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November 21, 2006

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1201 Constitution Avenue NW
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ATTN: 8(d) Health & Safety Reporting Rule (Notification/Reporting)

met
300365

CONTAIN NO CBI

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

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



8 6 0 7 0 0 0 0 0 1 7

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

Index of Health & Safety Studies submitted by Procter & Gamble
Page 2 of 2

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: 94-96-2
Identity: 2-Ethyl-1, 3-Hexanediol
Purity: 97% active
Remarks: Test substance identified as B0859.01.

Method

GLP: Yes
Report/Study Year: 1985
Method/Guideline Followed: Testing conducted in accordance with the protocol, as supplemented by Malcolm Pirnie's Standard Operating Procedures.
Test Type: Semi-Continuous Activated Sludge (SCAS) Removability
Sludge Source: Avondale, PA Sewage Treatment – Activated Sludge
Exposure Period: 7-days
Test Conditions: Test Material Loading: 20 mg/L as 97% active.
Test Material Addition: 1000 mg/L stock solution in deionized water
Total Organic Carbon (TOC) Level of Stock Solution: 0.676 mg C/mg active
Theoretical TOC for 2-Ethyl-1, 3-Hexanediol: 0.66 mg C/mg active
Temperature: 22 – 23.5°C
Analytical Monitoring: No

Results:

Results: Based upon Soluble Organic Carbon (SOC) over 7 days of exposure to 2-Ethyl-1, 3-Hexanediol, the average % removal of 2-Ethyl-1, 3-Hexanediol is 99.0% (95% confidence intervals $\pm 3.4\%$).

Remarks: There were no significant differences in % removal among controls or replicate test systems.

Data Quality

Reliability (Klimisch): 2

Reference

Report/Study Number: 85-029
Reference: The Procter & Gamble Company, 1985. Semi-Continuous Activated Sludge (SCAS) Removability Test on B0859.01. Accession # 31559

SEMI-CONTINUOUS ACTIVATED SLUDGE
(SCAS) REMOVABILITY TEST ON
B0859.01
THE PROCTER & GAMBLE COMPANY
CINCINNATI, OHIO

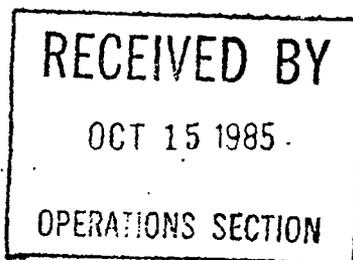
WESTON PROJECT No. 85-029

Kay H. Marks
Kay H. Marks 9/30/85
Study Director

Dianne S. Therry
Dianne S. Therry 10/5/85
Quality Assurance
Coordinator

Peter J. Marks
Peter J. Marks 10/8/85
Project Director

Prepared by:



WESTON
Weston Way
West Chester, PA 19380



Semi-Continuous Activated Sludge (SCAS)
Removability Test on B0859.01

1.0 EXPERIMENTAL PROTOCOL

The semi-continuous activated sludge test was determined according to:

Mr. K. W. Surowitz' protocol of 1 August 1985
entitled "SCAS Removability Test on B0859.01."

Organic carbon analyses were performed utilizing a Dohrmann Analyzer, Model DC 80.

Suspended solids analyses were performed per Standard Methods for the Examination of Water and Wastewater, 15th Edition, Method 209D.

Centrifugation of samples for soluble organic carbon analyses was performed per WESTON's SOP 83-E002; Centrifugation Procedure.

2.0 TEST MATERIAL

TSIN	-	B0859.01
Percent Active	-	97
Solubility	-	4.2% in H ₂ O (20°C)
Color	-	Colorless
Form	-	Liquid
Theoretical TOC	-	0.66 mg TOC/mg active
Date Received	-	8/21/85

3.0 TEST CONDITIONS

Temperature: 22°C - 23.5°C.

Activated sludge was obtained from the Avondale Sewage Treatment Plant, Avondale, Pennsylvania.

A stock solution of B0859.01 was prepared at 1000 mg/L active by weight/volume with deionized water. The stock was analyzed for total organic carbon (TOC) prior to the acclimation period and was re-analyzed for TOC after the last day of testing. The results were as follows:

1000 mg/L Stock Solution Preparation 0.676 mg/mg active

1000 mg/L Stock Solution Post Test 0.685 mg/mg active

Test material was added to two units (Test 1 and Test 2) at a concentration of 20 mg/L based on active ingredient. Two units (Control 1 and Control 2) received no test material and served as controls.

4.0 MISCELLANEOUS PROJECT INFORMATION

Sludge units were set up 19 August 1985 and acclimated to laboratory conditions until 29 August 1985. The sludge was then pooled and re-distributed, and the acclimation to test compound was started. The test period began 5 September 1985 and was completed 12 September 1985.

The testing was performed at WESTON's Laboratory in West Chester, PA. Kay H. Marks was the Study Director and carried out the test initiation and data collection.

Test data may be found in WESTON's Laboratory Notebook #611, pages 1-8.

5.0 RESULTS

Computer analysis may be found in Appendix A, including organic carbon analyses data for the daily effluent samples.

Appendix B provides a description of the quality assurance methods used to insure the quality of the data.



APPENDIX A

COMPUTER ANALYSES

ULTIMATE BIODEGRADABILITY--
SEMI-CONTINUOUS ACTIVATED SLUDGE (S.C.A.S.) TEST
STATISTICAL ANALYSIS OF DATA

CLIENT	--	THE PROCTER & GAMBLE COMPANY
TEST MATERIAL	--	B0850.01
TEST START DATE	--	9/5/85
TEST DURATION (DAYS)	--	7

TEST MATERIAL -- B0859.01

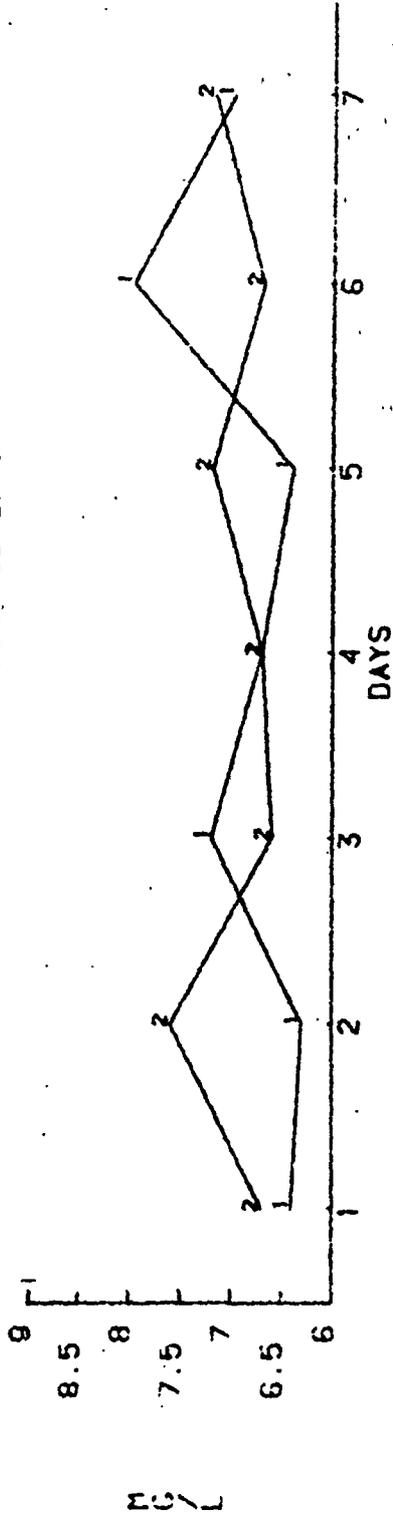
DATA ARE SOLUBLE ORGANIC CARBON (SOC) MEASUREMENTS IN mg/l

	DAYS						
	1	2	3	4	5	6	7
CONTROL 1	6.4	6.3	7.2	6.7	6.4	8.0	7.0
CONTROL 2	6.7	7.6	6.6	6.7	7.2	6.7	7.2
TEST 1	6.9	7.2	6.7	8.3	6.7	6.3	7.3
TEST 2	6.2	6.9	6.6	6.8	7.6	7.7	7.3

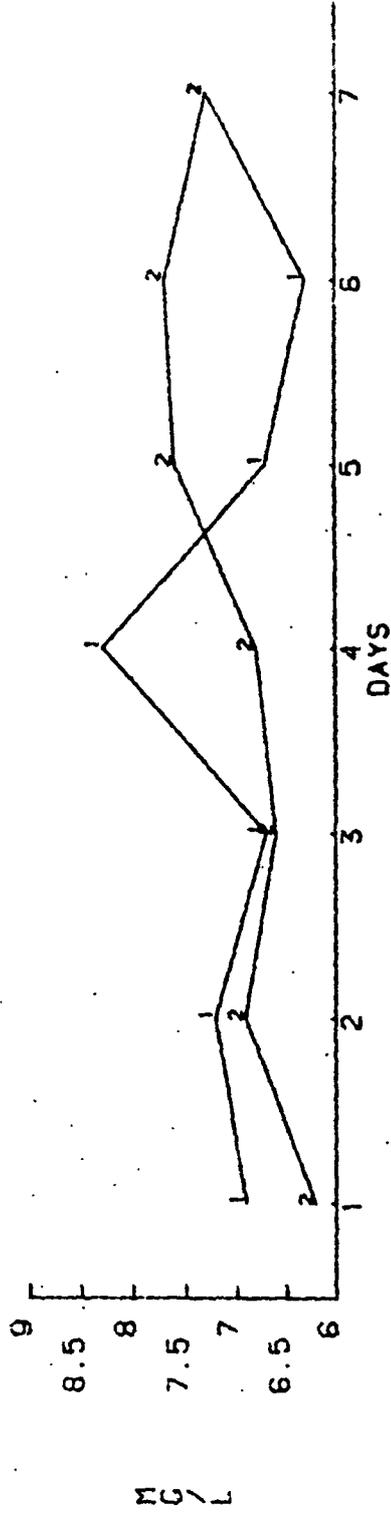
TEST MATERIAL CARBON ADDED--TMC (mg/l) 13.2

TEST MATERIAL -- B0850.01

INTERACTION PLOT OF CONTROL DATA



INTERACTION PLOT OF TEST DATA



TEST MATERIAL -- B0859.01

CONTROL DATA

TWO-WAY ANOVA
Factorial design (balanced)
Combination AB Pooled with Error

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SUM OF SQUARES	F RATIO
DAYS (FACTOR A)	0.8343	6	0.139	
CONTROLS (FACTOR B)	0.0350	1	0.035	0.095
ERROR + INTERACTION	2.2200	6	0.370	
TOTAL	3.0893	13	0.238	

FOR CONTROLS; $F_{.01}(1,6) = 13.70$

THEREFORE, AT A 1 PER CENT TEST LEVEL, IT CAN BE CONCLUDED THAT --

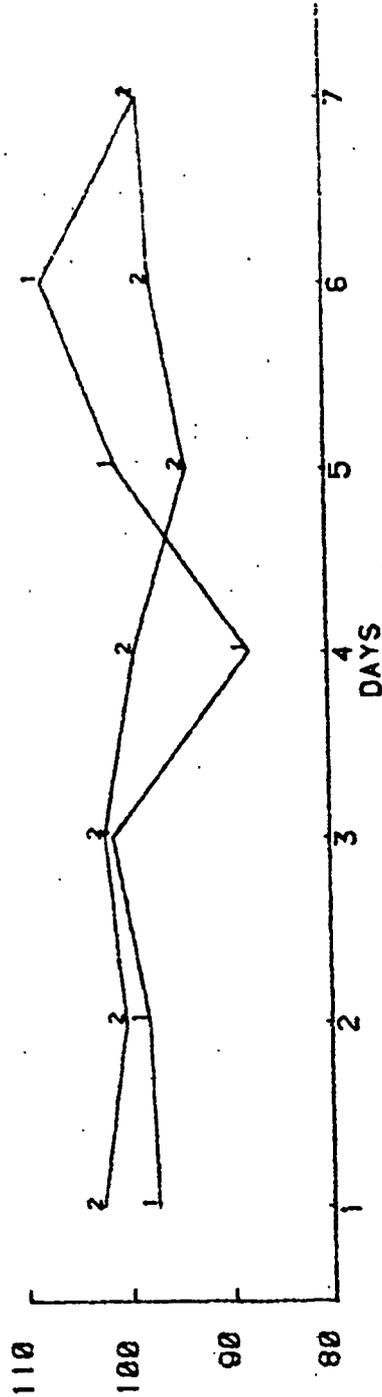
THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN CONTROLS

TEST MATERIAL -- B0859.01

PER CENT CARBON REMOVED

	1	2	3	4	5	6	7
TEST 1	97.35	98.11	101.52	87.88	100.76	107.95	98.48
TEST 2	102.65	100.38	102.27	99.24	93.94	97.35	98.48

PLOT OF PER CENT CARBON REMOVED



TEST MATERIAL -- B0859.01

PER CENT CARBON REMOVED

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM	MEAN SUM OF SQUARES	F RATIO

DAYS (FACTOR A)	110.6848	6	18.447	
TESTS (FACTOR B)	0.3689	1	0.369	0.014
ERROR + INTERACTION	160.6159	6	26.769	
TOTAL	271.6696	13	20.898	

FOR TESTS, $F_{01}(1,6) = 13.70$

THEREFORE, AT A 1 PER CENT TEST LEVEL, IT CAN BE CONCLUDED THAT --

THERE IS NO SIGNIFICANT DIFFERENCE BETWEEN TESTS

AND

AVERAGE % REMOVAL FOR ALL TEST UNITS WITH 95% CONFIDENCE LIMITS IS --

99.0 ± 3.4



APPENDIX B

QUALITY ASSURANCE METHODS



This report and related records have been audited by the Quality Assurance Coordinator for adherence to protocol, laboratory standard operating procedures, and pertinent EPA Good Laboratory Practices. If non-compliance items were identified, management and the Study Director were notified immediately for corrective action.

Audits for the study were conducted on the following dates:

24 August 1985

9 September 1985

5 October 1985

A handwritten signature in cursive script that reads "Dianne S. Therry".

Dianne S. Therry

Quality Assurance Coordinator

PROTOCOL

SPONSOR: The Procter & Gamble Company, Cincinnati, Ohio
Roy F. Weston, Inc.

LABORATORY: Weston Way
West Chester, PA 19380

TITLE: Semi-Continuous Activated Sludge (SCAS) Removability Test on B0859.01

OBJECTIVE: To determine the removability of the test material in the SCAS test system, as measured by soluble organic carbon.

JUSTIFICATION FOR TEST SYSTEM: Most of our products are disposed of through wastewater treatment systems and activated sludge is a common type of wastewater treatment process.

TEST MATERIAL:

Sample Code B0859.01 Color colorless Form liquid

% Active 97% Density 0.9325 g/cm³ Solubility 4.2% in water (20°C)

Expiration Date in progress Other _____

Theoretical TOC 0.66 mg TOC/mg active

Storage Conditions - room temperature

Safe Handling Precautions - harmful if inhaled or absorbed; irritant.

The Sponsor accepts full responsibility for appropriate characterization and stability verification of this test material.

TEST MATERIAL PREPARATION:

(indicate test concentration and check appropriate preparation procedure)

Test Material Concentration - 20 mg active/L

[x] For compounds soluble in water, prepare a stock solution of the test material at 1000 mg/L active by weight/volume with ASTM Type II water¹ or equivalent. Analyze the stock solution for total organic carbon (TOC) following the laboratory's standard operating procedures. If the measured TOC is not within 15% of theoretical, contact the Principal Investigator before initiating the study. Refrigerate the stock solution between uses. On the final day of testing, or as soon thereafter that is practical, reanalyze the stock solution for TOC. Any significant change (>15%) in the initial and final measured TOC concentration should be discussed with the the Principal Investigator and addressed in the final report.

[] For insoluble compounds, the attached procedure is to be followed.
(include procedures for test material addition).

SYNTHETIC SEWAGE:

Feed of the following composition is used as synthetic sewage:

D-glucose	30 g
Nutrient broth	20 g
K ₂ HPO ₄	13 g
Tap water	1 L

Prepare the synthetic sewage in small batches (1-2 L), bring to a boil on a hot plate, and store under refrigeration when not in use. Discard the batch if a scum forms on the surface or if the solution becomes turbid, indicating growth of microorganisms.

TEST ORGANISMS:

Activated sludge is to be obtained following the laboratory's standard operating procedures from a municipal treatment plant receiving predominantly domestic waste.

TEST PROCEDURE:

1. Setting up SCAS units. SCAS units are described in Figure 1 (attached).² Two units are needed for the test material and two for the controls. Pass the sludge through a 2 mm screen to remove large clumps. Take a suspended solids reading (in duplicate) on the screened sludge following laboratory's standard operating procedures. Based on this reading, distribute the sludge among the SCAS units such that when the volume in each unit is adjusted to 1.5 L with tap water, the suspended solids level will be approximately 2500 mg/L. Units are to be set up the same day sludge is obtained.
2. Daily Operation. Aerate the units, as illustrated in Figure 1, for 23 1/2 hours (+1/2 hr) at a rate of approximately 500 ml/min. At the end of this period, turn off the air and allow the sludge to settle for approximately 30 minutes. Then draw off 1 L of effluent (by opening the hose connected to a drain hole located at the 500 ml mark on the chamber). Replace with 1 L of the appropriate influent to bring the volume back to 1.5 L and turn on the air. This process is repeated on a daily basis. Aeration time for the first day of both the sludge and test material acclimation periods can be varied to select a convenient time for subsequent daily maintenance.
3. Sludge Acclimation Period. Feed all units (see Daily Operation) 10 ml synthetic sewage and 990 ml tap water daily for a minimum of four days prior to initiation of the test material acclimation period. With fresh sludge, it is sometimes difficult to obtain good settling during the first week or ten days. If after 30 minutes of settling the sludge level is not below the 500 ml mark, siphon effluent off the top until the sludge level is reached and bring the volume back to 1.5 L with synthetic sewage plus tap water. Double strength synthetic sewage (20 ml/day) may be fed to help maintain suspended solids at 2500 mg TSS/L. Units must be maintained at the standard feed level of 10 ml/day and good settling must occur for a minimum of four days prior to initiating the test material acclimation period. If good settling is not obtained within 21 days, new sludge should be obtained.
4. Sludge Distribution. On the day addition of the test material is to start, composite the settled sludge from all units. Take a suspended solids reading in duplicate on the settled sludge, and add the volume needed in each unit to bring the final suspended solids reading to 2500 mg/L. All units are then fed 10 ml of synthetic sewage, the appropriate amount of test material, and enough tap water to bring the final volume to 1.5 L.

5. Test Material Acclimation Period. The test material is added to the test units incrementally for a 7-day acclimation period (see Daily Operation). On the first day, add enough of the stock solution to bring the test material concentration in the influent to 20 percent of the final test concentration (see Test Material Preparation). Increase this by 20% on a daily basis for five days until the final test concentration is reached. Feed the final test concentration for two more days. This completes the test material acclimation period. The fourth feeding of the final test concentration begins the testing period.

During this acclimation period, all units, including the control units, are fed 10 ml synthetic sewage and enough tap water to bring the final volume to 1.5 L. Daily effluents are discarded.

6. Testing Period. On a daily basis, withdraw 1 L of effluent from each unit and save it for analysis. Replace the effluent with a comparable volume of influent which contains 10 ml synthetic sewage, appropriate amount of test material (except for control units), and enough tap water to bring the final volume to 1.5 L (see Daily Operation). Conduct the test for 7 days.
7. Effluent Analysis. Within one hour of collection, prepare effluent samples for SOC measurements following laboratory's standard operating procedures. Samples are to be refrigerated until analyzed.
8. Temperature and Lighting. Test temperature should be checked daily and be in a range of $23 \pm 3^{\circ}\text{C}$. SCAS units should not be in direct sunlight and room lighting should only be on during daily maintenance.

CALCULATIONS - STATISTICAL ANALYSIS:

To be performed by (check one): [] Laboratory [] Sponsor, after receipt of final report. If by Sponsor, Item 12 in the Reporting section is not applicable.

1. For the two blank (control) units, an analysis of variance (ANOVA) is performed on the carbon data to ascertain whether there is any significant difference between the two replicates. (The ANOVA includes sources to: replicates, days, and a replicate x day indication, which is the measure of inherent error.) The remainder of the calculations assumes no statistical difference between the control units. If there is a statistical difference, the Principal Investigator is to be contacted. Agreement to the acceptability of the study and alternate calculation methods should be documented and discussed in the report.
2. For each day of the test, calculate the % carbon remaining in solution by subtracting the average carbon in the control units (blank) from the carbon in the test unit. Next divide this number by the amount of test material carbon added (based on theoretical TOC) and multiply by 100, according to the following formula:

% carbon remaining =

$$100 \times \frac{\text{carbon in test unit (mg/L)} - \text{average carbon in blanks (mg/L)}}{\text{Test material carbon added to test unit (mg/L)}}$$

3. Calculate the percent removal by subtracting the % remaining from 100 percent. (If more carbon was removed in the test units than the control unit, the % removal will be reported as greater than 100%.)

4. For each test unit, perform an ANOVA as in #1, on the % removals to determine if the replicate units are significantly different. If they are not, an overall average removal can be computed for the test material. If these units are significantly different, an average % removal is to be reported for each unit. For each of these cases, a 95% confidence interval can be calculated for the average % removals, using the error term (replicate x day) from the ANOVA in the computations.

RECORDS TO BE MAINTAINED: All records necessary to reconstruct the study and demonstrate adherence to the Protocol.

PROTOCOL CHANGES: If a change in the approved protocol becomes necessary, verbal agreement should be made between the Study Director and Sponsor's Principal Investigator. As soon as practical thereafter, this change and the reasons for it should be put in writing, approved by both persons, and attached to the protocol as an addendum.

REPORTING: The report is to be a typed document in triplicate, describing the results of the study and is to be signed and dated by the Study Director, Quality Assurance Officer and Laboratory Manager. It is to include, but is not limited to, the following:

1. Identification of test material by sample code, percent active, theoretical TOC, color, form and date received.
2. Procedures followed for test material preparation and addition.
3. Test material concentrations.
4. Reference to the Protocol (title, author and date) and addenda if made, Test Methods, and any analytical or standard operating procedures used.
5. Any Protocol deviations and their implications.
6. Reference to laboratory notebook or other file containing raw data.
7. Starting and ending dates of study.
8. Procedures followed for preparation of effluent samples for carbon analyses.
9. TOC analysis on stocks reported as mg carbon/mg active.
10. Daily SOC results as mg SOC/L on all test and control units.
11. Daily % removals for all test units.
12. Average % removal with 95% C.I. for the test units and a summary of the statistical analysis.
13. Temperature range recorded during test period.
14. Activated sludge source.
15. Description of the quality assurance methods used to insure the quality of the data.

REFERENCES:

1. "Standard Specification for Reagent Water", ASTM Committee D-19 on Water, ASTM Designation D1193-74, June 27, 1974.
2. "A Procedure and Standards for the Determination of the Biodegradability of Alkyl Benzene Sulfonate and Linear Alkylate Sulfonate. J. Amer. Oil Chem. Soc. 42 (1965) 1-16.

ALTERNATE PRINCIPAL INVESTIGATOR R. H. Hall PHONE: (513) 530-3347

NOTED: J. W. Williams J.W. Williams 8/16/85 PHONE: (513) 245-2120
Operations/Logistics Date

APPROVED: K. G. Surowitz K.G. Surowitz 8/1/85 PHONE: (513) 530-3332
Principal Investigator Date

TO BE COMPLETED BY STUDY DIRECTOR:

Project No. 85-029

Estimated Starting Date 8/28/85 Defined As: acceleration of test material

Estimated Reporting Date 11/1/85

Date Test Material Received 8/21/85

Approved Kev & Michael 8/23/85 PHONE: 85 292 2020
Study Director Date

KGS003/pcj
8/1/85

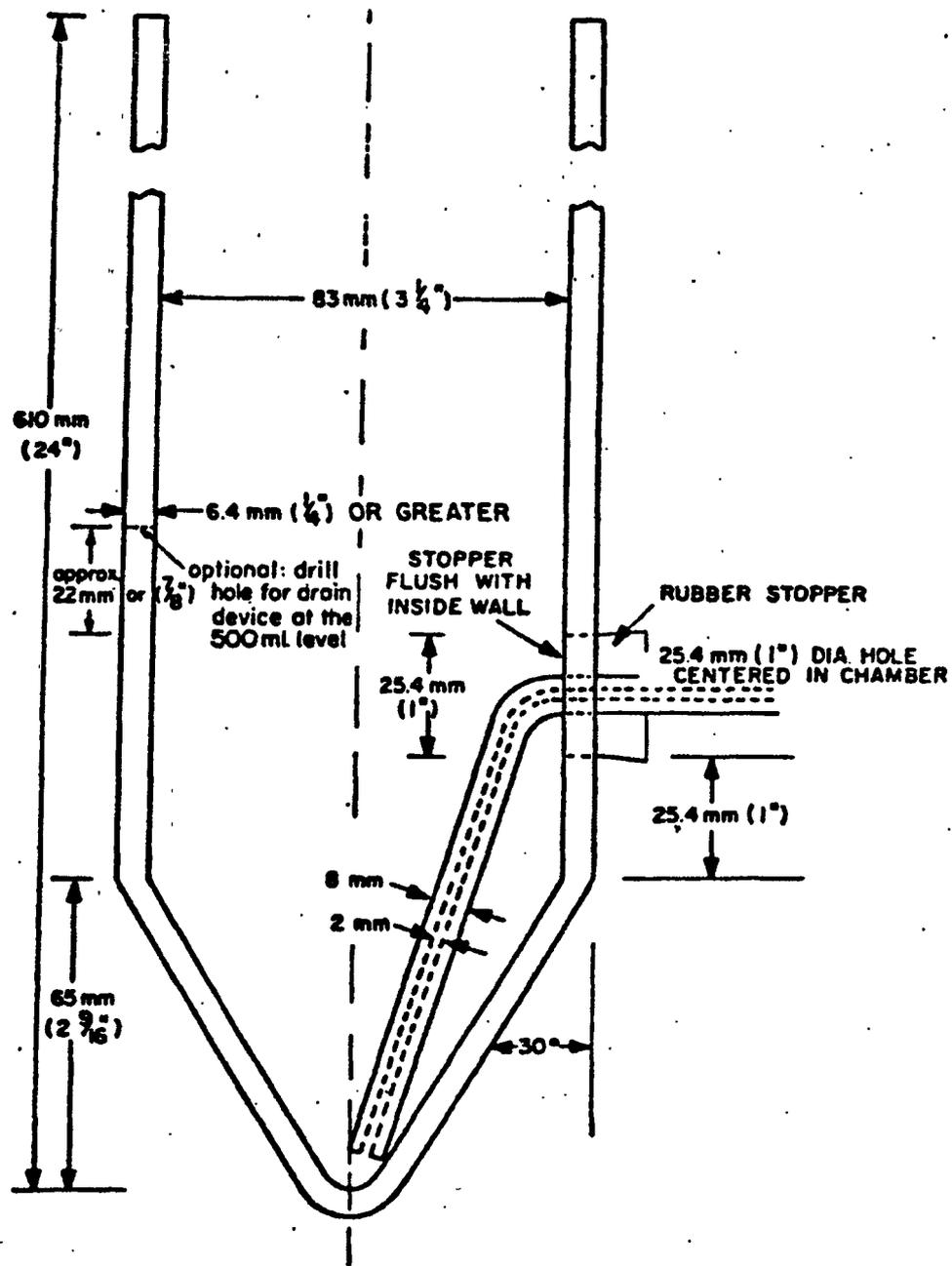


FIG. 1. Semicontinuous activated sludge aeration chamber.

