morgan: *[Signature]*
(Head of Quality Assurance)

Date: *[Signature]*

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1.0 SUMMARY

PKPS/PRP 5328 was tested in vitro by the Ames plate incorporation method for its ability to induce mutations in two histidine dependent auxotrophic mutants of Salmonella typhimurium strains TA98 and TA100.

Two independent mutation tests were performed, each in both the presence and absence of a metabolic activation system (S-9 mix) derived from the livers of Aroclor 1254 treated rats. The bacteria were exposed to the test substance dissolved in sterile deionized water, which was also the solvent control. Based on the results of a preliminary toxicity rangefinder, doses used in the mutation experiments were :

5000, 1000, 200, 40 and 8µg/plate

All solvent (negative) controls gave counts of revertants within normal ranges.

All positive controls gave numbers of induced revertants within expected ranges demonstrating the sensitivity of the assay and the metabolising activity of the S-9 mix.

In both experiments, PKPS/PRP 5328 has caused increased numbers of revertants in TA100 in both the absence and presence of S-9, with statistical significance of some of the increases. In addition, statistically significant increases in numbers of revertants scored were seen for TA98 in the absence of S-9 in Experiment 2.

It is concluded that PKPS/PRP 5328 has been found to be mutagenic in the Ames plate incorporation assay.

2.0 INTRODUCTION

The purpose of this study was to assess the mutagenic potential of PKPS/PRP S328 by testing reversion to prototrophy in two histidine auxotrophic mutants of Salmonella typhimurium. Two independent mutation tests were conducted, each in the presence and absence of a metabolic activation system derived from the livers of rats which had been treated with Aroclor 1254, an enzyme inducing agent. The test was performed in accordance with the plate incorporation method of Ames (references 1 and 2).

This study was part of an initial screen of eight test substances.

3.0 TEST SUBSTANCE

The test substance PKPS/PRP S328 was a clear viscous liquid in an amber/yellow clear plastic tube with a white snap-on cap. The tube was labelled 'PKPS/PRP S328'.

The test substance arrived at the testing facility on 3rd April 1990.

The reference M/AMES/24666 was given to PKPS/PRP S328 for this study. When not in use the substance was stored in a locked metal cupboard at room temperature.

The test substance is referred to as 'PKPS/PRP S328' throughout this report.

As the initial sample was found to be contaminated with bacteria, a second sample in a white plastic bottle with a white plastic screw cap was delivered to the testing facility on 22nd June 1990.

4.0 MATERIALS

4.1 Bacterial Strains

The following strains were used in the Ames test :

<u>S.</u>	<u>typhimurium</u>	TA98	his D3052 rfa ⁻	Δ uvr B ⁻ R ⁺
<u>S.</u>	<u>typhimurium</u>	TA100	his G46 rfa ⁻	Δ uvr B ⁻ R ⁺

Both Salmonella strains are defective in DNA repair capacity (Δ uvrB⁻) and have a defective lipopolysaccharide barrier on the cell wall (rfa⁻). These two properties confer extra sensitivity to DNA damage and also greater permeability of large molecules into the cell. They also contain a resistance transfer factor (plasmid pKM 101). This factor (R⁺), which confers resistance to ampicillin, enhances the operation of an error-prone repair system.

The strains are tested routinely for histidine dependence, cell membrane permeability and ampicillin resistance (reference 3) where appropriate.

TA100 is reverted to prototrophy by base substitution mutagens and TA98 and occasionally TA100 by frameshift mutagens.

Several days before the test, each bacterial strain was streaked from an overnight broth culture, prepared from freshly thawed frozen cells, onto nutrient agar plates. These plates were incubated at 37°C overnight to give isolated colonies and were then stored at 4°C until required.

On the day before the experiment, 50ml quantities of sterile nutrient broth were inoculated with a single colony of appropriate strain and incubated overnight at 37°C.

4.2 Sample Preparation

PKPS/PRP 5328 was miscible with water. It was mixed with sterile deionized water by addition of the water to a sterile plastic universal bottle containing the test substances, while the bottle was being agitated in a rotary mixer. The highest concentration solution was prepared in this way and lower concentrations were then prepared by serial dilutions.

