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The Procter & Gamble Company
Legislative & Regulatory Relations
PO Box 599 (C-06)
Cincinnati, OH 45201
www.pg.com

Re: TSCA Section 8(d) Submission (71 FR 47310, August 16, 2006)
[EPA-HQ-OPPT-2005-0055; FRL-7764-7]

CONTAIN NO CBI

Dear Sir or Madam:

This submission is being made by The Procter & Gamble Company (P&G) in accordance with TSCA Section 8(d) health and safety data reporting requirements.

We are submitting health and safety studies for substances listed in the TSCA 8(d) final rule originally published in the Federal Register on August 16, 2006 (71 FR 47310) and subsequently modified via two Federal Register Notices published September 15, 2006 (71 FR 54434) and September 29, 2006 (71 FR 57439). Please note that some of the studies being submitted are for substances that are used by P&G solely in FDA-regulated applications. While TSCA reporting obligations do not apply for these materials, we have included these safety data as information we believe is of interest to the Agency.

We have attached an index that lists the applicable chemical names and CAS Numbers listed in the final rule and the corresponding study titles/descriptions. We have also attached a summary to each study to facilitate the review of information being submitted.

If you have any questions regarding this submission, please do not hesitate to contact me.

Sincerely,
THE PROCTER & GAMBLE COMPANY

Richard J. Hackman
Associate Director
Regulatory & Technical External Relations
(513) 983-0534
hackman.rj@pg.com



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Index of Health & Safety Studies submitted by Procter & Gamble
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Chemical Name	CAS #	Lab Study #	Title	Company Acc #
1,3-Hexanediol, 2-ethyl-	94-96-2	85-029	Semi-Continuous Activated Sludge (SCAS) Removability Test on B0859.01	31559
1,3-Hexanediol, 2-ethyl-	94-96-2	BW-86-1-1928	Acute Toxicity of B0859.01 to Bluegill (<i>Lepomis macrochirus</i>)	32348
1,3-Hexanediol, 2-ethyl-	94-96-2	165-09-1100-1	Toxicity of B0859.01 to <i>Microcystis aeruginosa</i> .	32358
1,3-Hexanediol, 2-ethyl-	94-96-2	85-030	CO ₂ Production Test on B0859.01	32091
1,3-Hexanediol, 2-ethyl-	94-96-2	BW-85-11-1884	Acute Toxicity of B0859.01 to <i>Daphnia magna</i> .	32066
1,3-Hexanediol, 2-ethyl-	94-96-2	MVS1482	Testing 2-ethyl-1,3-hexanediol in the mouse <i>in vivo</i> skin micronucleus model	MVS1482
1,3-Hexanediol, 2-ethyl-	94-96-2	191-1215	Rabbit Eye Irritation (Low Volume Procedure)	31928
1,3-Hexanediol, 2-ethyl-	94-96-2	T4636.380	Test for Chemical Induction of Unscheduled DNA Synthesis in Primary Cultures of Rat Hepatocytes (by Autoradiography)	33081
1,3-Hexanediol, 2-ethyl-	94-96-2	851013	Repeated Insult Patch Test	44564
1,3-Hexanediol, 2-ethyl-	94-96-2	LSR 69	Human Repeat Insult Patch Test LSR 69 ECM BTS 1083, E2751.01	35365
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	4708-126/69	Delayed Contact Hypersensitivity Study in the Guinea Pig, (Buehler Test) Test Article RO 163	42321
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	2-5-253-85	Guinea Pig Sensitization Testing modified by Ritz and Buehler on R 0163	42322
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	78-368-21	Delayed Contact Hypersensitivity Study in Guinea Pigs of R0060-01	21114
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	WIL-1179-78	Delayed Hypersensitivity Study in Guinea Pigs of R0060-02.	20749
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	78-086-21	Delayed Contact Hypersensitivity Study in Guinea Pigs of R0060	19932
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	L08321-SNO9	Performance of the Murine Local Lymph Node Assay	36754
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	930/040	Magnusson & Kligman Maximisation Study in the Guinea Pig.	100345
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	V 92.392/352063	Sensitization study with xxx in guinea pigs (maximization test).	103104
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	TES810036	Guinea Pig Sensitization Study – Magnusson-Kligman Maximization Method – Positive Control	44906
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	50931	Delayed Contact Hypersensitivity Study in Guinea Pigs (Buehler Sensitization Test) of xxx	103074

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Chemical Name	CAS #	Lab Study #	Title	Company Acc #
Methanone, (2-hydroxy-4-methoxyphenyl)phenyl-	131-57-7	130	Drosophila Melanogaster Somatic Mutation and Recombination Test Assay of MV#2820-019	36113
Methanone, (2-hydroxy-4-methoxyphenyl)phenyl-	131-57-7	003-347-595-7	Test for Chemical Induction of Mutation in Mammalian Cells in Culture the L5178Y TK+/- Mouse Lymphoma Assay	25950
Methanone, (2-hydroxy-4-methoxyphenyl)phenyl-	131-57-7	T8880.105	Cytogenicity Study Rat Bone Marrow In-Vivo of MV# 2820-019, P89-018	36648
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	7L647	Chromosomal Aberration Study of RE1122.03 in Cultured Mammalian Cells	43693
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	7L646	Bacterial Reverse Mutation Study of RE1122.03	43692
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	7L747	Primary Dermal Irritation Study of RE1122.03 in Rabbits.	43696
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	7L748	Primary Eye Irritation Study of RE1122.03 in Rabbits (Low Dose Procedure).	43694
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	3029-2073	A Low Volume Eye Irritation Study in Rabbits with RE1122.01	40235
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	916-014	Oral (gavage) Chernoff-Kavlock Developmental Toxicity Assay of RE-0981.05, RE-1122.01, RE-1123.01, and RE-1125.01 in Rats	43658
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	L08607 SN15	Murine Local Lymph Node Assay	40229
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	L08607 SN23	Murine Local Lymph Node Assay	43675
Methanesulfonamide, N-[2-[(4-amino-3-methylphenyl)ethylamino]ethyl]-, sulfate (2:3)	25646-71-3	7L749	Dermal Single-Dose Toxicity Study of RE1122.03 in Rats	43695
Tannins	1401-55-4	B86-0168	Rabbit Skin Irritation (Modified Closed Patch Test)	32487

Test Substance

CAS Number: 131-57-7
Identity: Benzophenone-3
Purity: 100%
Remarks: Identified as MV# 2820-019, also as Spectra Sorb uv9

Method

Method/Guideline Followed: Somatic Mutation and Recombination Test (SMART).
Test Type: Drosophila Melanogaster Somatic Mutation and Recombination Test
Report Year: 1991
GLP: Yes
Species/Strain: Drosophila melanogaster
Route of Administration: Feeding
Exposure Period: 48 hour
Doses: 3000 and 3500 ppm
Remarks: The test substance was tested for somatic mutagenic and recombinant activity (the induction of aberrant wing hair spots) in the multiple wing hair somatic mutation and recombination test following the chronic feeding exposure of three day old Drosophila melanogaster larvae. A screening study was conducted to determine the larvae's tolerance to ethanol; based on this a maximum of 8% ethanol was chosen. Solubility of the test substance in ethanol provided a maximal test substance concentration of 3500 ppm. 25 ppm dimethylnitrosamine (DMN) was included as a positive control.

Results

Results:

<u>Compound</u>	<u>Small Spots</u>	<u>Large Spots</u>	<u>Twin Spots</u>	<u>Total</u>
3500 ppm	Negative	Negative	Negative	Negative
3000 ppm	Negative	Negative	Inconclusive	Negative
25 ppm DMN	Positive	Positive	Positive	Positive

Remarks: The test material did not induce mutations or genetic recombination in the somatic cells of Drosophila melanogaster following a chronic feeding.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Report Number: 130
Reference: Vicks Research Center, 1991. Drosophila Melanogaster Somatic Mutation and Recombination Test Assay of MV#2820-019. Accession #36113

Acc # 36113

DROSOPHILA MELANOGASTER
SOMATIC MUTATION AND RECOMBINATION TEST
ASSAY OF MV#2820-019

Sponsor
Vicks Research Center
Richardson-Vick, Inc.
100 Far Mill Crossing
Shelton, Connecticut 06484

Testing Laboratory
Zoology Department
University of Wisconsin
1117 West Johnson Street
Madison, Wisconsin 53562

Sponsor's Project Officer:
Albert Kraus, Ph.D.

Sponsor's Project No. P89-017

Testing Laboratory Project No. 130

Study Conducted September 12, 1989 to April 19, 1990

Report prepared by

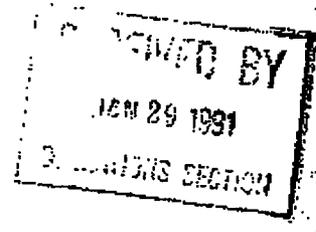
Polly Fourman
Study Director, Polly Fourman, Ph.D.

Date January 22, 1991

Report inspected by

Edward D. Bayer
Quality Assurance Officer, Edward Bayer

Date 01-22-91



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SUMMARY

The chemical, MV#2820-019, was tested for somatic mutagenic and recombinogenic activity (the induction of aberrant wing hair spots) in the multiple wing hair somatic mutation and recombination (SMART) test following the chronic feeding exposure of three day old Drosophila melanogaster larvae.

Feeding continued for approximately 48 hr. until the larvae pupated. Wings were removed from adults and the wing hairs were scored at 400x magnification (phase contrast) for aberrant spots. Spots are classified in one of three ways: (1) small single spots exhibiting either the mwh or flr⁺ phenotype and involving only one or two cells; (2) large single spots exhibiting either the mwh or flr⁺ phenotype and involving more than 2 cells; or (3) twin spots consisting of some mwh and some flr⁺ cells.

The results (shown below) show no difference in response between the exposed and control groups.

COMPOUND	SMALL SPOTS	LARGE SPOTS	TWIN SPOTS	TOTAL SPOTS
3500 ppm MV#2820-019	-	-	-	-
3000 ppm MV#2820-019	-	-	i	-
25 ppm DMN (positive control)	+	+	+	+

+ = positive; - = negative; w = weak positive; i = inconclusive

It is concluded that the chemical, MV#2820-019, does not induce mutations or genetic recombination in the somatic cells of Drosophila melanogaster larvae following a chronic feeding exposure.

1. Objective of the study

The purpose of this study was to evaluate the ability of the test article to exhibit mutagenic or recombinogenic activity in the somatic cells of Drosophila melanogaster. The wing hair "SMART", somatic mutation and recombination test, was used following feeding exposure of larvae that were approximately 72 hours old.

2. Chemical identification and characterization

2.1 Test chemical

The test substance is a powder, cream to white in color, identified by the sponsor as MV#2820-019. The stability of the test article under experimental conditions was not determined by the testing facility.

2.2 Positive control chemical

Dimethylnitrosamine (DIN) was used. The sample was aliquot 884244, 99+% pure, provided by Radian Corporation, Austin, Texas. It is a yellow liquid and is water soluble. It is highly toxic and carcinogenic and is a potent mutagen.

3. Exposures -- Range-finding and definitive tests

Preliminary toxicity work addressed the range of larvae's ethanol tolerance between 4% and 10%. A maximum exposure level of 8% was chosen. Because of the limited solubility of the test article, a maximum exposure of 3500 ppm was used in the definitive tests. The limit of solubility in pure ethanol was approximately 40,000 ppm, so dilutions of concentrations higher than this were deemed to be unacceptably inaccurate. At this level essentially all of the chemical was dissolved. An exposure range of 1000 ppm to 3500 ppm in increments of 500 ppm were used in the definitive tests. A delay in hatch relative to the ethanol control was observed for the higher concentrations in these tests.

Exposures to the positive control article were done at 25 ppm (in distilled water) only, since we have shown previously that this level is adequate to produce a highly significant positive result.

4. Method of administration of the chemical

Virgin females homozygous for mwh (multiple wing hair) were aged and mated en masse to males heterozygous for the marker allele flr^s (flare) and the balancer chromosome TM3, Ser (Third Multiple 3, marked with the dominant mutation Serrate). Females were allowed to lay eggs for approximately 8 hours. Approximately 72 hours later, larvae (68-79 hours old) were collected by washing from the medium with 20% NaCl solution. Exposure vials were made by adding 5 ml of 8% ethanol, distilled water, or exposure solution to 1.5 gm Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply). Larvae were weighed and put into the exposure vials. Vials were subsequently kept in an incubator. For the toxicity tests, the incubator was maintained at approximately 23-24° C. For the definitive tests, the incubator was maintained at 25 - 26° C. Temperature was monitored within the incubator. Because the larvae generally leave the medium and pupate at approximately day 5 of their life cycle, exposures are estimated to have continued for 48 hours. Exposure related changes in timing of the life cycle, however, as well as stability of the compound in the exposure medium could either decrease or extend this period.

5. Genetic testing procedure

The Drosophila melanogaster stocks used are laboratory lines obtained in 1988 from Dr. Alan Katz of Illinois State University at Bloomington, Illinois. The stocks are transferred weekly and observed for genetic purity.

The culture medium for the stocks is a standard Drosophila culture medium. The original formula is described in Drosophila Information Service, No. 34, p. 117, 1960. Our modified formula is as follows:

Water	4500 ml
Agar	72 gm
Molasses (unsulfured)	500 ml
Corneal	500 gm
Dried brewer's yeast	200 gm
Propionic acid (mold retardant)	12.5 ml
Propionic acid-resistant live yeast (sprayed on surface of cooled medium)	

The mixture is cooked at 190° F. A log book is maintained with all details of preparation of each batch of medium.

6. Scoring procedure

When adult flies emerged, they were collected and stored in 70% ethanol. Wings were removed and mounted in Faure's solution. They were examined at 400x phase contrast and occurrences of mutant wing hairs (specifically mwh or flr^s phenotypes) were recorded. These were classified in one of three ways: as (1) twin spots; (2) small single spots (involving 1-2 cells); or (3) large single spots (involving > 2 cells). Twin spots can only be produced (except in very rare circumstances) by mitotic recombination between the centromere and the flr^s gene. Both small and large single spots can be caused by mutation, deletion or recombination between the mwh and flr^s genes. (See Figure 1.) Spots were classified according to the criteria defined in Graf et. al., 1984.

7. Results

7.1 Data presentation

Tables 1 and 2 show the data obtained, represented as the total number of twin spots, small single spots, and large single spots noted among the number of wings scored. No historical control data are available with this solvent, but distilled water data are also presented for comparison (Table 2).

7.2 Statistical analysis

Statistical analysis is done by a multi-decisional process developed specifically for the wing hair SMART test by Frei and Wuerigler, 1988. Statistical analysis is handled by a computer program provided to us by Dr. F. Wuerigler, Zurich, named "SMART.SMS -- edition 1.0, 1-Jul-89". Parameters chosen included alpha = 0.05 for all endpoints, n = 3 for small single spots and total spots and n = 5 for large single spots and twin spots.

8. Discussion of results

8.1 Positive Control

As can be seen from Table 1, an exposure to 25 ppm DMN resulted in a significant increase compared to the control for all endpoints scored. A total of 12 wings scored was sufficient for this determination.

8.2 Test substance

As presented in Table 1, no evidence of an increase compared to the concurrent control was noted for either of the exposure levels tested for total spots (small single, large single, and twin spots combined) or for any single endpoint alone. The single non-negative result obtained, an inconclusive evaluation for twin spots following a 3000 ppm exposure to the test chemical, is almost certainly a result of the small numbers of twin spots obtained.

Table 2 shows the ethanol (8%) control compared the distilled water control. There is no evidence of an effect of the solvent for any of the endpoints studied. This is in agreement with results reported by Graf et. al. (1984) for 10% ethanol exposure.

9. Conclusion

No significant increase ($\alpha = 0.05$) in any endpoint was observed among those larvae exposed to MV#2820-019. It is concluded that MV#2820-019, does not induce somatic mutations or genetic recombination in the wing cells of Drosophila melanogaster following chronic feeding exposure of approximately 72 hour old larvae.

10. Data storage

Copies of all raw data will be sent to the sponsor along with this report. The original raw data, data summaries, slides, and reports will be kept on file in the testing laboratory.

11. References

Frei, H. and F.E. Würgler (1988). Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative, or inconclusive result. *Mutat. Res.* 203: 297-308.

Graf, U., F.E. Würgler, A.J. Katz, H. Frei, H. Juon, C.B. Hall, and P.G. Kale (1984). Somatic mutation and recombination test in *Drosophila melanogaster*. *Environ. Mutagen.* 6: 153-188.

12. Compliance

In the conduct of this study, the laboratory adhered to the Good Laboratory Practices Regulations for Nonclinical Laboratory Studies.

13. Personnel

In addition to the study director, two Research Specialists were involved in the study:

Dana Possin
Carmen Murach

A varying number of undergraduate students did routine work. Training records are on file.

174 *Genes and* **Figure 1. Origins of spot types.** (taken from Graf et al 1986).