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 1 of 2

PARS SECT. ED.

EX. Sect. for PARS
ORIG. SECT. ED.

Test Substance Identification Number (TSIN): B0859.01
Safety Test Request Number: BSRS- 85.05
Principal Investigator: K. G. Surowitz

Product or Ingredient: 2-ethyl-1,3-hexanediol (ETHED) Brand Notebook Ref:
Physical Description: clear, viscous liquid Solubility: 4.2% in water (20°C) pH: 7.1
Recommended Storage Conditions: room temperature Expiration Date: in progress
Hazards (i.e. flammability, toxic gases): harmful if inhaled or absorbed; irritant
Dept. of Transportation Hazard Classification: non-hazardous CAS No. (*): 94-96-2

Formulated Composition (b)

<u>Component (c)</u>	<u>Mol. Wt.</u>	<u>Nominal Level (X by Wt.)</u>	<u>Acceptable Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Number (NB-Ref.)</u>
2-ethyl-1,3 hexanediol (isomer mixture)	146.23	97%			Aldrich	3007LL

- (a) Include CAS number(s) for the three most major components of a formulation or for single chemical products. Footnote to the material with which the respective number is associated.
- (b) If information requested is not known, then the symbol NK will be entered.
- (c) Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-chemical names, such as Tergitol 15-S-0 or Yellow Dye #10, may not be acceptable but should be previewed with the responsible toxicologist. Nondefinitive identification (e.g. Arquad, BC-base) is not acceptable.

The above information provided by: KG Surowitz
(Name)

KG Surowitz
(Signature) 7/19/81
(Date)

The above information reviewed and accepted by:

Principal Investigator: KG Surowitz
(Name)

KG Surowitz
(Signature) 7/19/81
(Date)

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 2 of 2

Test Substance Identification Number (TSIN): B0859.01

Analyzed Composition

<u>Date Submitted</u>	<u>Submitter Code</u>	<u>Analysis Code/Analysis</u>	<u>Estimated Value</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
-----------------------	-----------------------	-------------------------------	------------------------	-----------------------	---------------------------

Commercial sample (Aldrich Chemical Co.) No Analysis performed.

Analytical Information Verified By: _____

(Signature)

Date: _____

This test substance is suitable for environmental (nonclinical) safety testing.

Principal Investigator: _____

(Signature)

Date: 7/15/85

This test substance is suitable for human (clinical) safety testing.

Principal Investigator: _____

(Signature)

Date: _____

KGS001/pcj
7/12/85

Test Substance

CAS Number: 94-96-2
Identity: 2-Ethyl-1,3-Hexanediol
Purity: 97% active
Remarks: Test substance identified as B0859.01

Method

GLP: Yes
Report/Study Year: 1986
Method/Guideline Followed: U.S. EPA, 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. Ecological Research Series (EPA-860/3-75-009).

Test Type:

Species: Acute Fish Toxicity – Static
Bluegill sunfish, *Lepomis macrochirus*
Total Length: mean = 38 mm, range = 26-46 mm; n = 10
Wet weight: mean = 0.69 g, range = 0.22-1.22 g; n = 30

Exposure Period:

Media: 96-hour
Freshwater – Deionized, reconstituted well water
Total hardness as CaCO₃: 42 mg/L
Total alkalinity as CaCO₃: 31 mg/L
pH: 7.6
Specific Conductivity: 170 µmhos/cm
Test Temperature: 22°C

Treatments:

1000, 600, 360, 130, 78 mg/L of 2-Ethyl-1,3-Hexanediol based on 98.6% active. Material appeared to be in solution at all treatment levels.
Remarks: Protocol Deviation: The protocol states that the test material is 97 % active. The sample container specified that the sample was 98.6 % active ingredient. During the study the nominal test concentrations were calculated based on 98.6 % active ingredient. This difference in reported % active ingredient of 1.67 % was considered an insignificant effect on the calculated nominal treatment concentrations.

Analytical Monitoring:

No

Results:

Results: The 96-hour LC50 for Bluegill sunfish magna was estimated by non-linear interpolation to be 280 mg/L with a 95% confidence interval calculated by binomial probability to be 220-360

Remarks:

Surviving fish at all treatments were surfacing.
Protocol Deviation: The protocol states that the dissolved oxygen concentration in the control solution must be maintained at >40% of saturation throughout the study period. During the initial 24 hours of the test, the dissolved oxygen concentration in all exposure solutions remained ≥ 45% of saturation. The dissolved oxygen concentrations after 24 hours of exposure ranged from 14 to 27% of saturation. Comparison of the 24 and 96 hour LC50 values (320 and 280 mg/L, respectively) demonstrates that the number of mortalities occurring after 24 hours was not significant. This deviation did not affect the results of the study.

Data Quality

Reliability (Klimisch): 1

Reference

Report/Study Number: BW-86-1-1928
Reference: The Procter & Gamble Company, 1986. Acute Toxicity of B0859.01 to Bluegill (*Lepomis macrochirus*). Accession # 32348

Acc# 32348

.04

ACUTE TOXICITY OF B0859.01
TO BLUEGILL
(Lepomis macrochirus)

TOXICITY TEST REPORT
SUBMITTED TO
THE PROCTER & GAMBLE COMPANY
CINCINNATI, OHIO

REPORT #BW-86-1-1928
STUDY #1011-0885-6142-100

Springborn Bionomics, Inc.
790 Main Street
Wareham, Massachusetts 02571
April, 1986

RECEIVED BY
MAY 12 1986
OPERATIONS SECTION

SUMMARY

96-Hour Static Acute (LC50) Test with Bluegill

Springborn Bionomics, Inc.
790 Main Street
Wareham, Massachusetts 02571

SPONSOR: The Procter & Gamble Company

TEST PROTOCOL: Static Acute Freshwater Fish Toxicity Study of
B0859.01; Principal Investigator, K.W. Surowitz,
8/1/85.

REPORT NUMBER AND DATE: #BW-86-1-1928, April, 1986

STUDY NUMBER: 1011-0885-6142-100

MATERIAL: B0859.01 DATE RECEIVED: 20 August 1985

DESCRIPTION: a clear colorless liquid tested as 98.6% active
ingredient

TEST DATE: 4-8 October 1985

SPECIES: Lepomis macrochirus

Total length: Mean = 38 mm; range = 28-46 mm; N = 30

Wet weight: Mean = 0.69 g; range = 0.22-1.22 g; N = 30

Source: Commercial fish supplier in Connecticut

DILUTION WATER: Deionized, reconstituted well water

pH: 7.6

Specific conductivity: 170 μ mhos/cm

Total hardness as CaCO₃: 42 mg/L

Total alkalinity as CaCO₃: 31 mg/L

TEST TEMPERATURE: 22°C

NOMINAL TEST CONCENTRATIONS: 1000, 600, 360, 220, 130, and 72 mg/L
of B0859.01

RESULTS: The 96-hour LC50 was estimated by nonlinear interpolation
to be 280 mg/L with a 95% confidence interval calculated by
binomial probability to be 220-360 mg/L.

INTRODUCTION

The purpose of this study was to estimate the acute toxicity (LC50) of B0859.01 to bluegill (Lepomis macrochirus) under static test conditions. The LC50 is defined as the concentration of the test material in dilution water which causes mortality of 50% in the exposed test population after a fixed period of time. This value is often used as a relative indicator of potential acute hazards resulting from release of the test material into aquatic environments. A 96-hour definitive test was conducted from 4-8 October 1985 at the Springborn Bionomics, Inc., laboratories in Wareham, Massachusetts. All raw data produced during the study are stored at the above location.

MATERIALS AND METHODS

The B0859.01, a clear colorless liquid tested as 98.6% active ingredient, was received from The Procter & Gamble Company, Cincinnati, Ohio, on 20 August 1985. Test concentrations are reported as milligrams of B0859.01 per liter of solution (mg/L).

Procedures used in this acute toxicity study followed those described in "Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians" (EPA, 1975) and the protocol entitled "Static Acute Freshwater Fish Toxicity Study of B0859.01" (K.W. Surowitz, Principal Investigator, 1 August 1985) issued to Springborn Bionomics, Inc., by the Procter & Gamble Company, Cincinnati, Ohio.

The bluegill (Bionomics lot #85A14A) were obtained from a commercial fish supplier in Connecticut and held in a 500-L fiberglass tank under a photoperiod of 16 hours light and 8 hours darkness. The well water which flowed into this tank had total hardness and alkalinity ranges as calcium carbonate (CaCO_3) of 32-33 mg/L and 24-26 mg/L, respectively, and a specific conductance range of 110-130 micromhos per centimeter ($\mu\text{mhos/cm}$) (Weekly Gravity Feed Tank Water Quality Analysis Logbook). Other parameters monitored in the holding tank were a pH of 6.8, a dissolved oxygen concentration range of 96-99% of saturation and a flow rate of 8.6-9.9 tank volume replacements/day (Weekly Record of Fish Holding Water Characteristics). The temperature range in the holding tank was 21°C during this period. Test fish were maintained under these conditions for a minimum of 14 days. The fish were fed a dry commercial pelleted food, ad libitum, daily except during the 48 hours prior to testing. There was no mortality of the test fish population during this 48-hour period (Daily Record of Fish Holding Conditions). The mean wet weight of the test fish population was 0.69 g (range 0.22-1.22 g, N=30) and the mean total length was 38 mm (range 28-46 mm, N=30) (Fish Weight and Length Log).

A sodium lauryl sulfate reference test was conducted with the test fish population from 16-20 September 1985. The resulting 96-hour LC50 was 6.8 mg/L (95% confidence interval 4.6-10 mg/L) (Reference Test Log).

The toxicity test was conducted in 19.6-L glass jars which contained 15 L of test solution. The test solution depth was 27.5 cm with a surface area of 545 cm². The dilution water used was soft water reconstituted from deionized water according to recommended procedures (EPA, 1975). This water had a total hardness and alkalinity as CaCO₃ of 42 mg/L and 31 mg/L respectively; pH of 7.6; and specific conductivity of 170 µmhos/cm (Reconstituted Water, Water Quality Analysis Log).

A cloudy stock solution of 150 mg/mL was prepared by diluting 38.034 grams of B0859.01 with distilled water to volume in a 250-mL volumetric flask. The appropriate volume of stock solution was then added to 15 L of dilution water in each test jar and mixed by stirring with a glass rod. One control jar was established containing the same dilution water and maintained under the same conditions as the test jars but containing no B0859.01.

All test solution temperatures were controlled by a system designed to maintain temperatures at 22 ± 1°C. Test solutions were not aerated. The photoperiod during testing was the same as that provided in the fish culture area.

Ten bluegill selected impartially from the holding tank were placed in each test jar within 15 minutes after the test solutions had been prepared. The resulting test organism loading concentration was 0.46 grams of biomass per liter of test solution. Fish were not fed during exposure.

All jars were examined after 0, 24, 48, 72 and 96 hours of exposure as follows: mortalities were recorded, dead fish were removed, and observations of the fish and the physical characteristics of the test solutions were recorded. Dissolved oxygen concentrations and pH were measured in the control and all test concentrations, and temperature was measured in the control jar.

Total hardness concentrations presented in this report were measured by the EDTA titrimetric method and total alkalinity concentrations were determined by potentiometric titration to an endpoint of pH 4.5 (APHA et al., 1985). Specific conductivities were measured with a Yellow Springs Instrument Company (YSI) Model #33 salinity-conductivity-temperature meter and probe. pH was measured with an Instrumentation Laboratory Model #175 pH meter and combination electrode. Dissolved oxygen concentrations were measured with a YSI Model #57 dissolved oxygen meter and probe. Temperatures were measured with a Brooklyn alcohol thermometer.

Statistics

The concentrations tested and the corresponding mortality data derived from the toxicity test were used to estimate median lethal concentrations (LC50) and 95% confidence intervals at each 24-hour interval of the exposure period. The LC50 is defined as the concentration of the test material in dilution water which caused mortality of 50% of the test organism population at the stated exposure interval. A computer program (Stephen, 1982 and 1977) was

used to obtain a point estimate of the LC50 by nonlinear interpolation (i.e., logarithm transformation of the concentration and the angle transformation of the percent dead). The 95% confidence interval was calculated by the binomial probability method.

RESULTS

The nominal test concentrations, the corresponding cumulative mortalities and the observations made during the test are presented in Table 1. Table 2 summarizes the 24-, 48-, 72- and 96-hour LC50's, and corresponding 95% confidence intervals. The 96-hour LC50 for bluegill exposed to B0859.01 was estimated by nonlinear interpolation to be 280 mg/L with a 95% confidence interval calculated by binomial probability to be between 220 and 360 mg/L. The pH's, dissolved oxygen concentrations and temperatures measured during the toxicity test are presented in Table 3.

Protocol Deviation

1. The protocol states that the test material is 97 percent active ingredient. The sample container specified that the sample was 98.6 percent active ingredient. During this study the nominal test concentrations were calculated based on 98.6 percent active ingredient (telephone communication -- J. Williams, 7 January 1986).
2. The protocol states that the dissolved oxygen concentration in the control solution must be maintained at >40 percent of saturation throughout the stud, period. During the initial 24 hours of the test, the dissolved oxygen concentration in all exposure solutions remained ≥45% of saturation. The dissolved oxygen concentrations after 24 hours of exposure ranged from 14 to 27% of saturation. Comparison of the 24- and 96-hour LC50 values (320 and 280 mg/L, respectively) demonstrates that the number of mortalities occurring after 24 hours was not significant. Based on these data, it is SBI's opinion that this deviation did not affect the results of the study and that the reported LC50 values accurately estimate the acute toxicity of B0859.01 to bluegill.

DC Surprenant

Donald C. Surprenant
Director, Aquatic Toxicology

4/30/86
date

Richard B. Nicholson

Richard B. Nicholson
Study Director

4/29/86
date

LITERATURE CITED

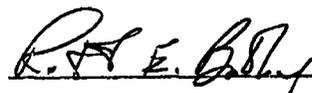
APHA, AWWA, WPCF. 1985. Standard Methods for the Examination of Water and Wastewater. 16th Edition, Washington, D.C., 1268 pp.

Stephan, C. E. 1977. Methods for calculating an LC50. Aquatic Toxicology and Hazard Evaluation, ASTM STP 634, F. L. Mayer and J. L. Hamelink, Eds., American Society for Testing and Materials, pp. 65-84.

Stephan, C. E. 1982. U.S. EPA, Environmental Research Laboratory, Duluth, Minnesota. Personal communication to Dr. Lowell Bahner, Chairman ASTM Task Group on Calculating LC50's.

U. S. EPA. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. Ecological Research series (EPA-660/3-75-009), 61 pp.

The data contained in this report were audited by the Quality Assurance Unit to assure compliance with the protocols, standard operating procedures and the pertinent EPA Good Laboratory Practice Regulations on the following dates: 10 November and 16 December 1985, 14 January and 7 April 1986. If discrepancies were found, reports were made immediately to the Study Director and management. It is the opinion of this Unit that these data accurately reflect the raw data generated during this study.

 30 Apr '86
Robert E. Bentley
Director, Quality Assurance
and Special Projects

SUBMITTED BY:

Springborn Bionomics, Inc.
790 Main Street
Wareham, Massachusetts 02571
April, 1986

STUDY DIRECTOR:

Richard B. Nicholson

Richard B. Nicholson 4/20/86
Aquatic Toxicologist

APPROVED BY:

Donald C. Surprenant

DC Surprenant 4/30/86
Director, Aquatic Toxicology

DATA AUDITED BY:

Robert E. Bentley

RE Bentley 30 Apr '86
Director, Quality Assurance
Unit

Table 1. Concentrations tested and corresponding cumulative mortalities of bluegill (*Lepomis macrochirus*) exposed to B0859.01 for 24, 48, 72 and 96 hours.

Nominal concentration (mg/L)	Cumulative Mortality (%)			
	24-hour	48-hour	72-hour	96-hour
control	0	0	0	0
78	0	0	0 ^a	0 ^a
130	0	0	0 ^a	0 ^a
220	0 ^a	0 ^a	0 ^a	0 ^a
360	70 ^a	90 ^a	100	100
600	100	100	100	100
1000	100	100	100	100

^aAll surviving fish were at the surface of the test solution.

Table 2. The LC50 values and 95% confidence intervals for bluegill (Lepomis macrochirus) exposed to B0859.01.

	LC50 (mg/L)	Confidence Limits	
		Lower (mg/L)	Upper (mg/L)
24-hour	320 ^a	220 ^b	600 ^b
48-hour	300 ^a	220 ^b	360 ^b
72-hour	280 ^a	220 ^b	360 ^b
96-hour	280 ^a	220 ^b	360 ^b

^aLC50 value estimated by nonlinear interpolation

^b95% confidence limits calculated by binomial probability

Table 3. pH, dissolved oxygen concentration, and temperature measured during 96-hour exposure of bluegill (Lepomis macrochirus) to B0859.01.

Nominal concentration (mg/L)	0-hour	24-hour	48 hour	72-hour	96-hour
A. pH					
control	7.6	6.7	6.6	6.5	6.5
78	7.4	6.6	6.6	6.4	6.5
130	7.4	6.7	6.6	6.3	6.3
220	7.3	6.6	6.5	6.5	6.5
360	7.3	6.7	6.6	6.5	---- ^a
600	7.3	6.7	---- ^a	---- ^a	---- ^a
1000	7.3	6.6	---- ^a	---- ^a	---- ^a
B. Dissolved Oxygen, mg/L (% saturation in parentheses)					
control	9.2 (104)	4.4 (50)	2.4 (27)	1.7 (19)	1.2 (14)
78	9.2 (104)	4.7 (53)	2.4 (27)	0.9 (10)	1.0 (11)
130	9.2 (104)	4.0 (45)	2.0 (23)	1.0 (11)	1.0 (11)
220	9.2 (104)	4.1 (46)	1.3 (15)	1.1 (12)	1.1 (12)
360	9.2 (104)	5.1 (58)	2.9 (33)	1.6 (18)	---- ^a
600	9.2 (104)	5.4 (61)	---- ^a	---- ^a	---- ^a
1000	9.2 (104)	4.2 (48)	---- ^a	---- ^a	---- ^a
C. Temperature (°C)					
control	22	22	22	22	22

^aMeasurement of parameter not required at stated time interval due to mortality of 100% of the test population at the previous 24-hour interval.

R-5

Laboratory Study No. 1011-0885-6142-100

PROTOCOL

SPONSOR: The Procter & Gamble Company; Cincinnati, Ohio
Springborn Biometrics, Inc.

LABORATORY: 790 Main St.
Wareham, MA 02571

TITLE: Static Acute Freshwater Fish Toxicity Study of B0859.01

OBJECTIVE: To determine the 96 hr LC₅₀ of the test material to a freshwater fish species.

JUSTIFICATION FOR TEST SYSTEM: The bluegill (Lepomis macrochirus) is a readily available freshwater fish species, on which a large amount of toxicity data exists, and is recommended by the USEPA¹ as a standard test organism.

TEST MATERIAL:

Sample Code B0859.01 Color colorless Form liquid
XActive 97X Density 0.9325 g/cm³ Solubility 4.2% in water (20°C)
Expiration Date in progress Other --

Storage Conditions - room temperature

Safe Handling Precautions - Harmful if inhaled or absorbed; irritant.

The Sponsor accepts full responsibility for appropriate characterization and stability verification of this test material.

TEST MATERIAL ADDITION/PREPARATION: All calculations and measurements are to be based on the active ingredient. The maximum concentration to be tested is 1000 mg/L active ingredient.

TEST ORGANISM:

Species - Bluegill (Lepomis macrochirus)
Age - All fish from the same year class.
Weight - Average is not to exceed 1.2 g.
Length - The largest fish is to be no more than twice the standard length of the shortest fish.

All fish must be in holding for a minimum of 14 days prior to starting the test. Fish are to be acclimated to the test temperature for a minimum of 48 hours in the "dilution water" or water of similar chemical composition to the "dilution water". Fish are not to be fed during this acclimation period or during the test period. A group of fish must not be used if individuals appear to be diseased or otherwise stressed or if more than 3% die during the 48 hours.

¹USEPA, Committee on Methods for Toxicity Tests with Aquatic Organisms (1975). Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. EPA-660/3-75-009.

TEST CONTAINERS: Wide mouth glass jars (approximately 20 L capacity) containing 15 L of test solution.

DILUTION WATER: Soft reconstituted water prepared as described in USEPA, p. 14-20. Do not use dechlorinated water.

TEST CONDITIONS:

- (1) Maintain temperature at $22 \pm 1^\circ\text{C}$.
- (2) The dissolved oxygen concentration in each test container must be at least 90% of saturation on Day 0, and for the control, must remain greater than 40% of saturation throughout the test.
- (3) The biomass to water ratio must not exceed 0.8 g/L.
- (4) A control and at least five concentrations of the test material are to be used. Except for the control, the concentration of test material in each treatment must be at least 56% of the next higher one.

OPERATION:

Ten fish are randomly assigned to each test container, following the laboratory's standard operating procedures, within 30 minutes after the addition of the test material. One treatment must have killed or affected more than 65% of the fish, and one treatment (not the control) must have killed or affected less than 35% of the exposed fish. The test is conducted for 96 hours, commencing when the test fish are first exposed to the test material.

If any additive (e.g. solvent) is used to solubilize the test material, another control containing the greatest amount of solvent present in any other container is also required. A test is not valid if there is greater than 10% mortality in either control.

WATER CHEMISTRY: Dissolved oxygen (DO) and pH are determined at 0, 24, 48, 72, and 96 hours in all test concentrations, including the controls. When 100% mortality is observed in a test concentration, determinations are to be made, but further determinations for that concentration are discontinued.

SPECIAL INSTRUCTIONS (If any, for operation, sampling, analyses, or monitoring):

RANGE-FINDING TEST: To be conducted at the discretion of the laboratory unless otherwise specified.

OBSERVATIONS: Observations concerning mortality, morbidity, and behavior are to be recorded for all test concentrations, of specific interest, but not exclusive to, are those discussed in USEPA, p. 38-39. Observations are made at 24, 48, 72, and 96 hours, at which time dead fish are removed.

CALCULATIONS: Test results are used to calculate the 96 hour LC_{50} . The 24, 48, and 72 hour LC_{50} 's are calculated when possible. The LC_{50} is defined as the calculated concentration of the test material which causes 50% mortality in populations of test fish at the specified time of exposure. Results are to be calculated and reported on the basis of added (nominal) test concentrations or from concentrations confirmed by actual analysis, if requested.

RECORDS TO BE MAINTAINED: All records necessary to reconstruct the study and demonstrate adherence to the Protocol.

PROTOCOL CHANGES: If a change in the approved protocol becomes necessary, verbal agreement should be made between the Study Director and Sponsor's Principal Investigator. As soon as practical thereafter, this change and the reasons for it should be put in writing, approved by both persons, and attached to the protocol as an addendum.

REPORTING: The report is to be a typed document in triplicate, describing the results of the study and is to be signed and dated by the Study Director, Quality Assurance Officer and Laboratory Manager. It is to include, but is not limited to, the following:

- 1) Identification of test material by sample code, percent active, color, form, and date received.
- 2) Procedures followed for test material preparation and addition.
- 3) Reference to laboratory notebook or other file containing raw data.
- 4) Date definitive test was conducted.
- 5) Species tested, source, mean and range of the length and weight.
- 6) Percentage mortality in all test containers, including the control.
- 7) Calculated LC₅₀ values, 95% confidence intervals, and reference to the method used to calculate these values.
- 8) Description of dilution water used, including a range of the measured pH, hardness, alkalinity, and conductivity.
- 9) All temperature, pH, and DO determinations and all visual observations.
- 10) Laboratory Study Number.
- 11) Reference to Protocol (title, author, and date) and addenda if made, and any analytical procedures used.
- 12) Any Protocol deviations and their implications.
- 13) Results of reference toxicant and date conducted.
- 14) Description of the quality assurance methods used to insure the quality of the data.