PKPS/PRP 5328 was tested at the following concentrations :-

Rangefinder : 50, 10, 2, 0.4, 0.08 and 0.016 mg/ml
Mutation Experiments : 50, 10, 2, 0.4 and 0.08mg/ml

These concentrations gave dose levels of :-

Rangefinder : 5000, 1000, 200, 40, 8 and 1.6μg/plate
Mutation Experiments : 5000, 1000, 200, 40 and 8μg/plate

4.3 Metabolic Activation System (S-9 Mix)

The liver microsomal fraction (S-9) was prepared according to the method of Ames et al (reference 1) from Fischer 344 rats which had been induced with Aroclor 1254 (see Appendix 4).

The protein content, estimated by the method of Lowry et al (reference 4) was found to be 51.6 mg/ml. For use, the S-9 protein content must not be less than 35mg/ml.

The metabolic activation system (S-9 mix) used in this study was made as described in Appendix 4 and was kept on ice throughout the experiment.

4.4 Positive Controls

(a) Without S-9 mix

<u>Strain</u>	<u>Positive Control</u>
TA100	Sodium azide (SA) at 1µg/plate
TA98	2-Nitrofluorene (2NF) at 0.5µg/plate

(b) With S-9 mix

<u>Strain</u>	<u>Positive Control</u>
TA98)	2-Aminoanthracene (2AA) at 2µg/plate
TA100)	

5.0 METHODS

5.1 Toxicity Rangefinder

In order to determine if PKPS/PRP 5328 was toxic to the tester bacteria a preliminary rangefinder was carried out using Salmonella typhimurium strain TA98. Only one Salmonella strain was used as in our experience all strains used show a similar toxic response. For this purpose, an Ames test was carried out with the above strain in the presence and absence of S-9 mix as described in 5.2. The concentrations used for the rangefinder were 5000, 1000, 200, 40, 8 and 1.6µg/plate. After 24 hours incubation at 37°C the plates were examined for the appearance of a complete bacterial lawn as seen under a dissecting microscope.

5.2 Ames Test

All procedures were carried out under low intensity yellow lighting in a class II cabinet.

PKPS/PRP 5328 was tested against the above (4.1) bacterial strains at five dose levels, both with and without S-9 mix, in triplicate in two separate experiments. The test substance was assayed in both experiments at the dose levels given in Section 4.2.

5.2.1 Without metabolic activation

The following components were added sequentially to 2ml of histidine and biotin supplemented molten top agar :-

- 0.1ml of a dilution of PKPS/PRP 5328
- or 0.1ml of appropriate positive control
- or 0.1ml of sterile deionized water (negative control)
- 0.1ml of appropriate overnight bacterial culture (approximately 10^8 organisms)
- 0.5ml of sterile 0.2M phosphate buffer (pH 7.4)

The components were rapidly mixed on a Whirlimixer and poured onto Vogel Bonner E minimal agar plates. Three plates were prepared for each dose point or control. When the agar had set, the plates were inverted and incubated at 37°C for 48 hours. Numbers of revertants per plate were counted using a Biotran III automatic colony counter and the results recorded on form MU20F1.

5.2.2 With metabolic activation

Methodology was as described in 5.2.1 except that 0.5ml of S-9 mix (see Appendix 4) was added to test tubes instead of sterile phosphate buffer.

5.3 Evaluation of Results

Mean and standard deviations for each treatment group are calculated and recorded on form MU20F1.

Dunnett's test is used to determine the statistical significance of the data for each strain with and without S-9 and for each experiment, by obtaining the t statistic for combined treatments with the control (reference 5). This analysis is used as recommended by UKEMS (reference 6)

These are recorded on Form MUSF.2.

A reproducible statistically significant increase in the mean number of revertants plus a dose response relationship with PKPS/PRP 5328 is considered necessary to indicate a positive result.