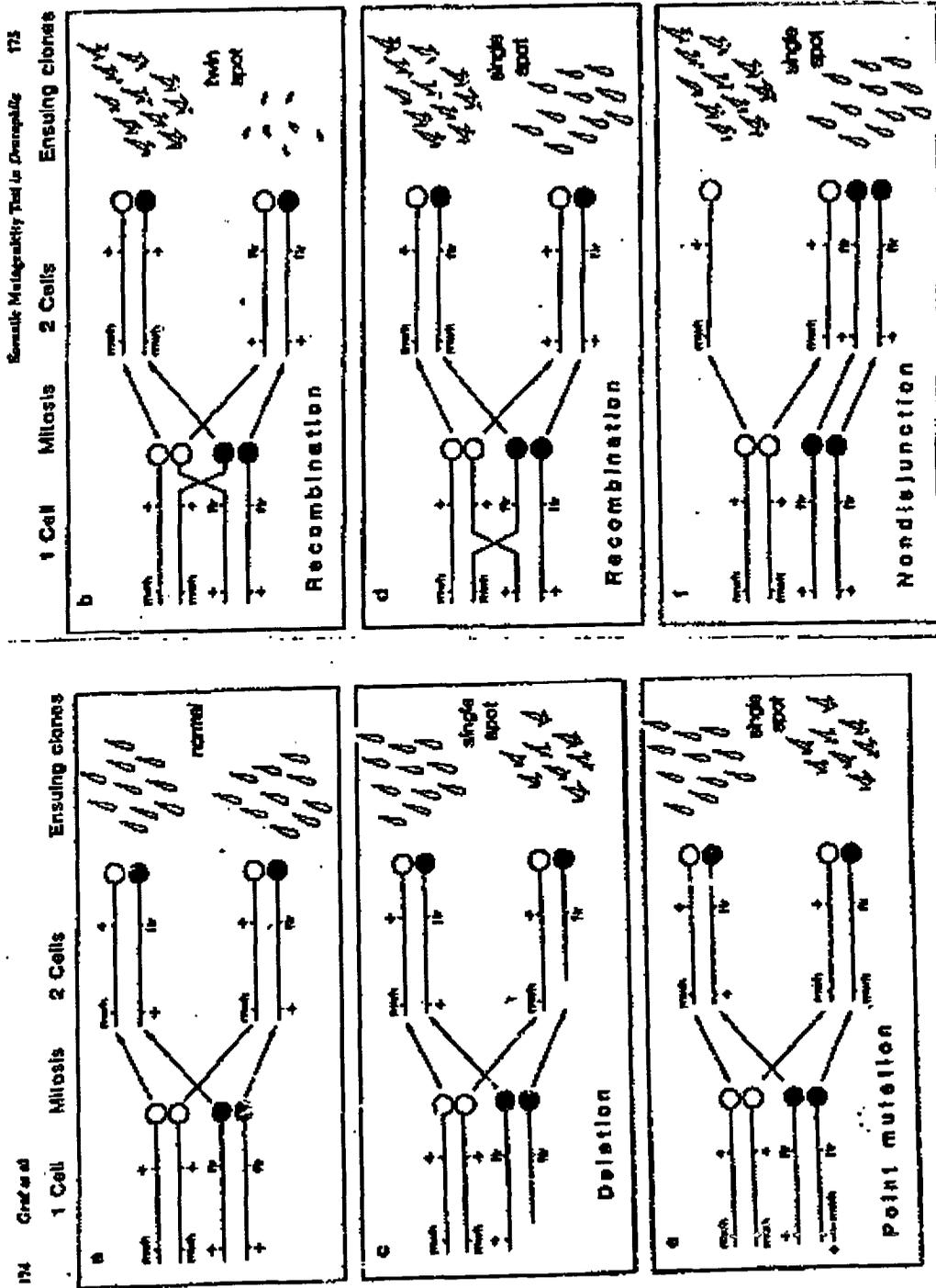
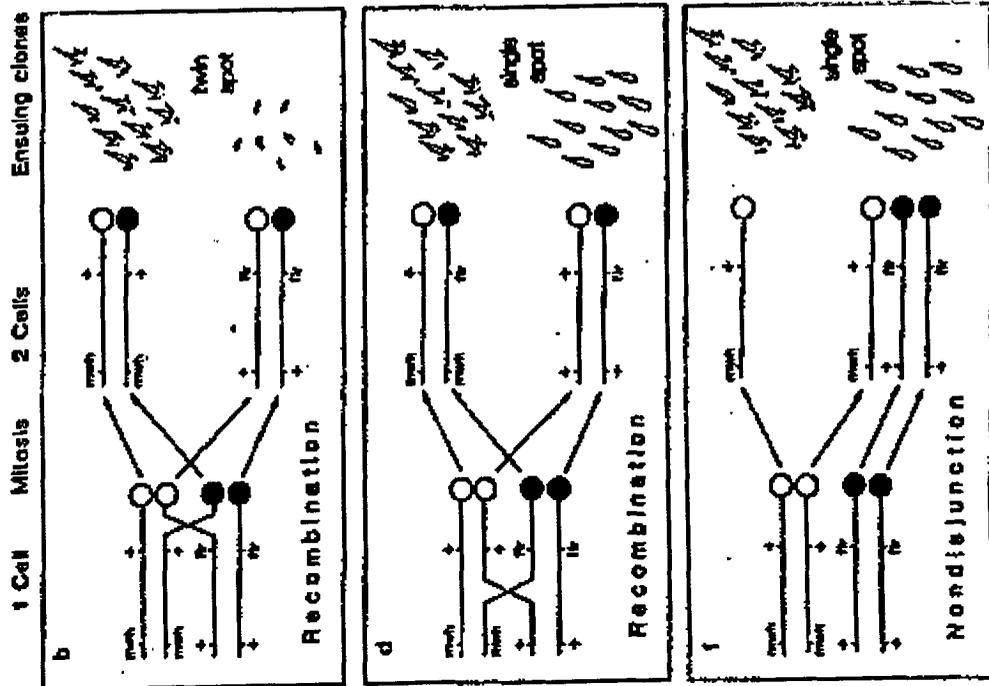


Fig. 1. Genetic schemes illustrating various ways of spot formation in the somatic mutation and recombination area with the wing cell markers multiple wing hairs (mwh) and discs (D). Each spot is obtained by recombination (vertical to the fly marker D), while arrows show recombination products (mwh single spots only (a). Deletions (c, e), point mutations (d) and nondisjunction events (f) give rise



to mwh single spots, or to analogous ways to the single spots (not illustrated). Only the relevant left arms of chromosomes 3 are shown in the panels.

TABLE 1. SUMMARY OF RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST.
ANALYZED VS. CONCURRENT CONTROL.

Com- pound	Num- ber of wings	Spots per Wing (Number of Spots)			Stat. Diagn. *
		Small single spots (1-2 cells) m = 3.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.00	
<u>CONCURRENT CONTROL (ETOH 8%)</u>					
	312	0.07 (23)	0.03 (8)	0.01 (3)	0.11 (34)
<u>MV#2820-019</u>					
3500ppm	200	0.08 (15)-	0.04 (7)-	0.00 (1)-	0.12 (23)-
<u>MV#2820-019</u>					
3000ppm	160	0.04 (6)-	0.03 (4)-	0.02 (3)i	0.08 (13)-
<u>DIMETHYLNITROSAMINE (DMN)¹</u>					
25ppm	12	2.56 (31)+	1.50 (18)+	0.17 (2)+	4.25 (51)+

* Statistical diagnosis according to Frei and Wuergler (1968):
+ = positive; - = negative; w = weak positive; i = inconclusive.
m = multiplication factor
Probability levels : alpha = beta = 0.05.
One-sided statistical tests.

1. DMN analyzed vs. concurrent distilled water control.

TABLE 2. SUMMARY OF RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST.
 ETHANOL (8%) CONTROL VS. WATER CONTROL.

Com- pound	Num- ber of wings	Spots per Wing (Number of Spots)			Stat. Diagn. *
		Small single spots (1-2 cells) m = 3.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.00	
<u>ETHANOL CONTROL</u>					
	312	0.07 (29)	0.03 (8)	0.01 (3)	0.11 (34)
<u>DISTILLED WATER CONTROL</u>					
	200	0.09 (17)-	0.02 (4)-	0.01 (3)i	0.12 (24)-

* Statistical diagnosis according to Frei and Wuergler (1988):
 + = positive; - = negative; w = weak positive; i = inconclusive.
 Probability levels: alpha = beta = 0.05.
 One-sided statistical tests.

University of Wisconsin-Madison
Zoology Research Building

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: *Drosophila melanogaster* SMART assay of MV # 2820 019
SPONSOR: Richardson - Vicks, Inc.
SPONSOR'S STUDY NUMBER: 789-017
TEST SUBSTANCE: MV # 2820-019
STUDY NUMBER: d 130

Quality Assurance inspections for the study and/or review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to the study director on the following dates:

<u>Inspection/Date</u>	<u>Findings Reported</u>	<u>Auditor</u>
General Facility 8/3/89	9/26/89	A. Gilbertson
Initial Inspection 8/3/89	9/26/89	A. Gilbertson
Dosage Prep 8/31/89	9/26/89	A. Gilbertson
Exposure to Tend System 8/31/89	9/26/89	A. Gilbertson
Adult Collection 8/31/89	9/26/89	A. Gilbertson
Larval Collection 8/31/89	9/26/89	A. Gilbertson
Smart Scoring 9/26/89	10/3/89	A. Gilbertson
Draft Final Report Review 7/5/90	7/5/90	E. Bayer

Edward D. Bayer
Quality Assurance Officer

7-5-90
Date

ZOOLOGY DEPARTMENT, UNIVERSITY OF WISCONSIN

STUDY PROTOCOL

April, 1989

I. STUDY TITLE

Drosophila Somatic Mutation and Recombination Test

II. PROJECT IDENTIFICATION

- A. Sponsor's project number PR9-012 (HW# 2820-019)
- B. Testing laboratory's project number #130

III. MANAGEMENT OF STUDY

A. Sponsor's name and address

Vicks Research Center
Richardson-Vick, Inc.
100 Far Hill Crossing
Shelton, Connecticut 06484

B. Sponsor's project officer

Christina Gamba-Vitalo, Ph.D.
Staff Toxicologist

C. Testing laboratory's name and address

Zoology Department
1117 West Johnson Street
University of Wisconsin
Madison, WI 53706

D. Study director

Polly Foureman, Ph.D.

E. Quality Assurance Officer

Amy Gilbertson

IV. TEST MATERIAL INFORMATION (to be provided by the sponsor)

A. Identification MU# 2820-019

B. Physical-chemical description

CREAM TO WHITE POWDER.

C. Handling precautions

D. Purity (including instructions regarding expression of concentration)

E. Units of measurement preferred by sponsor

mg/ml of test solution

MU 2820-019 is not water soluble. It is soluble in ethanol to 40%; acetone to 10%. may have to test a "Tween-suspension"

LOT H2714

V. TEST SYSTEM

A. Description of test principles

The *mwh-flr* SMART assay detects the occurrence of gene mutations, chromosome deletions, mitotic recombination, and nondisjunction in the somatic wing cells of the insect *Drosophila melanogaster*. Recombination between the *mwh* and *flr* markers produces a twin spot, all other events produce single spots.

B. Stocks

The *Drosophila melanogaster* stocks to be used are laboratory strains developed in F.E. Wurgler's laboratory in Schwerzenbach, CH and obtained from Alan Katz in Bloomington, Ill. Virgin females to be used in the P1 mating will be collected from a "multiple wing hair" (*mwh*) stock. P1 males will be collected from a *flr* /*TM3, Ser* stock. The "flare" (*flr*) mutation is homozygous lethal, so it can only be kept in a heterozygous condition. Small spots of homozygous *flr* cells, however, are not lethal.

C. Culture medium

The culture medium for the stocks as well as for the P1 matings, is a standard *Drosophila* medium. The original formula is described in *Drosophila Information Service*, No. 34, p. 117, 1960.

Our modified formula is as follows:

Water	4500 ml
Agar	72 gm
Molasses (Grandma's unsulfured)	300 ml
Cornmeal	500 gm
Dried brewer's yeast	200 gm
Propionic acid (mold retardant)	12.5 ml
Propionic acid-resistant live yeast (sprayed on surface of cooled medium)	

The mixture is cooked at 190 F. A log book is maintained with all details of preparation of each batch of medium.

An alternative food, *Drosophila* Instant Medium Formula 4-24 (supplied by Carolina Biological Supply), is used for larval exposures and to grow larvae after they have been collected from regular culture medium.

VI. TESTING PROCEDURE

A. Route of administration

The route of administration will be chosen, based on:

1. The method or methods most appropriate for the chemical, considering its physical state, solubility, stability, and volatility.

2. The route by which humans might be exposed.

3. The sponsor's request.

The routes of choice are:

1. Chronic larval feeding. Larvae are collected and put into vials containing 1.5 gm Drosophila Instant Medium and 5 ml of the exposure solution. An attempt is made to put a standard volume and/or weight of larvae in each exposure vial of a given run, but larvae are not counted. The larvae mature to the imago stage on the exposure mixture.

2. Acute larval feeding. Larvae are collected and put directly into the exposure solution. The volume is such that the larvae cannot escape from the solution. The exposure time as well as the exposure concentration can be varied. The exposure solution can also be mixed with cellulose to provide a more solid medium. At the end of the specified exposure time, the larvae are washed from the solution and placed on fresh food vials.

3. Inhalation. Larvae are treated in sealed hypovials. The amount of gas in the vial and the duration of exposure are adjusted as needed, considering toxicity, narcosis and other effects.

For this study the route of administration will be:

Chronic larval feeding

B. Solubility tests (for liquids and solids)

Possible solvents, in order of preference, are: water, ethyl alcohol, Tween 20, DMSO and combinations of the above solvents. Other solvents or carriers (e.g. oil or butter) may be used occasionally.

Poc: solubility may limit the concentration of the compound that can be used to treat the flies. For larval exposures, fine suspensions of particals are considered adequate for exposure.

The solvent used in this study will be chosen based on the chemical information supplied and the results of preliminary solubility observations.

C. Sample preparation

For each run, an appropriate series of approximately 3-10 concentrations will be used. The actual number will depend on the compound solubility, compound toxicity, instructions from sponsor, information from previous runs, and the number of larvae available.

1. Chronic larval feeding. Five (5) ml of the chemical, including any other solvent(s) needed to dissolve the chemical, are mixed with 1.5 gm Drosophila Instant Food (or melted standard food.) The concentration recorded will be that of the original dosing solution.

2. Acute larval feeding: Larvae are forced to swim for a period of time in a solution of the chemical, plus any solvent(s) necessary to dissolve the chemical. Concentrations will be expressed in terms of the concentration of the dosing solution per duration of exposure (e.g. ppm x hr). A series of exposures varying in concentration of dosing solution and/or length of exposure will be performed.

3. Inhalation. The gas is administered in air. Concentrations are expressed in concentration of chemical in air times the duration of the exposure.

D. Toxicity Testing

Toxicity due to exposure will be estimated as a part of every run by comparing the number of imagoes hatched in exposure vials to the number hatched in control vials. An approximately equal volume and weight of larvae (or a known fraction of the control larval volume or weight) will be placed in each exposure vial.

E. Choice of exposure concentration(s) to study

The chemical concentration ("dose") that can be adequately administered may be limited by any one or more of several factors, namely solubility, toxicity, or palatability. As a general rule, the following criteria will be used to determine which exposed flies to analyze for the study:

1) First, analyze flies collected from the vial with the highest exposure concentration that produces normal-sized imagoes (as defined by the size of control imagoes). Approximately 100 to 200 wings will be scored from this dose to substantiate a negative response. These will be pooled from more than one exposure run. If toxicity is too high to permit analysis at this dose, the dose to be analyzed is that with a toxicity estimated to be approximately 75-50% of the control.

2) If the exposure defined in the paragraph above is negative, flies from the vial with the next highest exposure concentration will be scored. If that vial cannot be used, flies from the vial with the next lowest concentration will be scored. In the absence of a clearly positive result, a minimum of 50 to 100 wings will be scored from this exposure level.

3) At the request of the sponsor or at the discretion of the study director, wings from other exposure levels may be analyzed.

4) Other factors to be considered in the choice of dose to analyze are delayed hatch and estimated toxicity.

H. Genetic test plan

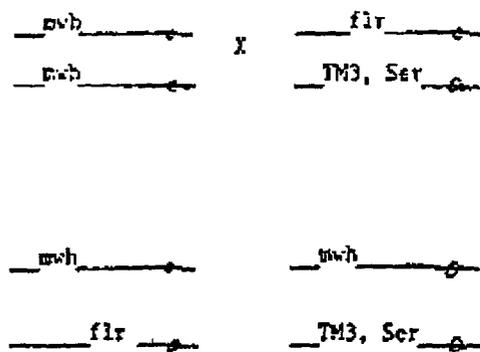
1. The genetic scheme is shown in Figure 1.
2. Parental flies are collected and mated en masse in bottles. Parents are put on fresh food at appropriate intervals. To obtain larvae to be used for a test, parents are shaken over to fresh food for a timed interval, usually 8 hours. Larvae are collected and exposed at the age specified in the protocol.

The age of larvae to be exposed in this study is: 72 hr

3. Emerging imagoes will be collected at appropriate intervals, counted, and stored in 70% ethanol. Wings of flies will be removed and mounted on slides. The wings will be examined at 400X magnification (or above) by a trained specialist and scored for aberrant wing hairs including small single spots of *w^h* and *flr* phenotypes, large single spots of *w^h* and *flr* phenotypes, and *w^h* - *flr* twin spots according to the criterion detailed in Murgler et al (1984).

4. The running time will usually be approximately 15-20 weeks. See flow chart, Figure 2.

Figure 1.
GENETIC SCHEME FOR SMART (mwh) ASSAY



The wings of *mwh/flr* flies (phenotypically *Ser*+) are examined for the presence of *mwh* spots and *mwh-flr* twin spots.

Description of markers:

flr = flare - a recessive chromosome III mutation, homozygous lethal

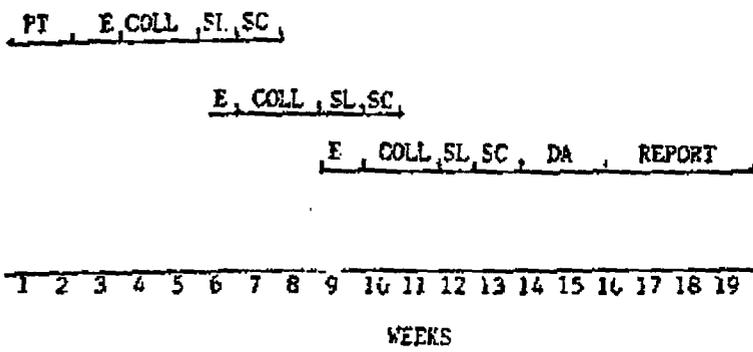
mwh = multiple wing hair - a recessive chromosome III mutation, homozygous viable

TM3 = a third chromosome balancer stock

Ser = Serrate - a dominant chromosome III mutation

Figure 2.