ALTERNATE PRINCIPAL INVESTIGATOR R. H. Hall PHONE: (513) 530-3347

NOTED: J. W. Williams J.W. Williams 8/16/85 PHONE: (513) 245-2120
Operations/Logistics Date

APPROVED: K. G. Surowitz KG Surowitz 8/1/85 PHONE: (513) 530-3332
Principal Investigator Date

TO BE COMPLETED BY STUDY DIRECTOR:

Study No. 1011-0685-6192-100

Estimated Starting Date 13 September 1985

Estimated Reporting Date 15 November 1985

Date Test Material Received 21 August 1985

Approved Richard B. Nilsen RBN 8/21/85 PHONE: 617 295-2550
Study Director Date

KGS004/pcj
8/1/85

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 1 of 2
Page 1 of 1
ORIG. INVT./MD.

Test Substance Identification Number (TSIN): B0859.01
Safety Test Request Number: BSRTS- 85.05
Principal Investigator: E. G. Surowitz

Product or Ingredient: 2-ethyl-1,3-hexanediol (ETHED) Brand Notebook Ref: _____
Physical Description: clear, viscous liquid Solubility: 4.2% in water (20°C) pH: 7.1
Recommended Storage Conditions: room temperature Expiration Date: in progress
Hazards (i.e. flammability, toxic gases): harmful if inhaled or absorbed; irritant
Dept. of Transportation Hazard Classification: non-hazardous CAS No. (M): 94-96-2

Formulated Composition (b)

<u>Component (c)</u>	<u>Mol. Wt.</u>	<u>Nominal Level (X by Wt.)</u>	<u>Acceptable Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Number (S-Ref.)</u>
2-ethyl-1,3 hexanediol (isomer mixture)	146.23	97%			Aldrich	3007LL

- (a) Include CAS number(s) for the three most major components of a formulation or for single chemical products. Footnote to the material with which the respective number is associated.
- (b) If information requested is not known, then the symbol NK will be entered.
- (c) Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-chemical names, such as Tergitol 15-S-0 or Yellow Dye #10, may not be acceptable but should be previewed with the responsible toxicologist. Nondefinitive identification (e.g. Arquad, BC-base) is not acceptable.

The above information provided by: KG Surowitz (Name) [Signature] (Signature) 7/17/85 (Date)

The above information reviewed and accepted by:
Principal Investigator: KG Surowitz (Name) [Signature] (Signature) 7/17/85 (Date)

TSCR1

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 2 of 2

Test Substance Identification Number (TSIN): 90859.01

Analyzed Composition

<u>Date Submitted</u>	<u>Submitter Code</u>	<u>Analysis Code/Analysis</u>	<u>Estimated Value</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
-----------------------	-----------------------	-------------------------------	------------------------	-----------------------	---------------------------

Commercial sample (Aldrich Chemical Co.) No Analysis performed.

Analytical Information Verified By: _____

(Signature)

Date: _____

This test substance is suitable for environmental (non-clinical) safety testing.

Principal Investigator: _____

(Signature)

Date: 7/13/85

This test substance is suitable for human (clinical) safety testing.

Principal Investigator: _____

(Signature)

Date: _____

KGS001/pcj
7/12/85

Test Substance

CAS Number: 94-96-2
Identity: 2-Ethyl-1, 3-Hexanediol
Purity: 97% active
Remarks: Test substance identified as B0859.01

Method

GLP: Yes
Report/Study Year: 1986
Method/Guideline Followed: Not stated.
Test Type: Algistatic Determination
Species: Microcystis aeruginosa

Inoculums came from a 7-day old stock culture which had previously been maintained in AAP medium for at least three transfers.

Exposure Period: 14-days
Media: APP (Algal Assay Procedure Medium)
pH: 7.5 ± 1.0
Test Conditions: Light level: constant 2153 ± 323 lumens / m²
Temperature: 24 ± 2 °C

Treatments: Flasks were continuously shaken at 100 ± 10 oscillations / minute
Definitive Test - 100, 180, 320, 560, and 1000 mg/L of 2-Ethyl-1, 3-Hexanediol based on 97% active. Material appeared to be in solution at all treatment levels.

Remarks: A range-finding test conducted using test material at concentrations from 0.1 to 1000 mg/L had indicated that test concentrations from 100 to 1000 mg/L would be appropriate for definitive testing.

Analytical Monitoring: No

Results: Based upon the hemacytomter counts after 5 days of exposure to 2-Ethyl-1, 3-Hexanediol, the algicidal concentration was determined to be 144 mg/L (95% confidence limits 55-336 mg/L).

Remarks: Algal cultures exposed to definitive testing concentrations were subjected to the recovery phase. All transferred cultures exhibited growth, but the degree of recovery was limited and was inversely related to the exposure concentration.

A factor contributing to the relatively low population densities in the transferred cultures may have been the small number of cells used to begin the recovery phase. However, since at least some degree of recovery was observed at all test concentrations, the algicidal concentration is greater than 1000 mg/L

Data Quality

Reliability (Klimisch): 2

Reference

Report/Study Number: 165-09-1100-1
Reference: The Procter & Gamble Company, 1986. The Toxicity of B0859.01 to *Microcystis aeruginosa*. Accession # 32358

Acc # 32358

Aquatic Toxicology
MPI-QA-SIGN-1

Malcolm Pirnie, Inc.
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Cincinnati, Ohio 45217

Title of Report: The Toxicity of B0859.01 to
Microcystis aeruginosa

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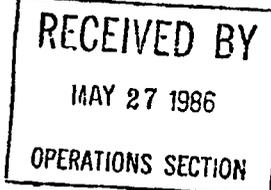


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I. SUMMARY

An algal toxicity test was conducted for the Procter and Gamble Company, Cincinnati, Ohio by Malcolm Pirnie, Inc., White Plains, NY during the period March 7 to March 21, 1986. The objective of the study was to determine the algistatic concentration of the test material B0859.01 to the freshwater blue-green alga, Microcystis aeruginosa. Algal cultures were exposed to a series of nominal concentrations (100 to 1000 mg/L) of the test material for five days. At the end of five days, algal cells from cultures with populations less than or similar to the initial inoculum level were transferred to test material-free medium. Transferred cultures were then incubated for a nine-day recovery period. Biomass was estimated during the assay by cell counts using an electronic particle counter and also by using a hemacytometer and microscope.

The algistatic concentration of B0859.01 to Microcystis aeruginosa was determined to be 144 mg/L (95% confidence limits 55-336 mg/L). Cultures that had been exposed to 180, 320, 560 and 1000 mg/L were subjected to the recovery phase, in addition to the control. All transferred cultures exhibited growth, but the degree of recovery was inversely related to the exposure concentration. The algicidal concentration is greater than 1000 mg/L.

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II. INTRODUCTION

Payne and Hall (1979) developed an algal toxicity test specifically for use in the environmental safety assessment of new chemicals. The procedure, based upon the Algal Assay Procedure Bottle Test (EPA, 1971; Miller et al., 1978), employs a five-day exposure of algal cultures to a range of test material concentrations followed by a nine-day recovery period in the absence of test material. Biomass estimates are made by cell counts, in vivo fluorescence, or both. The primary response sought in this method is the algistatic concentration of the test material, defined as the concentration that causes no net increase in cell number after the 5-day exposure period but permits regrowth when the cells are resuspended in test material-free medium. The algicidal concentration is the lowest concentration tested which causes no net increase in cell number during either the exposure or recovery period; i.e. cells do not recover when transferred to test material-free medium.

The objective of the study was to determine the algistatic concentration of the test material B0859.01 to Microcystis aeruginosa. This representative freshwater blue-green alga, is readily available, easily cultured and has ecological importance.

The study was conducted by Malcolm Pirnie, Inc. during the period March 7 to March 21, 1986. The study was conducted for the Procter and Gamble Company, Cincinnati, Ohio under MPI Project No. 165-09-1100-1. Methods were according to the protocol prepared by Mr. K.G. Surowitz of the Procter and Gamble Co., dated February 25, 1986, as supplemented by Malcolm Pirnie's Standard Operating Procedures.

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III. METHODS AND MATERIALS

All testing was conducted at the laboratory of Malcolm Pirnie, Inc. in White Plains, New York.

Test Organism

Microcystis aeruginosa used in this test came from laboratory stock cultures. The original culture was obtained from the University of Texas Culture Collection, Austin, TX (UTEX L2061). Stock cultures are maintained in algal assay procedure medium (AAP medium) (Appendix A) in Erlenmeyer flasks under constant illumination of 2152 ± 323 lumens/m² and temperature of $24 \pm 2^\circ\text{C}$. Flasks are continuously shaken at 100 ± 10 oscillations/minute. Transfers into fresh medium are conducted weekly according to Standard Operating Procedures.

Preparation of Glassware - All glassware used in testing was thoroughly scrubbed with non-phosphate detergent and rinsed with tap water. This was followed by a rinse with acetone, further rinses with tap water, a rinse in 10 percent reagent grade hydrochloric acid, and thorough rinsing in tap water and distilled deionized water. Since the Coulter Counter was used, the final rinse was in distilled deionized water passed through a 0.22 micron pore size membrane-filter. Glassware was dried in an oven at $50-70^\circ\text{C}$. Foam plugs were inserted and the glassware was autoclaved for 20 minutes at 1.1 Kg/cm^2 and 121°C .

Preparation of Medium - AAP medium was prepared by adding 1 mL of each of the macronutrient stock solutions and 1 mL of the micronutrient stock solution, in the order listed in Appendix A, to 900 mL of distilled deionized water, with mixing after each addition. The volume was brought to 1 liter and the pH was adjusted to 7.5 ± 0.1 with 0.1N sodium hydroxide or hydrochloric acid. The medium was immediately filtered through a 0.22 micron porosity membrane filter into a sterile

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container. Medium was stored in the dark at 4°C and brought to room temperature prior to use.

Preparation of Test Material

The test material was received on February 26, 1986. It is characterized by the sponsor as B0859.01 and is a colorless liquid. The test material is 97% active ingredient and the solubility is 4.29% weight/volume in water. It was stored at room temperature.

A range-finding test conducted using test material concentrations from 0.1 to 1000 mg/L had indicated that test concentrations of 100 to 1000 mg/L would be appropriate for the definitive test.

All calculations and measurements are based on the percent active ingredient. To begin the definitive test, 100 mL of a stock solution of 10 mg a.i./mL was prepared in AAP medium. The test treatments were prepared by adding calculated amounts of stock solution to AAP medium in labelled 250 mL volumetric flasks. The volume was then brought to 250 mL in each flask with AAP medium. The test concentrations were 100, 180, 320, 560, and 1000 mg/L.

The pH of the highest test treatment was checked and fell within the prescribed range of 7.5 ± 1. Therefore, no pH adjustment of the test concentrations was required. After thorough mixing, 50 mL of each test concentration was added to each of three replicate test vessels (250 ml Erlenmeyer flasks). The control contained AAP medium with no additions.

Inoculation

The inoculum of Microcystis aeruginosa came from a 7-day old stock culture which had previously been maintained in AAP medium for at least three transfers. Population density in the stock culture was determined with a Model ZBI Coulter Counter equipped with a C-1000 Channelyzer and MHR Computer.

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The stock culture contained 7,960,000 cells/mL. A 0.314 mL volume of this culture was aseptically added to 50 mL medium in each flask, yielding a nominal initial concentration of 50,000 cells/mL.

Incubation

Test flasks were kept in a Psycrotherm Controlled Environment Incubator Shaker, Model G-27, at a temperature of $24 \pm 2^\circ\text{C}$. Temperature was recorded daily. Flasks were continuously shaken at 100 ± 10 oscillations/minute. Continuous illumination of 2152 ± 323 lumens/m² was provided by overhead cool-white fluorescent lights. Flasks were randomly repositioned each day to minimize spatial differences in the incubator.

Biomass Estimation

Biomass measurements during the assay were conducted by cell counts using an electronic particle counter on days 3 and 5 of the exposure period and days 2, 6, and 9 of the recovery period. In addition, cell counts were made using a hemacytometer and microscope on selected flasks on selected days. This was done to obtain more accurate cell counts, as the test material contained particulates which interfered with the electronic particle counter.

Cell counts made using an electronic particle counter employed a Model ZBI Coulter Counter with C-1000 Channelyzer and MHR Computer. The Coulter Counter operates on the principle that cells are poor electrical conductors. The algal cells, suspended in an electrolyte, can be sized and counted by passing them through an aperture with a specific path of current flow. As cells pass through the aperture and displace a volume of electrolyte equal to their volume, the resistance changes, causing current and voltage changes which are translated into number and size of cells. On each counting day, a

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sample was collected aseptically from each flask using an automatic micropipette with a sterile tip. Flasks were swirled prior to pipetting to ensure representative sampling. The samples were placed in individual particle-free disposable containers and diluted with the electrolyte Isoton II (Coulter Electronics, Inc.). Three counts per replicate flask were made. All counts were multiplied by the appropriate conversion factors (for sample dilution and volume counted) to yield cells/mL. The Coulter Counter was calibrated, using an organic calibration material, as per Standard Operating Procedures. The instrument settings used are given in Appendix B.

The electronic counts on day 5 indicated a higher population density in the 1000 mg/L test concentration than in the 560 mg/L test concentration. Although not visible to the eye, possible interference from particulates present in the test material was suspected. Thus, population density was also determined with a microscope and hemacytometer on day 5. For microscopic counting, an improved Neubauer hemacytometer, 0.1 mm deep, was used. Two samples were taken from each flask, and two counts were made for each sample. Whenever feasible, 400 cells per flask were counted in order to obtain ± 10 percent accuracy at the 95 percent confidence level.

Recovery Phase

After five days' exposure of the algal cultures to the test material, the recovery phase was initiated. Algal cells from cultures with day 5 cell concentrations similar to or less than the initial inoculum level were washed by centrifugation and resuspended in test material-free medium for a nine-day recovery phase. The test concentrations that were submitted to the recovery phase were 180, 320, 560 and 1000 mg/L.

To conduct the recovery phase, the contents of the replicate flasks were pooled for each treatment, yielding approxi-

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mately 120-150 mL. This was divided into aliquots for centrifuging in centrifuge tubes of approximately 40 mL capacity. Cells were centrifuged for 10 minutes at approximately 1000 x g. The supernatant was removed and the cells resuspended in test material-free medium. Again the cells were centrifuged, the supernatant removed and the cells resuspended. The divided aliquots were then re-combined. Cell density in the washed suspensions was determined with the Coulter Counter.

Except for the control, an insufficient number of algal cells was available to begin the recovery phase with the original inoculum level of 50,000 cells/mL. For the four test concentrations, the available volumes of washed cell suspension were divided equally among the three replicate flasks to be inoculated. The population density used to begin the recovery phase was not actually determined but was less than 5000 cells/mL. For each test concentration and the control, the appropriate volume was aseptically added to each of three replicate flasks per test concentration containing test material-free medium. The flasks were incubated for nine days under the same conditions previously described for the exposure phase.

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IV. RESULTS - DISCUSSION

Data Analysis

The algistatic concentration with its associated 95% confidence intervals is determined by linear regression analysis of \log_{10} of the ratio of the cell numbers (at the end of the exposure period) to the initial inoculum level regressed against the \log_{10} of the concentration of material tested, using linear model. The algistatic concentration is then selected by "inverse estimation" as that concentration of test material that corresponds to a Day 5 ratio of one. Algistatic concentrations and 95% confidence intervals were calculated using a computer program developed by Procter and Gamble.

Discussion

Electronic cell counts during the assay are presented in Table 1 and depicted graphically in Figure 1. Hemacytometer cell counts are given in Table 2. Only one replicate for each concentration was counted on day 9 of the recovery phase, as these counts confirmed the accuracy of the Coulter Counter on the test material-free medium.

As indicated by the hemacytometer counts, the mean population densities in the 180, 320, 560, and 1000 mg/L test concentrations after five days' exposure to B0859.01 were less than or similar to the initial inoculum level of 50,000 cells/mL. Based upon the hemacytometer counts, the algistatic concentration was determined to be 144 mg/L (95% confidence limits 55-336 mg/L). Algal cultures exposed to these concentrations and the control were subjected to the recovery phase. All transferred cultures exhibited growth, but the degree of recovery was limited and was inversely related to the exposure concentration.

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A factor contributing to the relatively low population densities in the transferred cultures may have been the small number of cells used to begin the recovery phase. However, since at least some degree of recovery was observed at all test concentrations, the algicidal concentration is greater than 1000 mg/L.

Deviations from Protocol

There were no deviations from protocol.

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REFERENCES

Environmental Protection Agency, National Eutrophication Research Program, 1971, Algal Assay Bottle Test.

Miller, W.E., J.C. Greene and T. Shiroyama, 1978, The Selenastrum capricornutum Printz Algal Assay Bottle Test, EPA-600/78-018, 126 pp.

Payne, A.G. and R.H. Hall, 1979, "A method for measuring algal toxicity and its application to the safety assessment of new chemicals," Aquatic Toxicology, ASTM STP 667, L.L. Marking and R.A. Kimerle, eds., American Society for Testing and Materials, 1979, pp. 171-180.

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The Toxicity of B0859.01 to *H. seruginosa*

Table 1 - Cell counts (cells/mL) during assay, using electronic particle counter

Nominal Concen- tration mg/L		Exposure Day 3 3-10-86	Exposure Day 5 3-12-86	Recovery Day 2 3-14-86	Recovery Day 6 3-18-86	Recovery Day 9 3-21-86
0	A	88,000	270,000	213,000	3,340,000	7,640,000
	B	116,000	418,000	215,000	3,360,000	7,800,000
	C	113,000	400,000	194,000	3,180,000	7,920,000
	Mean	106,000	363,000	207,000	3,290,000	7,790,000
	SD ¹	1.2E4	8.00E4	1.2E4	9.9E4	1.4E5
	Var ²	1.58E8	6.52E9	1.34E8	9.73E9	1.97E10
100	A	92,000	150,000	NT ³	NT	NT
	B	96,000	167,000	NT	NT	NT
	C	96,000	162,000	NT	NT	NT
	Mean	95,000	160,000	--	--	--
	SD	2.3E3	8.7E3	--	--	--
	Var	5.33E6	7.63E7	--	--	--
180	A	82,000	109,000	12,000	27,000	400,000
	B	81,000	96,000	12,000	27,000	328,000
	C	82,000	103,000	12,000	23,000	318,000
	Mean	82,000	103,000	12,000	26,000	349,000
	SD	<1.0E3	6.5E3	0	2.3E3	4.5E4
	Var	3.33E5	4.23E7	0	5.33E6	2.00E9
320	A	53,000	52,000	8,000	16,000	150,000
	B	42,000	47,000	9,000	19,000	243,000
	C	44,000	51,000	8,000	15,000	161,000
	Mean	46,000	50,000	8,000	17,000	185,000
	SD	5.8E3	2.6E3	<1.0E3	2.1E3	5.1E4
	Var	3.43E7	7.00E6	3.33E5	4.33E6	2.58E9
560	A	55,000	32,000	7,000	24,000	76,000
	B	60,000	37,000	8,000	28,000	92,000
	C	51,000	34,000	11,000	33,000	101,000
	Mean	55,000	34,000	9,000	28,000	90,000
	SD	4.5E3	2.5E3	2.9E3	4.5E3	1.3E4
	Var	2.03E7	6.33E6	8.67E6	2.03E6	1.60E8

(continued)

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The Toxicity of B0959.01 to M. aeruginosa

Table 1 - Cell counts (cells/mL) during assay, using electronic particle counter (Cont'd)

Nominal Concen- tration mg/L	Exposure Day 3 3-10-86	Exposure Day 5 3-12-86	Recovery Day 2 3-14-86	Recovery Day 6 3-18-86	Recovery Day 9 3-21-86
1000 A	64,000	54,000	5,000	21,000	26,000
B	64,000	45,000	7,000	28,000	65,000
C	72,000	50,000	5,000	33,000	50,000
Mean	67,000	50,000	6,000	27,000	47,000
SD	4.6E3	4.5E3	1.1E3	6.0E3	2.0E4
Var	2.13E7	2.0E37	1.33E6	3.63E7	3.87E8

¹SD = Standard deviation

²Var = Variance

³NT = Not transferred

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The Toxicity of B0859.01 to M. aeruginosa

Table 2 - Cell counts (cells/mL) on selected days, using hemacytometer

Nominal Concentration, mg/L	Exposure Day 5 3-12-86	Recovery Day 9 3-21-86
0	A	296,000
	B	412,000
	C	452,000
	Mean	387,000
	SD ¹	8.1E4
	Var ²	6.57E9
100	A	116,000
	B	122,000
	C	109,000
	Mean	116,000
	SD	7.0E3
	Var	4.23E7
180	A	51,000
	B	43,000
	C	46,000
	Mean	47,000
	SD	1.0E3
	Var	1.63E7
320	A	7,000
	B	7,000
	C	8,000
	Mean	7,000
	SD	<1.0E3
	Var	3.33E5
560	A	3,000
	B	4,000
	C	3,000
	Mean	3,000
	SD	<1.0E3
	Var	3.33E5

(continued)

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The Toxicity of B0859.01 to M. aeruginosa

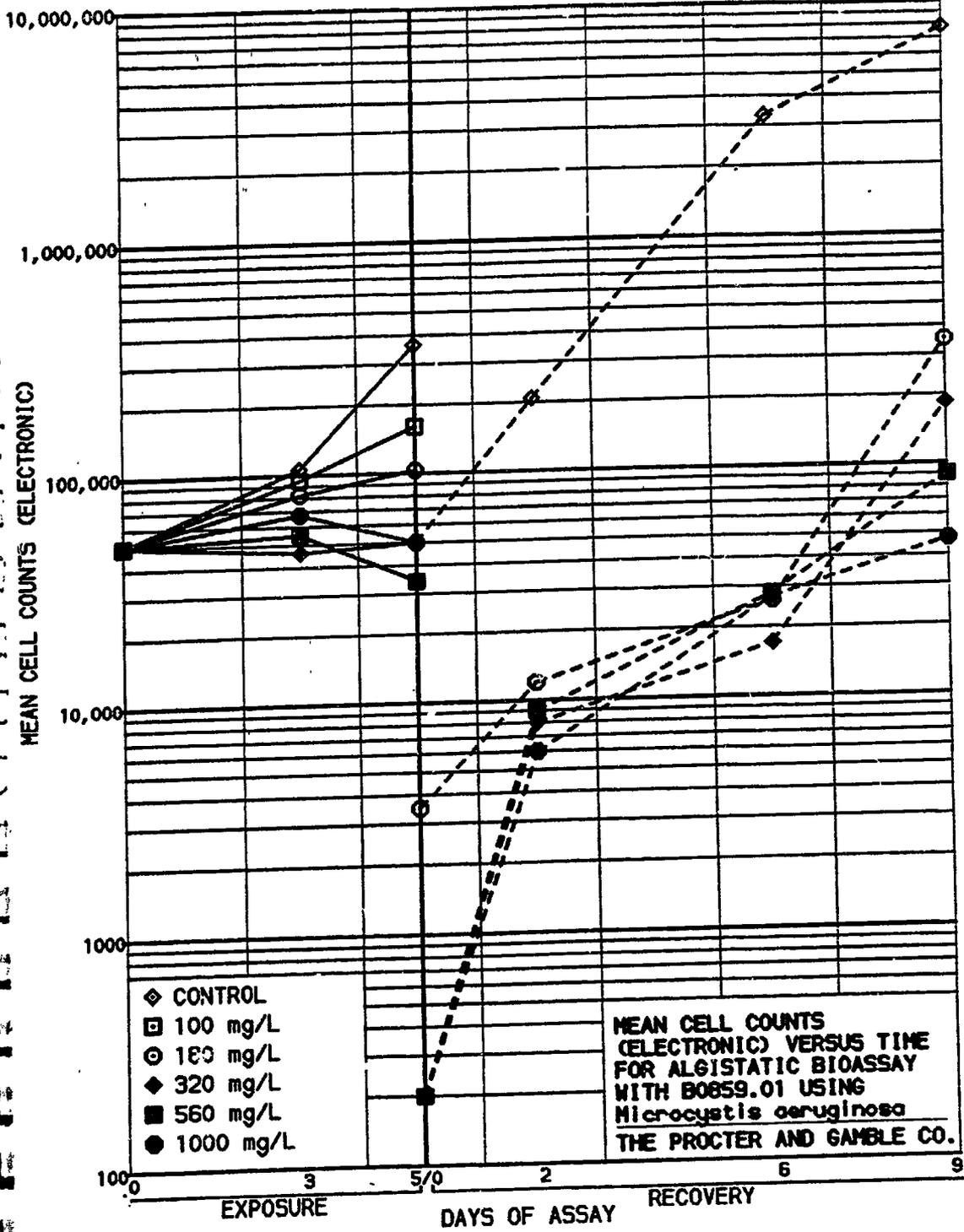
Table 2 - Cell counts (cells/mL) on selected days, using hemacytometer
(continued)

Nominal Concentration, mg/L	Exposure Day 5 3-12-86	Recovery Day 9 3-21-86
1000 A	6,000	24,000
B	3,000	NC
C	6,000	NC
Mean	5,000	--
SD	2.0E3	--
Var	3.00E5	--

- ¹SD = Standard deviation
²Var = Variance
³NC = Not counted
⁴NT = Not transferred

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FIGURE 1



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APPENDIX A. COMPOSITION OF ALGAL ASSAY PROCEDURE (AAP) NUTRIENT MEDIUM (AFTER MILLER ET AL, 1978)

a. Macronutrients

Stock solution		Nutrient composition of prepared medium	
Compound	Concentration, g/L	Element	Nominal Concentration, mg/L
NaNO_3	25.500	N	4.200
NaHCO_3	15.000	Na	11.001
		C	2.143
K_2HPO_4	1.044	K	0.469
		P	0.186
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	14.700	S	1.911
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	12.164	Mg	2.904
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	4.410	Ca	1.202

b. Micronutrients

Stock solution		Nutrient composition of prepared medium	
Compound	Concentration, mg/L	Element	Nominal Concentration, $\mu\text{g/L}$
H_3BO_3	185.520	B	32.460
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	415.610	Mn	115.374
ZnCl_2	3.271	Zn	1.570
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1.428	Co	0.354
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.012	Cu	0.004
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	7.260	Mo	2.878
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	160.000	Fe	33.051
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	300.000

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The Toxicity of B0859.01 to M. aeruginosa

APPENDIX B. OPERATING PARAMETERS FOR THE COULTER COUNTER

For counting algae using the Model ZBI Coulter Counter with C-1000 Channelyzer and MHR Computer, the following parameters were used:

Aperture tube	100 um
Volume	500 uL
Separate/Locked switch	Separate
1/aperture	1/2
1/amplification	1/2
Matching switch	20K
Gain trim	3
Lower threshold	8
Upper threshold	100
MHR threshold	8
Base channel threshold	5
Window width	100
Edit switch	On
Mean Threshold factor	1.252 (n = 3)
Range of cell volumes included in count	10-140 um ³

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Aquatic Toxicology
MPI-QA-CS-2

Quality Assurance Compliance Statement

The study entitled The Toxicity of B0859.01 to

Microcystis aeruginosa

conducted and reported by Malcolm Pirnie, Inc., White Plains, NY for

The Procter & Gamble Co.

is in compliance with EPA Good Laboratory Practice Standards under the Federal Insecticide, Fungicide and Rodenticide Act and the Toxic Substances Control Act (Fed. Reg. Vol. 48, No. 230, 11/29/83), except as follows:

Study conducted in compliance with EPA Good

Laboratory Practice Standards

Inspections were conducted during the course of this study for compliance verification.