5.4 Archiving

Copies of the final report, study protocol and protocol amendments and all raw data relating to the study will be archived at Toxicol Laboratories Ltd.

This documentation will be retained in the archive for 7 years.

At the end of the archive period, after due consultation, the documentation will be returned to the sponsor or destroyed by Toxicol Laboratories.

6.0 RESULTS6.1 Toxicity Rangefinder

After 24 hours incubation, growth assessment of the bacterial background lawn following treatment with PKPS/PRP 5328 was as follows :-

Strain	Z	Concentration of Test Substance ($\mu\text{g}/\text{plate}$)*						
	S-9	0	1.6	8	40	200	1000	5000
TA98	0	+	+	+	+	-	+	+
TA98	10	+	+	+	+	+	+	+

* These are actual amounts of test substance added and correspond to solutions of test compound with concentrations from 0.015 to 50 mg/ml.

+ = normal growth

PKPS/PRP 5328 was not toxic to the bacteria at any concentration tested, as shown by normal growth of the background lawns.

PKPS/PRP 5328 was therefore tested in the mutagenicity experiments at dose levels of :-

5000, 1000, 200, 40 and 8 $\mu\text{g}/\text{plate}$ in both the absence and presence of S-9.

t - Statistic from Dunnett's test for each dose in Experiment 1

PKPS/PRP 5328

M/AMES/24668

Strain	I S-9	Concentration of test substance (µg/plate)					Degrees of Freedom	
		8	40	200	1000	5000	N	D
TA 98	0	2.94	2.49	1.70	1.67	-0.30	5	12
TA 100	0	8.50*	8.35*	7.89*	9.00*	7.47*	5	12
TA 98	10	-0.61	0.22	-0.004	0.25	0.81	5	12
TA 100	10	4.49*	4.94*	4.03*	4.92*	5.55*	5	12

* = P<0.01

MU20.F1

BACTERIAL MUTATION TESTS

Date of Experiment :- 25/6/90

Ref. No. :- M/AMES/24668

Study Investigators :- JA/VM

Test Substance :- PKPS/PRP 5328

Bacterial Strain :- TA98

Positive Control :- 2NF -S-9
2AA +S-9

Solvent :- H₂O

S-9 0%	Concentration (µg/plate)						
	Solvent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	0.5
No. of revertants per plate	25	40	30	31	31	31	219
	26	30	35	29	34	21	203
	23	33	33	30	25	20	246
Mean	24.7	34.3	32.7	30.0	30.0	24.0	222.7
S.D.	1.53	5.13	2.52	1.00	4.58	6.08	21.73

S-9 10%	Concentration (µg/plate)						
	Solvent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	2
No. of revertants per plate	29	31	42	33	38	30	446
	36	34	36	41	32	41	415
	39	31	29	30	37	44	436
Mean	34.7	32.0	35.7	34.7	35.7	38.3	432.3
S.D.	5.13	1.73	6.51	5.69	3.21	7.37	15.82

MU20.21

BACTERIAL MUTATION TESTS

Date of Experiment :- 25/6/90

Ref. No. :- M/AMES/24668

Study Investigators :- JA/VM

Test Substance :- PKPS/PRP 5328

Bacterial Strain :- TA100

Positive Control :- SA -S-9
2AA +S-9

Solvent :- H₂O

S-9 07	Concentration (µg/plate)						
	Soivent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	1
No. of revertants per plate	87	174	142	162	165	161	449
	80	167	183	169	192	163	526
	101	179	192	168	181	161	565
Mean	89.3	173.3	172.3	166.3	179.3	161.7	513.3
S.D.	10.69	6.03	26.65	3.79	13.58	1.15	59.03

S-9 107	Concentration (µg/plate)						
	Soivent Control	Test Substance					Positive Control
	0	8	40	200	1000	5000	2
No. of revertants per plate	119	149	150	148	155	173	697
	105	141	150	151	161	164	690
	107	153	155	132	139	136	648
Mean	110.3	147.7	151.7	143.7	151.7	157.7	678.3
S.D.	7.57	6.11	2.89	10.21	11.37	19.30	26.50