FLOW CHART FOR SMART (mwh) ASSAY



- PT Preliminary Testing
- E Exposure of larvae
- COLL Collect adults
- SL Slide Preparation
- SC Score slides
- DA Data analysis

VII. DATA TO BE COLLECTED

Recorded during exposure: Number of imagoes emerging from each exposure vial.

Recorded during scoring: Number of wings scored; number of small single mwh spots (size = 1-2); number of large single mwh spots (size > 2); and number of mwh-flr twin spots.

VIII. POSITIVE CONTROLS

For feeding exposures, the positive control compound will be dimethylnitrosamine (DMN), a powerful mutagen requiring metabolic activation.

For inhalation experiments, 1,2-dibromoethane (DBE) will be used.

Positive controls may not be run concurrently with all experiments but will be concurrent with at least one experiment.

IX. ANALYSIS OF SMART DATA

Data from treated and control wings will be analyzed according to methods described in Frei and Wurgler (1988).

X. RECORDS TO BE RETAINED

All original data (or copies, if original is sent to sponsor), microscope slides, and copies of all reports will be retained for not less than five years after completion of the study.

XI. GOOD LABORATORY PRACTICE

This study will be conducted according to FDA Good Laboratory Practice Regulations (21 CFR 58.1-58.219, 1979).

XII. QUALITY ASSURANCE

An in-house QA officer oversees quality assurance procedures in the laboratory and, if desired, collaborates with the sponsor's QA officer.

XIII. ALTERATIONS OF STUDY DESIGN

Alterations of this protocol may be made as the study progresses. No changes will be made without consultation with the sponsor. Any change will be documented and signed by the sponsor's project officer and the laboratory's study director.

XIV. REPORTS

A. Interim reports

If requested, interim oral (telephone) reports will be made to the sponsor's designated representative.

B. Final report

At the termination of the study, a final report which includes the following information will be submitted:

1. Experimental design and methods
2. Results
3. Interpretation of the results
4. Conclusions

XV. PROPOSED STUDY SCHEDULE

A. Initiation

Sept. 12, 1989

B. Final report

Approximately 21 weeks after initiation of study. 2-9-90

XVI. REFERENCES

allows for extended preliminary testing period

Graf, H., F.E. Wurgler, A.J. Katz, H. Frei, H. Juon, C.B. Hall, and P.G. Kale. (1984) Somatic mutation and recombination test in *Drosophila melanogaster*. *Environ. Mutagen.* 6:153-164.

Frei, H. and F.E. Wurgler. (1988) Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative, or inconclusive result. *Mutation Research* 203, 297-308.

PROTOCOL APPROVAL

A. Study director

By Polly Lawrence

Date 9-11-89

B. Sponsor

By Cristina Gamba Vitale

Date August 23, 1989

RICHARDSON-VICKS, USA
DEPARTMENT OF PHARMACOLOGY, TOXICOLOGY AND HUMAN SAFETY

PROJECT NO.: 5758

STUDY NO.: P89-017

CONTROL NO.: 515

INVESTIGATOR: University of Wisconsin, Zoology Department

INVESTIGATOR'S STUDY NO.: 130

DATE: September 27, 1989

STUDY: Drosophila Somatic Mutation and Recombination Test

REVISION:

The Richardson-Vicks, U.S.A. representative at the initiation of this study was Christina Gamba-Vitalo. She has been replaced as Richardson-Vicks, USA representative by Albert Kraus, Ph.D. who is now responsible for acceptance of the completed final report.

APPROVAL


STUDY DIRECTOR

10/2/89
DATE


RICHARDSON-VICKS, USA REPRESENTATIVE

9/27/89
DATE

UNIVERSITY OF WISCONSIN
ZOOLOGY RESEARCH BUILDING
DROSOPHILA MUTAGENESIS LABORATORY

STUDY NO: UW #130

SPONSOR'S STUDY NO: P89-017

PROTOCOL AMENDMENTS

REVISION:

Edward Bayer took over as Quality Assurance Officer on this study in May, 1990. He replaces Amy Gilbertson.

APPROVAL


Polly Foursman, Ph.D.
Study Director

7-7-70
Date

VICKS RESEARCH CENTER PRODUCT PREPARATION REPORT
 Personal Care Products Division
 THEORETICAL FORMULA

PRODUCT TYPE: OTC Drug IND/INDA Drug _____ Device _____ Cosmetic _____
 Product Name: SPECTRA-SORB UV-9 MV #: 2820-19
 Project # / Name: B05 5758
 Issued By: Joseph Cray Date: 8-24-89
 Verified By: D.D. Stett Date: 8/24/89

List ingredients in descending order of predominance.
 For drug products list active ingredients first and indicate with an asterisk (*).

VIC Code #	Trade or Common Name (Identify Supplier for Specialty Items)	Compasial (Drugs) or CTFA (Collected) Name	Percentage Composition
01569 T1	SPECTRA-SORB UV-9	BENZOPHENONE-3 (oxybenzone)	100%

FOR COSMETIC DRUG PRODUCTS INDICATE AMOUNT OF EACH ACTIVE INGREDIENT PER DOSAGE UNIT OR PER UNIT OF WEIGHT OR MEASURE OF THE PRODUCT AND A STATEMENT OF THE TOTAL WEIGHT OR MEASURE OF ANY DOSAGE UNIT:

Benzophenone-3 100%
 (oxybenzone)

Test Substance

CAS Number: 131-57-7
Identity: Benzophenone-3
Purity: 100%
Remarks: Identified as TSIN#TO899.01

Method

GLP: Yes
Report/Study Year: 1981
Method/Guideline Followed: Modification of Clive, D. and Spector, J.F.S. Laboratory procedure for assessing specific locus mutations at the TK locus in culture 5178Y Mouse Lymphoma cells. Mutation Research 31:17-29, 1975.

Test Type: L5178Y TK+/- Mouse Lymphoma Mutagenicity Assay

Conditions: *in vitro* Mouse lymphoma cells

Doses: 6.7 – 500 µg/mL.

Remarks: Complete toxicity was observed in both the nonactivated and S-9 activated cultures at 500 µg/mL. Tested with and without metabolic activation (S-9). After a two day expression period, seven cultures without activation and six cultures with exogenous metabolic activation were selected for cloning based on the degree of toxicity. Nonactivated cultures were cloned at 50, 38, 28, 21, 16, 12 and 8.9 µg/mL. Activated cultures were cloned at 28, 21, 16, 12, 8.9 and 6.7 µg/mL.

Results

Result: For the non-activated cultures, the two highest clonable cultures exhibited mutant frequencies which were 2.3 and 2 times greater than that of the solvent control. For the cultures treated with S-9, the highest dose exhibited a mutant frequency which was 2-fold greater than the solvent controls.

Conclusion: Under these test conditions, the test material did induce a weak, but significant increase over background in the mutant frequencies in both the presence and absence of S-9. This is considered weak because it was exhibited only in cultures where high toxicity was observed.

Data Quality

Reliability (Klimisch): 2

Reference

Laboratory Study Number: 003-347-595-7

Reference: Procter and Gamble, 1981. Test for Chemical Induction of Mutation in Mammalian Cells in Culture the L5178Y TK+/- Mouse Lymphoma Assay. Accession # 25950.

Acc # 25950

EG&G MASON RESEARCH INSTITUTE

1530 EAST JEFFERSON STREET, ROCKVILLE, MARYLAND 20852 • TEL. (301) 770-4400

Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK+/- Mouse Lymphoma Assay

Sponsor: The Procter and Gamble Company
P.O. Box 39175
Cincinnati, Ohio 45247

Study No.: 003-347-595-7

Test Article I.D.: T0889.01

Test Article Lot No.: 1

Test Article Description: Light Yellow Powder

Storage Conditions: Room Temperature with Desiccation

Date Received: 1/16/81

Date Study Started: 1/27/81

Date Study Completed: 4/22/81

Date of Report: 4/24/81

RECEIVED BY
APR 29 1981
OPERATIONS SECTION

Lab. Coordinator: J. B. Oster, Ph.D.
The Procter and Gamble Company

Study Director: Paul E. Kirby, Ph.D.
EG&G Mason Research Institute

Paul E. Kirby 4/22/81 Rose F. Pizzarello 4/22/81
Paul E. Kirby, Ph.D. Date Rose F. Pizzarello Date
Study Director Laboratory Manager

Rebecca A. Vega 4/22/81 Patricia E. Williams 4/22/81
Rebecca A. Vega Date Patricia E. Williams Date
Group Leader Group Leader

Gerry L. Reichard 4/22/81 Richard E. Wattam 4/22/81
Gerry L. Reichard Date Richard E. Wattam Date
Data Technician II Biologist

Jane J. Clarke 4-22-81 Paula A. Sikora-Ricketts 4/22/81
Jane J. Clarke Date Paula A. Sikora-Ricketts Date
Biologist Biologist

 **EG&G MASON RESEARCH INSTITUTE**

1530 EAST JEFFERSON STREET, ROCKVILLE, MARYLAND 20852 • TEL. (301) 770-4900

QUALITY ASSURANCE UNIT STATEMENT

The L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay has been divided into a series of critical phases. Using a random sampling approach, the QAU monitors each of these in process phases over a series of studies. It examines procedures, documentation, equipment, etc. to assure that the test is conducted according to the protocol and in compliance with the Good Laboratory Practice Regulations. Findings are reported to the study director on the day of each inspection and on the day of the review of the final report.

The following are the inspection dates, critical phases inspected and report dates of QAU monitors of the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay of Study No.: 003-347-595-7; TOBBS.G1

DATE OF INSPECTION	CRITICAL PHASES INSPECTED	REPORT SUBMITTED TO MANAGEMENT
1/20/81	Solubility Determination	1/30/81
1/27/81	Initial Toxicity: Preparation of Cultures	1/30/81
	Dilution of Test Article	
	Treatment of Cultures	
2/9/81	Dilution of Test Article & Positive Controls	2/13/81
	Treatment of the Cultures	
2/11/81	Cell Dilution & TPT Addition to the Cloning Flasks	2/13/81
2/23/81	Counting the Plates	2/27/81

To assure that the reported results accurately reflect the raw data of the study and that the final report is in compliance with the GLP's, the final report was reviewed on April 22, 1981.



Nona S. Karten
QAU Manager

4/22/81

Date

Summary

Procter and Gamble Company's test article T0889.01 (MRI #595) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay with and without metabolic activation by Aroclor induced rat liver S-9. The cultures treated without activation were cloned over a range of concentrations which produced from 12% to 77% Suspension Growth, and the cultures receiving S-9 metabolic activation were cloned over a range of concentrations which produced from 12% to 93% Suspension Growth.

The results of the assay indicate that under these test conditions, test article T0889.01 (MRI #595) did induce a significant increase over background in the mutant frequencies of test cultures treated both in the presence and absence of Aroclor induced rat liver S-9.

Introduction

Test article T9889.01 (MRI #595) was received on January 16, 1981, for testing in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay with and without exogenous metabolic activation by Aroclor induced rat liver microsomes.

Experimental Methods

The experimental protocol (see Appendix) is a modification of that described by Clive, D. and Spector, J. F. S. Laboratory procedure for assessing specific locus mutations at the TK locus in culture L5178Y Mouse Lymphoma cells. Mutation Research 31: 17-29, 1975

- Note:
1. Some of the numbers generated by the test data, whether it is Toxicity, Mutant Frequency, Induced Mutant Frequency, etc., are computed using non-rounded numbers. This may, in some instances, cause what appear to be errors in calculation if only the rounded numbers are used when checking the data.
 2. All of the raw data generated by the assay and the original final report will be maintained in EG&G Mason Research Institute's archives located in our Rockville, Maryland facilities.
 3. The stability of the test article under the actual experimental conditions used in this study was not determined by EG&G Mason.

All test article stock solutions were freshly prepared immediately before their use in each procedure.

Results

The Initial Toxicity Test conducted on test article T0889.01 (MRI# 595) indicated threshold levels of complete toxicity at 500 µg/ml for both the nonactivated and S-9 activated cultures. Based on this data, the test article was tested in the mutagenesis assay over a range of concentrations from 500 µg/ml to 6.7 µg/ml.

After a two day expression period, seven cultures without activation and six cultures with exogenous metabolic activation were selected for cloning based on their degree of toxicity. The nonactivated cultures were cloned at 50, 38, 28, 21, 16, 12 and 8.9 µg/ml. The cultures receiving exogenous metabolic activation were cloned at 38, 21, 16, 12, 8.9, and 6.7 µg/ml. The results of the Cloning Data are presented in Tables 2, 3 and 4 and the results of the Total Compound Toxicity Data are presented in Tables 5, 6 and 7. The results of the Cloning Data and Toxicity Data are also presented graphically in Figure 1 (without activation) and in Figure 2 (with S-9 activation).

For the nonactivated cultures, the two highest clonable cultures exhibited mutant frequencies which were approximately 2.3 and 2 times greater than that of the solvent control cultures. Both cultures exhibited 11% Total Growth. The rest of the cultures which were cloned exhibited mutant frequencies which were not significantly different than that of the solvent control cultures. The Total Growth exhibited by the nonactivated cultures ranged from 9% to 77%.

For the cultures treated in conjunction with induced rat liver S-9, the highest treated clonable culture exhibited a mutant frequency which was two-fold greater than that of the average mutant frequency of the solvent controls. The

Total Growth of this culture was 12%. The rest of the cultures which were cloned exhibited mutant frequencies which were not significantly different than those of the solvent controls. The Total Growth exhibited by the S-9 activated cultures ranged from 12% to 102%.

Study No. 008 347 595 7

Table 1

EDG MASON RESEARCH INSTITUTE

7.0 L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

INITIAL COMPOUND TOXICITY TEST

003-347-595-7
Study NumberPaul E. Kirby
Study Director0.005 ug/ml to 5000 ug/ml
Dose RangeT0889.01 (MR1^F 595)
Test Article IdentityDMSO
Solvent

	Compound Concentration	Cell Concentration (X 10 ⁶)			Suspension Growth	
		Day 1	Day 2	Day 3	Total*	% of Control
Without Activation	5000 ug/ml	0.003	0.007		0.0	0%
	500 ug/ml	0.003	0.000		0.0	0%
	50 ug/ml	0.457	1.157		5.9	35%
	5.0 ug/ml	1.287	1.235		18.4	108%
	0.5 ug/ml	1.322	1.266		18.6	109%
	0.15 ug/ml	1.349	1.239		18.6	109%
	0.005 ug/ml	1.280	1.231		17.5	103%
	Solvent 1	1.311	1.208		17.6	17.0
	Solvent 2	1.199	1.221		16.3	
5-9 Activation	5000 ug/ml	0.007	0.020		0.0	0%
	500 ug/ml	0.005	0.003		0.0	0%
	50 ug/ml	0.467	1.236		6.4	37%
	5.0 ug/ml	1.055	1.399		16.4	95%
	0.5 ug/ml	1.105	1.308		16.1	93%
	0.05 ug/ml	1.092	1.264		15.3	89%
	0.005 ug/ml	1.142	1.415		18.0	104%
	Solvent 1	1.108	1.393		17.1	17.3
	Solvent 2	1.112	1.412		17.4	

$$* \text{ Total Growth} = \frac{(\text{Day 1 Conc.})}{0.3 \times 10^6} + \frac{(\text{Day 2 Conc.})}{0.3 \times 10^6} + \frac{(\text{Day 3 Conc.})}{0.3 \times 10^6}$$

+ Culture Lost

Table Prepared By: Rose F. Riggarello

Signature

2/4/81
DateWorkbook Page No. 12Report Page No. 6

Table 2
 A.E.C. 4. RESEARCH INSTITUTE
 15178Y MC LYMPHOMA MUTAGENIC ASSAY
 CLONING DATA

Compound Conc.	No. of Colonies/R.N. Plate			R.N. #/ Plate	No. of Colonies/M.C. Plate			Ave. #/ Plate	Mutant Frequency	Induced Mutant Frequency
	1	2	3		1	2	3			
50 ug/ml	58	52	47	52	127	121	112	120	0.9	0.5
38 ug/ml	47	48	32	42	117	119	96	111	0.8	0.4
28 ug/ml	40	22	30	31	132	132	132	132	0.5	0.1
21 ug/ml	45	49	50	48	143	144	140	142	0.7	0.3
16 ug/ml	43	50	39	44	161	157	163	160	0.5	0.1
12 ug/ml	25	25	17	22	156	136	145	146	0.3	0.1
8.9 ug/ml	44	35	44	41	167	164	162	164	0.5	0.1
Solvent 1	41	43	44	43	195	179	182	187	0.5	0.4
Solvent 2	24	22	21	22	147	139	150	142	0.3	0.1

Study Number 003-347-595-7
 Experiment Number 59581

Test Article Identity T0889,01 (MRI*595)
 Metabolic Activation NONE

Solvent DMSO

Signature & Label
Richard F. Cingolillo
 Calculations Performed By: Richard F. Cingolillo
 Signature & Label