I) Instrumentation Inspection

Date of Inspection	Inspector	Date Findings Reported	Date Corrective Action Taken	Comments
<u>3/17/86</u>	<u>J.S. Reed</u>	<u>3/17/86</u>	<u>N/A</u>	<u>None</u>
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

II) Study Inspection (Test System and Raw Data)

Date of Inspection	Inspector	Date Findings Reported	Date Corrective Action Taken	Comments
<u>3/11/86</u>	<u>J.S. Reed</u>	<u>3/11/86</u>	<u>N/A</u>	<u>None</u>
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

Quality Assurance Inspector: *J.S. Reed*

Date: 5-23-86

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TEST PROCEDURE: Algal cultures are exposed to the test material under continuous fluorescent lighting and mixing for five days. At the end of this five-day period, algal cells from cultures that have populations less than or similar to the initial inoculum level are washed and transferred into test material free medium. Cells from the control cultures are also washed and transferred. The transferred cultures are then incubated for a recovery period of nine days under the same conditions as the exposure period.

Biomass measurements - Algal growth is determined by cell counts following laboratory's SOP on days 3 and 5 of the exposure period and days 2, 6 and 9 of the recovery period. Chlorophyll a relative fluorescence measurements are also made on each of these days following laboratory's SOP. (Note: Fluorescence measurements are not required for Microcystis)

Cell washing procedure -

- 1) pool replicates from cultures to be transferred;
- 2) centrifuge at $\leq 1,000 \times g$ to form a pellet;
- 3) decant the supernatant;
- 4) resuspend cells in test material free medium;
- 5) repeat steps 2, 3, and 4.

Washed cells are added at the original inoculum level to clean Erlenmeyer flasks containing test material free medium. If a sufficient number of cells is not available, the entire quantity is divided equally into the replicate flasks. Under these circumstances, washed cells from the control culture are added at both the original inoculum level and the lowest level included in the recovery period.

Test conditions are as follows:

- 1) Test containers and solution volume - ~~250 ml Erlenmeyer flasks containing 100 ml test solution, or 125 ml and 50 ml, respectively.~~ *as per laboratory's SOP. June 2/19/76*
- 2) Number of replicates - minimum of three.
- 3) Inoculum source and age - laboratory stock cultures 7 to 14 days old and previously maintained in the respective test medium for a minimum of three transfers.
- 4) Inoculum level - 2×10^4 cells/ml for Selenastrum, Navicula, and Dunaliella
- 5×10^4 cells/ml for Microcystis.
- 5) Temperature - as per laboratory's SOP.
- 6) Light level - 400 footcandles \pm 15% for Selenastrum, Navicula, and Dunaliella
- 200 footcandles \pm 15% for Microcystis.
- 7) Shaker speed - as per laboratory's SOP's.
- 8) Glassware cleaning - as per laboratory's SOP.

CALCULATIONS: The algistatic concentration, with its associated 95% confidence intervals, is determined by linear regression analysis of \log_{10} of the ratio of the cell numbers (at the end of the exposure period, that is, Day 5) to the initial inoculum level regressed against the \log_{10} of the concentration of material tested, using a linear model. The algistatic concentration is then selected by "inverse estimation" as that concentration of test material that corresponds to a Day 5 ratio of one (i.e., where the \log_{10} of the ratio is zero). When test concentrations causing 100% die-off are used in the calculation, an arbitrary value representing one half of the minimum count possible (as per laboratory's SOP) is used in place of zero.

The algicidal concentration is reported as the lowest concentration tested demonstrating inhibitory effects (equal to or below the original inoculum level) and from which cells do not recover when transferred into test material free medium.

RECORDS TO BE MAINTAINED: All records necessary to reconstruct the study and demonstrate adherence to the Protocol.

PROTOCOL CHANGES: If a change in the approved protocol becomes necessary, verbal agreement should be made between the Study Director and Sponsor's Principal Investigator. As soon as practical thereafter, this change and the reasons for it should be put in writing, approved by both persons, and attached to the protocol as an addendum.

REFERENCES:

1. USEPA 1978. The *Selenastrum capricornutum* Prutz Algal Assay Bottle Test, Environmental Research Laboratory, Corvallis, OR, 126 pp.
2. USEPA 1974. Marine Algal Assay Procedure: Bottle Test, Pacific Northwest Environmental Research Laboratory, Corvallis, OR, 43 pp.

REPORTING: The report is to be a typed document in triplicate, describing the results of the study and is to be signed and dated by the Study Director, Quality Assurance Officer and Laboratory Manager. It is to include, but is not limited to, the following:

1. Identification of test material by sample code, percent active, color, form and date received.
2. Procedures followed for test material preparation and addition.
3. Reference to laboratory notebook or other file containing raw data.
4. Laboratory Study Number.
5. Date definitive test was conducted.
6. Species tested, including the source and age of the inoculum.
7. Calculated algistatic concentrations with associated 95% confidence intervals and the algicidal concentration.
8. Plots and tables of biomass measurements across the 14-day testing period.
9. Description of the test conditions including test media, temperature, shaker speed, and light intensity.
10. Reference to the Protocol (title, author and date), and any standard test methods or any analytical procedures used.
11. Any Protocol deviations and their implications.
12. Description of the quality assurance methods used to insure the quality of the data.

ALTERNATE PRINCIPAL INVESTIGATOR R. H. Hall PHONE: (513) 530-3347

NOTED: J. W. Williams 2/19/86 PHONE: (513) 245-2120
Operations/Logistics Date

APPROVED: K. G. Surowitz 1/29/86 PHONE: (513) 530-3716
Principal Investigator Date

TO BE COMPLETED BY STUDY DIRECTOR:

Study No. 165-09-1100-1
Estimated Starting Date 3/7/86 Defined As: Definitive test
Estimated Reporting Date 4/4/86
Date Test Material Received 2/25/86
Approved Jane S. Hughes 2/26/86 PHONE: 914-684-2100
Study Director Date

KGS008/pcj

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 1 of 2
3685 SECT. NO.
EX-111 in box
ORIG. SECT. NO.

Test Substance Identification Number (TSIN): B0859.01
Safety Test Request Number: BSST- 85.05
Principal Investigator: K. G. Surovitz

Product or Ingredient: 2-ethyl-1,3-hexanediol (ETHED) Brand Notebook Ref: _____
Physical Description: clear, viscous liquid Solubility: 4.2% in water (20°C) pH: 7.1
Recommended Storage Conditions: room temperature Expiration Date: in progress
Hazards (i.e. flammability, toxic gases): harmful if inhaled or absorbed; irritant
Dept. of Transportation Hazard Classification: non-hazardous CAS No.: 94-96-2

Formulated Composition (b)

<u>Component (c)</u>	<u>Mol. Wt.</u>	<u>Nominal Level (X by Wt.)</u>	<u>Acceptable Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Number (NB-Ref.)</u>
2-ethyl-1,3-hexanediol (isomer mixture)	146.23	97%			Aldrich	3007LL

- (a) Include CAS number(s) for the three most major components of a formulation or for single chemical products. Footnote to the material with which the respective number is associated.
- (b) If information requested is not known, then the symbol NK will be entered.
- (c) Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-chemical names, such as Tergitol 15-S-O or Yellow Dye #10, may not be acceptable but should be previewed with the responsible toxicologist. Nondefinitive identification (e.g. Arquad, BC-base) is not acceptable.

The above information provided by: K.G. Surovitz
(Name)

[Signature]
(Signature) 7/1/85
(Date)

The above information reviewed and accepted by:

Principal Investigator: K.G. Surovitz
(Name)

[Signature]
(Signature) 7/1/85
(Date)

TSCR1

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 2 of 2

Test Substance Identification Number (TSIN): B0859.01

Analyzed Composition

<u>Date Submitted</u>	<u>Submitter Code</u>	<u>Analysis Code/Analysis</u>	<u>Estimated Value</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
-----------------------	-----------------------	-------------------------------	------------------------	-----------------------	---------------------------

Commercial sample (Aldrich Chemical Co.) No Analysis performed.

Analytical Information Verified By: _____

(Signature)

Date: _____

This test substance is suitable for environmental (non-clinical) safety testing.

Principal Investigator: _____

(Signature)

Date: 7/15/85

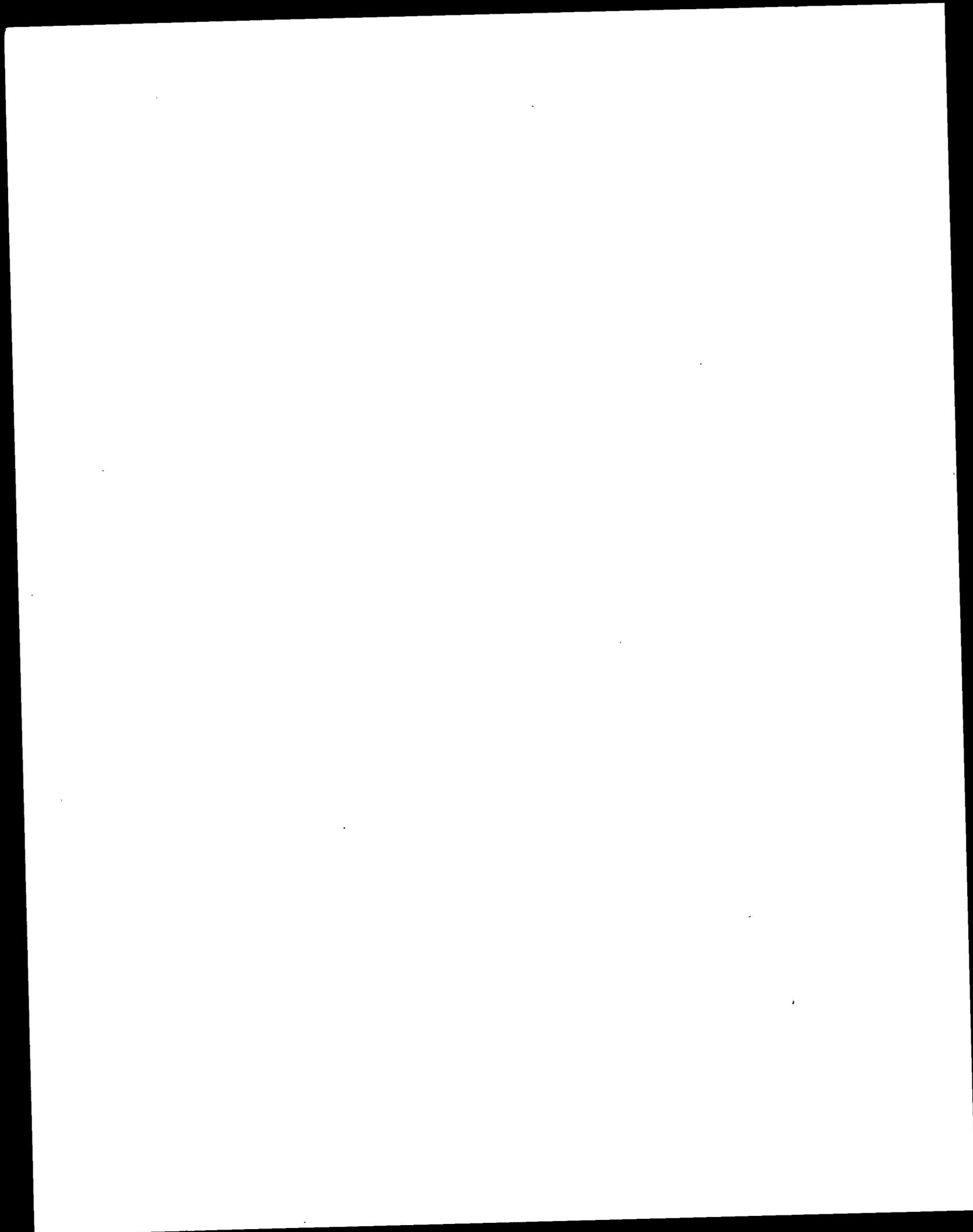
This test substance is suitable for human (clinical) safety testing.

Principal Investigator: _____

(Signature)

Date: _____

KGS001/pcj
7/12/85



Test Substance

CAS Number: 94-96-2
Identity: 2-Ethyl-1, 3-Hexanediol
Purity: 97% active
Remarks: Test substance identified as B0859.01

Method

GLP: Yes
Report/Study Year: 1985
Method/Guideline Followed: According to protocol
Test Type: CO₂ Production Test
Exposure Period: 28-days
Inoculum: 1% by volume supernatant of homogenized activated sludge from Semi-Continuous Activated Sludge (SCAS) Removability Test control units.
Test Conditions: Inoculum Loading: 1.6×10^7 organisms/ml.
Total Organic Carbon (TOC) Level of D-Glucose Stock Solution: 0.392 mg C/mg active
Total Organic Carbon (TOC) Level of 2-Ethyl-1, 3-Hexanediol Stock Solution: 0.676 mg C/mg active
Theoretical TOC for 2-Ethyl-1, 3-Hexanediol: 0.66 mg C/mg active
TCO₂ for 2-Ethyl-1, 3-Hexanediol: 2.40 mg CO₂/mg active
Temperature: 22 - 24°C
Analytical Monitoring: No

Results:

Test Material	Conc. Mg active/L	Final %TCO ₂	Final mg SOC/L	Asymptote %TCO ₂	Rate Day-1	Lag Days
Control	n/a	n/a	1.0	n/a	n/a	n/a
D-Glucose	20	94.4	1.1	90.7±3.0	0.16±0.02	0.0±0.0
CAS # 94-96-2	10	77.5	1.5	80.9±1.6	0.12±0.01	3.2±0.2
CAS # 94-96-2	20	81.9	1.3	87.3±1.9	0.11±0.01	2.9±0.2

Data Quality

Reliability (Klimisch): 1

Reference

Report/Study Number: 85-030
Reference: The Procter & Gamble Company, 1985. CO₂ Production Test on B0859.01. Accession # 32091



102
Acc # 32091

THE PROCTER & GAMBLE COMPANY
CINCINNATI, OHIO

CO₂ PRODUCTION TEST ON B0859.01

Kay H. Marks
Kay H. Marks 11/21/85
Study Director

WESTON Project #85-030

Dianne S. Therry
Dianne S. Therry 11-26-85
Quality Assurance Coordinator

Peter J. Marks
Peter J. Marks 12/5/85
Project Director

Prepared by:

RECEIVED BY
DEC 19 1985
OPERATIONS SECTION

WESTON
Weston Way
West Chester, PA 19380



CO₂ PRODUCTION TEST ON B0859.01

1.0 EXPERIMENTAL PROTOCOL

The measurements of CO₂ evolution were determined in accordance with the following protocol and referred test method.

Dr. K. W. Surowitz' protocol of 1 August 1985, entitled "CO₂ Production Test on B0859.01."

Ms. K. H. Marks' protocol addendum, dated 18 November 1985: Extending study three additional days.

Total and soluble organic carbon analyses were performed utilizing a Dohrmann Analyzer, Model DC-80.

Bacteriological enumeration was performed per Standard Methods, 15th Edition, Standard Plate Count, Section 907. The test was incubated at a temperature of 23.0°C.

Centrifugation of samples for soluble organic carbon analyses was performed per WESTON's SOP 83-E002; Centrifugation Procedure.

2.0 TEST MATERIAL

TSIN	-	B0859.01
% Active	-	97
Solubility	-	4.2 % in water (20°C)
Color	-	Colorless
Form	-	Liquid
Theoretical TOC	-	0.66 mg TOC/mg active
TCO ₂	-	2.40 mg CO ₂ /mg active
Date Received	-	8/21/85

3.0 TEST CONDITIONS

Temperature Range: 22° - 24°C.

Inoculum was obtained from the supernatant of homogenized activated sludge generated in an unacclimated semi-continuous activated sludge system being run at WESTON.

The inoculum for this study was taken from the Control units and was added to the test flasks at a concentration of 1% v/v.

A stock solution of the test material was prepared at 1000 mg/L active ingredient by weight/volume dilution with deionized water. The stock solution was volumetrically added to the test flasks at concentrations of 10 and 20 mg active/L.

4.0 MISCELLANEOUS PROJECT INFORMATION

The testing was performed at WESTON's Laboratory in West Chester, PA. Kay H. Marks was the Study Director, and carried out the test initiation and data collection.

The CO₂ evolution performed on the compound listed in Section 2.0 began 28 August 1985 and was completed 27 September 1985.

Bacteriological enumeration using inoculum from the Control units of the SCAS system was 1.6×10^7 CFU/mL.

Test data may be found in WESTON Laboratory Notebook 610, pages 1-8.

5.0 RESULTS

Table 1 summarizes CO₂ production for the test carboys throughout the test period.

Table 2 provides a summary of TOC and SOC analyses.

Appendix A - A description of the quality assurance methods used to ensure the quality of the data.

Appendix B - Statistical Analysis.

Appendix C - A copy of the Protocol Addendum

TSIM B0859.01

Table 1
CO₂ Test

Author Kay H. Marks Date 8/31/85

Study Director Kay H. Marks Date 9/27/85

Cumulative mg CO₂
Control Corrected % TC02

mL of Std. 0.05N HCl/100 mL Ba(OH)₂

Date	Day	Control	20 mg/L Dextrose	10 mg/L	20 mg/L	Initial	20 mg/L Dextrose	10 mg/L	20 mg/L	20 mg/L Dextrose	10 mg/L	20 mg/L	20 mg/L Dextrose	10 mg/L	20 mg/L	Cumulative mg CO ₂ from Control per liter	Initials	Comments
8/31/85	2	39.7	22.8	40.0	39.8	45.0	18.6	-0.3	-0.1	31.7	-0.6	-0.1	2.9	KM				
9/2/85	4	40.6	29.9	37.3	32.9	45.0	30.4	3.3	8.4	51.8	6.9	8.8	5.3	KM				
9/5/85	7	41.2	36.4	30.4	19.6	45.6	35.7	15.2	32.2	60.8	31.7	33.5	7.7	KM				
9/8/85	10	44.1	40.3	36.8	31.4	45.5	39.9	23.2	46.2	68.0	48.3	48.1	8.5	KM				
9/11/85	13	43.8	41.6	40.7	37.2	46.0	42.3	26.6	53.5	72.1	55.4	55.7	9.7	KM				
9/14/85	16	44.0	40.8	41.3	36.9	46.4	45.8	29.6	61.3	78.1	61.7	63.9	11.0	KM				
9/17/85	19	44.0	40.4	41.4	36.2	46.5	49.8	32.5	69.9	84.9	67.7	72.8	12.4	KM				
9/19/85	21	44.5	42.3	42.0	40.9	46.0	52.2	35.3	73.9	89.0	73.5	77.0	13.2	KM				
9/23/85	25	44.9	43.5	43.8	42.5	46.2	53.7	36.5	76.5	91.5	76.0	79.7	13.9	KM				
9/26/85	28	45.5	44.3	44.9	44.2	46.2	55.0	37.2	77.9	93.7	77.5	81.1	14.3	KM				
9/27/85	29	45.5	45.1	45.5	44.9	46.8	55.4	37.2	78.6	94.4	77.5	81.9	15.0	KM				



THE PROCTER & GAMBLE COMPANY

TABLE 2

ORGANIC CARBON ANALYSES

<u>SAMPLE DESCRIPTION</u>	<u>RFW #</u>	<u>mgC/mg Active</u>
Dextrose 1000 mg/L	8508-885-0010	0.392
B0859.01 1000 mg/L	8508-885-0020	0.676

DAY 26 RESIDUAL

<u>SAMPLE DESCRIPTION</u>	<u>RFW #</u>	<u>SOC mg/L</u>
Control	8509-020-0010	1.0
Dextrose 20 mg/L	8509-020-0020	1.1
B0859.01 10 mg/L	8509-020-0030	1.5
B0859.01 20 mg/L	8509-020-0040	1.3



APPENDIX A

QUALITY ASSURANCE METHODS



QUALITY ASSURANCE METHODS

This report and related records have been audited by the Quality Assurance Coordinator for adherence to protocol, laboratory standard operating procedures, and pertinent EPA Good Laboratory Practices. If non-compliance items were identified, management and the Study Director were notified immediately for corrective action.

Audits for the study were conducted on the following dates:

9 September 1985

26 November 1985

Dianne S. Therry

Dianne S. Therry

Quality Assurance Coordinator



APPENDIX B

STATISTICAL ANALYSIS

REGRESSION TITLE
 PROCTER 2 GAMBLE CURVE FIT - LOSS9.61 F = P1 * (1 - EXP(-K * (DAY - C)))

REGRESSION NUMBER 0
 INDEPENDENT VARIABLE (FOR BUILT-IN FUNCTION) DAY
 WEIGHTING VARIABLE DEXTR0SE
 NUMBER OF PARAMETERS 3
 NUMBER OF CONSTRAINTS 0
 TOLERANCE FOR PIVOTING0000001000
 TOLERANCE FOR CONVERGENCE00001000000
 MAXIMUM NUMBER OF ITERATIONS 30
 MAXIMUM NUMBER OF INCREMENT HALVINGS 5
 NUMBER OF DATA PASSES PER CASE 1
 COMPUTE LOSS FUNCTION NO

**** FUN PARAGRAPH IS USED ****

USING THE ABOVE SPECIFICATIONS THIS PROGRAM COULD PROCESS 1168 CASES.

BASED ON INPUT FORMAT SUPPLIED I RECORDS READ PER CASE.

NUMBER OF CASES READ 11

VARIABLE NO. NAME	MEAN	STANDARD DEVIATION	MINIMUM	MAXIMUM
2 DAY	15.818182	9.453234	2.000000	29.000000
3 DEXTR0SE	74.161616	19.916668	31.700000	94.400000
4 K	52.327271	28.135952	-.600000	77.500000
5 TWENTY	54.763636	29.263349	-.100000	61.900000

PARAMETER MAXIMA2126765+038 .2126765+038 .2126765+038 .2126765+038

PARAMETER MINIMA30.000000 .00000000 .00000000 .00000000

ITERATION INCREMENT RESIDUAL SUM OF SQUARES

ITERATION NUMBER	INCREMENT	RESIDUAL SUM OF SQUARES	P1	K	C
0	0	.738740+003	100.000000	.100000	.100000
1	0	.465207+003	58.643181	.141952	.000000
2	0	.276467+003	91.016651	.157720	.000000
3	0	.276904+003	90.769605	.161353	.000000
4	0	.276830+003	90.683422	.162328	.000000
5	0	.276826+003	90.661521	.162433	.000000
6	0	.276825+003	90.656332	.162480	.000000
7	0	.276825+003	90.655123	.162491	.000000
8	0	.276825+003	90.654842	.162494	.000000
9	0	.276825+003	90.654777	.162495	.000000
10	0	.276825+003	90.654762	.162495	.000000

ITERATION 10 HAS THE SMALLEST RESIDUAL SUM OF SQUARES (SUBJECT TO CONSTRAINTS, IF ANY).

PAGE 3 BMDP3K PROCTER & GAMBLE CURVE FIT - B0859.01 F = P1 * (1 - EXP(-K * (DAY - C)))

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

P1 K C

P1	1	1.0000	
K	2	-.7415	1.0000
C	3	.0000	.0000

RESIDUAL MEAN SQUARE 30.7584

DEGREES OF FREEDOM 9

PARAMETER	ESTIMATE	ASYMPTOTIC STANDARD DEVIATION	TOLERANCE
P1	90.654762	2.956886	.4501837026
K	.162495	.019983	.4501837026
C	.000000	.000000	.0000000000

IF LINEAR DEPENDENCE IS FOUND OR IF A PARAMETER IS ON THE BOUNDARY IT IS ASSIGNED A STANDARD DEVIATION OF ZERO

CASE NO. LABEL	PREDICTED DEXTROSE	STD DEV OF PRED VALUE	OBSERVED DEXTROSE	RESIDUAL	COOK DISTANCE	DAY	TEN	TWENTY
1 01	25.153515	2.083500	31.700000	6.546485	.688859	2.000000	7.600000	7.100000
2 02	43.327812	2.894730	51.600000	8.472188	.400321	4.000000	6.900000	8.800000
3 03	61.587981	2.907611	69.800000	-7.812019	.503517	7.000000	31.700000	33.500000
4 04	72.802824	2.408775	66.000000	-4.802824	.071632	10.000000	48.300000	48.100000
5 05	79.690641	1.972199	72.000000	-7.590641	.103476	13.000000	55.400000	55.700000
6 06	83.920931	1.040713	79.000000	-4.920931	.051084	16.000000	61.700000	63.900000
7 07	86.519047	1.968144	84.900000	-1.619047	.004658	19.000000	67.700000	72.799999
8 08	87.666562	2.105057	89.000000	1.333437	.003789	21.000000	73.500000	77.000000
9 09	89.094751	2.386127	91.500000	2.405248	.017477	25.000000	76.500000	79.700000
10 10	89.696651	2.553590	93.700000	4.003349	.059299	28.000000	77.500000	81.099999
11 11	89.840347	2.599726	94.400000	4.559652	.081317	29.000000	77.500000	81.900000

SERIAL CORRELATION701

PAGE 7 BMDP3R PROCTER & GAMBLE CURVE FIT - BU859.01 F = P1 * (1 - EXP(-K * (DAY - C)))

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

	P1	K	C
P1	1.0000		
K	-.8737	1.0000	
C	-.4303	.6720	1.0000

RESIDUAL MEAN SQUARE 1 2.79999

DEGREES OF FREEDOM 8

PARAMETER	ESTIMATE	ASYMPTOTIC STANDARD DEVIATION	TOLERANCE
P1	80.864334	1.584643	.1918096740
K	.122847	.008628	.1290805463
C	3.159658	.202157	.4444048053

CASE NO. LABEL	PREDICTED TEN	STD DEV OF PRED VALUE	OBSERVED TEN	RESIDUAL	COOK DISTANCE	DAY	DEXTROSE	TWENTY
1 01	.000000	.000000	.600000	-.600000	.000000	2.000000	31.700000	-.100000
2 02	7.931462	1.547807	6.500000	-1.031462	5.198167	4.000000	51.800000	8.800000
3 03	33.413521	.697818	31.700000	1.286479	.111853	7.000000	60.800000	33.500000
4 04	45.965331	.914049	43.300000	2.334669	.393335	10.000000	68.000000	48.100000
5 05	56.723188	.658413	55.400000	-1.323189	.101034	13.000000	72.099999	55.700000
6 06	64.164862	.726953	61.700000	-2.464863	.207415	16.000000	78.099999	63.900000
7 07	69.312569	.631866	67.700000	-1.612569	.060046	19.000000	84.900000	72.799999
8 08	71.829000	.630525	73.500000	1.670999	.064111	21.000000	89.000000	77.000000
9 09	75.336725	.760614	76.000000	-.663275	.017192	25.000000	91.500000	79.700000
10 10	77.040648	.903746	77.500000	-.459351	.014605	28.000000	93.700000	81.099999
11 11	77.482670	.951450	77.500000	.017330	.000025	29.000000	94.400000	81.900000

SERIAL CORRELATION

.233

PAGE 5 BMDP3K PROCTER & GAMBLE CURVE FIT - BU959.01 F = P1 * (1 - EXP(-K * (DAY - C1)))
 PLOTS OF VARIABLE 21 VERSUS PREDICTED AND OBSERVED VARIABLES 91 AND VERSUS RESIDUALS.

DAY	RESIDUALS	PREDICTED	OBSERVED
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CPU TIME USED 4.933 SECONDS DAY

PAGE 11 BMDP3R PROCTER 4 GAMBLE CURVE FIT - B0859.D1 F = PI * (1 - EXP(-K * (DAY - C)))

ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS

PI 1 2 3

PI 1 1.0000
 K 2 -.9027 1.0000
 C 3 -.4909 .7069 1.0000

RESIDUAL MEAN SQUARE 2.88924

DEGREES OF FREEDOM 8

PARAMETER	ESTIMATE	ASYMPTOTIC STANDARD DEVIATION	TOLERANCE
P1	87.285423	1.933519	.1417380496
K	.108333	.007978	.09334245783
C	2.889053	.226267	.3831523766

CASE NO. LABEL	PREDICTED TWENTY	STD DEV OF PRED VALUE	OBSERVED TWENTY	RESIDUAL	COOK DISTANCE	DAY	DEXTROSE	TEN
1 01	600000	600000	600000	-.100000	.000000	2.000000	31.700000	6.600000
2 02	9.697444	1.553734	6.600000	-1.097444	4.295808	4.000000	51.800000	6.900000
3 03	31.370411	.697307	33.500000	2.129589	.280236	7.000000	60.800000	31.700000
4 04	46.685239	.903037	46.100000	1.214761	.693272	10.000000	66.000000	48.300000
5 05	58.095143	.673796	55.700000	-2.395143	.323110	13.000000	72.099999	55.400000
6 06	66.194616	.758660	63.900000	-2.294617	.188707	16.000000	78.099999	61.700000
7 07	72.046717	.655808	72.799999	.753282	.013452	19.000000	84.900000	67.700000
8 08	75.015230	.642440	77.000000	1.984769	.088365	21.000000	89.000000	73.500000
9 09	79.330097	.769810	79.700000	.369902	.005124	25.000000	91.500000	76.000000
10 10	81.537475	.934635	81.099999	-.437475	.013716	28.000000	93.700000	77.500000
11 11	82.127623	.992367	81.900000	-.227624	.004689	29.000000	94.400000	77.500000

SERIAL CORRELATION .147

APPENDIX C

PROTOCOL ADDENDUM



PROTOCOL ADDENDUM

Re: CO₂ PRODUCTION TEST ON B0859.01

Per a phone conversation on 23 September 1985, the Study Director recommended extending the test three additional days to assure that the 10 and 20 mg/L test flasks reached a plateau. The Principal Investigator agreed that the study be terminated on Day 28.

K. W. Surowitz
K. W. Surowitz (Date)
Principal Investigator

K. H. Marks 11/12/85
K. H. Marks (Date)
Study Director

Laboratory Project No. 85-070

PROTOCOL

SPONSOR: The Procter & Gamble Company, Cincinnati, Ohio
Roy F. Weston, Inc.

LABORATORY: Weston Way
West Chester, PA 19380

TITLE: CO₂ Production Test on B0859.01

OBJECTIVE: To determine the rate and extent of the ultimate biodegradation of the test material.

JUSTIFICATION FOR TEST SYSTEM: Most of our products are disposed of through wastewater treatment systems where microorganisms can biodegrade the organic product components. Since activated sludge is a common wastewater treatment process and contains a variety of microbial species, it has been chosen to provide the inoculum for this test.

TEST MATERIAL:

Sample Code B0859.01 Color Colorless Form liquid

% Active 97% Density 0.9325 g/cm³ Solubility 4.2% in water(20°C)

Expiration Date in progress Other _____

TCO₂ 2.40 mg CO₂/mg active Theoretical TOC 0.66 mg TOC/mg active

Storage Conditions - room temperature

Safe Handling Precautions - harmful if inhaled or absorbed; irritant

The Sponsor accepts full responsibility for appropriate characterization and stability verification of this test material.

TEST MATERIAL PREPARATION/ADDITION:

(check one and indicate test concentrations)

[x] For compounds soluble or dispersible in water, prepare a stock solution or homogeneous suspension at 1,000 mg/L active ingredient by weight/volume dilution with ASTM Type II water¹ or equivalent. Determine the total organic carbon (TOC) concentration in the stock solution following laboratory's standard operating procedures. If the measured TOC is not within 15% of theoretical, contact the Principal Investigator before initiating the study. Store the stock solution under refrigeration for a maximum of three days prior to test initiation. If greater than three days elapses, another stock solution should be prepared. At test initiation, determine the pH of an aliquot of the stock solution. If outside the range of 4.0-10.0, adjust the pH of the stock solution to 7.0 (+1.0) with HCl or NaOH. To avoid contaminating the stock solution, aliquots taken for pH measurements should be discarded. Add the appropriate volume of the stock solution to the respective flasks to obtain 10 and 20 mg active/L test concentrations.

¹"Standard Specification for Reagent Water", ASTM Committee D-19 on Water, ASTM Designation D1193-74, June 27, 1974.

[] For compounds insoluble in water, add the appropriate amount of test material directly to the respective flasks on a [] weight (analytical balance) or [] volume (microliter syringe) basis to obtain ___ and ___ mg active/L test concentrations. Initial pH determinations are not required.

GLUCOSE PREPARATION/ADDITION:

Prepare a stock solution of standard reagent-grade glucose at 1,000 mg/L in ASTM Type II water or equivalent. Determine the TOC concentration of the stock solution. The criteria for acceptable use and storage requirements are the same as those given for the preparation/addition of soluble compounds. Initial pH determinations are not required. Add 40 ml of the stock solution to the respective flask to obtain 20 mg/L glucose.

TEST ORGANISMS: (check one; for acclimated inoculum, indicate test concentration and complete SCAS protocol information.

[] Acclimated inoculum obtained on the final day of test from SCAS units tested at 20 mg/L active ingredient. See Protocol: "Semi-Continuous Activated Sludge (SCAS) Removability Test on _____", Principal Investigator _____, Date _____.

[x] Unacclimated inoculum obtained from SCAS units that have not received any test material. These units should be maintained following laboratory's standard operating procedures.

The procedure for preparation of the inoculum is as follows:

Equal volumes of mixed liquor are collected from duplicate SCAS units and composited. The mixed liquor is then homogenized at room temperature for approximately two minutes at medium speed in a Waring blender or equivalent. This homogenized sample is poured into a beaker and allowed to settle for 15-30 minutes before the supernatant is carefully decanted. Carryover of sludge solids should be avoided since this may significantly increase background carbon and endogenous CO₂ production. Sufficient volumes of mixed liquor should be collected and treated at one time to provide enough inoculum for all flasks. Inoculum is used on the same day of preparation.

CO₂ Scrubbing Apparatus:

For a series of 12 flasks or less:

Five 1-liter plastic bottles filled with 700 ml 10 N NaOH

One 1-liter Erlenmeyer flask filled with 700 ml 0.024 N Ba(OH)₂ to serve as a CO₂ indicator trap

One empty 1-liter Erlenmeyer flask to prevent liquid carryover

These containers are connected in series with Tygon tubing to a pressurized air source (~5 psi) and air is sparged through the scrubbing solution at a constant rate. The flow rate is adjusted to insure that all test flasks are receiving CO₂-free air (see Test Procedure 7, page 3).

CO₂ PRODUCTION APPARATUS:

Erlenmeyer flasks are connected by tubing to the CO₂-free air source. Each flask is also connected by tubing to a series of three 4-oz. "French squares" to serve as Ba(OH)₂ traps. The flasks and traps are to be equipped with 2-hole rubber stoppers with solid plastic or glass tubing inserted to force the air through the test media and Ba(OH)₂ solutions.

TEST PROCEDURE

1. Testing is conducted in 4-L Erlenmeyer flasks. The final volume of medium + test material + inoculum in each flask is 2 liters.
2. The test medium is modified BOD water which contains, per liter of distilled water, the following standard BOD reagent solutions;
 - 1 ml of standard magnesium sulfate solution (Fisher #So-M-109 or equivalent)
 - 1 ml of standard calcium chloride solution (Fisher #So-C-10 or equivalent)
 - 2 ml of standard phosphate buffer (Fisher #So-P-341 or equivalent)
 - 4 ml of standard ferric chloride solution (Fisher #So-F-97 or equivalent)
 - 1 ml of a 4% (w/v) solution of $(\text{NH}_4)_2\text{SO}_4$.
3. Flasks containing the appropriate amount of test medium are aerated overnight with CO_2 -free air to purge the system of carbon dioxide.
4. After the aeration period, three CO_2 absorber bottles are filled with 100 ml of 0.024N $\text{Ba}(\text{OH})_2$ and connected in series to the exit air line of each flask. The $\text{Ba}(\text{OH})_2$ solution should be filtered through E&D 617 filter paper, or equivalent, before use. All bottles are to be filled from one $\text{Ba}(\text{OH})_2$ solution.
5. Test material is added to two of the four flasks to achieve the concentrations specified on page 1 or 2 under Test Material Preparation/Addition. The third flask receives no test material (blank) and the last receives glucose from the 1000 mg/L stock solution to achieve the final concentration of 20 mg/L.
6. Each flask receives a 1% inoculum (10 ml/L) of the activated sludge preparation. A bacterial plate count is performed on the sludge preparation following laboratory's standard operating procedures to ascertain viability of the test organisms. Plates are incubated at test temperature. The sludge preparation is thoroughly mixed before taking aliquots for inoculation and enumeration.
7. The test is started by aerating the headspace of each flask at 50-100 ml/min (2-4 bubbles/sec in the $\text{Ba}(\text{OH})_2$ traps). The CO_2 produced in each carboy reacts with the $\text{Ba}(\text{OH})_2$ and precipitates as BaCO_3 . The amount of CO_2 produced is determined by titrating the remaining $\text{Ba}(\text{OH})_2$ with 0.05N standardized HCl. Periodically, (every 2 or 3 days or before BaCO_3 is observed in the second trap) the CO_2 absorber nearest the flask is removed for titration.

The remaining two absorbers are each moved one place closer to the test flask, and a new absorber with 100 ml of 0.024N $\text{Ba}(\text{OH})_2$ is placed at the far end of the series. With every change of traps, an extra 100 ml of $\text{Ba}(\text{OH})_2$ solution is titrated to allow CO_2 production from the blank test flask (no test material) to be monitored. All respective absorbers within a test are titrated on the same day. All respective absorber bottles are to be filled from one $\text{Ba}(\text{OH})_2$ solution.
8. The test is conducted for 25 days. If CO_2 production in either the test flasks or the glucose flask does not reach a plateau by this time, contact the Principal Investigator before terminating the study. If CO_2 production in the glucose flask does not plateau at $>70\%$ of theoretical, the acceptability of the study should be discussed in the final report.

9. After CO_2 production reaches a plateau, the pH of each flask is measured and 1 ml of concentrated HCl is added to each flask to drive off inorganic carbonate. The flasks are aerated overnight and the final titration is made the following day.
10. Titrations of the $\text{Ba}(\text{OH})_2$ solution are made after removing the bottles closest to the flasks. The entire bottle is emptied into a 400 ml beaker and titrated to a phenolphthalein end point with standardized 0.05N HCl (Fisher #CS-126-1 or equivalent) from a 50 ml burett. Back titration with standard 0.05N NaOH (Fisher #So-S-278 or equivalent) from a 5 ml burett is performed if over-titration occurs. The amount of base used in the back-titration is subtracted from the volume of acid titrated to get a corrected figure.
11. After acidification and overnight aeration, the amount of soluble organic carbon remaining in each flask is determined following laboratory's standard operating procedure.
12. Temperature must not fall below 20°C , exceed 28°C , or vary more than 4°C during the test period as measured in a flask containing 2 liters of water in close proximity to the test. Flasks should not be in direct sunlight and room lighting should only be on during daily maintenance. Flasks are agitated on a rotary platform shaker at 110 ± 10 rpm for the duration of the test.

CALCULATIONS - STATISTICAL ANALYSIS

1. Determine the amount of CO_2 produced by the test material and glucose for each day of titration by the following equation:

$$\text{mg CO}_2 = \text{ml titrant for blank trap} - \text{ml titrant for experimental trap} \\ (\text{test material or glucose}) \times 1.1$$
2. Determine the cumulative mg CO_2 produced for both test flasks and the glucose at each titration day.
3. Determine the cumulative % of theoretical CO_2 (TCO_2 given on page 1) for each titration day by the following equation:

$$\% \text{ TCO}_2 = \frac{\text{cumulative mg CO}_2 \text{ produced}}{(\text{mg material added}) (\text{TCO}_2)} \times 100$$

4. Computer analysis - The cumulative % TCO_2 values obtained on the respective days are analyzed by a non-linear regression analysis on the form:

$$y = a(1 - e^{-b(x-c)}) \text{ for } x > c \text{ or } 0 \text{ for } x \leq c$$

where

- a = asymptote of curve (% TCO_2)
- b = rate constant (day^{-1})
- c = lag time before CO_2 production occurs (day)
- x = days
- y = cumulative % TCO_2

The constants a, b and c, along with their associated 95% confidence intervals are generated for each concentration of test material and for the glucose control. Computer plots of the cumulative % TCO_2 vs. time data are also made for each test material concentration and the glucose control.

RECORDS TO BE MAINTAINED: All records necessary to reconstruct the study and to demonstrate adherence to the Protocol.

PROTOCOL CHANGES: If a change in the approved protocol becomes necessary, verbal agreement should be made between the Study Director and Sponsor's Principal Investigator. As soon as practical thereafter, this change and the reasons for it should be put in writing, approved by both persons, and attached to the protocol as an addendum.

REPORTING: The report is to be a typed document in triplicate, describing the results of the study and is to be signed and dated by the Study Director, Quality Assurance Officer and the Laboratory Manager. It is to include, but is not limited to, the following:

1. Identification of test material by sample code, percent active, TCO₂, Theoretical TOC, color, form and date received.
2. Procedures followed for test material preparation and addition.
3. Test material concentrations.
4. Reference to the Protocol (title, author and date) and addenda if made, Test Methods, and any analytical or standard operating procedures used.
5. Any Protocol deviations and their implications.
6. Reference to laboratory notebook or other file containing raw data.
7. Starting and ending dates of study.
8. Cumulative % TCO₂ on each day of analysis for all test units and a graphical representation of the results.
9. A summary of the statistical analyses including the % TCO₂ asymptote, rate constant, lag time, and R² values.
10. TOC analysis on stocks (including glucose) reported as mg carbon/mg active.
11. All final SOC results as mg SOC/L.
12. Temperature range recorded during test period.
13. Inoculum source.
14. Colony-forming units of original inoculum.
15. Description of the quality assurance methods used to insure the quality of the data.

ALTERNATE PRINCIPAL INVESTIGATOR R. H. Hall PHONE: (513) 530-3347

NOTED: J. W. Williams J.W. Williams 8/16/85 PHONE: (513) 245-2120
Operations/Logistics Date

APPROVED: K. G. Surowitz K.G. Surowitz 8/17/85 PHONE: (513) 530-3332
Principal Investigator Date

TO BE COMPLETED BY STUDY DIRECTOR:

Project No. - 85-030

Estimated Starting Date 8/28/85 Defined As: addition of test material

Estimated Reporting Date 11/15/85

Date Test Material Received 8/21/85

Approved Karl M. ... 8/23/85 PHONE: 715 692 3030
Study Director Date

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 1 of 2
PARS SECT. HD.
[Signature]
ORIG. SECT. HD.

Test Substance Identification Number (TSIN): B0859.01
Safety Test Request Number: BSETS- 85.05
Principal Investigator: K. G. Surowitz

Product or Ingredient: 2-ethyl-1,3-hexanediol (ETHED) Brand Notebook Ref: _____
Physical Description: clear, viscous liquid Solubility: 4.2% in water (20°C) pH: 7.1
Recommended Storage Conditions: room temperature Expiration Date: in progress
Hazards (i.e. flammability, toxic gases): harmful if inhaled or absorbed; irritant
Dept. of Transportation Hazard Classification: non-hazardous CAS No. ^(a): 94-96-2

Formulated Composition (b)

<u>Component (c)</u>	<u>Mol. Wt.</u>	<u>Nominal Level (% by Wt.)</u>	<u>Acceptable Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Num (NB-Ref.)</u>
2-ethyl-1,3 hexanediol (isomer mixture)	146.23	97%			Aldrich	3007LL

- (a) Include CAS number(s) for the three most major components of a formulation or for single chemical products. Footnote to the material with which the respective number is associated.
- (b) If information requested is not known, then the symbol NK will be entered.
- (c) Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-chemical names, such as Tergitol 15-S-0 or Yellow Dye #10, may not be acceptable but should be previewed with the responsible toxicologist. Nondefinitive identification (e.g. Arquad, BC base) is not acceptable.

The above information provided by: KG Surowitz *[Signature]* 7/15/8
(Name) (Signature) (Date)

The above information reviewed and accepted by:

Principal Investigator: KG Surowitz *[Signature]* 7/15/8
(Name) (Signature) (Date)

TSCR1

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 2 of 2

Test Substance Identification Number (TSIN): B0859.01

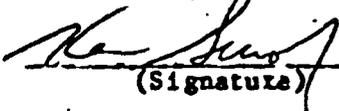
Analyzed Composition

<u>Date Submitted</u>	<u>Submitter Code</u>	<u>Analysis Code/Analysis</u>	<u>Estimated Value</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
-----------------------	-----------------------	-------------------------------	------------------------	-----------------------	---------------------------

Commercial sample (Aldrich Chemical Co.) No Analysis performed.

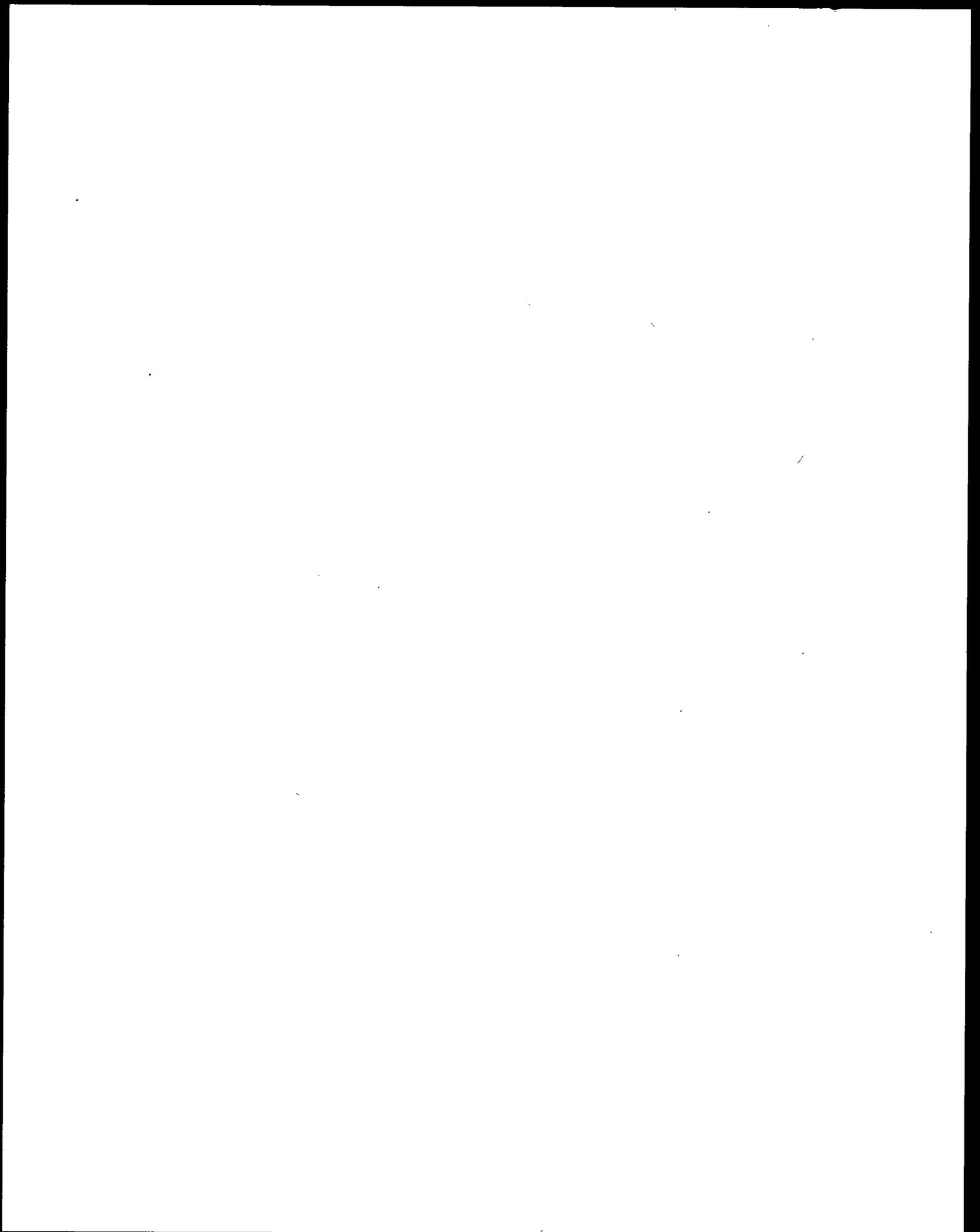
Analytical Information Verified By: _____ Date: _____
(Signature)

This test substance is suitable for environmental (non-clinical) safety testing.