* For 10⁴ surviving cells + Culture Lost

Table 4
REGIS L. C. RESEARCH INSTITUTE
L5178Y TK-1-10C LYMPHOMA MUTAGENESIS ASSAY
LONING DATA

Study No. 003 347 595 7

Study Number		Study Director						Experiment Number	
003-347-595-7		L. C. Regis						595B1	
Test Article Conc.	No. of Colonies/R.M. Plate	Ave. #/Plate	No. of Colonies/v.c. Plate			Ave. #/Plate	Mutant Frequency	Induced Mutant Frequency	
	1	2	3	1	2	3			
Ethylmethanesulfonate (No Activation)									
1.0ul/ml	185	184	196	188	16	17	15	25.7	25.3
0.5ul/ml	297	297	288	294	95	80	85	6.9	6.5
SOLVENT 1	28	26	26	27	139	119	134	0.43	0.4
SOLVENT 2	19	20	14	18	98	91	98	0.43	0.43
7,12 Dimethylbenz(a)anthracene (With Activation)									
7.5 ug/ml	+	+	+		+	+			
5.0 ug/ml	183	169	160	171	41	40	43	7.9	7.5
SOLVENT 1	20	20	17	19	75	99	96	0.4	
SOLVENT 2	+	+	+		+	+			
Mutations Assayed by: <i>Richard A. Vega</i>		Calculations Performed by: <i>R. M. F. Dugganella</i>		2-23-81		Date			
(Signature & Date)		(Signature & Date)		(Signature & Date)		(Signature & Date)			

*Per 10⁶ surviving cells
+ Culture lost

Table 5
 MASON RESEARCH INST. VI.
 GEN. LYS. PHOMA MUTAGENESIS GEN
 TOX. GEN. TOXICITY D. 72

Study No. 003 347 595 7

Test Article Concentration	Cell Concentration x 10 ⁶ cells/ml			Suspension Growth Total	Suspension Growth % Control	Cloning Growth Ave. V. C. % Control		% Growth
	Day 1	Day 2	Day 3			++	++	
89 ug/ml	0.004	0.005		0.0	0%	++	++	++
67 ug/ml	0.014	0.018		0.0	0%	++	++	++
50 ug/ml	0.399	0.890		3.4	16%	12.0	73%	11%
38 ug/ml	0.338	0.904		3.4	16%	11.1	67%	11%
28 ug/ml	0.255	0.887		2.5	12%	12.2	80%	9%
21 ug/ml	0.468	1.194		6.2	29%	14.2	86%	25%
16 ug/ml	0.533	1.155		6.7	32%	16.0	97%	31%
12 ug/ml	0.773	1.396		12.0	56%	14.6	88%	50%
8.9 ug/ml	1.018	1.459		16.5	77%	16.4	99%	77%
SOLVENT 1	6.298	1.602		23.1	26.3	18.7	16.5	
SOLVENT 2	1.081	1.613		19.4		14.2		

003-347-595-7
 Study Number
 10889.01 (HR. # 595)
 Test Article Identity

Paul E. Kirby
 Study Director
 DMSO
 Solvent

595 B1
 Experiment Number
 None
 Metabolic Activation

Tables prepared and calculations performed by: Jose J. Cappanello 3/3/81
 (Signature & Date)

* % Growth = (S Suspension Growth / S Cloning Growth) x 100
 + Culture lost
 ++ Too toxic to clone

INSTITUTO VAREZCACHA INSTITUTE

LS17BY T(+/-) - MOUSE LYMPHOMA MUTAGENESIS ASSAY

TABLE FOUND TOXICITY DATA

003-347-995-7 Eric J. Farby Experiment Number 59581
 Study Number DM50 Study Director DM50 Induced Rat Liver S-9
 Test Article Identity DM50 Solvent DM50 Metabolic Activation

Test Article Concentration	Cell Concentration x 10 ⁶ cells/ml		Suspension Growth Total	Cloning Growth Ave. V. C. & Control	Growth* & Growth*
	Day 1	Day 3			
50 ug/ml	0.041	0.071	0.0	0%	++
38 ug/ml	0.128	0.256	0.4	2%	++
27 ug/ml	0.244	0.785	2.1	12%	142
21 ug/ml	0.424	1.366	6.4	37%	153
16 ug/ml	0.728	1.551	12.5	72%	168
12 ug/ml	0.869	1.641	15.8	91%	158
9.9 ug/ml	0.923	1.577	16.2	93%	145
6.7 ug/ml	0.904	1.534	15.4	99%	139
SOLVENT 1	0.943	1.670	17.5	17.1	148
SOLVENT 2	0.960	1.625	17.3	17.1	134

* Growth = $\frac{\% \text{ Suspension Growth}}{\% \text{ Cloning Growth}} \times 100$

* Culture Lent

** Too Toxic to Clone

Tables prepared and calculations performed by: Eric J. Farby (Signature & Date)

187/01/01/91

Study No. 003 347 595 7

Table 7

LS178X TR+/ - M. J. LYMPHOMA MUTAGENESIS ASSAY
TOTAL COP AND TOXICITY DATA

Compound Concentration	Cell Concentration x 10 ⁶ cells/ml			Suspension Growth		Cloning Growth		Experiment No.
	Day 1	Day 2	Day 3	Total	% Control	Ive. V.C.	% Growth*	
Echymethanesulfonate (No Activation)								
1.0µl/ml	0.580	0.930		6.0	33%	15	13%	4%
0.5µl/ml	0.873	1.214		16.8	64%	85	73%	47%
SOLVENT 1	1.101	1.403		17.2	18.4	134	116	
SOLVENT 2	1.157	1.527		19.6		98		
7,12 Dimethylbenz(a)anthracene (With Activation)								
7.5µg/ml	0.195	0.148		0.3	2%	++	++	++
5.0µg/ml	0.429	0.701		3.3	11%	43	45%	9%
SOLVENT 1	0.932	1.558		16.1		94		
SOLVENT 2	0.894	2.370		+	+	+	+	+

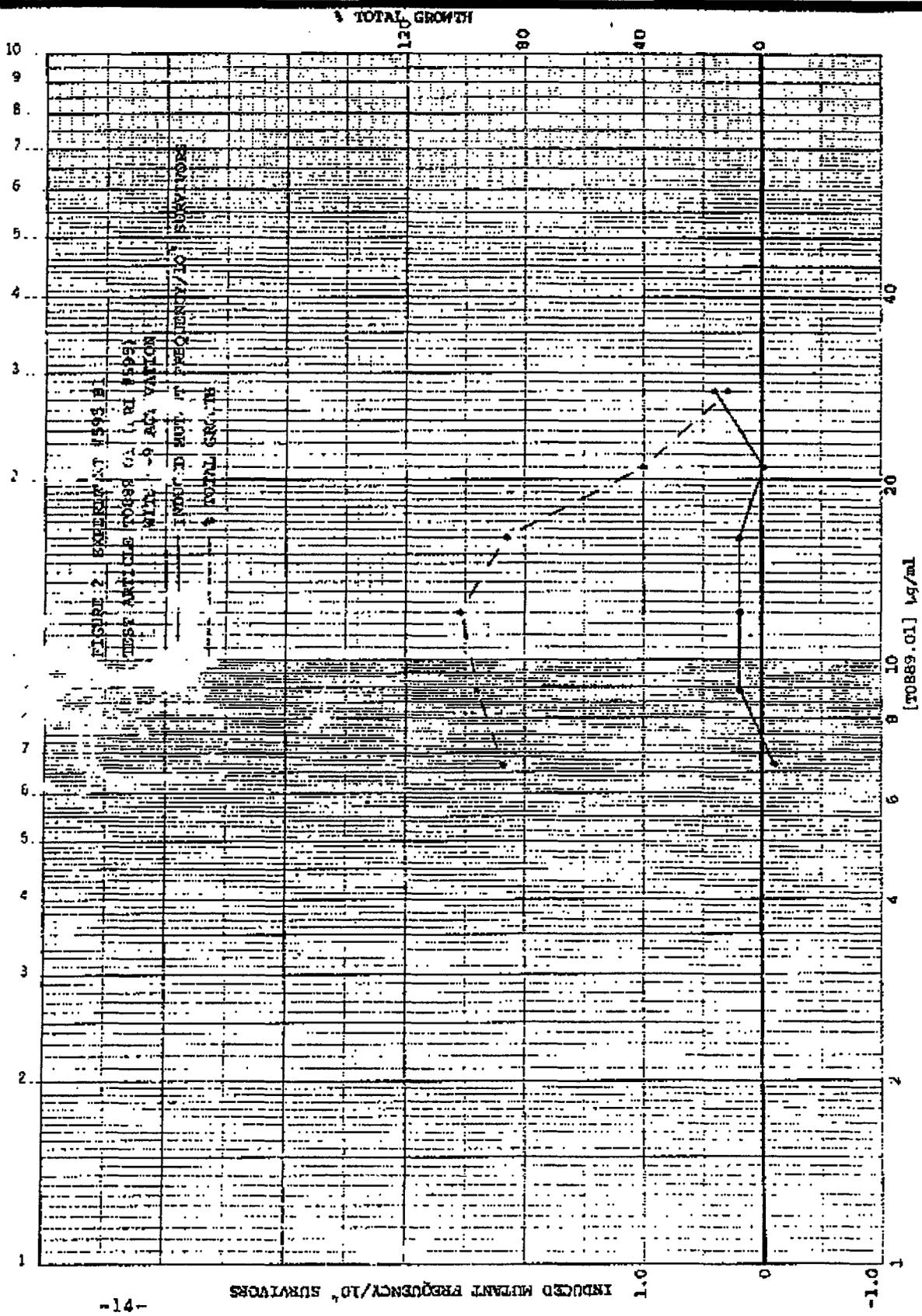
Tables prepared and calculations performed by: Ray J. Pignatello
(Signature & Date)

* Growth = (Σ Suspension Growth) / (Σ Cloning Growth) / 100
+ Culture Lost
++ Too Toxic to Clone

Study No. 008 347 595 7

Study Number 003-347-595-7
Experiment No. 595B1

Chad J. Kirby
Station: to



Discussion

The test article appears to have induced a weak positive response in cultures treated both in the presence and absence of Aroclor induced rat liver S-9. The response is considered to be weak because only cultures which were treated with highly toxic doses of the test article exhibited mutant frequencies which were two-fold greater than background.

In both the presence and absence of exogenous metabolic activation, the two-fold increase was observed in cultures which exhibited 11-12% Total Growth. The cultures having greater than 12% Total Growth exhibited mutant frequencies which were within the range of the mutant frequencies of the solvent controls.

Conclusion

Procter and Gamble Company's test article T0889.01 (MRI# 595) was tested in the L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay in the presence and absence of Aroclor induced rat liver S-9. A weak positive response was observed under both conditions.

APPENDIX

**Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay**

Issue Date: September 15, 1980
Supersedes Issue Dated: January 1, 1980

Test Substance Identification Number (TSIN) # T0889.01 AND T0890.01

Divisional Request Document Number (DRD) # JG 1800

Sponsor: The Procter & Gamble Company
Cincinnati, Ohio

Testing Facility:
(To be filled in by
Operations Section)

EO&G Mason Research Institute
1530 E. Jefferson Street
Rockville, Maryland 20852

003-347-595-7
Study # *003-347-595-7*
(To be filled in by
Testing Facility)

Purpose: To determine the potential of a chemical compound to induce mutations at the thymidine kinase (TK) locus of cultured L5178Y TK⁺ mouse lymphoma cells. 1, 2, 3

System for Selection of Test Cell:

The L5178Y/TK⁺ mouse lymphoma cells clone 3.7.2C is the system of choice due to the amount of background data available.

Route of Administration of Test Substance and Reason for Choice:

IN VITRO with and without metabolic activation.
Route specified by test procedure.

Records to be Maintained:

Receipt of and/or preparation and storage of L5178Y/TK⁺ mouse lymphoma cell cultures, S-9, and restrictive medium. Documentation of test substance preparation, preparation of cells, preparation of S-9 mix, dosing, washing, cell count, cloning, colony counts, and sizing. Include any other records that would be required to reconstruct the study and demonstrate adherence to protocol.

Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: September 15, 1980

Test Substance(s)		Description		Expiration Date
TSIN #	DRD Number	Color	Physical Form	
T0889.01	TG 1800	Light Yellow	Powder	11/14/82
T0890.01	TG 1800	Light Yellow	Powder	11/14/82

Storage Conditions: (Check one)

Room temperature Refrigerator Freezer
 Other

Hazards: (Check one)

None known. Take ordinary precautions in handling.
 As follows:

Special Instructions: (Check one)

None
 As follows:

Dose Preparation:

Vehicles in order of preference

Water or FOP*
 DMSO
 EtOH T0890.01
 Acetone
 Other T0890.01

Solubility: 20% in EtOH T0890.01 T0890.01
>20% in N-methylpyrrolidone OR
52% in Toluene

Unless the solubility properties of the test substance are provided by the Sponsor or the solubility properties are available from another source, a suitable solvent must be found for the test substance prior to testing using the Standard Operating Procedures of the Test Facility.

*See Appendix 1 for abbreviations and glossary of terms.

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PROTOCOL NO. C29 (Cont'd)

Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: September 15, 1980

Dose Preparation
(Cont'd):

The preferred solvents, in order of preference, are water (or F₀), dimethyl-sulfoxide, ethanol, and acetone. Any other solvent which shows no toxic effect to the L5178Y cells and no significant increase in background mutation frequency at the levels used is acceptable subject to approval by the Sponsor.

Chemicals:

Positive controls and other chemicals to be used for testing will be purchased from a commercial source or obtained from the Sponsor. Chemicals are stored according to the recommendations of the commercial supplier or Sponsor. After completion of the assay, unused commercially obtained chemicals may be saved for future use. Excess chemicals obtained from a Sponsor, however, will be either returned or discarded at the discretion of the Sponsor.

Dosage Levels:

All solutions of the test substance are prepared fresh on the day of the test. Doses are chosen on the basis of the toxicity test described in Toxicity Test Section. A complete mutagenicity assay consists of at least five cloned doses of the test substance (see mutagenicity test below for criteria used for selection of doses to be cloned) and a solvent control all tested with and without activation, a positive control of ethyl methanesulfonate (EMS), a mutagen that does not require activation, and a positive control either of 2-acetylaminofluorene (2-AAF), 7,12 dimethyl benzanthracene (DMBA), or benzo(a)pyrene (Bap), mutagens that require metabolic activation with an S-9 fraction obtained from the livers of rodents induced with a chemical such as Aroclor. In some special cases the S-9 fraction used will be obtained from the livers of uninduced rodents. In these cases, the positive control used will be dimethylnitrosamine (DMN). All dosing solutions will be prepared as 100X concentrated stock solutions.

[] Other, specify:

**Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
The L5178Y TK⁺ Mouse Lymphoma Assay**

Issue Date: September 15, 1980

Dosage Level (Cont'd): Note

A concentration analysis of the test substance - vehicle mixture(s) will ; will not be required.

If a concentration analysis is required:

- Prepare a sufficient quantity of the most concentrated test substance - vehicle mixture(s) so that a portion can be returned to the Sponsor's Divisional Toxicologist.

Shipping Instructions

Send approximately _____ ml. Send frozen;
 under ambient conditions; other _____

- Analyze the test substance - vehicle mixture(s) for test substance concentration using the analytical method in Appendix _____.

Identification:

Individual cultures and cloning plates are to be identified according to the Standard Operating Procedures of the Test Facility.

Test System:

L5178Y/TK⁺, clone 3.7.2C mouse lymphoma cells obtained from D. Clive, Research Triangle Park, N.C., Burroughs-Wellcome Co.

Test System Storage:

Frozen stocks of the L5178Y clone 3.7.2C cells are prepared and maintained in a liquid nitrogen freezer according to the Standard Operating Procedures of the Test Facility.

Methods:Cell Line

The TK⁺ clone 3.7.2C L5178Y cell line is maintained as growing suspension cultures according to the Standard Operating Procedures of the Test Facility. The medium used is Fischer's Medium for Leukemic Cells of Mice containing approximately 10% (v/v) horse serum and supplemented according to the Standard Operating Procedures of the Test Facility. Medium may be obtained from a suitable commercial supplier as a powder, or 1X or 10X liquid.

Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: September 15, 1980

Methods (Cont'd):Cell Line (Cont'd)

Cells are periodically cleansed free of spontaneous TK⁻ mutants by treatment of stock cultures with THMG according to the Standard Operating Procedures of the Test Facility. TK⁻ cells are sensitive to the toxic effects of methotrexate in the presence of exogenous thymidine, hypoxanthine and glycine (THMG treatment). TK⁺ cells, however, are not killed by THMG treatment since they can use the exogenous thymidine, hypoxanthine, and glycine (THG) to overcome the block to folate metabolism imposed by the methotrexate. Cultures used for the assay are cleansed within the two week period prior to initiation of the project.

Preparation of the Microsomal Enzyme (S-9)
Metabolic Activation SystemNon-Induced S-9 Fraction

A liver microsomal enzyme (S-9) activation system is employed in this assay to detect promutagens. S-9 is prepared by the homogenization of minced livers from commercially obtained male, Sprague-Dawley rats (200-275 gms) according to the Standard Operating Procedures of the Test Facility. The liver is homogenized in a mixture consisting of one part liver in gms wet weight to three parts solvent in ml. (either 0.15N KCl or 0.25 M sucrose according to the Standard Operating Procedures of the Test Facility).

Aliquots of the S-9 are stored frozen below -70°C until used.

Induced S-9 Fraction

Induced S-9 fraction is prepared from rats given a single intraperitoneal injection of a polychlorinated biphenyl (Aroclor) in corn oil five days prior to sacrifice. The standard dose of Aroclor is 500 mg/kg body weight. The Aroclor used for injection may be either a 2:1 mixture of Aroclor 1242:Aroclor 1254 or Aroclor 1254 alone according to the Standard Operating Procedures of the Test Facility.

Toxicity Test:

In addition to limitations imposed by the solubility of a substance, the levels at which it can be tested for mutagenicity are determined by its toxic effect on L5178Y cells. As a result the toxicity of a compound is first tested over a wide range of concentrations.