Principal Investigator:  Date: 7/12/85
(Signature)

This test substance is suitable for human (clinical) safety testing.

Principal Investigator: _____ Date: _____
(Signature)



Test Substance

CAS Number: 94-96-2
Identity: 2-Ethyl-1,3-Hexanediol
Purity: 97% active
Remarks: Test substance identified as B0859.01

Method

GLP: Yes
Report/Study Year: 1985
Method/Guideline Followed: U.S. EPA, 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. Ecological Research Series (EPA-860/3-75-009).

Test Type: Acute Invertebrate Toxicity – Static

Species: *Daphnia magna* ≤ 24-hours old

Exposure Period: 48-hour

Media: Freshwater – fortified well water
Total hardness as CaCO₃: 160 mg/L
Total alkalinity as CaCO₃: 120 mg/L
pH: 8.0
Specific Conductivity: 600 µmhos/cm
Test Temperature: 21°C

Treatments: 1000, 600, 360, 130, 78 mg/L of 2-Ethyl-1,3-Hexanediol based on 98.6% active. Material appeared to be in solution at all treatment levels.

Remarks: The test protocol states that the test material is 97%

Analytical Monitoring: No

Results:

Results: The 48-hour LC50 for *Daphnia magna* was calculated by non-linear interpolation to be 720 mg/L with a 95% confidence interval calculated by binomial probability of 600-1000 mg/L.

Remarks: Test organism surfacing was observed at all test concentrations. Report referenced test substance purity at 98.6%, but test substance characterization mentions 97%. This minor deviation did not appreciable affect the study.

Data Quality

Reliability (Klimisch): 1

Reference

Report/Study Number: BW-85-11-1884
Source Reference: The Procter & Gamble Company, 1985. Acute Toxicity of B0859.01 to *Daphnia magna*. Accession #32066

Acc # 32066

.01

ACUTE TOXICITY OF B0859.01
TO Daphnia magna

TOXICITY TEST REPORT
SUBMITTED TO
THE PROCTER & GAMBLE COMPANY
CINCINNATI, OHIO

BIONOMICS REPORT #BW-85-11-1884
BIONOMICS STUDY #1011-0885-6142-110

Springborn Bionomics, Inc.
Aquatic Toxicology Laboratory
790 Main Street
Wareham, Massachusetts
December, 1985

*Rec'd 12-16-85
by Oper. Section*

1

SUMMARY

48-Hour Static Toxicity Test: Freshwater
Invertebrates

Springborn Bionomics, Inc.
Aquatic Toxicology Laboratory
790 Main Street
Wareham, Massachusetts

CLIENT: The Procter & Gamble Company

PROTOCOL CITED: Static Acute Freshwater Invertebrate
Toxicity Study of B0859.01; Principal
Investigator, K.W. Surowitz, 8/1/85.

REPORT NUMBER AND DATE: #BW-85-11-1884, November, 1985

STUDY NUMBER: 1011-0885-6142-110

MATERIAL: B0859.01

DESCRIPTION: a clear-colorless liquid, tested as 98.6%
active ingredient

DATE MATERIAL RECEIVED: 20 August 1985

TEST DATE: 2-4 October 1985

SPECIES: Daphnia magna

Age: <24 hours old

source: Bionomics culture facility

DILUTION WATER: fortified well water

Total hardness as CaCO₃: 160 mg/L

Total alkalinity as CaCO₃: 120 mg/L

pH: 8.0

Specific conductivity: 600 umhos/cm

TEST TEMPERATURE: 21°C

METHOD OF TEST MATERIAL ADDITION : stock solution.
100 mg of B0859.01/mL distilled water.

TEST CONCENTRATIONS: 1000, 600, 360, 220, 130, 78
mg/L of B0859.01

RESULTS: Results are reported as nominal concentrations of B0859.01 based on 98.6% active ingredient. The 48-hour LC50 was calculated by non-linear interpolation to be 720 mg/L with a 95% confidence interval calculated by binomial probability to be 600-1000 mg/L.

INTRODUCTION

The purpose of this study was to estimate the acute toxicity of B0859.01 to daphnids (Daphnia magna) under static conditions. A 48-hour definitive test was conducted from 2-4 October 1985, at the Aquatic Toxicology Laboratory of Springborn Bionomics, Inc., Wareham, Massachusetts. All raw data generated are stored at the above location.

MATERIALS AND METHODS

The B0859.01, a clear-colorless liquid tested as 98.6% active ingredient, was received from The Procter & Gamble Company, Cincinnati, Ohio, on 20 August 1985. Nominal test concentrations are reported as milligrams of B0859.01 active ingredient per liter of solution (mg/L).

Procedures used in this acute toxicity study followed those described in "Methods for acute toxicity tests with fish, macro-invertebrates, and amphibians" (U.S. EPA, 1975) and the protocol entitled "Static Acute Freshwater Invertebrate Toxicity Study of B0859.01" (K. W. Surowitz, Principal Investigator, 1 August 1985).

The daphnids used in this toxicity test were obtained from

laboratory stocks cultured at Springborn Bionics, Inc., Wareham, Massachusetts. The water was prepared by fortifying well water based on the formula for hard water (U.S. EPA, 1975) and filtering it through carbon and Amberlite XAD-7 resin columns to remove any potential organic contaminants. This water had total hardness and alkalinity ranges as calcium carbonate (CaCO_3) of 160-180 mg/L and 110-130 mg/L, respectively, a pH range of 7.9-8.3, a temperature of $20 \pm 1^\circ\text{C}$, a dissolved oxygen concentration of greater than 60% of saturation and a specific conductance range of 400-600 micromhos per centimeter (umhos/cm).

The daphnid culture area received a regulated photoperiod of 16 hours of light and eight hours darkness. Light at an intensity of 5-10 hectolux at the culture solutions' surface was provided by a combination of Sylvania Growlux^R and Cool White^R fluorescent bulbs. Daphnids were fed a solution of green algae (Ankistrodesmus sp.) and yeast suspension once daily. The air temperature was controlled to maintain the culture solutions at $20 \pm 1^\circ\text{C}$.

A sodium lauryl sulfate reference test was conducted with the daphnid test population from 19-21 September 1985. The 48-hour LC50 and 95% confidence interval was 6.7 (5.6-8.3) mg/L (Reference Test Log).

The toxicity test was conducted in 250-milliliter (mL) glass beakers. The dilution water used (#IRC 499, IWQ-6 Log Book) was

from the same source as the culture water previously mentioned and was characterized as having a total hardness and alkalinity of 160 and 120 mg/L as CaCO₃, respectively, a pH of 8.0 and a specific conductivity of 600 umhos/cm.

A cloudy stock solution of 100 mg/mL was formulated by dissolving 5.070 grams of B0859.01 in distilled water to volume in a 50-mL volumetric flask. Test solutions were formulated by adding the appropriate volumes of the stock solution to dilution water to total 1000 mL. Each solution was mixed on a magnetic stirrer for one minute and then divided into three beakers to provide replicate exposure treatments each containing 200 mL. The excess 400 mL of test solution was discarded. The test solution depth was 6.2 centimeters (cm) with a surface area of 33 cm². Three control beakers containing the same dilution water and maintained under the same conditions as the exposure concentrations, but containing no B0859.01, were established. The air temperature in the laboratory was controlled in order to maintain test solution temperature at 20 ± 1°C. Test solutions were not aerated. The test area was illuminated with Sylvania Growlux^R and Cool White^R fluorescent lights at an intensity of 7 hectolux at the solutions' surface. The photoperiod during the test was the same as in the culture area.

Fifteen daphnids, <24 hours old, were impartially distributed to each concentration (five daphnids per replicate) within 15 minutes after the test solutions had been prepared.

Daphnids were not fed during the exposure. Mortalities in replicate test solutions were recorded at 24 and 48 hours of exposure. Biological observations and observations of physical characteristics of each replicate test solution were also made and recorded at 0, 24, and 48 hours. The pH's and dissolved oxygen concentrations were measured at 0 and 48 hours in one replicate vessel of each test concentration and the control. Temperature was measured in one replicate vessel of the control at 0, 24, and 48 hours of exposure.

Total hardness concentrations presented in this report were measured by the EDTA titrimetric method and total alkalinity concentrations were determined by potentiometric titration to an endpoint of pH 4.5 (APHA et al., 1985). Specific conductivities were measured with a Yellow Springs Instrument Company (YSI) Model #33 salinity-conductivity-temperature meter and probe; the pH's were measured with an Instrumentation Laboratory Model #175 pH meter and combination electrode; the dissolved oxygen concentrations were measured with a YSI Model #57 dissolved oxygen meter and probe and the temperatures were measured with a Brooklyn alcohol thermometer. Light intensity was measured with a General Electric type 213 light meter.

Statistics

The concentrations tested and the corresponding mortality data derived from the toxicity test were used to estimate 24- and 48-hour median lethal concentrations (LC50) and 95% confi-

dence intervals. The LC50 is defined as the concentration of the test material in dilution water which caused mortality of 50% of the test animal population at the stated exposure interval. If sufficient toxicant-related mortality occurred during the test (e.g., presence of at least one test concentration causing mortality of $\geq 50\%$ of the animals in the test population), then a computer program (Stephan, 1982, personal communication) was used to calculate the LC50 values. The computer program scanned the data base, identified the most appropriate statistical methods and performed the analysis. Three statistical methods, in the following order of preference, were available in the computer program: moving average angle analysis, probit analysis, binomial probability (Stephan, 1977). Binomial probability method calculates only the 95% confidence interval. In such a case, a point estimate of the LC50 is obtained by non-linear interpolation (i.e., logarithm transformation of the concentration and the angle transformation of the percent dead) (Stephan, 1982). The method selected was determined by the above order of preference and by the characteristics of the data base (e.g., presence or absence of several test concentrations causing mortality of a partial number of animals in the respective test population).

RESULTS

The concentrations tested, corresponding mortalities and observations made during the test are presented in Table 1. The 48-hour LC50 for D. magna exposed to B0859.01 was calcu-

lated by non-linear interpolation to be 720 mg/L with a 95% confidence interval calculated by binomial probability of 600-1000. Table 2 summarizes the 24-hour and 48-hour LC50 values. The results of the water quality analysis are presented in Table 3.

LITERATURE CITED

APHA, AWWA, WPCF. 1985. Standard methods for the examination of water and wastewater. 16th Edition, Washington, D.C., 1268 pp.

U.S. EPA. 1975. Methods for acute toxicity tests with fish, macroinvertebrates, and amphibians. Ecological Research Series (EPA-660/3-75-009), 61 pp.

Table 1. Concentrations tested, corresponding cumulative mortalities and observations made during the 48-hour exposure of daphnids (*Daphnia magna*) to B0859.01

Nominal concentration (mg/L)	Cumulative Mortality (%)							
	24-hour				48-hour			
	A	B	C	X	A	B	C	X
2800	100	60	80	80 ^a	100	100	100	100
600	0	0	0	0 ^b	20	0	20	13 ^a
360	0	0	0	0	0	0	0	0 ^a
220	0	0	0	0 ^c	0	0	0	0 ^a
130	0	0	0	0 ^b	0	0	0	0 ^a
78	0	0	0	0 ^b	0	0	0	0 ^a
control	0	0	0	0	0	0	0	0

^aAll surviving daphnids were at the surface of the test solutions.

^bSeveral daphnids were at the surface of the test solutions.

^cOne daphnid was at the surface of the test solution.

Table 2. The estimated 24- and 48-hour LC50 values for daphnids (*Daphnia magna*) exposed to B0859.01

24-hour	LC50 ^a (mg/L)	48-hour
850 (600-1000)		720 (600-1000)

^aLC50 values were estimated by non-linear interpolation, 95% confidence intervals were calculated by binomial probability.

Table 3. The pH's, dissolved oxygen concentrations and temperatures measured during the 48-hour exposure of Daphnia magna to B0859.01

	Nominal concentration (mg/L)	0-hour	24-hour	48-hour
pH	1000	7.6	---	7.9
	600	7.5	---	7.9
	360	7.5	---	7.9
	220	7.5	---	7.9
	130	7.4	---	7.9
	78	7.4	---	7.9
	control	7.3	---	7.8
Dissolved oxygen (mg/L)	1000	8.3 (92) ^a	---	7.3 (81)
	600	8.3 (92)	---	7.2 (80)
	360	8.3 (92)	---	7.1 (79)
	220	8.3 (92)	---	7.0 (78)
	130	8.3 (92)	---	7.0 (78)
	78	8.3 (92)	---	7.0 (78)
	control	8.3 (92)	---	7.2 (80)
Temperature (°C)	control	21	21	21

^aPercent of saturation.

^bMeasurement not required at stated time interval.

The data contained in this report were audited by the Quality Assurance Unit to assure compliance with the protocols, standard operating procedures and the pertinent EPA Good Laboratory Practice Regulations on the following dates: 10 and 13 November and 10 December 1985. If discrepancies were found, reports were made immediately to the Study Director and management. It is the opinion of this Unit that these data accurately reflect the raw data generated during this study.

R. E. Bentley 16 Dec. '85
Robert E. Bentley
Director, Quality Assurance and
Special Projects

SUBMITTED BY:

Springborn Bionomics, Inc.
Aquatic Toxicology Laboratory
790 Main Street
Wareham, Massachusetts
December, 1985

STUDY DIRECTOR:

Richard B. Nicholson

Richard B. Nicholson 12/13/85
Aquatic Toxicologist

APPROVED BY:

Donald C. Surprenant

DC Surprenant 12/12/85
Director, Aquatic Toxicology

DATA AUDITED BY:

Robert E. Bentley

R. E. Bentley 10 Dec 85
Director, Quality Assurance Unit

R-5

Laboratory Study No. 1011.0885-0142-110

PROTOCOL

SPONSOR: The Procter & Gamble Company; Cincinnati, Ohio
Springborn Bionomics, Inc.

LABORATORY: 790 Main St.
Wareham, MA 02571

TITLE: Static Acute Freshwater Invertebrate Toxicity Study of B0859.01

OBJECTIVE: To determine the 48 hr LC₅₀ of the test material to a freshwater invertebrate species.

JUSTIFICATION FOR TEST SYSTEM: Daphnia magna is a readily available freshwater invertebrate species, on which a large amount of toxicity data exists, and is recommended by the USEPA¹ as a standard test organism.

TEST MATERIAL:

Sample Code B0859.01 Color colorless Form liquid

% Active 97% Density 0.9325 g/cm³ Solubility 4.2% in water (20°C)

Expiration Date in progress Other _____

Storage Conditions - room temperature

Safe Handling Precautions - Caution: Harmful if inhaled or absorbed; irritant.

The Sponsor accepts full responsibility for appropriate characterization and stability verification of this test material.

TEST MATERIAL ADDITION/PREPARATION: All calculations and measurements are to be based on the active ingredient. The maximum concentration to be tested is 1000 mg/L active ingredient.

TEST ORGANISM:

Species - Daphnia magna

Handling and Preparation - On the day preceding test initiation, reproductively mature adults are to be isolated from any young to assure only adult sizes are present in the stock cultures. The young produced by these isolated adults are to be removed from the stock cultures and used for testing. The maximum time to initiate a test following isolation is 36 hours.

TEST CONTAINERS: 250 ml glass beakers containing 200 ml of test solution. Beakers are to be covered with glass or clear plastic during the test period to minimize evaporation.

¹USEPA, Committee on Methods for Toxicity Tests with Aquatic Organisms (1975) Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians. EPA-3-75-009.

DILUTION WATER: Water of the same characteristics as the water normally used for culturing Daphnia in the laboratory.

TEST CONDITIONS:

- (1) Maintain temperature at $21 \pm 1^{\circ}\text{C}$.
- (2) The dissolved oxygen concentration in each test container must be at least 90% of saturation on Day 0.
- (3) A control and at least five concentrations of the test material are to be used. Except for the control, the concentration of test material in each treatment must be at least 56% of the next higher one.

OPERATION:

Five Daphnia are randomly assigned to each test container, following the laboratory's standard operating procedure, within 30 minutes after the addition of the test material. A minimum of three test containers are to be used for each test concentration and the control. One treatment must have killed or affected more than 65% of the Daphnia, and one treatment (not the control) must have killed or affected less than 35% of the Daphnia. The test is conducted for 48 hours, commencing when the test Daphnia are first exposed to the test material. Daphnia are not to be fed during the test period.

If any additive (e.g. solvent) is used to solubilize the test material, another control containing the greatest amount of solvent present in any other container is also required. A test is not valid if there is a greater than 10% mortality in either control.

WATER CHEMISTRY: Dissolved oxygen (DO) and pH are measured at 0 and 48 hours in at least one replicate for all test concentrations, including the controls. The 0 hour measurements are taken prior to the addition of the test organisms. If 100% mortality is observed in a test concentration at 24 hours, determinations are to be made at that time, but further determinations for that concentration are not required.

SPECIAL INSTRUCTIONS (If any, for operation, sampling, analyses, or monitoring):

RANGE-FINDING TEST: To be conducted at the discretion of the laboratory unless otherwise specified.

OBSERVATIONS: Mortality observations are to be made and recorded at 24 and 48 hours for all test containers, including the control. The criteria for death is the lack of reaction to gentle prodding.

CALCULATIONS: Test results are used to calculate the 48 hour LC_{50} . The 24 hour LC_{50} is calculated when possible. The LC_{50} is defined as the calculated concentration of the test material which causes 50% mortality in populations of test organisms at the specified time of exposure. Results are to be calculated and reported on the basis of added (nominal) test concentrations or from concentrations confirmed by actual analysis, if requested.

RECORDS TO BE MAINTAINED: All records necessary to reconstruct the study and demonstrate adherence to the Protocol.

PROTOCOL CHANGES: If a change in the approved protocol becomes necessary, verbal agreement should be made between the Study Director and Sponsor's Principal Investigator. As soon as practical thereafter, this change and the reasons for it should be put in writing, approved by both persons, and attached to the protocol as an addendum.

REPORTING: The report is to be a typed document in triplicate, describing the results of the study and is to be signed and dated by the Study Director, Quality Assurance Officer and Laboratory Manager. It is to include, but is not limited to, the following:

- 1) Identification of test material by sample code, percent active, color, form, and date received.
- 2) Procedures followed for test material preparation and addition.
- 3) Reference to laboratory notebook or file containing raw data.
- 4) Date definitive test was conducted.
- 5) Species tested, including the control.
- 6) Percentage mortality in all test groups including the control.
- 7) The calculated LC₅₀ values, 95% confidence intervals, and a reference to the method used to calculate these values.
- 8) Description of dilution water used, including a range of the measured pH, hardness, alkalinity, and conductivity.
- 9) All temperature, pH, and DO determinations and all visual observations.
- 10) Laboratory Study Number.
- 11) Reference to the Protocol (title, author, and date) and addenda if made, and any analytical procedures used.
- 12) Any Protocol deviations and their implications.
- 13) Results of reference toxicant and date conducted.
- 14) Description of the quality assurance methods used to insure the quality of the data.

ALTERNATE PRINCIPAL INVESTIGATOR R. H. Hall PHONE: (513) 530-3347

NOTED: J. W. Williams J.W. Williams 8/1/85 PHONE: (513) 530-2120
Operations/Logistics Date

APPROVED: K. G. Surowitz K.G. Surowitz 8/1/85 PHONE: (513) 530-3332
Principal Investigator Date

TO BE COMPLETED BY STUDY DIRECTOR:

Study No. 1011-0885-6142-110

Estimated Starting Date 13 September 1985

Estimated Reporting Date 15 November 1985

Date Test Material Received 21 August 1985

Approved Richard B. Kuehler 8/21/85 PHONE: (617) 295-2550
Study Director Date

KGS005/pcj
8/1/85

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 1 of 2
PASS SECT. NO.
ORIG. SECT. NO.

Test Substance Identification Number (TSIN): 80839.01
Safety Test Request Number: BSRS- 85.05
Principal Investigator: E. C. Surowitz

Product or Ingredient: 2-ethyl-1,3-hexanediol (ETHED) Brand Notebook Ref:
Physical Description: clear, viscous liquid Solubility: 4.2% in water (20°C) pH: 7.1
Recommended Storage Conditions: room temperature Expiration Date: in progress
Hazards (i.e. flammability, toxic gases): harmful if inhaled or absorbed; irritant
Dept. of Transportation Hazard Classification: non-hazardous CAS No. 94-96-2

Formulated Composition (b)

<u>Component (c)</u>	<u>Mol. Wt.</u>	<u>Nominal Level (X by Wt.)</u>	<u>Acceptable Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Number (NB-Ref.)</u>
<u>2-ethyl-1,3 hexanediol (isomer mixture)</u>	<u>146.23</u>	<u>97%</u>			<u>Aldrich</u>	<u>3007LL</u>

- (a) Include CAS number(s) for the three most major components of a formulation or for single chemical products. Footnote to the material with which the respective number is associated.
- (b) If information requested is not known, then the symbol NK will be entered.
- (c) Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-chemical names, such as Tergitol 15-S-0 or Yellow Dye #10, may not be acceptable but should be previewed with the responsible toxicologist. Nondescriptive identification (e.g. Arquad, BC-base) is not acceptable.

The above information provided by: KG Surowitz (Name) [Signature] (Signature) 7/1/68 (Date)

The above information reviewed and accepted by:
Principal Investigator: KG Surowitz (Name) [Signature] (Signature) 7/1/68 (Date)

TSCR1

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 2 of 2

Test Substance Identification Number (TSIN): B0859.01

Analyzed Composition

<u>Date Submitted</u>	<u>Submitter Code</u>	<u>Analysis Code/Analysis</u>	<u>Estimated Value</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
-----------------------	-----------------------	-------------------------------	------------------------	-----------------------	---------------------------

Commercial sample (Aldrich Chemical Co.) No Analysis performed.

Analytical Information Verified By: _____ Date: _____
(Signature)

This test substance is suitable for environmental (non-clinical) safety testing.

Principal Investigator: *[Signature]* Date: 7/15/85
(Signature)

This test substance is suitable for human (clinical) safety testing.

Principal Investigator: _____ Date: _____
(Signature)

KG8001/pcj
7/12/85

Test Substance

CAS Number: 94-96-2
Identity: 2-ethyl-1,3-hexanediol
Purity: Not stated

Method

GLP: No
Report/Study Year: 2004
Method/Guideline Followed: Mouse *in vivo* Skin Micronucleus Assay (based on Nishikawa et al, Mutation Research 444 (1999) 159-166)
Test Type: Mouse *in vivo* skin micronucleus
Species: Mouse
Strain: SKA-1
Sex: Male (3/group)
Route of administration: Dermal
Exposure period: Up to 5 days.
Doses: Maximum amount applied - 0.1 mL/6cm² as indicated below.

Preliminary Study: 2.5, 5, 10, 20 and 40% test substance in ethanol. Negative control – ethanol. Positive control – vinblastin sulfate (VB) (0.05%) in ethanol

Main Study: 10, 20 and 40% test substance in ethanol, negative control (ethanol) and positive control (VB in ethanol).

Remarks: Hairless mice were treated two times at 24 hour intervals. The highest dose tested was based on the maximum soluble dose. Twenty-four hours after the last treatment the animals were sacrificed and the skin collected. The skin was placed in cold trypsin overnight. The dermis and epidermis were separated and the cells dissociated by a gently stirring in complete medium. The cells were pelleted, rinsed and fixed, then placed on slides. The slides were stained with acridine orange and the number of micronuclei scored from 2000 cells/animal. Study design included positive and negative controls.

Results

Result: 2-ethyl-1,3-hexanediol (EHD) did not induce an increase in micronuclei (MN) in mouse skin after two applications.

Group (animal #)	#MN / group	MN/1000 cells
Ethanol	4/6000	= 0.67/1000
VB 0.05% in EtOH	34/6000	= 5.67/1000*
10% EHD	1/6000	= 0.17/1000
20% EHD	4/6000	= 0.67/1000
40% EHD	3/6000	= 0.5/1000

* significant with Dunnett's adjustment

Conclusion: The test substance is negative under the conditions of this assay.
Remarks: Responses of positive and negative controls were appropriate.

Data Quality

Reliability (Klimisch): 2

Reference

Laboratory Study Number: MVS-1482
Reference: Procter and Gamble, 2004. Testing 2-ethyl-1,3-hexanediol in the mouse *in vivo* skin micronucleus model. Notebook MVS-1482, pages 117-124. Accession # MVS-1482

Date 1-13-04

P&G Restricted

Subject Dosing Animals with 2-Ethyl-1,3-Hexanediol

Chemical 2 Ethyl-1,3-Hexanediol mixture of isomers 97%
 a clear liquid - viscous - soluble in Ethanol

Objective: To test the above chemical in the mouse
 SKIN IN VIVO micronucleus assay.

This chemical was chosen because it was
 reported as negative in SKIN carcinogenesis assay
 and Ames (-) but with CA+

Solubility: Solubility was determined by weighing
 ~4gms of material ^(app 4.48 gm) and adding
 ethanol and vortexing - sonicating until into
 solution.

Step 1 4gm add 1ml - insoluble
 add 1ml - insoluble
 " " "
 " " "
 " " "
 " " Soluble

So 4gms soluble in about what of 10mls containing
 the volume of ethanol & chemical this = a 40% sol.

Oral LD50 for this chemical in mice is ~47.5 g
 so this soluble dose is 84% of that oral LD50

Dose will be Ethanol
 UB 0.05%

Chemical	40%	2 animals for all groups
	20"	except Ethanol which
	10"	has 3
	5"	run
	2.5"	

Worker's Signature D. Miller

Date 1/13/04

Corroborating Witness

Date

Subject cont from 117 On 10/17/06

Prep of chemical continued

From 40% stock = A

1.5mls A + 1.5mls Ethanol = 20% = B

1.5" B + " " = 10% = C

1.5" C + " " = 5% = D

1.5" D + 1.5ml " = 2.5 = E

VB - made new stock here 10mg/bottle

add = 2mls Ethanol = 5mg/ml this is

not in solution after vortexing & sonicating

I made a 1:10 dilution of this 0.2mls + 1.8mls Ethanol

again I vortexed & sonicated by it still is

not in solution. But matches VB look from

p. with graph study which appeared to work

based on an inverse in MN.

Again each animal gets 0.1ml via area
of app. 2x3 cm.

All animals looked good after dosing
with no physical signs of distress

Supplies pitched to carcinogen waste after
dosing.

Worker's Signature

D. Hill

Date

10/17/06

Corroborating Witness

Date

120

Date _____

P&G Restricted

Subject Cent for 119 Oz 10/18/06

1.20.2004

Scoring Slide

Slides stained with acridine orange

1:50 dilution of stock for 1 min.

1st - places slide in PBS for 1 min then stain 1 min

then in 3 changes of Buffer - let air dry

then wet in buffer & mount with cover slip.

Control #1 3/2000

$9/4000 = 1.5/1000$

Control #2 3/4000

Control # 3/2000

VB1 0.05% 10/2000 $25/4000 = 6.25/1000$

VB2 0.05% 15/2000

2.5% #1 2/2000 $5/4000 = 1.25/1000$

2.5% #2 3/2000

5.0% #1 2/2000 $3/4000 = 0.75/1000$

5.0% #2 1/2000

10% #1 1/2000 $3/4000 = 0.75/1000$

10% #2 2/2000

20% #1 2/2000 $2/4000 = 0.50/1000$

20% #2 0/2000

40% #1 1/2000 $2/4000 = 0.50/1000$

40% #2 1/1000

Worker's Signature *[Signature]*

Date 2/12/04

Corroborating Witness _____

Date _____

Date 1-27-04

P&G Restricted

121

Subject Definitive Study with 2-ethyl-1,3-Hexanediol 97%

Objective: To determine potential of chemical above to induce micronuclei in skin of SKH-1 male mice

Animals are sweetened for this study

Dose levels same as pg 117 except VB used at 0.1%

EH Dose levels

40%

20%

10%

5%

25%

VB 0.1% 0.05% Dg 9/29/2004

Ethanol

Procedure same as for all studies

Dose Day 1

Dose Day 2-24hr later

Day 3 - Sacrifice animals collect skin - ^{Rinse} Place in 70% ethanol

Day 4 - Dissociate cells - fix and prepare cells slides

Prep of Chemical

Chemical for definitive study is weighed

1 weight 1.89gms need 40% top dose
 $\frac{4}{10} \times \frac{1.89}{x} = \frac{1.89}{4}$ 4.725 - 1.89 = Need 2.835gms of Ethanol

Ethanol Add - drop by drop until weight is ~ 4.73gms
Storing bar added and sample stored until ~ 30min before use.

For VB have 5mg/L Stock in Ethanol diluted

1:5 0.2mls + 0.8mls Ethanol = 1mg/ml = 0.1%

100mg/100ml = 0.1%

over

Worker's Signature

J. Hill

Date

10/17/04

Corroborating Witness

Date

122

Date 1-28-04

P&G Restricted

Subject Cont from 121

Right before dosing - other dilutions were made
 by 1.5 mls of 40% + 1.5 mls of Ethanol = 20% = B vortex
 1.5 mls of 20% + " " " = 10% = C vortex
 1.5 mls of 10% + 1.5 ml " = 5% D

Animals dosed at ~ 1:15-1:30 0.1 mls / animal

After dosing samples are discarded to carcinogen waste
 in 61A7.

The animals looked fine after dosing with no
 apparent ill effects from the chemical.

Animals are placed in Thoren Rack for this study

1-28-04 - Day 2 - dosing

Samples prepared again by wgt.

weigh - 1.7 gm of Chem-1

$$\frac{4}{6} \times \frac{1.7}{x} = \frac{1.7}{4} = 2.55 \text{ need } 2.55 \text{ gm ethanol}$$

$$\frac{1.7}{4.25} = 4.25 \text{ gm total}$$

Ethanol added to

scintillation vial with ~ 1.7 gm of Chemical until wt was
 ~ 4.25 gm = 40%

Next stir bar added - sample capped and sealed
 and placed on stirrer until ~ right before dosing.

VB prepared same as pg 121 (0.2 + 0.8)

All samples diluted with Ethanol

20%, 10% and 5% samples prepared exactly as on pg 121.

Animal dosed starting at ~ 1:10 pm 0.1 ml / in 6 cm area
 of mice skin. Again there did not appear
 to be any ill effects to mice from dosing.

After dosing samples discarded to carcinogen waste
 in 61A7. Animals still in Thoren Cage Single housed

Worker's Signature

D. Hill

Date

4-16-04

Corroborating Witness

Date

Date 1-29-04

P&G Restricted

Subject Cent fire 122

1-29-04

Animals were sacrificed by CO₂ asphy. and
 SKIN collected where dozed. Skin is clipped and
 placed in DPBS w Pen/Strep - 3 animals/ep/one 100mm
 Petri dish
 Animals placed in freezer for disposal or liquidation
 SKINS - taken in DPBS to 24075 and
 processed like this

1 - cut into small sections

2 - rinse in 3rd DPBS with Pen/Strep

3. 1st rt in DPBS with 0.02% EDTA for 5min.

4. place in cold DPBS with Pen/Strep and 2.5% Trypsin

5. place in corb overnight.

Next day ~~skin~~ skin has been rinsed
 then epidermis / dermis separated.

Then placed separated section into ~35ml total

Dmem + 10% FBS (pen/strep) stir at ~250rpm with stirring bar
 for at least 1hr. Pass filter through nylon mesh

then 40mm strainer Spin for 5min at ~1000rpm
 decant, add ~10ml fresh media transport - spin for 5min
 then decant add 0.075% KCl (pH 7.5) for at least 10min place

at 37°C. After ~10min add 2.5mls 3:1 media/serum + add
 spin & decant add ~10mls ^{col} 3:1 fix - spin

decant - resuspend cells add enough 3:1 to have
 cloudy suspension. make 3 dishes / animal
 let air dry

Worker's Signature

J. M.

Date

4.16.04

Corroborating Witness

Date

124

Date

P&G Restricted

Subject cont from 123 day 10/17/06

7-12-04

Control #1	1/2000	
#2	4/2000	1 cell - irregular - 1 MN not counted
#3	1/2000	

4/14/04

EAS #1	0/2000	
#2	1/2000	1 apoptotic cell
#3	1/2000	

EH10 #1	1/2000	
#2	0/2000	
#3	0/2000	

EH20 #1	1/2000	
#2	1/2000	
#3	2/2000	

EA40 #1	2/2000	
#2	1/2000	
#3	0/2000	

4-16-04

UB # 1	14/2000	
UB # 2	11/2000	
UB # 3	7/2000	

10/14/2005
wrong cell
day 0.16

Worker's Signature

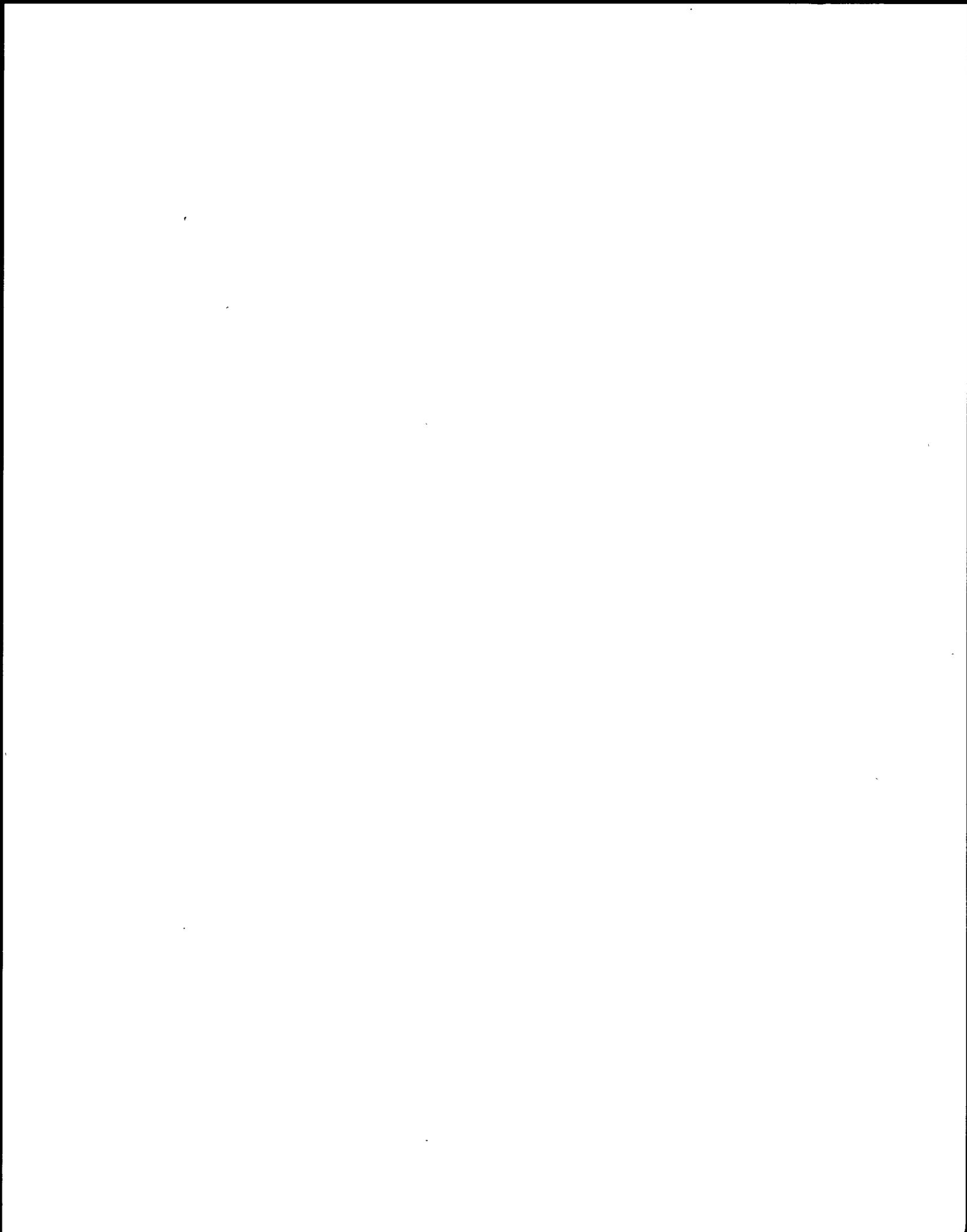
D. Del

Date

4-16-04

Corroborating Witness

Date



Test Substance

CAS Number: 94-96-2
Identity: 2-Ethyl-1,3-Hexanediol
Purity: 100%
Remarks: Test substance identified as B0941-01

Method

Method/Guideline Followed: Primary Eye Irritation Rabbit – Low Volume Method
GLP: Yes
Report Year: 1986
Species: Rabbit (New Zealand White)
Gender: Male/Female
Number of Animals: 6
Concentration: Undiluted
Remarks: 10 µL was placed directly on the cornea of one eye on each animal. There was no rinse in one group of 3 animals. There was rinse in other group of 3 animals.

Results

Results: Moderate to Severe Irritant
Remarks: 2-Ethyl-1,3-Hexanediol (10 ul, unrinsed) produce moderate to significant reversible irritation in 3/3 rabbits. Corneas (2/3), Iris (3/3), and conjuctiva (3/3) were affected, all eyes cleared by day 14. The maximum average score (MAS) for the unrinsed group was 20.3 on day 1. Rinsed eyes showed almost no irritation, one animal displayed conjuctival redness on day 1 only. The MAS was 0.7 on day 1, all eyes cleared by day 2.

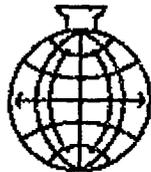
Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Report Number: 191-1215
Reference: Procter & Gamble, 1986. Rabbit Eye Irritation (Low Volume Procedure). Accession #31928

Acc # 31928

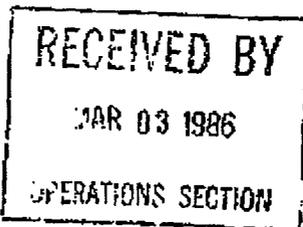


International Research
and Development Corporation

MATTAWAN, MICHIGAN, U.S.A. 49001 TELEPHONE (616) 668-3326

TO: The Procter and Gamble Company
SUBJECT: Rabbit Eye Irritation (Low Volume Procedure)
D&D NO: BSBTS 955
TSIN: B0941-01
REPORT NO.: 191-1215
DATE OF SUBMISSION: February 27, 1986

Reviewed and Accepted by BS&HCF/P&RS
W. Bruce Gibson Date: 3/20/86
W. B. GIBSON



191-1215



"credence through research"

Study No: 191-1215
Sponsor Ref: BSSTB 953

Rabbit Eye Irritation
(Low Volume Procedure)

Report of a biological test performed at:
International Research and Development Corporation
Marlaven, Michigan 49071

Deviation From
Protocol: None

During the period:
January 31, 1986 to February 14, 1986
According to the attached protocol
(P&G NO. C28)

Issue Date: November 20, 1985

Test Substance(s) (TS #)	Color	Physical Form	Storage Conditions
100-01	colorless	liquid	room temperature

Sponsor's Divisional Toxicologist: W. E. Gibson

Source and Strain of Animals Used: Kuiper's Rabbit Ranch, Gary, Indiana
New Zealand White rabbits

Concentration and Amount of Test Substance Dosed:
Administered undiluted as received, 10 mcl per right eye
(unwashed and washed), using a 100 mcl, Hamilton syringe.

RESULTS

TREATMENT GROUP	MAXIMUM AVERAGE SCORE/TIME	NUMBER OF EYES AFFECTED/TREATED			NUMBER OF DAYS FOR CLEARANCE*	MEDIAN NUMBER OF DAYS FOR CLEARANCE
		CORNEA	IRIS	CONJUNCTIVAE		
10 mcl/right eye, unwashed	20.3/DAY 1	2/3	3/3	3/3	14,14,14	14.0
10 mcl/right eye, washed	0.7/DAY 1	0/3	0/3	1/3	2,a,a	1.0

a - No irritation was observed; for the median number of days calculation,
a = the first observation interval reported in terms of days.
* All scores to return to zero; reported for individual animals.

Technical Supervisory Staff,

Acute Toxicology and Special Studies:

Stephen W. Allen, B.S.
Group Supervisor

Linda L. Fleetwood, B.A.
Unit Supervisor

PREPARED BY:

Brian T. Walker
Brian T. Walker, B.S.
Report Writer
Acute Toxicology and
Special Studies

2/24/86
Date

REVIEWED BY:

Dale E. Johnson
Dale E. Johnson, Pharm.D., Ph.D.
Associate Director, Division
of Toxicology

2/24/86
Date

STUDY DIRECTOR'S STATEMENT

The study used in IRAC Study Number 191-1215 followed the experimental criteria specified in the protocol.

To the best of my knowledge, there were no significant deviations from the Good Laboratory Practice Regulations which affected the quality or integrity of this study. This study was conducted in conformance with the Good Laboratory Practice Regulations. This report accurately reflects the raw data obtained during the performance of this study.

All data including the final study report are stored in the International Research and Development Corporation Archives, Mattawan, Michigan.

James R. Myer
James R. Myer, B.S.
Manager of Acute Toxicology and
Special Studies
Study Director

2/27/86
Date

191-1215

TABLE 1.
 B0941-01
 10 mcl/right
 eye, unwashed

RABBIT EYE IRRITATION

RABBIT NUMBER	OBSERV. TIME	CORNEA			IRIS		CONJUNCTIVAE			SCORE (A+B+C)*2
		A	B	SCORE AKBKS	A	SCORE AKS	A	B	C	
29391 M	DAY 1	0	0	0	1	5	3	2	2	14
	DAY 2	1	1	5	1	5	3	1	0	8
	DAY 3	1	1	5	1	5	2	1	0	6
	DAY 4	1	1	5	1	5	2	2	0	8
	DAY 7	0	0	0	0	0	1	1	0	4
	DAY 14*	0	0	0	0	0	0	0	0	0
29441 M	DAY 1	0	0	0	1	5	3	3	3	18
	DAY 2	1	1	5	0	0	3	2	3	16
	DAY 3	1	1	5	1	5	3	2	1	12
	DAY 4	1	1	5	0	0	3	2	0	10
	DAY 7	0	0	0	0	0	0	1	0	2
	DAY 14*	0	0	0	0	0	0	0	0	0
29398 F	DAY 1	0	0	0	1	5	3	2	2	14
	DAY 2	0	0	0	1	5	3	1	0	8
	DAY 3	0	0	0	1	5	3	2	0	10
	DAY 4	0	0	0	1	5	3	2	0	10
	DAY 7	0	0	0	1	5	3	1	0	8
	DAY 14*	0	0	0	0	0	0	0	0	0

*All scores zero; animal removed from study

TABLE 1. CONT.
60941-01
10 mcl/right
eye, unwashed

RABBIT EYE IRRITATION

<u>TIME</u> <u>PERIODS</u>	<u>TOTAL</u> <u>GROUP</u> <u>SCORE</u>	<u>GROUP</u> <u>AVERAGE</u> <u>SCORE</u>
DAY 1	61	20.3
DAY 2	52	17.3
DAY 3	53	17.7
DAY 4	48	16.0
DAY 7	19	6.3
DAY 14	0	0.0

MAXIMUM AVERAGE SCORE: 20.3/DA . 1

TABLE 1. CONT.

80941-01
10 mcl/right
eye, washed

RABBIT EYE IRRITATION

RABBIT NUMBER	OBSERV. TIME	CORNEA			IRIS		CONJUNCTIVAE			SCORE (A+B+C)/2
		A	B	SCORE AXBK5	A	SCORE AX5	A	B	C	
29469 M	DAY 1*	0	0	0	0	0	0	0	0	0
29380 F	DAY 1*	0	0	0	0	0	0	0	0	0
29400 F	DAY 1	0	0	0	0	0	1	0	0	2
	DAY 2*	0	0	0	0	0	0	0	0	0

TIME PERIODS	TOTAL GROUP SCORE	GROUP AVERAGE SCORE
DAY 1	2	0.7
DAY 2	0	0.0

MAXIMUM AVERAGE SCORE: 0.7/DAY 1

*All scores zero; animal removed from study

TABLE 2.
 BQ941-01
 10 ucl/right
 eye, unwashed

RABBIT EYE IRRITATION
 (OTHER FINDINGS)

RABBIT NUMBER	OBSERV. TIME	FINDINGS
29391 M	DAY 1	b, i, s(50%)
	DAY 2	d
	DAY 3	d, t(50%)
	DAY 4	d
	DAY 7	t(0%)
	DAY 14*	t(0%)
29441 M	DAY 1	a, d, s(50%)
	DAY 2	b, c, d
	DAY 3	b, c, d, t(50%)
	DAY 4	c, d
	DAY 7	t(0%)
	DAY 14*	t(0%)
29398 F	DAY 1	a, c, d, s(40%)
	DAY 2	h, s(40%)
	DAY 3	c, d, h, s(40%), t(15%)
	DAY 4	c, d, h, s(30%)
	DAY 7	c, h, t(>5%)
	DAY 14*	t(0%)

a - Purulent discharge
 b - Mucous discharge
 c - redness
 d - Blepharitis

h - Pannus
 s - Corneal dulling (% of area)
 t - Sodium fluorescein exam (% stained)

*All scores zero; animal removed from study

TABLE 2. CONT.

B0941-01
10 mcl/right
eye, washed

RABBIT EYE IRRITATION
(OTHER FINDINGS)

RABBIT NUMBER	OBSERV. TIME	FINDINGS
29469 M	DAY 1 *	
29380 F	DAY 1 *	
29400 F	DAY 1 DAY 2 *	

*All scores zero; animal removed from study

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION

PROTOCOL REVISION OR CLARIFICATION

Protocol Sheet No. 1 Study No. 191-1215 (IRDA BS&IS 950) (ISIRI B0941-01)

TITLE: RABBIT EYE IRRITATION (LOW VOLUME PROCEDURE)

Page 1 of 2

<u>ITEM</u>	<u>JUSTIFICATION</u>
1	Study initiation.
2	Identification of the test article.
3	Identification of the source and age of the test animals.
4	Clarification of statistical analysis.
5	Animal selection.
6	Identification of diet used.

<u>ITEM</u>	<u>PROTOCOL REVISION OR CLARIFICATION</u>
1	Conduct study in accordance with the attached protocol. The letter of authorization is considered part of the protocol.
2	The test article is identified as B0941-01, IRDC 8814.
3	The source of the rabbits will be Kuipers Rabbit Ranch, Gary, Indiana. The animals will be 3-4 months of age at study initiation.
4	No statistical analyses are required on this study, therefore, none will be performed.

Study Director James R. Myer, B.S.

James R. Myer . 1/28/86
Signature Date

INTERNATIONAL RESEARCH AND DEVELOPMENT CORPORATION

PROTOCOL REVISION OR CLARIFICATION

Protocol Sheet No. 1

Study No. 191-1215 (DRD# 9585 955)

(ISIR# 80941-01)

TITLE: RABBIT EYE IRRITATION (LOW VOLUME PROCEDURE)

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ITEM

PROTOCOL REVISION OR CLARIFICATION

5

Acceptable animals will be placed on study with the aid of a computer-generated random number table.

6

The diet used in the study is Certified Rabbit Chow® #5322, Ralston Purina Company.

Study Director James E. Hyer, B.S.

Signature

James E. Hyer

1/28/86

Date

PROTOCOL NO. 028

Rabbit Eye Irritation (Low Volume Procedure)

Issue Date: November 20, 1985
Supersedes Issue Dated: May 1, 1984

Test Substance Identification Number (TSIN) # 50941-01

Divisional Request Document Number (DRD) # 955

Sponsor: The Procter & Gamble Company
Cincinnati, Ohio

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

International Research
& Development Corporation
Mattawan, MI 49071

Study # 191-1215
(To be filled in by
Testing Facility)

Purpose: To determine the level of eye irritation of a test substance in the rabbit so that it may be compared with more familiar substances.

Justification for
Selection of Test
Animal:

The New Zealand albino rabbit is the animal of choice based on large orbit and nonpigmented iris.

Route of Administration
of Test Substance and
Reason for Choice:

Place the test substance directly on the cornea of one eye. The route of exposure and dose volume used in this procedure are more likely to approximate human accidental exposure and to be retained within the eye, eliciting responses that are more consistent with human experience than would larger dose volumes.

Diet and/or Water
Analysis Required:

None (no known contaminants expected which would interfere with this study)

Records to be
Maintained:

All records that would be required to reconstruct the study and demonstrate adherence to protocol.

Rabbit Eye Irritation (Low Volume Procedure)

Issue Date: November 20, 1965

Test Substance(s)

<u>TSIN #</u>	<u>DRD Number</u>	<u>Description</u>		<u>Expiration Date</u>
		<u>Color</u>	<u>Physical Form</u>	
B0941-c1	8075 965	colorless	liquid	12/6/66

Storage Conditions: (Check one)

Room temperature Refrigerator Freezer
 Other

Hazards: (Check one)

None known. Take ordinary precautions in handling.
 As follows: severe eye irritant

Instructions: (Check one)

None
 If two or more test substances will be tested, dose all comparable groups from all samples on the same day. If possible, dose all groups from all samples on the same day.
 If possible, use the same grade throughout the study.
 As follows:

Animals:

Rabbits, New Zealand albino of either sex weighing ≥ 1.5 kg.

note only date 11/30/65.

avg
11/30/65

(Check one)

Six (6) animals divided into two test groups; three (3) no rinse, three (3) rinse
 Three (3) animals per test group, no rinse
 Six (6) animals per test group, no rinse
 Nine (9) animals divided into two test groups: six (6) no rinse, three (3) rinse

Animal Care:

Follow the approved Standard Operating Procedures of the Test Facility. (Acclimation period must be a minimum of seven (7) days.)

Environmental Conditions:

Follow the approved Standard Operating Procedures of the Test Facility.

AnimalIdentification:

Follow the approved Standard Operating Procedures of the Test Facility.

Rabbit Eye Irritation (Low Volume Procedure)

Issue Date: November 20, 1985

Pre-Dose Requirements: Examine eyes prior to testing using the Standard Operating Procedures of the Test Facility. Use only animals showing no eye abnormalities. One eye of each animal is used for test and the other eye for control.

Dose Preparation: (Check appropriate box)

(1) Liquids

Dose test substance as received using a 100 µl Hamilton or equivalent 100 µl microsyringe (without a needle or other fittings). Fill microsyringe with a 10 µl volume of sample. Dose one animal. Repeat this procedure for remaining animals. Use a clean microsyringe for each test substance. Specify manufacturer of microsyringe in the report.

Dose according to special instructions (page 2)

(2) Solids

Dose as described below

Dose according to special instructions (page 2)

1. Measure a convenient volume (at least 2 ml) of the solid granules or powder in a small graduate cylinder. Do not grind. (Tap gently, record the weight in grams and the volume in milliliters.) Calculate the bulk density (d) of the test substance to the nearest 0.1 gm/ml using the following calculation:

$$\frac{\text{Weight of test substance (gm)}}{\text{Volume of test substance (ml)}} = \text{density (d)}$$

2. Calculate the weight of test substance which occupies a 10 µl volume, using the following calculation:

$$\text{density (d)} \times 10 = \text{X mg of test substance to be used in the eye}$$

3. Grind a representative sample of test substance with a mortar and pestle. Sieve ground substance through a #40-mesh sieve to assure adequate grinding before weighing for dosing. The entire sample aliquot must be sufficiently ground to pass through the sieve.