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Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: September 15, 1980

Toxicity Test (Cont'd): Toxicity is measured by the ability of a given dose of substance to inhibit the suspension growth of treated cultures. The method and length of exposure of cells to chemical and incubation conditions are similar to those used in the Mutagenicity Test Section. The exact procedure is conducted according to the Standard Operating Procedures of the Test Facility.

From the information obtained in the toxicity test, the doses of substance to be tested in the mutagenicity test are determined. Based on the results of the toxicity test, the highest dose of test chemical to be used in the mutagenicity test is chosen to give substantial or complete toxicity relative to the solvent control. Within the limits of predictability of the toxicity test, subsequent doses are chosen to span the range of relative toxicity to a level where little or no relative toxic effect is observed.

Mutagenicity Test:S-9 Mix (Metabolic Activation System)

Prior to dosing the cells S-9 mix will be prepared by combining S-9 fraction with a neutralized solution of NADP and sodium isocitrate. The final concentrations of each component in the cultures during treatment are 100 µl/ml S-9, 2.4 mg/ml NADP, and 4.5 mg/ml sodium isocitrate in F_{OP}. Alternatively, the amount of any given batch of S-9 liver fraction used may be determined from its protein concentration, or the amount required to elicit a standard response in a mutagenicity test with a positive control chemical according to the Standard Operating Procedures of the Test Facility. The S-9 mix will be prepared shortly before use from freshly thawed S-9 fraction. Unused portions should be discarded at the end of the day.

Dosing, Expression Growth and Cloning of Cells

Each sample will be prepared for dosing by transferring 6 ml L5178Y/TK⁺ clone 3.7.2C cells at a concentration of 1×10^6 cells/ml to a labeled, sterile 50 ml centrifuge tube or T25 tissue culture flask. Four ml of F_{OP} are then added to each sample to be tested without metabolic activation, and 4 ml of the S-9 mix to each sample to be tested with metabolic activation. Each sample is mixed gently and 0.1 ml of the 100X concentrated dose of test substance, solvent, or positive control chemical is added. Any major change in pH (by color change of phenol red in culture medium) caused by addition of the test substance is noted, and

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PROTOCOL NO. C29 (Cont'd)

Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: September 15, 1980

Mutagenicity Test
(Cont'd):

Dosing, Expression Growth and Cloning of Cells (Cont'd)

the pH is quickly brought back to approximate neutrality (normal red-orange color of medium) by dropwise addition of 0.5-1.5N NaOH or HCl. Each sample vessel is then gassed with 5% CO₂-in-air, sealed and incubated at 37 ± 0.5°C on a roller drum (centrifuge tube) or gyrotory shaker (tissue culture flask) for four hours. The cell samples are then centrifuged, the supernatant discarded and the cells washed twice with fresh F_{10P}. The cells are then resuspended in F_{10P} at a concentration of approximately 3 X 10⁵ cells/ml, based on the original cell number of 6 X 10⁶ per culture prior to treatment with chemical, and all samples incubated as described above for a two or three day expression period according to the Standard Operating Procedures of the Test Facility. During the expression period, the cell concentration is determined daily and all cultures are diluted to 3 X 10⁵ cells/ml, if necessary, in order to keep the cells in an active state of growth.

At the end of the expression period, doses are chosen for cloning based on the relative toxicity shown during the expression period. In general, dose levels which exhibit from 10 to 90% relative growth inhibition during the expression period are chosen for cloning. However, if that level of toxicity is not achieved within the solubility limits of the compound, then dose(s) showing less than 10% inhibition may be cloned. Dose levels showing greater than 90% growth inhibition will not be cloned. A portion of each culture is centrifuged and resuspended in F_{10P}. The appropriate dilutions are then made and a portion of each sample is plated on petri dishes in soft agar medium with and without the selective agent, TFI according to the Standard Operating Procedures of the Test Facility. Three dishes of each sample at 1 x 10⁶ cells/plate are prepared in TFI medium. Three dishes of each sample at an estimated cell number from 100-200 cells/plate are prepared in cloning medium without selective agent. All petri dishes are then incubated at 37.0 ± 0.5°C for 10-14 days to allow colonies to form from individual cells. At the end of this time, the number of colonies on each plate is counted. The number of viable cells (survivors) originally placed on the plates containing the TFI medium is determined from the number of colonies in dishes containing the non-selective medium. The number of TK⁺ mutants is determined from the number of colonies in dishes containing the TFI medium.

PROTOCOL NO. C29 (Cont'd)

Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: September 15, 1980

Protocol Changes:

If it becomes necessary to change the approved protocol, verbal agreement to make this change should be made between the Study Director and the Sponsor. As soon as practical, this change and the reasons for it should be put in writing and signed by both the Study Director and the Sponsor's Divisional Toxicologist. This document is then attached to the protocol as an addendum.

Results:

The raw data are reported for each negative and positive control and each dose of substance. Raw data consist of dose preparation information, the daily cell concentrations, the number of viable, colony-forming cells on each petri dish containing non-selective medium, and the number of TFT-resistant colony-forming cells on each dish. A mutation frequency (the number of TFT-resistant colony-forming cells per unit survivor) and the fold increase in mutation frequency relative to the solvent control is determined for each sample. The induced mutation frequency, the mutation frequency of each sample minus the spontaneous mutation frequency shown in the solvent controls may also be determined. In addition to the mutation frequencies, the percent survival relative to the control is reported for each sample for both the expression period growth in suspension and the overall growth (the relative suspension growth corrected for viability as determined by the plating efficiency in non-selective medium).

Results of each test are considered independently, but in order to be considered a valid test, the spontaneous mutation frequencies observed for the negative controls should be no higher than 150 mutants per 10⁶ survivors. In addition, the mutation frequencies observed for the positive controls should fall within acceptable ranges as determined by a developing historical base.

Report:

Final Report

A report of the results will be prepared for this study by the contract laboratory within 30 days from the completion of the study. The report will include, but not be limited to, the following:

1. Name and address of the facility performing the study and the dates on which the study was initiated and completed.
2. Objectives as stated in the approved protocol, and a copy of the original protocol.

Test for Chemical Induction of Mutation
in Mammalian Cells in Culture
the L5178Y TK⁺ Mouse Lymphoma Assay

Issue Date: September 15, 1980

Report (Cont'd): Final Report (Cont'd)

3. A detailed description of all methods used.
4. Statistical methods employed for analysis of the data, if any.
5. Deviations from the Test Facility's Standard Operating Procedures or the approved protocol.
6. A summary of the results as they relate to the study's objective.
7. The location where all raw data will be stored.
8. The daily cell concentrations, all colony counts, and results of calculations.

This report shall conform to all requirements outlined in Section 58.185, Subpart J, Good Laboratory Practices Regulations.

Sponsor: J. R. Oster
0 Divisional Toxicologist

Date approved by Sponsor's Divisional Toxicologist: 12/19/80

Proposed Starting Date: January 27, 1981

Proposed Completion Date: March 12, 1981
(Final Report Available)

)To be completed
)by the Test
)Facility

Study Director: Paul S. Kish

Date: 1/19/81

Study Cost: \$5,000

Abbreviations and Glossary of Terms:

1. 2-AAF - 2-acetylaminofluorene
2. BUdR - bromodeoxyuridine
3. Cloning medium- Fischer's Medium for Leukemic Cells of Mice supplemented as described below for F_{OP} and with approximately 20% (v/v) horse serum and 0.32-0.37% noble agar according to the Standard Operating Procedures of the Test Facility
4. DMN - dimethylnitrosamine
5. DMSO - dimethylsulfoxide
6. EMS - ethyl methanesulfonate
7. F_{OP} - Fischer's Medium for Leukemic Cells of Mice supplemented with sodium pyruvate, Pluronic F68, and penicillin-streptomycin according to the Standard Operating Procedures of the Test Facility
8. F_{10P} - F_{OP} plus approximately 10% (v/v) horse serum
9. Gassing - Replacement of the air in a culture vessel with 5% CO₂-in-air by purging with CO₂-air mixture
10. NADP - β-nicotinamide adenine dinucleotide phosphate
11. selective cloning medium - Cloning medium containing TFT according to the Standard Operating Procedures of the Test Facility
12. S-9 - The supernatant obtained by centrifugation of a homogenate of liver at 9000 X g according to the Standard Operating Procedures of the Test Facility
13. TFT - trifluorothymidine
14. TK - thymidine kinase

REFERENCES

- ¹Clive, D. and J. F. S. Spector. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. *Mutation Res.*, 31: 17-29 (1975).
- ²Clive, D., K. O. Johnson, J. F. S. Spector, A. G. Batson, and M. H. M. Brown. Validation and characterization of the L5178Y/TK mouse lymphoma mutagen assay system. *Mutation Res.* 59: 61-108 (1979).
- ³Clive, D., W. G. Flamm, and J. B. Patterson. Specific locus mutational assay systems for mouse lymphoma cells. In A. Hollaender (ed.), *Chemical Mutagens: Principles and Methods for their Detection*. Volume 3, Plenum Press, New York, 1973, pp. 79-103.
- ⁴Ames, B. N., W. E. Durston, E. Yamasaki, and F. D. Lee. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Nat. Acad. Sci. USA* 70: 2281-2285 (1973).

TOILET GOODS TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

For Tox Office Use Only:

DND # T61800

TSIN # TO 289.01

11. Characterization, Microbial and Properties Information

	Date Submitted	Submitter Code # or Lab Notebook #	Component or Property	Measured Value	Allowable Limits	Testing Lab or Data Source
1	11/14/80	TES 527	% Volatile		2%	T.G. Anal.
2	11/14/80	TES 527	M.P.	63°C	61-65°C	T.G. Anal.
3	11/14/80	TES 527	ppm Fe	3 ppm	70 ppm	T.G. Anal.
4	11/14/80	TES 527	I.R.	Pass	Must pass	T.G. Anal.
5	11/14/80	TES 527	U.V. Scan		Must pass	T.G. Anal.
6	11/14/80	TES 527	Microbial	Pass	Must pass	REB
7	11/14/80	TES 527	Vis. Scan		Must pass	T.G. Anal.
8						
9						
10						
11						
12						
13						
14						
15						

Above data verified by: [Signature] 11/19/80
 s.: [Signature] 11/19/80 GC Anal. for # I-6 2, 3, 4
 t.: [Signature] 11/19/80 Microbial (MCT) # 6
 Other # _____
 (Signature) (Date)

12. Approvals

a. Development W.L. Maier W.L. MAIER 12/18/80
 (Signature) (Name) (Date)
 b. Products Research R. H. Bednarz R.H. BEDNARZ 12/18/80
 (Signature) (Name) (Date)
 c. GMP-Quality Assurance J.C. Fix J.C. FIX 12/18/80
 (Signature) (Name) (Date)

Finished product samples will be retained by Quality Assurance.
 (no. samples)

13. The characterization tests requested are appropriate and the test substance is acceptable for: animal safety test _____; human safety test _____; in vitro safety test .

J.B. Oster J.B. Oster 12/18/80
 (Toxicologist's Signature) (Name) (Date)

Test Substance

CAS Number: 131-57-7
Identity: Benzophenone-3
Purity: 100%
Remarks: Identified as MV# 2820-019 and Spectra-Sorb UV-9

Method

GLP: Yes
Report/Study Year: 1990
Method/Guideline Followed: Not indicated
Test Type: In Vivo Cytogenetics
Species: Rats
Strain: Sprague-Dawley
Sex: Male and female
Route of administration: Oral gavage
Exposure period: Up to 5 days.
Doses: Acute: Single gavage administration of 0.5, 1.67 or 5 g/kg body weight
Subchronic: five daily gavage administrations of 5 g/kg body weight.
Remarks: Bone marrow cells, arrested in metaphase and collected 8 and 12 hours after a single treatment and 12 hours after the last of five daily treatments, were examined microscopically for chromosome aberrations. The bone marrow harvest times were selected according to findings of the cell cycle kinetic study to assure analysis of post-treatment, first-division metaphase cells. Study design included positive (cyclophosphamide) and negative (water) controls. Cell cycle kinetic study involved 3 male animals / group. Cytogenetic study involved 10 male and 10 female animals / group.

Results

Result: No statistically significant increase in the percentage of aberrant cells were observed in the test article treated groups, regardless of sex or treatment regimen. The test material was negative in the rat bone marrow cytogenicity study under the conditions of this study
Remarks: Responses of positive and negative controls were appropriate.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Study Number: T8880.105
Reference: Microbiological Associates, Inc. Cytogenicity Study Rat Bone Marrow In-Vivo of MV# 2820-019, P89-018, Accession #36648

Acc #36648

**CYTOGENICITY STUDY
RAT BONE MARROW IN-VIVO**

FINAL REPORT

TEST ARTICLE

**MV# 2820-019
PS9-018**

AUTHORS

**Donald L. Putman, Ph.D.
James M. Melhorn, B.S.**

STUDY COMPLETION DATE

February 20, 1990

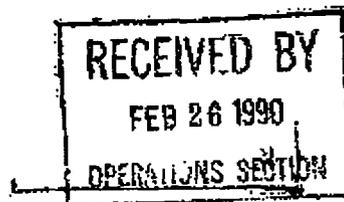
LABORATORY STUDY NUMBER

T8880.105

PERFORMING LABORATORY

**MICROBIOLOGICAL ASSOCIATES, INC.
5221 RIVER ROAD
BETHESDA, MARYLAND 20816**

PAGE 1 OF 41



 **MICROBIOLOGICAL
ASSOCIATES INC.**

9

STATEMENT OF COMPLIANCE

To the best of my knowledge, T8880.105, Cytogenicity Study - Rat Bone Marrow In-Vivo, was conducted in compliance with the Good Laboratory Practice Regulations as published in 21 CFR 58, 40 CFR 160 and 40 CFR 792 in all material aspects with the following reservations:

The identity, strength, purity and composition or other characteristics to define the test or control substance have not been determined by the testing facility (Section 105 (a)).

The stability of the test or control substance under the test conditions has not been determined by the testing facility and is not included in the final report (Sections 105 (a) and (b) and 185 (a) (5)).

Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility (Section 113 (a)).


Donald L. Putman, Ph.D.
Study Director

1-12-50

Date

QUALITY ASSURANCE STATEMENT

Study Title: CYTOGENICITY STUDY
RAT BONE MARROW IN-VIVO

Study Number: T8880.105

Study Director: Donald L. Putman, Ph.D.

Initiation Date: 89/09/13

Review Completed Date: 90/02/20

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice regulations (21CFR58), the U.S. EPA GLPs (40CFR792 and 40CFR160), and the OECD guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of the study.

INSPECT ON 89/09/14 - 89/09/14, TO STUDY DIR 89/09/14, TO MGMT 89/09/14
PHASES: PROTOCOL REVIEW

INSPECT ON 89/10/17 - 89/10/17, TO STUDY DIR 89/10/18, TO MGMT 89/10/20
PHASES: RANDOMIZATION

INSPECT ON 89/12/20 - 89/12/22, TO STUDY DIR 89/12/22, TO MGMT 89/12/28
PHASES: DRAFT REPORT

INSPECT ON 90/02/20 - 90/02/20, TO STUDY DIR 90/02/20, TO MGMT 90/02/20
PHASES: DRAFT REPORT TO FINAL REPORT

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

Donald L. Putman
Quality Assurance
RA/QA Department

2/20/90
Date

CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

FINAL REPORT

Test Article I.D.: MV# 2820-019

Divisional Request Document No.: P89-018

MBA Study No.: T8880.105

Test Article Description: Cream to white powder

Purity: Not listed

Storage Conditions: Room temperature;
Protected from light and humidity

Date Samples Received: 8-30-89 and 10-13-89

Initiation Date: 9-13-89

Sponsor: The Procter and Gamble Company
P.O. Box 39175
Cincinnati, Ohio 45247

Authorized Representative: Christina Gamba-Vitalo

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.
5221 River Road
Bethesda, Maryland 20816

Study Director: Donald L. Putman 2-20-90
Donald L. Putman, Ph.D. Date

Lab Supervisor: James H. Nelhorn 2-20-90
James H. Nelhorn, B.S. Date

Biologist: Jennifer A. Jennings 2-20-90
Jennifer A. Jennings, B.S. Date

Cytogeneticist: William P. Nash 2-20-90
William P. Nash, Ph.D. Date

Cytogeneticist: Hesed P. Nash 2-20-90
Hesed P. Nash, Ph.D. Date

MBA Study T8880.105

4

 MICROBIOLOGICAL
ASSOCIATES INC.

SUMMARY

Male and female Sprague-Dawley rats were dosed with MV# 2820-019 by a single gavage administration of 0.5, 1.67 or 5 gm/kg body weight as well as by five daily gavage administrations of 5 gm/kg body weight. The acute and subchronic dose levels were established by the Sponsor based on existing toxicity data. Bone marrow cells, arrested in metaphase and collected 8 and 12 hours after a single treatment and 12 hours after the last of five daily treatments, were examined microscopically for chromosome aberrations. The bone marrow harvest times were selected according to the findings of the cell cycle kinetic study in order to assure analysis of post-treatment, first-division metaphase cells. No statistically significant increases in percentage of aberrant cells were observed in the test article treated groups, regardless of sex or treatment regimen. The results of the assay indicate that, under the conditions described in this report, MV# 2820-019 was negative in the rat bone marrow cytogenicity study.

INTRODUCTION

Cytogenetic analysis of rodent bone marrow cells provides a valuable technique for evaluation of mutagenic potential based upon the direct observation and classification of chemical-induced aberrations. These aberrations result from a variety of DNA lesions which lead to helix alterations or to interference with DNA replication. Alteration of the chromosome number and structure in somatic cells is implicated in the production of tumors in experimental animals and man and is closely linked to the appearance of genetic disorders.

PURPOSE

The purpose of this study is to evaluate the clastogenic potential of the test article administered in vivo as manifested by induced chromosomal aberrations in bone marrow cells of rodents.

TEST ARTICLE CHARACTERIZATION

The test article, MV# 2820-019, was received by Microbiological Associates, Inc. on 10-13-89 and was assigned the code number T8880. The test article was characterized by the Sponsor as a cream to white powder which should be stored at room temperature, with an expiration date of 7-31-90.

Upon receipt, the test article was described as an off-white to pale yellow powder and was stored at room temperature under desiccation in the dark. At the time of use, the test article was suspended in corn oil, lot JUN1990J obtained from Giant Food Company, at a concentration of 500 mg/ml for use as the high dosing solution. The stability of the test article under the experimental conditions was not determined by Microbiological Associates, Inc. An aliquot of the most concentrated dosing solution was sent to the Sponsor for chemical analysis.

Cyclophosphamide (CP, CAS No. 6055-19-2), lot 67P-0153, was obtained from Sigma Chemical Company, St. Louis, MO, and was dissolved in sterile distilled water at a concentration of 4 mg/ml for use as the positive control.

MATERIALS AND METHODS

Materials

Animals: Sprague-Dawley Rats,
Males, 286-311 gms at dosing for cell cycle kinetics
Males, 269-337 gms at dosing for cytogenetics
Females, 185-229 gms at dosing for cytogenetics
8-10 weeks old upon receipt
Harlan Sprague Dawley, Inc.
Frederick, Maryland

Materials: Hank's balanced salt solution (HBSS)
Colchicine, 0.25 mg/ml in HBSS
Potassium chloride, 0.075 M
Methanol:acetic acid fixative
Carbon dioxide
Giemsa stain
Permunt
Glass slides and coverslips
Screw-capped centrifuge tubes
Pipettes, assorted sizes

Chemicals: Carrier vehicle for test article (corn oil)
Cyclophosphamide (CP)
2'-5'-Bromodeoxyuridine (BrdU)

Methods

The assay was conducted according to the methodology described in detail in the protocol (Appendix I) with the following deviations: group mean body weights differed by more than 3 grams at the time of dose administration. The following deviations from standard operating procedures were documented in the raw data: animal scale was calibrated using standard weights of 100 and 200 gms but not 500 gms; relative humidity of the animal room deviated from the acceptable range on several occasions.

Evaluation of Test Results

The mitotic index, percent polyploid and percent endoreduplicated cells, and total number and types of aberrations found for each animal are presented and discussed. The percentage of damaged cells (including cells scored as unanalyzable) in the total population of cells examined was calculated for each treatment group. Single and isochromatid gaps are presented in the data but not included in the total percentage of cells with one or more aberrations. The severity of damage within the cells is reported as the number of aberrations per cell for each

treatment group. Single and isochromatid gaps are presented in the data but are not included in the average number of aberrations per cell.

Male and female animals were analyzed separately. The Fisher's Exact Test was used to compare the percentage of aberrant cells between each animal and the vehicle control group. The Wilcoxon Rank Sum Test was used to compare the mean aberrations per cell per animal in each treatment group to the appropriate vehicle control. If a vehicle other than water was used, statistical comparisons were made between the vehicle and water control groups.

The test article is considered to induce a positive response when the mean aberrations per cell per animal is significantly increased relative to the vehicle control or when the percentage of aberrant cells of the treated group is significantly increased relative to the vehicle control group.

Criteria for Determination of a Valid Test

The percentage of cells in the vehicle control group demonstrating aberrations of any type, other than gaps, must not exceed 4%.

Records

All raw data, the final report, and bone marrow slides used for analysis are maintained at Microbiological Associates Inc.'s archives located at 9900 Blackwell Road, Rockville, Maryland 20850.

RESULTS AND DISCUSSION

Sacrifice intervals for the cytogenetics assay were determined following a preliminary cell cycle kinetics test (Table 1). MV# 2820-019 was administered once by gavage to three male rats at 5 gm/kg for the acute study and at 5 gm/kg/day for five consecutive days for the subchronic study. A water control group of three male rats was included for comparison in both the acute and subchronic study. Water and test article suspended in corn oil were administered in a constant volume of 10 ml/kg body weight. All animals were sacrificed 24 hours after implantation of an agar coated 100 mg BrdU pellet. Bone marrow cells were prepared, stained by a modified fluorescence-plus-Giemsa technique and scored for first-, second- and third-division metaphase cells. The average generation time (AGT) was calculated according to protocol. Since MV# 2820-019 did not extend the AGT beyond that observed for the water control, bone marrow collection times were

set at 0 and 12 hours after a single administration for the acute study and at 12 hours after the last of five daily administrations for the subchronic study.

For the acute cytogenetics assay, male and female rats were dosed once by gavage with 0.5, 1.67 and 5 gm MV# 2820-019/kg body weight which was administered suspended in corn oil at a rate of 10 ml/kg body weight (Table 2). The positive control, CP, was administered by gavage as a single administration of 20 mg/kg body weight and as five daily administrations of 20 mg/kg/day. CP was administered in a total volume of 4 ml/kg body weight. Clinical signs of toxicity observed within 6 hours of dose administration included diarrhea (4/5 males at 1.67 gm/kg, 2/5 males at 5 gm/kg) and lethargy (1/5 males at 5 gm/kg). Clinical signs at about 12 hours after dose administration included diarrhea (1/5 males and 1/5 females in the corn oil control, 1/5 males at 0.5 gm/kg, 1/5 males in the cyclophosphamide group), piloerection (1/5 males and 4/5 females at 1.67 gm/kg and 1/5 males and 1/5 females at 5 gm/kg), crusty nose (2/5 males at 1.67 gm/kg), lethargy (1/5 males and 4/5 females at 1.67 gm/kg), hypothermia (4/5 females at 1.67 gm/kg), and ataxia (2/5 females at 1.67 gm/kg). No marked changes in body weight were observed over the 12 hour observation period. For the subchronic cytogenetics assay, male and female rats were dosed by gavage daily for five consecutive days with 5 gm MV# 2820-019/kg body weight which was given in a total volume of 10 ml/kg body weight (Table 3). Clinical signs during the five day dosing period included diarrhea (4/5 males and 3/5 females at 5 gm/kg/day), ruffled fur (2/5 males and 5/5 females) and crusty nose (1/5 females at 5 gm/kg/day). Corn oil and water control animals appeared normal throughout the dosing period. No marked changes in body weight were observed over the five day dosing period. One female rat receiving 5 gm/kg/day was found dead prior to the fifth dose administration. Gross observation at necropsy revealed no unusual findings.

The percentage of damaged cells in the total population of cells scored and the mean number of aberrations per cell are presented for each treatment group by sex in Table 4 for the acute study and Table 5 for the subchronic study. The mean aberrations per cell per animal was not significantly increased in the test article-treated groups, regardless of sex or treatment regimen ($p > 0.05$, Wilcoxon's rank sum test). The percentage of aberrant cells was not significantly increased in the test article-treated groups, regardless of sex or treatment regimen ($p > 0.05$, Fisher's exact test). In the cyclophosphamide-treated groups, both the mean aberrations per cell and the percentage of cells containing one or more aberrations were statistically elevated above that observed in the water control in both the acute and subchronic studies.

The mitotic index, percent polyploid and endoreduplicated cells and the number and types of aberrations in each animal are

presented by sex and treatment regimen for the acute study in Tables 6-9 and the subchronic study in Tables 10-11. NV# 2820-019 had no apparent effect on the mitotic index. The percentage of cells containing one or more aberrations was not significantly increased relative to the vehicle control group in any of the test article treated animals ($p > 0.05$, Fisher's Exact Test).

CONCLUSIONS

The negative and positive controls fulfilled the requirements for determination of a valid test.

Under the conditions of the assay described in this report, the test article, NV# 2820-019, was negative in the acute and subchronic cytogenetics assay using male and female Sprague-Dawley rats.

TABLE 1

EFFECT OF MW 2820-019 ON CELL CYCLE KINETICS
IN BONE MARROW CELLS OF MALE SPRAGUE DAWLEY RATS

Treatment	Animal Number	Cell Cycle Kinetics			Average Generation Time (AGT) ¹
		N ₁	N ₂	N ₃	
ACUTE STUDY					
Water 10 ml/kg	14	24	24	2	15.4
	9	25	24	1	15.8
	16	25	22	3	15.4
				Mean	15.5
MW 2820-019 5 gm/kg	10	17	32	1	14.3
	6	19	30	1	14.6
	11	15	34	5	12.8
				Mean	13.9
SUBCHRONIC STUDY					
Water 10 ml/kg	12	16	24	10	12.8
	1	13	32	5	13.0
	15	11	34	5	12.8
				Mean	12.9
MW 2820-019 5 gm/kg	5	9	35	6	12.4
	8	7	38	5	12.2
	3	6	34	10	11.5
				Mean	12.1

$$^1AGT = \frac{26 \text{ hr. BrdU incorporation}}{1(N_1 \text{ frequency}) + 2(N_2 \text{ frequency}) + 3(N_3 \text{ frequency})} \times 100$$

TABLE 2

EFFECT OF MVA 2820-019 ON BODY WEIGHTS OF SPRAGUE DALEY RATS AFTER A SINGLE TREATMENT

Treatment	Sex	Group Mean Body Weight (gms) ¹			% Change ²	
		Pre-treatment	8 Hr	12 Hr	8 Hr	12 Hr
Metar 10 ml/kg	M	311 ± 13	302 ± 14	317 ± 13	-2.9%	1.9%
	F	206 ± 11	206 ± 13	208 ± 9	0.0%	1.0%
Corn Oil 10 ml/kg	M	316 ± 12	312 ± 13	314 ± 12	-1.3%	-0.6%
	F	209 ± 10	205 ± 8	215 ± 12	-1.9%	2.9%
OP 20 mg/kg	M	314 ± 15		312 ± 15		-0.6%
	F	207 ± 13		207 ± 12		0.0%
MVA 2820-019 5 gm/kg	M	312 ± 16	310 ± 16	306 ± 13	-0.6%	-1.9%
	F	208 ± 10	207 ± 7	207 ± 12	-0.5%	-0.5%
1.67 gm/kg	M	309 ± 12	304 ± 13	301 ± 12	-1.6%	-2.6%
	F	205 ± 12	205 ± 15	204 ± 6	0.0%	-0.5%
0.5 mg/kg	M	310 ± 10	306 ± 11	317 ± 15	-1.3%	2.3%
	F	208 ± 12	210 ± 12	213 ± 12	1.0%	2.4%

¹ Reported as mean ± standard deviation² % Change = $\frac{\text{Post-treatment weight} - \text{Pre-treatment weight}}{\text{Pre-treatment weight}} \times 100$

TABLE 3

EFFECT OF MMS 2820-019 ON BODY WEIGHTS OF SPRAGUE DAWLEY RATS AFTER FIVE DAILY TREATMENTS

Treatment	Sex	GROUP MEAN BODY WEIGHTS (gms) ¹					Sacrifice	% Change ²
		Pre-treatment	Day 2	Day 3	Day 4	Day 5		
Water 10 ml/kg/day	M	288 ± 13	295 ± 16	302 ± 18	305 ± 20	311 ± 18	304 ± 19	6%
	F	203 ± 10	205 ± 9	205 ± 9	208 ± 11	210 ± 11	210 ± 11	3%
Corn Oil 10 ml/kg/day	M	297 ± 13	297 ± 13	302 ± 14	306 ± 12	309 ± 13	309 ± 14	4%
	F	202 ± 8	204 ± 7	204 ± 6	208 ± 8	209 ± 7	211 ± 8	4%
CP 20 mg/kg/day	M	296 ± 10	297 ± 11	298 ± 8	297 ± 8	295 ± 8	291 ± 8	-2%
	F	200 ± 7	197 ± 10	202 ± 10	201 ± 7	200 ± 9	204 ± 9	2%
MMS 2820-019 5 mg/kg/day	M	292 ± 16	286 ± 8	276 ± 9	280 ± 14	283 ± 16	286 ± 12	-2%
	F	203 ± 8	196 ± 8	194 ± 9	195 ± 13	200 ± 6	207 ± 7	2%

¹ Reported as mean ± standard deviation² % Change = $\frac{\text{Post-treatment weight} - \text{Pre-treatment weight}}{\text{Pre-treatment weight}} \times 100$

TABLE 4
 CHROMOSOMAL DAMAGE IN SPRAGUE-DAWLEY RATS FOLLOWING TREATMENT WITH RIV 2820-019
 ACUTE STUDY

Treatment ¹	Sex	Time (hr)	Total Number of Cells			Percent Aberrant Cells ^{2,4}	Total Number of Aberrations				Aberrations Per Cell ^{3,5}	
			Scored	Not Analyzable	With Aberrations		Chromatid-type Gaps	Chromosome-type Breaks	Exchanges	Exchanges		
Water												
10 ml/kg	M	8	250	0	1	0.4	0	1	0	0	0	0.004 ± 0.009
	F	8	250	0	0	0.0	0	0	0	0	0	0.000 ± 0.000
	M	12	250	0	0	0.0	1	0	0	0	0	0.000 ± 0.000
	F	12	250	0	0	0.0	0	0	0	0	0	0.000 ± 0.000
Corn Oil												
10 ml/kg	M	8	250	0	0	0.0	1	0	0	0	0	0.000 ± 0.000
	F	8	250	0	1	0.4	0	1	0	0	0	0.004 ± 0.009
	M	12	250	0	0	0.0	1	0	0	0	0	0.000 ± 0.000
	F	12	250	0	1	0.4	0	1	0	0	0	0.004 ± 0.009
CP												
20 mg/kg	M	12	250	0	38	15.2*	2	68	10	0	1	0.316 ± 0.209**
	F	12	250	0	31	12.4*	3	41	3	0	0	0.176 ± 0.095**
Test Article												
5000 mg/kg	M	8	250	0	1	0.4	1	1	0	0	0	0.004 ± 0.009
	F	8	250	0	0	0.0	0	0	0	0	0	0.000 ± 0.000
	M	12	250	0	2	0.8	0	2	0	0	0	0.008 ± 0.011
	F	12	250	0	1	0.4	1	1	0	0	0	0.004 ± 0.009
1670 mg/kg	M	8	250	0	1	0.4	0	1	0	0	0	0.004 ± 0.009
	F	8	250	0	1	0.4	1	1	0	0	0	0.004 ± 0.009
	M	12	250	0	1	0.4	1	1	0	0	0	0.004 ± 0.009
	F	12	250	0	0	0.0	2	0	0	0	0	0.000 ± 0.000
500 mg/kg	M	8	250	0	0	0.0	2	0	0	0	0	0.000 ± 0.000
	F	8	250	0	0	0.0	0	0	0	0	0	0.000 ± 0.000
	M	12	250	0	0	0.0	2	0	0	0	0	0.000 ± 0.000
	F	12	250	0	0	0.0	1	0	0	0	0	0.000 ± 0.000

¹Water, vehicle and test article in vehicle were delivered in a volume of 10 ml/kg body weight.

²Includes unanalyzable cells.

³Excluding gaps; reported as mean ± standard deviation.

⁴*, p<0.025, Fisher's exact test

⁵***, p<0.05, Rank Sum test

TABLE 5

CHROMOSOMAL DAMAGE IN SPRAGUE-DAWLEY RATS FOLLOWING TREATMENT WITH MMS 2820-019
SUBCHRONIC STUDY

Treatment ¹	Time (hr)	Sex	Total Number of Cells			Percent Aberrant Cells ^{2,4} (%)	Total Number of Aberrations					Aberrations Per Cell ^{3,5}
			Scored	Not Analyzable	With Aberrations		Chromatid-type		Chromosome-type			
Water	12	M	250	0	0	0.0	0	0	0	0	0	0.000 ± 0.000
		F	250	0	1	0.4	1	1	0	0	0	0.004 ± 0.009
Corn Oil	12	M	250	0	1	0.4	1	1	0	0	0	0.004 ± 0.009
		F	250	0	2	0.8	0	2	0	0	0	0.008 ± 0.011
CP	12	M	250	3	50	20.9 ⁶	5	99	12	1	0	0.448 ± 0.092 ⁶
		F	250	2	44	17.9 ⁶	0	87	13	2	1	0.412 ± 0.248 ⁶
MMS 2820-019	12	M	250	0	0	0.0	0	0	0	0	0	0.000 ± 0.000
		F	200	0	0	0.0	0	0	0	0	0	0.000 ± 0.000

¹Water, vehicle and test article in vehicle were delivered in a volume of 10 ml/kg body weight.

²Includes unanalyzable cells.

³Excluding gaps; reported as mean ± standard deviation.

⁴p<0.025, Fisher's exact test

⁵p<0.05, Rank Sum Test

TABLE 6

CHROMOSOMAL DAMAGE IN MALE RATS 8 HOURS FOLLOWING ACUTE TREATMENT WITH MWP 2820-019

Treatment ¹	Animal Number	Mitotic Index	# of Cells		% Aberrant Cells		Gaps			Chromatid Type			Chromosome Type		
			Scored	UA ²	Aber ²	Structural	Numerical	Single Iso ²	Breaks	Ex ²	Del ²	Dic ²	Rg ²		
								Single	Iso ²	Ex ²	Del ²	Dic ²	Rg ²		
Water															
10 ml/kg	55	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	57	0.8	50	0	1	2.0	0.0	0	0	1	0	0	0	0	
	158	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	39	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	65	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
Corn Oil															
10 ml/kg	151	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	28	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	149	0.6	50	0	0	0.0	0.0	1	0	0	0	0	0	1	
	40	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	2	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
5000 mg/kg															
	52	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	15	0.8	50	0	0	0.0	0.0	1	0	0	0	0	0	0	
	47	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	38	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	36	1.0	50	0	1	2.0	0.0	0	0	1	0	0	0	0	
1670 mg/kg															
	56	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	18	1.0	50	0	1	2.0	0.0	0	0	1	0	0	0	0	
	156	2.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	31	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	51	0.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
500 mg/kg															
	42	0.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	133	0.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	148	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	30	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	
	156	0.6	50	0	0	0.0	0.0	2	0	0	0	0	0	0	

¹Water, vehicle and test article in vehicle were delivered in a volume of 10 ml/kg body weight.²UA = Unanalyzable cells; Aber = Cells with aberrations; Iso = Isochromatid; Ex = Exchange(s); Del = Deletion(s); Dic = Dicentric(s); Rg = Ring(s)³Includes unanalyzable cells; *, Percent aberrant cells significantly greater than vehicle control group (p<0.05, Fisher's Exact Test).⁴Includes polyploid and endoreduplicated cells.

TABLE 7

CHROMOSOMAL DAMAGE IN FEMALE RATS & MICE FOLLOWING ACUTE TREATMENT WITH WWS 2820-019

Treatment ¹	Animal Number	Mitotic Index	N of Cells		% Aberrant Cells			Chromatid Type			Chromosome Type			
			Scored	UA ²	Aber ²	Structural	Numerical	Group	Single	Is ²	Ex ²	Del ²	Dic ²	Rg ²
Water														
10 ml/kg	86	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	137	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	160	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	104	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	140	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0
Corn Oil														
10 ml/kg	120	1.0	50	0	1	2.0	0.0	0	0	1	0	0	0	0
	171	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	133	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	118	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	84	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0
5000 mg/kg														
	114	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	117	0.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	130	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	89	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	142	2.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0
1670 mg/kg														
	129	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	138	0.6	50	0	1	2.0	0.0	0	0	1	0	0	0	0
	119	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	134	0.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	112	1.4	50	0	0	0.0	0.0	0	1	0	0	0	0	0
500 mg/kg														
	168	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	141	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	75	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	116	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0
	132	0.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0

¹Water, vehicle and test article in vehicle were delivered in a volume of 10 ml/kg body weight.²UA = Unanalyzable cells; Aber = Cells with aberrations; Is = Isochromatid; Ex = Exchange(s); Del = Deletion(s); Dic = Dicentric(s); Rg = Ring(s)³Includes unanalyzable cells; *, Percent aberrant cells significantly greater than vehicle control group (p < 0.05, Fisher's Exact Test).⁴Includes polyploid and endoreduplicated cells.

TABLE 8

CHROMOSOMAL DAMAGE IN MALE RATS 12 HOURS FOLLOWING ACUTE TREATMENT WITH DMS 2820-019

Treatment ¹	Animal Number	Necrotic Tissue	# of Cells Scored		% Aberrant Cells		Gaps		Chromatid Type Breaks			Chromosome Digs				
			UA ²	Aber ²	Structural (%) ³	Numerical (%) ⁴	Single	Is ²	Single	Is ²	Ex ²	Del ²	Dic ²	Rg ²		
Water 10 ml/kg	68	2.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	64	2.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	159	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	5	1.2	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0	0
	71	1.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
Corn Oil 10 ml/kg	66	2.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	66	1.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	20	2.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	49	1.6	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0	0
	5	2.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
CP 20 mg/kg	19	0.4	50	0	8	16.0*	0.0	0	0	11	0	2	0	0	0	0
	58	1.2	50	0	9	18.0*	0.0	0	0	29	0	3	0	0	0	0
	146	1.8	50	0	8	16.0*	0.0	0	0	10	0	2	0	0	0	0
	70	1.4	50	0	6	12.0*	0.0	1	0	7	0	0	0	0	0	0
	60	1.4	50	0	7	14.0*	0.0	1	0	11	0	1	0	0	0	1
100 mg/kg	35	1.8	50	0	1	2.0	0.0	0	0	1	0	0	0	0	0	0
	155	2.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	72	3.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	41	1.0	50	0	1	2.0	0.0	0	0	1	0	0	0	0	0	0
	68	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
1670 mg/kg	37	2.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	145	2.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	36	2.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	43	1.2	50	0	1	2.0	0.0	1	0	1	0	0	0	0	0	0
	152	1.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
500 mg/kg	61	2.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	44	1.2	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0	0
	54	1.2	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0	0
	26	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	59	3.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0

¹Water, vehicle and test article in vehicles were delivered in a volume of 10 ml/kg body weight.²UA = Unanalyzable cells; Aber = Cells with aberrations; Iso = Isochromatid; Ex = Exchange(s); Del = Deletion(s); Dic = Dicentric(s); Rg = Ring(s)³Includes unanalyzable cells; *, Percent aberrant cells significantly greater than vehicle control group (p<0.05, Fisher's Exact Test).⁴Includes polyploid and endoreduplicated cells.

TABLE 9

CHROMOSOMAL DAMAGE IN FEMALE RATS 12 HOURS FOLLOWING ACUTE TREATMENT WITH MWF 2820-019

Treatment ¹	Animal Number	Mitotic Index	# of Cells		% Aberrant Cells		Gaps		Chromatid Type			Chromosome Type			
			Scored	Un ²	Aber ²	Structural (%) ³	Numerical (%) ⁴	Single	iso ²	Single	iso ²	EX ²	Del ²	Dic ²	Rg ²
Water															
10 ml/kg	113	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	124	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	91	0.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	111	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	170	1.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
Corn Oil															
10 ml/kg	136	1.6	50	0	1	2.0	0.0	0	0	1	0	0	0	0	0
	123	2.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	78	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	144	1.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	162	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
CP															
20 mg/kg	90	1.0	50	0	10	20.0*	0.0	0	0	15	1	1	0	0	0
	94	1.0	50	0	6	12.0*	0.0	1	0	6	0	0	0	0	0
	89	1.2	50	0	4	8.0*	0.0	1	0	6	0	0	0	0	0
	108	0.8	50	0	9	18.0*	0.0	0	0	5	0	1	0	0	0
	128	1.0	50	0	6	12.0*	0.0	1	0	8	0	1	0	0	0
500 mg/kg															
	143	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	127	0.2	50	0	1	2.0	0.0	0	0	1	0	0	0	0	0
	73	0.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	121	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	139	1.4	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0
1670 mg/kg															
	87	0.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	115	1.4	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0
	122	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	169	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	141	0.4	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0
500 mg/kg															
	166	2.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	167	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	164	0.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	83	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	97	0.4	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0

¹Water, vehicle and test article in vehicle were delivered in a volume of 10 ml/kg body weight.

²Un = Unanalyzable cells; Aber = Cells with aberrations; iso = Isochromatid; EX = Exchange(s); Del = Deletion(s); Dic = Dicentric(s); Rg = Ring(s)

³Includes unanalyzable cells; *, Percent aberrant cells significantly greater than vehicle control group (p<0.05, Fisher's Exact Test).

⁴Includes polyploid and endoreduplicated cells.

TABLE 10

CHROMOSOMAL DAMAGE IN MALE RATS 12 HOURS FOLLOWING FIVE DAILY TREATMENTS WITH MWF 2820-019

Treatment ¹	Animal Number	Mitotic Index	# of Cells		% Aberrant Cells			Gaps			Chromatid Type Breaks			Chromosome Type		
			Scored	UA ²	Aber ³	Structural	Numerical	Single	Is ⁴	Ex ²	Single	Is ²	Ex ²	Del ²	etc ²	Rg ²
Water																
10 ml/kg/day	13	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	11	0.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	33	1.6	50	0	0	0.0	0.0	0	0	0	2	0	0	0	0	0
	10	2.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	23	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
Corn Oil																
10 ml/kg/day	23	2.0	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0	0
	12	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	14	1.4	50	0	1	2.0	0.0	0	0	1	0	0	0	0	0	0
	17	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
23	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0	
CP																
20 mg/kg/day	4	1.0	50	0	12	24.0*	0.0	0	0	25	0	1	1	0	0	0
	16	1.4	50	1	9	19.6*	0.2	4	0	22	0	2	0	0	0	0
	9	0.6	50	2	11	25.0*	0.0	0	0	22	0	4	0	0	0	0
	27	1.0	50	0	9	18.0*	0.0	1	0	15	0	3	0	0	0	0
	29	1.4	50	0	9	18.0*	0.2	0	0	14	1	2	0	0	0	0
MWF 2820-019																
5 gm/kg/day	21	1.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	24	2.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	32	2.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	6	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0
	8	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0	0

¹Water, vehicle and test article in vehicle were delivered in a volume of 10 ml/kg body weight.²UA = Unanalyzable cells; Aber = Cells with aberrations; Is = Isochromatid; Ex = Exchange(s); Del = Deletion(s);

Dic = Dicentric(s); Rg = Ring(s)

³Includes unanalyzable cells; *, Percent aberrant cells significantly greater than vehicle control group (p<0.05, Fisher's Exact Test).⁴Includes polyploid and endoreduplicated cells.

TABLE 11

CHROMOSOMAL DAMAGE IN FEMALE RATS 12 HOURS FOLLOWING FIVE DAILY TREATMENTS WITH MW 2820-019

Treatment ¹	Animal Number	Mitotic Index	# of Cells		% Aberrant Cells		Gross		Chromatid Type			Chromosome Type			
			Scored	UA ²	Aber ²	Structural	Numerical	Single	Iso ²	Single	iso ²	Ex ²	Del ²	Dic ²	Rp ²
Water															
10 ml/kg/day	85	0.8	50	0	0	0.0	0.0	1	0	0	0	0	0	0	0
	79	1.0	50	0	1	2.0	0.0	0	0	1	0	0	0	0	0
	105	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	98	0.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	74	1.0	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
Corn Oil															
10 ml/kg/day	76	1.2	50	0	1	2.0	0.0	0	0	1	0	0	0	0	0
	81	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	92	1.4	50	0	1	2.0	0.0	0	0	1	0	0	0	0	0
	99	1.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	77	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
CP															
20 mg/kg/day	96	1.0	50	1	7	13.7	0.0	0	0	9	0	2	0	0	0
	100	0.6	50	0	5	10.0	0.0	0	0	7	0	3	0	0	0
	80	0.6	50	0	9	18.0	0.0	0	0	20	0	1	1	0	0
	93	0.6	50	1	13	27.5	0.0	0	0	25	0	1	1	0	0
	103	0.4	50	0	10	20.0	0.0	0	0	29	0	7	0	1	0
MW 2820-019															
5 gm/kg/day	104		0												
	82	0.4	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	107	1.6	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	101	1.8	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0
	99	1.2	50	0	0	0.0	0.0	0	0	0	0	0	0	0	0

¹Water, vehicle and test article in vehicle were delivered in a volume of 10 ml/kg body weight.²UA = Unanalyzable cells; Aber = Cells with aberrations; Iso = Isochromatid; Ex = Exchange(s); Del = Deletion(s); Dic = Dicentric(s); Rp = Ring(s)³Includes unanalyzable cells; *, Percent aberrant cells significantly greater than vehicle control group (p<0.05, Fisher's Exact Test).⁴Includes polyploid and endoreduplicated cells.

REFERENCES

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Appendix I
Study Protocol

NBA Study T8880.105

23

 MICROBIOLOGICAL
ASSOCIATES INC.

Received by RA/QA 9-13-89

APPROVED
9/13/89

PROTOCOL C27A
CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988
Supersedes Issue Dated: July 9, 1987

Purpose: To evaluate the clastogenic potential of a test substance administered *in vivo* as manifested by induced chromosomal aberrations in bone marrow cells of rodents.

Justification for Selection of Test System: Direct examination of chromosome aberrations in bone marrow cells of rodents is an acceptable and reliable system for the detection of substances with clastogenic activity.¹

This protocol is in compliance with the EPA Guidelines (1985 and 1986)^{2,3} and the Organization of Economic Cooperation and Development (OSCD, 1984)⁴.

per RUI procedure
8/23/89

Test Substance Identification Number (TSIN) # 8820-019

Divisional Request Document Number (DRD) # PS9-018

Sponsor: The Procter & Gamble Company
Cincinnati, Ohio

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC. Study # T8880.105
(To be filled in by ROCKVILLE, MD 20850 (To be filled in by Operating Division) Testing Facility)

Sponsor: Christina Gamble-Valde
Divisional Toxicologist

Date Approved by Sponsor's Divisional Toxicologist: 8/23/89

Proposed Starting Date: 9-13-89

Defined as granted approval date

Proposed Completion Date: 12-22-89

Defined as request date) To be completed by the Test Facility

Study Director: Donald J. [Signature]

Date: 9-13-89

Study Cost: ~~\$24,500~~ + \$3,900

error
9/13/89

PROTOCOL C27A (Cont'd)
 CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

Test Substance(s) (Information to be filled in by Operating Division)

Description		Expiration Date
Color	Physical Form	
CREAM TO WHITE	POWDER	7/31/90 <i>mk SR 9/11/89</i>

A concentration analysis of the test substance -
 vehicle mixture will ; will not be required.

If a concentration analysis is required:

Prepare a sufficient quantity of the most concentrated
 test substance - vehicle mixture so that a portion can
 be returned to the Sponsor's Divisional Toxicologist.
 Store solution/mixture at room temperature;
 refrigerator; freezer; other _____

Shipping Instructions - ~~Shipped Same Week Research Center~~
6/26/89 MJA 9-6-89
 Send approximately 50 ml. Send frozen;
 under ambient conditions; other _____

Analyze the test substance - vehicle mixture(s)
 for test substance concentration using the
 analytical method in Appendix _____.

Storage Conditions: (Check one)

Room temperature Refrigerator Freezer
 Other

Hazards: (Check one)

None known. Take ordinary precautions in handling.
 As follows:

PROTOCOL C27A (Cont'd)
CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

Special Instructions for Preparation of Test Substance: (To be filled in by Operating Division, check one). The test substance is prepared the same as in the maximum tolerated dose (MTD) finding study.

None

As follows:

A slurry of the test substance in mineral oil can be obtained by adding 125g of chemical to 5ml corn oil.

Test Substance

Preparation: (To be filled in by Operating Division)

The test substance is prepared fresh each day as per the instructions in this protocol. ~~The route of administration, solubility, vehicle, dose and dose volume, are the same as those used in the maximum tolerated dose (MTD) finding study.~~ The animals are weighed before the first dose in the acute study and before each dosing in the subchronic study. All dosing calculations are made on these weight values. NSA 4-7-89

Route of Administration of Test Substance: (refer to MTD study)

- By gavage, p.o.
- Diet
- Intravenous injection, i.v.
- By intraperitoneal, i.p.
- Other (specify) _____

Vehicle: (refer to MTD study)

- Distilled water
 - Corn oil
 - Mineral oil
 - Ethanol
 - Other (specify) _____
- Dose volume _____

Records to be Maintained:

Purchase order and receipt of animals, body weights of animals upon receipt, food identification, dose preparation records, body weights of animals before first dosing, time and date of individual dosings, daily toxicity observation data, body weights of animals before colchicine dosing, date of preparation of colchicine, time and date of colchicine dosings, time and date of individual animal sacrifice, preparation of bone

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PROTOCOL C27A (Cont'd)
CTTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

Records to be
Maintained (cont'd):

marrow, slide coding and preparation, staining methods, person(s) and microscope(s) used in scoring, and all other records that would be required to reconstruct the study and demonstrate adherence to protocol. All animals in all treatment groups are weighed at each colchicine treatment time. Slides are maintained.

Animals: Sexually mature (10-12 wk) outbred albino rats (Sprague-Dawley) from a source approved by the Test Facility, are used. Before dosing, female animals shall weigh in the range of 190-250 gm and males shall weigh 270-350 gm and the animals shall not exceed 12 weeks of age.

Animal Care: The animals are allowed to acclimate for at least seven days before beginning of the study. Animals are not fasted at anytime before or during the study. All other animal care procedures are according to the approved Standard Operating Procedures of the Test Facility.

Diet: A single lot of Rodent Chow as specified by the Test Facility's Standard Operating Procedures and water from a municipal water supply.

**Diet and/or
Water Analyses
Required:** None (no known contaminants expected which would interfere with this study)

**Environmental
Conditions:** The animals are housed individually with a 12-hour on-off light cycle. All other environmental conditions are according to the approved Standard Operating Procedures of the Test Facility.

Animal Ident. Upon receipt, each animal is weighed and assigned a number by which it is identified throughout the study. Follow the approved Standard Operating Procedures of the Test Facility.

Randomization: Within each sex, the animals are randomly assigned to one of the groups following the approved Standard Operating Procedures of the Test Facility such that the average body weights of each group differ by no more than 3 grams.

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**PROTOCOL C27A (Cont'd)
CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO**

Issue Date: December 12, 1988

Experimental Design:

Two dosing regimens are shown below, acute (single dose) and an optional subchronic (five doses given 24 hrs apart). The subchronic dosing regimen may be used if pharmacological/toxicological data indicates the necessity for repeated dosing to obtain a maximal dose to the bone marrow.

Acute Dosing (Test Substance): A single, high dose which is the acute maximum tolerated dose (MTD) is used. It has been shown that the LD1 value approximates the MTD⁶. The LD1 is determined by performing an acute maximum tolerated dose study using nonfasted male and female animals. A separate protocol for determining the MTD is available upon request. If the test article is nontoxic, as determined by an MTD finding study, the high dose level is 5 g/kg.

Doses:	High dose	Mid dose	Low dose
Female	5/kg	1.67/kg	0.5/kg
Male	5/kg	1.67/kg	0.5/kg

Subchronic Dosing (Test Substance): The subchronic MTD is used. It has been determined that the 0.5 x LD1 value from acute MTD studies approximates the MTD for subchronic cytogenetic studies⁶. The 0.5 x LD1 value is determined by performing an acute MTD finding study using nonfasted male and female animals. A separate protocol for determining the MTD is available upon request. If the test article is nontoxic as determined by an MTD finding study, the dose is 5 g/kg.

Doses:	High dose	Low dose
Female	5/kg	0.5/kg
Male	5/kg	0.5/kg

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PROTOCOL C27A (Cont'd)
CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

Controls: Positive Control: Cyclophosphamide 20 mg/kg animal body weight. Dosing volume about 1 ml/animal.

Acute and Subchronic Dosing Regimens

Prepare a 5 mg/ml (distilled water) solution and dose as follows:

- p.o. female
- p.o. male
- i.p. female
- i.p. male

Vehicle Control: If the test substance is prepared in a vehicle other than water, a vehicle control must be included.

- Corn Oil
- Mineral Oil
- Ethanol
- Other (specify)

Sacrifice Times: The sacrifice times are determined on the basis of a bone marrow cell cycle kinetic study. For the acute dosing regimen, three male rats receive the high dose of test compound by the appropriate route of administration approximately 2 h following subcutaneous implantation of an agar-coated BrdU pellet (approximately 100 mg pure BrdU powder per pellet). A control group of 3 male rats receive distilled water. For the subchronic dosing regimen, three male rats receive the high dose of test compound and three animals receive distilled water for four consecutive days by the usual subchronic dosing regimen. Two hours prior to the fifth dose, BrdU is implanted in both treatment groups. All animals are sacrificed 24 h after BrdU implantation. Two hours prior to sacrifice, the rats receive an i.p. injection of colchicine (1.0 mg/kg body weight). Bone marrow preparations are made and cells will be stained by a fluorescence-plus-Giemsa technique to obtain differentially stained chromosomes. The number of cells in first (M1), second (M2) and third or greater (M3+) division is determined in 50 cells per animal. The average generation time (AGT) for each treatment group is calculated by the formula $AGT = 24 \text{ hr} \times \frac{\text{BrdU incorporation}}{1 (\text{frequency M1}) + 2 (\text{frequency M2}) + 3 (\text{frequency M3+})}$ (Ivett and Tice, 1982). The AGT of the distilled water control group should be around 12 h. If the AGT of the treated group is in the range of 10-14 hr the two harvest times for the cytogenetic study are 8 and 12 h after chemical treatment. If the AGT of the treated group is 15 h or greater, a 12 hr harvest and a delayed harvest equal to the AGT of the treated group is used.

PROTOCOL C27A (Cont'd)
CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

Acute Dosing Regimen

<u>Group</u>	<u>No. of Males</u>	<u>No. of Females</u>	<u>Dose Levels</u>	<u>Sacrifice Time</u>
<u>Cell Cycle Kinetic Study</u>				
1. Negative Control	3	-	Distilled Water	24 h
2. Test High Dose	3	-	MTD	24 h

<u>Group</u>	<u>No. of Males</u>	<u>No. of Females</u>	<u>Dose Levels</u>	<u>Sacrifice Time</u>
<u>Cytogenetic Study</u>				
1. Negative Control	10	10	Distilled water	**
2. Vehicle* Control	10	10	Vehicle Control	**
3. Positive Control	5	5	Cyclophosphamide (see Positive Control section)	12 or 24 h
4. Test - High Dose	10	10	MTD (Max. tolerated dose) - LD1 value	**
5. Test-mid dose	10	10	1/2 MTD	**
6. Test-low dose	10	10	1/10 MTD	**

* A vehicle control is necessary if dosing is performed in any solvent other than water.

** Determined from the cell cycle kinetic study.

PROTOCOL C27A (Cont'd)
CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

Subchronic Dosing Regimen

<u>Group</u>	<u>No. of Males</u>	<u>No. of Females</u>	<u>Dose Levels</u>	<u>Sacrifice Time</u>
<u>Cell Cycle Kinetic Study</u>				
1. Negative Control	3	-	Distilled Water	24 h
2. Test High Dose	3	-	MTD	24 h
<u>Group</u>	<u>No. of Males</u>	<u>No. of Females</u>	<u>Dose Levels</u>	<u>Sacrifice Time</u>
<u>Cytogenetic Study</u>				
1. Negative Control	5	5	Distilled water	**
2. Vehicle Control*	5	5	Vehicle control	**
3. Positive Control	5	5	Cyclophosphamide (see Positive Control section)	12 or 24 h
4. Test - High Dose	5	5	MTD (Max. tolerated dose)- 0.5 X LD1	**

* A solvent control is necessary if dosing is performed in any solvent other than water.

** Determined from the cell cycle kinetic study.

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PROTOCOL C27A (Cont'd)
CITOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

Preparation of
Bone Marrow Cells
and Slides:

Two hours before each sacrifice time, the rats receive an intraperitoneal injection of colchicine (1.0 mg/kg) based on terminal weight to arrest cells in metaphase of mitosis. Dissolve the colchicine in Hank's balanced salts solution or Eagles minimal essential medium at a concentration of 0.25 mg/ml.

The animals are sacrificed with CO₂ at the times indicated in each dosing regimen. If the average body weight of any group of animals is less than 80% of the negative control group, continue to process that group, but notify the Sponsor's Divisional Toxicologist immediately.

Immediately following sacrifice, skin and muscle tissues are removed from the femurs. The bone marrow cells are aspirated from both femurs into a 5 or 10 cc syringe equipped with an 18-20 gauge needle. Add a small volume of the Hank's balanced salts solution (HBSS) or Eagles minimal essential medium (MEM) to the syringe before aspirating the cells. If a sufficient bone marrow plug is not retrieved from the femurs, aspirate the tibiae. The cells are transferred to a centrifuge tube containing HBSS. The tubes are stored in an ice bath (approximately 4°C) until centrifugation. The bone marrow cells are centrifuged approximately 200 x g and the supernatant is discarded. Resuspend the cells in an appropriate hypotonic solution like 0.075 N KCl and process according to the test facilities standard practice to obtain well spread metaphases. Prepare a test slide for each treatment group to assess the quality of metaphase spreads. If poorly spread metaphases are obtained in some groups washing the cells in 1:1 (glacial acetic acid:methanol) fixative may be used. If metaphase quality is still poor, the slides can be flamed as a

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PROTOCOL C27A (Cont'd)
CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

Preparation of
Bone Marrow Cells
and Slides: (Cont'd)

last resort. Slides can be made at this point, or the cells can be stored in fixative in capped centrifuge tubes in a refrigerator (approximately 4°C). Before preparing slides, the fixed cells are washed in fresh fixative. At least 3 slides per animal are prepared.

Place any remaining cells back in the refrigerator until all of the slides have been examined and the final report has been received by the Sponsor. (If some slides are unacceptable or additional metaphases are required, new slides may be prepared from these cells.) Stained slides are air-dried then spreads are stained with Giemsa. Slides are air-dried and then cover slipped. The slides are labeled such that they are scored blind. The slides are distributed evenly to each scorer such that every scorer analyzes cells from each experimental point. Do not score any slides from a group whose average terminal body weight is less than 80% of the negative control group unless specifically instructed to do so by the Sponsor's Divisional Toxicologist.

Protocol Changes:

If it becomes necessary to change the approved protocol, verbal agreement to make this change should be made between the Study Director and the Sponsor. As soon as practical, this change and the reasons for it should be put in writing and signed by both the Study Director and the Sponsor's Divisional Toxicologist. This document is then attached to the protocol as an addendum. Any change to the starting date, completion date, or the date for communicating a verbal result, and the reasons for the change are put in writing to the Sponsor.

Analysis of Slides:

A total of 50 metaphase spreads for each animal are scored if possible. Slides are scanned with low magnification and cells that appear suitable are analyzed at high magnification.

Once a mitotic figure is observed at high magnification, it can only be rejected for analysis for the following reasons:

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Analysis of
Slides: (cont'd)

- 1) the cell contains fewer centromeres than $2n \pm 1$.
- 2) the chromosomes are not sufficiently well spread and chromosome overlap prohibits accurate analysis or
- 3) non-chromosomal material, such as dirt or stain crystals prevents analysis
- 4) a majority of the chromosomes have undergone anaphase separation whereby the chromatids have divided at the centromere resulting in a scattering of the individual chromatid arms.
- 5) the staining of the chromosomes is such that they have a "fuzzy" or "puffy" morphology which does not allow for the identification of individual chromatid arms.

Mitotic Index/
Percent Polyploid/
Percent Endore-
duplicated:

The mitotic index (M.I.) for each animal is to be determined. This is achieved by randomly selecting an area on a slide and determining the proportion of cells in mitosis. A minimum of 500 (excluding erythrocytes) cells are counted and the ratio of the number of cells in mitosis/total number of cells counted X 100 is defined as the mitotic index. The percent polyploid and endoreduplicated cells are also analyzed from the 500 cells counted for the M.I.

Classification of
Chromosome
Aberrations:

Each metaphase figure is scored for the number of chromosomes ($2n \pm 1$) and aberrations are categorized as shown below. The vernier position (horizontal and vertical) is recorded for any mitotic figure which has aberration(s) and/or gaps or is questionable in any manner.

A sample score sheet is available upon request.

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PROTOCOL C27A (Cont'd)

MITOGENICITY STUDY - RAT BONE MARROW IN-VIVO

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Chromatid-type Aberrations

Chromatid gap an achrismatic (unstained) region in one chromatid less than or equal to the width of the chromatid. These are recorded but not included in the final analysis.

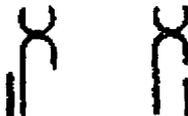


Isochromatid gap achrismatic regions less than the width of the chromatid at apparently the same locus in both sister chromatids. If one fragment is displaced or misaligned it is classified as a chromosome deletion. These gaps are recorded but not included in the final analysis.



Chromatid Break

a) **Terminal deletion** - an achrismatic region in one chromatid greater than the width of the chromatid. The fragment may be partially or completely displaced from the deleted chromosome.

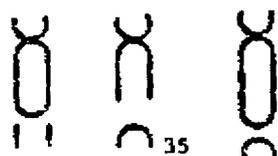


b) **Interstitial deletion** - a small single ring or fragment lying next to the deleted chromosome as indicated below. These deletions may not be easily observed if the fragment is separated from the chromosome.



Isochromatid Break

an achrismatic region at the same locus in both sister chromatids where the region is greater than the width of either chromatid and there is some form of rejoining. The chromosome or fragment can rejoin in a variety of ways (see below) and may be displaced. If there is no apparent rejoining this aberration is indistinguishable from a chromosome deletion and should be categorized as such.



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PROTOCOL C27A (Cont'd)

CYTOTOXICITY STUDY - RAT BONE MARROW IN-VIVO

Issue Date: December 12, 1988

**Chromosomal
exchange**

- a) **symmetrical interchange** - an exchange involving two chromosomes. The exchange can be complete or some pieces may fail to rejoin (incomplete).



- b) **asymmetrical interchange** - an exchange involving two chromosomes which have rejoined as shown below. There may be some incomplete rejoining.



- c) **asymmetrical intrachanges** - an exchange within a chromosome. There are various forms as shown below.



- d) **triradial** - an exchange involving two chromosomes which appears as a 3-armed configuration. An acentric fragment is associated with the first type of triradial. Indicate - F if no fragment is observed.



- e) **complex exchange**-involving more than two chromosomes. Every effort should be made to analyze the number of exchanges involved. Consult the study director if necessary. If an analysis cannot be made, record the cell at the bottom of the score sheet under "LI" unanalyzable.

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Chromosomal Aberrations (Cont'd)

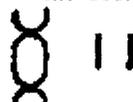
Centromere Break

An acentric fragment which can be of any size and can be displaced.



Dicentric

An exchange involving two chromosomes that results in a chromosome with two centromeres. There is an associated acentric fragment with this aberration. If no fragment is found indicate -F on the score sheet.



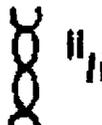
Ring

A chromosome which forms a double circle, with or without a centromere. A ring with a centromere will have an associated fragment. Indicate -F on the score sheet if no fragment is observed with a centric ring.



Other

a) Multicentric - an exchange involving three chromosomes results in a trivalent and two fragments, an exchange with four chromosomes results in a quadrivalent and three fragments, etc. Indicate which figure is observed.



b) Translocation - these are difficult to score accurately without G-banded preparations but sometimes there is an obvious exchange between two chromosomes which results in two chromosomes of unusual size for the karyotype. Consult with the study director when this is observed. Indicate a "T" in the "Other" category.

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PROTOCOL C27A (Cont'd)
CITOGENICITY STUDY - RAT BONE MARROW IN-VIVO

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Cells recorded
at the bottom
of the score sheet:

These cells are recorded but not computed in the analysis of aberration frequency.

Pulverized/shattered cell - the chromosomes are extremely fragmented and unanalyzable. Record under "PU".

Unanalyzable "UA" - those cells that can not be analyzed due to the number/complexity of the aberrations involved.

During the analysis of the 500 cells for the mitotic index determination the number of polyploids and endoreduplicated cells are recorded:

Polyploid - a cell containing multiple copies of the haploid (n) number of chromosomes. Record under "poly".

Endoreduplicated - a cell containing 4n chromosomes where the duplicated chromosomes are lying next to each other in a "paired" configuration.

Data Presentation:

Data are summarized in tables and will include individual animal results and treatment group results at the different time points. A table of the mean group body weights at each weighing time and the percent change will be presented. For the assessment of aberrations, gaps are recorded in individual animal results but not included in total aberration results per animal nor in group summary results.

The number of aberrations per cell per animal is calculated. The mitotic index, percent polyploid percent endoreduplicated cells and percent aberrant cells is calculated and included in individual and group results.

The number of aberrations in pulverized/shattered and unanalyzable metaphases cannot accurately be calculated. These cells are included in the percent aberrant cells but not in the number of aberrations per cell. In this case a "+" is placed next to the total number of aberrations per cell per animal or per group to indicate that the number is a minimum.

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Statistical Analysis: A statistical comparison is made between each animal and the appropriate control group (see below) and some indication is made of any animal with a statistically significant number of aberrations/cell or percent aberrant cells. The Fischer's Exact Test will be used in a statistical analysis of percent aberrant cells. The Wilcoxon's rank sum test⁸ and percent aberrant cells should be used in the analysis of aberrations/cell.

The average number of aberrations per cell and percent aberrant cells per animal for each treatment group is used in a statistical comparison of each treatment group to the control group. If a vehicle other than water was used, a comparison is made between the vehicle and water control groups. The treatment groups are compared to the vehicle group. Male and female results are analyzed separately and compared to each other to determine any sex differences. A Cochran-Armitage^{9,10} trend test for the percent aberrant cells is performed between the groups to test for evidence of a dose response. Any positive response is indicated with the corresponding p value where a p value <0.05 for a two-tailed test and $p <0.025$ for a one-tailed test is considered positive. The criteria for a positive response is a dose(s) that is statistically increased compared to control. A significant trend test only with no single dose statistically increased is considered equivocal.

Criteria for a Valid Assay:

In the vehicle control, the percent cells with aberrations (excluding gaps) shall not exceed 4%.

Report:

A report of the results is prepared for this study by the contract laboratory. The report will include, but not be limited to, the following:

1. Name and address of the facility performing the study and the dates on which the study was initiated and completed.
2. Objectives as stated in the approved protocol, and any changes to the original protocol.

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PROTOCOL C27A (Cont'd)
CYTOGENICITY STUDY - RAT BONE MARROW IN-VIVO

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Report (Cont'd):

3. A detailed description of all methods used. Protocol may be appended.
4. Statistical methods employed for analysis of the data.
5. The number of animals in the study, sex, body weights, (and body weight changes throughout the study,) source of supply, species, strain, and used for individual animal identification.
6. Deviations from the Test Facility's Standard Operating Procedures or the approved protocol.
7. A summary of the results as they relate to the study's objective.
8. The location where all raw data will be stored.
9. Raw data of the slide scoring. To be attached as an appendix.

This report shall conform to all requirements outlined in Section 58.185, Subpart J, Good Laboratory Practices Regulations.

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REFERENCES

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4. OECD Guidelines (1984) In vivo mammalian bone marrow cytogenetic test - chromosomal analysis. Supplement to OECD Guidelines for Testing Chemicals (1981) P.475, OECD Publications Office, 2-Rue Andre-Pascal 75775, Paris, Code 16, France.
5. Thompson, E.D. and D.P. Gibson, (1984) A method for determining the maximum tolerated dose for acute in vivo cytogenetic studies. *Fd. Chem. Toxic.*, 22, No. 8, p. 665-676.
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7. Ivett, J. L. and R. R. Tice (1982) Average generation time: A new method of analysis and quantitation of cellular replication kinetics. *Environ. Mutagen.*, 4, 358-359.
8. Snedecor, G. W. and W. G. Cochran (1967) *Statistical methods* 6th Ed. p. 130-132.
9. Cochran, W. G. (1954) *Biometrics* 10:417-451.
10. Armitage, P. (1955) *Biometrics* 11:375-386.

VICKS RESEARCH CENTER PRODUCT PREPARATION REPORT
 Personal Care Products Division
 THEORETICAL FORMULA

PRODUCT TYPE: OTC Drug INDINDA Drug _____ Device _____ Cosmetic _____

Name: SPECTRA - SOAP UV-9 MV #: 2820-19

/ Name: 905 5758

By: Joseph Craig Date: 8-24-89

By: R.D. Smith Date: 8/24/89

Ingredients in descending order of predominance.
 Drug products list active ingredients first and indicate with an asterisk (*).

AC Code #	Trade or Common Name (Identify Supplier for Specialty Items)	Compndial (Drug) or CIPA (Toiletries) Name	Percentage Composition
569 T1	SPECTRA - SOAP UV-9	Benzophenone-3 (oxybenzone)	100%

FOR COSMETIC DRUG PRODUCTS INDICATE AMOUNT OF EACH ACTIVE INGREDIENT PER DOSAGE UNIT OR PER UNIT OF WEIGHT OR MEASURE OF THE PRODUCT AND A STATEMENT OF THE TOTAL WEIGHT OR MEASURE OF ANY DOSAGE UNIT:

Benzophenone-3 100%
 (oxybenzone)