The quantity of test substance equivalent to a 10 µl volume is weighed to at least 1% accuracy using a suitable analytical balance.

Rabbit Eye Irritation (Low Volume Procedure)

Issue Date: November 20, 1985

Dose Preparation (Cont'd):Note

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

If a concentration analysis is required:

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

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 _____.

Instillation Instructions:

The eyelids are held gently open and 10 ul, or weight equivalent, of test substance is placed directly on the cornea of one eye of each rabbit. The eyelid is released immediately after instillation without forced blinking or manipulation. If test substance was or may have been incorrectly dosed, i.e., test substance was placed on palpebral conjunctiva or nictitating membrane, replace the rabbit and properly administer the test substance. Document all replacements and state reason(s) for replacement. If rinsing is required, rinse approximately four (4) seconds after application of the test substance by spraying 20 ml of lukewarm water from a hypodermic syringe, fitted with a snubbed 16- or 18-gauge needle, into the eye under moderate pressure. Observe and record whether or not the animals exhibit any response indicative of discomfort, e.g., squealing, etc., upon instillation of substance.

Observations:

Examine the eyes for corneal opacity, iritis, and conjunctivitis, and score the treated eyes. Report results according to the method of Draize⁸ (Appendix 1). Score at one (1), two (2), three (3), four (4), seven (7), fourteen (14), and twenty-one (21) days

⁸Draize, J. H. (1955) Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics. Assoc. of Food & Drug Officials of the U.S., Editorial Committee, Baltimore, Maryland, 40-52.

Rabbit Eye Irritation (Low Volume Procedure)

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Observations (Cont'd): following treatment. If by twenty-one (21) days all eyes have not cleared, the Sponsor's Divisional Toxicologist should be contacted regarding an extension of the observation period.

Scoring is discontinued at any time during the 21-day observation period if there is no evidence of irritation. Once eyes have cleared remove animals from study according to Standard Operating Procedures of the Test Facility. After removal of animals from study, assume all future scores would have been zero. Remove all remaining animals from study after 21 days unless otherwise instructed by the Sponsor. Discuss severely irritated eyes with the Sponsor's Divisional Toxicologist.

Note:

All animals that have an eye damaged which is producing undue stress or discomfort as judged by the Study Director and the Test Facility Veterinarian, with the concurrence of the Divisional Toxicologist, should be sacrificed immediately for humane reasons.

A necropsy is not required on animals that die or are sacrificed during the study. Record and describe all eye abnormalities not covered by Appendix 1, using Appendix 2 as a guide.

Questionable lesions or areas of involvement may be confirmed with a magnifying binocular head band/fluorescein staining. For more detailed observation, a slit lamp biomicroscope, or other devices, can be used.

At study termination, surviving animals should be removed from study following the Standard Operating Procedures of the Test Facility.

Calculations:

Calculate the Primary Irritation Score at each observation period for each group of animals by totaling all of the cornea, iris, and conjunctiva scores and dividing by the original number of animals in the group (3 or 6). Determine the Maximum Average Score (MAS) for each test group. The MAS is the highest Primary Irritation Score recorded for any given observation period.

Rabbit Eye Irritation (Low Volume Procedure)

Issue Date: November 20, 1985

Calculations (Cont'd): The median number of days for eyes to clear of all effects is determined for each group by arranging the days for each rabbit to clear in increasing numerical sequence. For any group with an even number of animals remaining, the middle two animals in the series are averaged (rounded off to the nearest 0.1 day); for any group with an odd number of animals remaining, the middle animal is used.

Protocol Change : 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.

Report The report for the study shall include how the study was conducted, date of study initiation and termination, the MAS (calculated and reported according to the method on page 5), the number of corneas, irides, and conjunctivas affected, and the individual and median number of days for the eyes to clear. The daily scores for individual animals are to be appended to the report. Any special observations such as squealing when the test substance is placed in the eye should also be included. This report shall conform to all requirements outlined in Section 56.185, Subpart J, Good Laboratory Practices Regulations.

Sponsor: W. Bruce Gibson W. Bruce Gibson
Divisional Toxicologist

Date Approved by Sponsor's Divisional Toxicologist 12/20/85

Proposed Starting Date: 1/31/86

Defined as day of dosing

Proposed Completion Date: 2/07/86

Defined as tentative date of last observation

To be completed
by the Test
Facility

Study Director: James R. Myer

James R. Myer, B.S.

Date: 1/28/86

Study Cost: _____

PROTOCOL - APPENDIX 2

DEFINITIONS OF TERMS

Rabbit Eye Irritation

Cornea

- | | |
|-----------------------|---|
| 1. Slight curling | Lack of normal corneal luster |
| 2. Sloughing | The visible shedding of corneal epithelium |
| 3. Stippling | Multiple small focal lesions in the corneal epithelium giving the appearance of small dots on the corneal surface |
| 4. Neovascularization | Invasion of the cornea by blood vessels |
| 5. Pigmentation | Appearance of melanin in cornea |
| 6. Scar | Gross infiltration of the cornea by granulation tissue |
| 7. Edema | Apparent increase in normal fluid content |
| 8. Ulceration | A loss of epithelium and damage to substantia propria |

Conjunctiva

- | | |
|---------------|------------------------------|
| 1. Hemorrhage | Leakage of blood into tissue |
| 2. Ulceration | Loss of epithelial surface |
| 3. Necrosis | Presence of dead tissue |

International Research and Development Corporation

QUALITY ASSURANCE STATEMENT

Study Title: Rabbit Eye Irritation (Low Volume Procedure)

Test Article: 80941-01

This report has been reviewed by the International Research and Development Corporation Quality Assurance Department in accordance with the United States Environmental Protection Agency Good Laboratory Practice Standards of May 2, 1984.

An inspection of the protocol for this study was conducted on February 4, 1986. A randomly sampled phase of the conduct of the study was inspected on January 31, 1986. Findings resulting from inspections, from a data audit, and from a review of the report were reported to management and the Study Director on February 19, 1986.

Approved And
Submitted By:



Wilson P. Dean, M.S.
Director of Quality Assurance

2-27-86
Date

191-1215

"credence through research"

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 1 of 2
 P&AS SECT. En.
 C.M. Bargholz
 ORIG. SECT.
 P.C. Pfeil

Test Substance Identification Number (TSIN): B0941-01
 Safety Test Request Number: BSBTS- 955, 955S
 Principle Investigator: W. Bruce Gibson

Product or Ingredient: 2-Ethyl-1,3-Hexanediol Brand Notebook Ref: TE 1229-16
 Physical Description: clear liquid Solubility: SS/S, O pH: 5.52
 Recommended Storage Conditions: room temperature Expiration Date: 12/6/86
 Hazards (i.e. flammability, toxic gases): combustible (and toxic)
 Dept. of Transportation Hazard Classification: non-hazardous CAS No. (a): 94-96-2

Formulated Composition (b)

<u>Component</u>	<u>Mol. Wt.</u>	<u>Nominal Level (X by Wt.)</u>	<u>Acceptable Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Number (WB-Ref.)</u>
<u>BYRD (1)</u>	<u>146</u>	<u>100%</u>	<u>N.K.</u>	<u>N.K.</u>	<u>Union Carbide</u>	<u>025884</u>

(1) 2-Ethyl-1,3-Hexanediol

- (a) Include CAS number(s) for the three most major components of a formulation or for single chemical products. Footnote to the material with which the respective number is associated.
- (b) IF information requested is not known, then the label NK will be entered.
- (c) Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-chemical names, such as Tergitol 15-S-0 or Yellow Dye #10, may not be acceptable but should be provided with the responsible toxicologist. Nondefinitive identification (e.g. Arquad, BC-base) is not acceptable.

The above information provided by: Lisa A. DeHoyes (Name) Lisa A. DeHoyes (Signature) 12/6/85 (Date)

The above information reviewed and accepted by:

Principle investigator: W. Bruce Gibson (Name) W. Bruce Gibson (Signature) 12/6/85 (Date)

TSCRID: ja

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 1 of 2

Test Substance Identification Number (TSIN): B0941-01

Analyzed Composites

<u>Date Submitted</u>	<u>Submitter Code</u>	<u>Analysis Code/Analysis</u>	<u>Estimated Value</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
12/26/85	YTB 3059 (955)	4199/Special GC (Solvent verification & purity)	100%		BS&HCPD

Analytical used not be completed prior to start of test.

12/26/85	YTB 3060 (9558)	3299/Hydrogen peroxide	less than 50ppm		
		/Formaldehyde	" 10ppm		
		/Zinc	" 200ppm		
		/Cadmium	" 20ppm		
		/Lead	" 5000ppm		
		/Arsenic	" 20ppm		
		/Mercury	" 40ppm		
		/Aluminum	" 5000ppm		
		/Chromium	" 20ppm		

Osmolality will be determined at MVL

4117/Methanol	0	BS&HCPD
2137/pH 0.5%	6.4 - 8.0	

Analytical Information Verified By: G. McCabe

(Signature)

Date:

This test substance is suitable for animal (non-clinical) safety testing.

Principle Investigator:

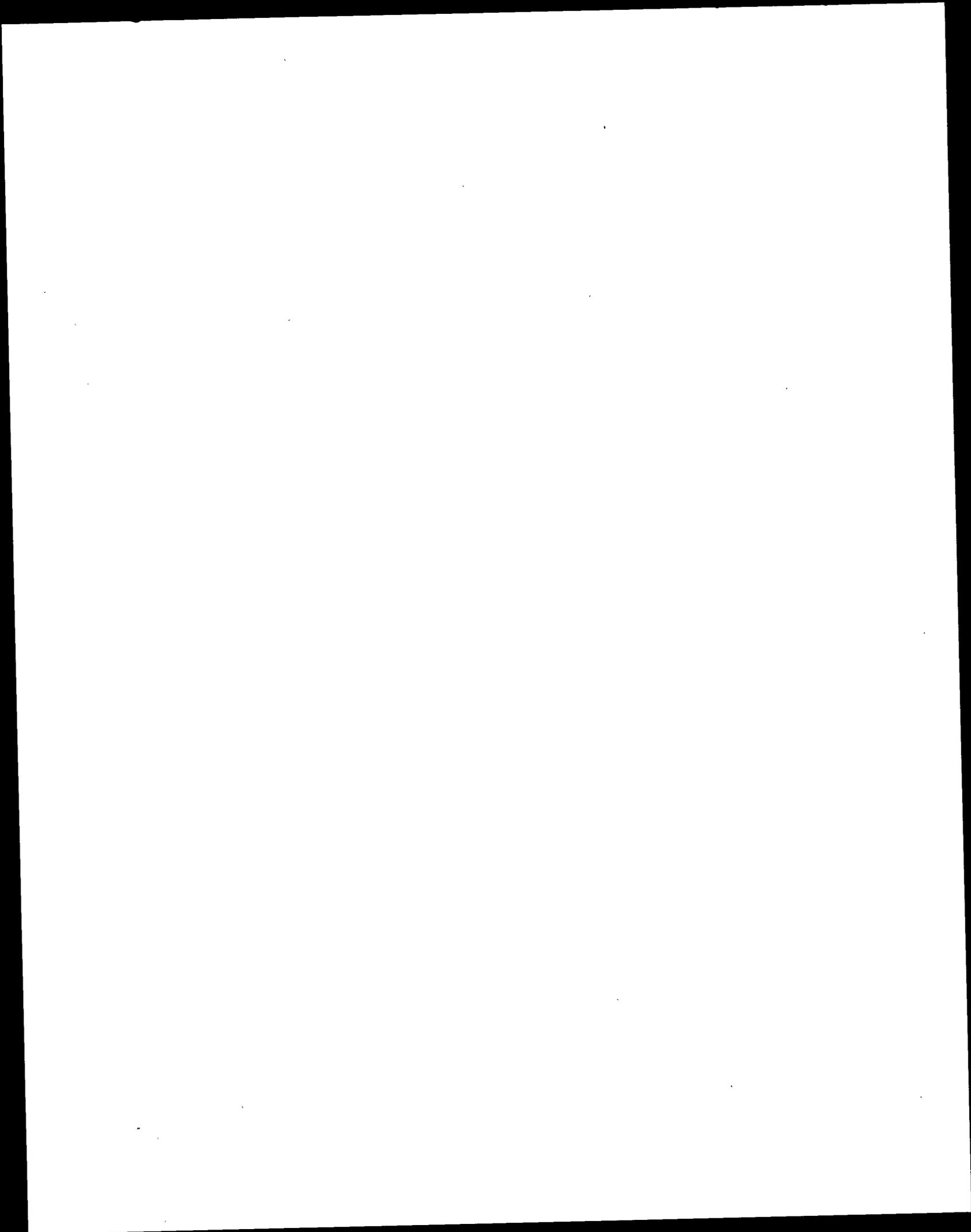
(Signature)

Date:

This test substance is suitable for human (clinical) safety testing.

Principle Investigator: W. Bruce Gibson

Date:



Test Substance

CAS Number: 94-96-2
Identity: 1,3-Hexanediol, 2-ethyl-
Purity: 100%
Comments: Test substance was identified B0941-01

Method

Method / Guideline: Unscheduled DNA Synthesis according to Williams, GM (1977) Cancer Research 37:1845-1851.

Report Year: 1986

GLP: Yes

Test System: Primary rat hepatocytes from livers of normal adult male Sprague-Dawley rats.

Study Design: The test substance was tested at 8 dose levels ranging from 0.0005 to 1.0 µl/ml in diluted Williams Media E. Five doses were fully evaluated for unscheduled DNA synthesis. The study included 7,12-dimethylbenzanthracene (DMBA) as a positive control. A preliminary cytotoxicity test was performed to establish the appropriate dose range for the main study. For the main study, three replicate plates were seeded and treated with test substance. Each test article and control dish received ³H-thymidine at a final concentration of 10 µCi/mL with parallel toxicity test plates. The cells were treated for 18-20 hours, then washed, swelled and fixed prior to measuring UDS by autoradiography.

Results

Result: The test material did not cause a significant increase in the mean number of net nuclear grain counts at any dose level, and was considered negative in this study. Positive control gave an appropriate response.

Data Quality

Reliability (Klimisch): 2

Reference

Laboratory Report Number: T4636.380
Reference: Procter & Gamble, 1986. Test for Chemical Induction of Unscheduled DNA Synthesis in Primary Cultures of Rat Hepatocytes (by Autoradiography). Accession #33081

Acc # 33081

TEST FOR CHEMICAL INDUCTION OF UNSCHEDULED
DNA SYNTHESIS IN PRIMARY CULTURES OF
RAT HEPATOCYTES (BY AUTORADIOGRAPHY)

TEST ARTICLE

B0941-01

DIVISIONAL REQUEST DOCUMENT NUMBER

BSBIS 9556

FINAL REPORT

FOR

Procter & Gamble, Co.
Miami Laboratories, P.O. Box 39175
Cincinnati, Ohio 45247

Reviewed and Accepted by BS&HCP/P&RS

BY W. Bruce Gibson
W. B. GIBSON

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

RECEIVED BY
AUG 06 1986
OPERATIONS SECTION

RECEIVED
SEP 5 1986
BY
HUMAN SAFETY
SECTION

 MICROBIOLOGICAL
ASSOCIATES INC.

TEST FOR CHEMICAL INDUCTION OF UNSCHEDULED DNA SYNTHESIS IN PRIMARY CULTURES OF RAT HEPATOCYTES (BY AUTORADIOGRAPHY)

FINAL REPORT

Test Article : B0941-01

Divisional Request Document No.: 85BIS 955S

MA Study No.: T4636.380

Test Article Description: Colorless liquid

Storage Conditions: Room Temperature; Protected from Light

Date Sample Received: January 27, 1986

Initiation Date: February 20, 1986

Completion Date: July 30, 1986

Sponsor: Procter & Gamble, Co.
Miami Laboratories, P.O. Box 39175
Cincinnati, Ohio 45247

Sponsor's Investigator: Dr. W. Bruce Gibson

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

Study Director: Rodger D. Curren 7/30/86
Rodger D. Curren, Ph.D. Date

Laboratory Technician: Linda L. Dunn 7/30/86
Linda Dunn, B.S. Date

Laboratory Technician: Rodger Curren (for M.E.) 7/30/86
Mary Ernst, B.S. Date

Laboratory Technician: Rose Postinus 7/30/86
Rose Postinus, M.T. Date

Laboratory Technician: Virginia Fortner 7/30/86
Virginia Fortner, B.S. Date

Laboratory Technician: Kathleen Wallace (for KW) 7/30/86
Kathleen Wallace, B.S. Date

 MICROBIOLOGICAL ASSOCIATES INC.

Study No. T4636.380

SUMMARY

Procter & Gamble, Co.'s test article, B0941-01, was tested in the Unscheduled DNA Synthesis Test using rat primary hepatocytes. Based on the results of a preliminary cytotoxicity test, the test article was tested at eight dose levels ranging from 0.0005 to 1.0 ul/ml. Five doses were fully evaluated for Unscheduled DNA Synthesis.

The results of the UDS assay indicate that under the test conditions, the test article did not cause a significant increase in the mean number of net nuclear grain counts (i.e., an increase of at least 5 counts over the control), at any dose level. Therefore, the test article is considered negative in this study.

Study No. T4636.380

INTRODUCTION

The laboratory phase of this study was conducted from February 20, 1986 to June 30, 1986 at Microbiological Associates, Inc. The experimental procedure employed was essentially that of Williams, G.M. (Cancer Research 37:1845-1851, 1977) and is described in detail in the specific protocol for this study (see Appendix).

The purpose of the study was to evaluate the test article, B0941-01, for its ability to induce Unscheduled DNA Synthesis in rat primary hepatocytes as measured by autoradiographic methods.

MATERIALS AND METHODS

Indicator Cells

Primary rat liver cell cultures derived from the livers of normal adult male Sprague-Dawley rats were used in this study. The animals were obtained from the Charles River Laboratories and were quarantined for at least one week prior to the initiation of the study. The animals were maintained on standard laboratory diet throughout the quarantine period.

The procedure used for obtaining rat hepatocyte cultures (HPC) was essentially that of Williams, et al., (In Vitro 13:809-817, 1977). Each rat used was sacrificed by inhalation of metofane. The animal was dissected and perfused first with 0.5M EGTA solution and then with a collagenase solution. The liver was removed from the animal and the cells were dissociated, counted, and seeded into 35 mm dishes containing coverslips (5.0×10^5 viable cells/dish). The cells were seeded in Williams Medium E (WME) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin and 100 ug of streptomycin/ml. The cultures were incubated at $37 \pm 1^\circ\text{C}$ in a humidified 5% CO_2 incubator for 90-120 minutes, washed and refed with serum-free medium and used in the test.

Test and Control Articles

The test article, B0941-01, was received on January 27, 1986, and stored at room temperature protected from light. The test article was dissolved and diluted in Williams Media E (Quality Biological, lots 701397, 701444 and 12-141-5) to make up the stock solutions. 7,12-Dimethylbenzanthracene (DMBA) (Kodak Lot C9C) was dissolved in DMSO and used as a positive control in this study.

The test article was diluted to appropriate concentrations immediately prior to use. Approximately 20 to 30 minutes elapsed between the time the test article was dissolved and the final treatment of cells. All test article and control treatments were done under subdued yellow lights to avoid possible problems of photoinactivation.

The sponsor has assumed responsibility for documentation of the derivation, characterization and stability testing of the test substances.

Identification of Test System

All culture plates were labeled with pen with a code system which clearly identifies the test article or control, test phase, and the experiment number. Slides were similarly labeled with pencil or pen.

Study No. T4636.380

Initial Cytotoxicity Test

A preliminary cytotoxicity test was performed to establish an appropriate dose range for the test article. Ten doses ranging from 0.0005 to 10.0 $\mu\text{l/ml}$ were tested. The test article was tested by treating replicate cultures of HFC 90-120 minutes after seeding. Eighteen to twenty hours later the cells were washed with Ca^{++} , Mg^{++} free phosphate buffered saline (PBS), trypsinized, exposed to trypan blue and counted in a hemacytometer. Two replicate plates were used for counting at each dose level. The relative survivals were obtained by comparing the treated to control groups.

Unscheduled DNA Synthesis Test

Based on the results of the initial cytotoxicity test, the test article, B0941-01, was tested at eight dose levels. Three replicate plates seeded with 5.0×10^5 HFC/plate were treated with 0.0005 to 1.0 $\mu\text{l/ml}$ of test article. DMSO, at 3 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$, was used as the positive control. Williams Media E, which was used to dissolve the test article and the positive control, was used as the solvent control. Each test article and control dish received ^3H -thymidine at a final concentration of 10 $\mu\text{Ci/ml}$. In parallel with the test plates, three cultures per dilution were treated with the same compound for a parallel toxicity test.

The cells were treated for 18-20 hours as described earlier. The parallel toxicity plates were harvested by trypsinization and viable cell counts were made as described in the initial cytotoxicity test to obtain the relative survivals and relative toxicities.

After eighteen to twenty hours of exposure, the cells in the Unscheduled DNA Synthesis assay plates were washed in serum-free WME, swelled in 1% sodium citrate and fixed in ethanol-acetic acid fixative. The coverslips were air-dried, mounted cell side up on glass slides, and allowed to dry. The slides were coated with Kodak NTB emulsion and stored for eight days at 4°C in light tight boxes with desiccant. The slides were then developed in Kodak D-19 developer, fixed in Kodak fixer and stained in hematoxylin-sodium acetate-eosin stain.

Study No. T4636.380

Scoring

The slides were read "blind" on an Artek Colony Counter. Nuclear grains were counted in 25 cells in random areas on each of two coverslips per treatment. The net nuclear counts were determined by counting three nucleus-sized areas adjacent to each nucleus and subtracting the average cytoplasmic count from the nuclear count. Replicative synthesis was identified by nuclei completely blackened with grains and such cells were not counted. Nuclei exhibiting toxic effects of treatment, such as dark staining disrupted membranes or irregular shape, were not counted.

Presentation of Data

For each treatment slide, the net nuclear counts were averaged and a standard deviation (S.D.) determined and recorded on a summary form. Also reported are the grand mean and S.D. for each dose level as well as the percent of cells in repair (cells with ≥ 5 net nuclear grains). Means, standard deviations and percent survivals were computed using a LOTUS 1-2-3 program on an IBM PC or compatible computer.

Criteria for Evaluation of Test Results

The results of this study were evaluated according to the criteria described below.

If the mean net nuclear count was increased by at least five counts over the control, the results for a particular dose level were considered significant. A test article was judged positive if it induced a dose-related response and at least one dose produced a significant increase in the average net nuclear grains when compared to that of the control. In the absence of the dose response, a test article which showed a significant increase in the mean net nuclear grain count in at least two successive doses was considered positive. If a test article showed a significant increase in the net nuclear grain count at one dose level without any dose response, the test article was considered to have a marginal positive activity. The test article was considered negative if no significant increase in the net nuclear grain counts at any dose level was observed.

Records

All raw data, final report and stained slides of this study are maintained in the archives of Microbiological Associates, Inc. located at 5221 River Road, Bethesda, Maryland 20816.

RESULTS AND DISCUSSION

Results of the preliminary cytotoxicity test are recorded in Table 1. The test article, B0941-01, was very toxic at the two highest doses, moderately toxic in the next five doses and slightly toxic in the remaining doses. The assay was repeated due to excess toxicity. Doses for the repeat assay were selected based on the results of the initial assay. The maximum dose of test article tested was 1.0 ul/ml.

The results of the parallel cytotoxicity are recorded in Table 2. The highest three dose levels caused RTs of 54.6%, 58.6% and 34.8%, whereas the remaining doses fluctuated about a plateau value of approximately 20%.

The results of the UDS assay are summarized in Table 3. Slides treated with B0941-01 or DMBA were compared to the appropriate negative control. According to the criteria set for evaluating the test results, both doses of the positive control compound, DMBA, induced a significant increase in the average net nuclear count of silver grains. None of the test article doses caused a significant increase in the mean net nuclear counts. All criteria for a valid test were met.

Study No. T4638.380 A7

TABLE 1
PRELIMINARY CYTOTOXICITY ASSAY
UNSCHEDULED DNA SYNTHESIS

TREATMENT	DISHES COUNTED	% VIABLE CELLS	VIABLE CELLS/DISH (X10 ⁵)	SURVIVAL INDEX	RELATIVE SURVIVAL	RELATIVE TOXICITY
B0941-01						
10 ul/ml	2	1.8%	0.030	0.6%	1.4%	98.6%
5 ul/ml	2	3.4%	0.045	0.9%	2.1%	97.9%
1.7 ul/ml	2	66.0%	0.675	13.5%	31.0%	69.0%
0.5 ul/ml	2	69.5%	0.990	19.8%	45.5%	54.5%
0.2 ul/ml	2	64.3%	1.245	24.9%	57.2%	42.8%
0.05 ul/ml	2	67.6%	1.260	25.2%	57.9%	42.1%
0.02 ul/ml	2	62.0%	1.140	22.8%	52.6%	47.4%
0.005 ul/ml	2	76.0%	1.800	36.0%	82.8%	17.2%
0.002 ul/ml	2	70.3%	1.905	38.1%	87.6%	12.4%
0.0005 ul/ml	2	71.7%	1.740	34.8%	80.0%	20.0%
WME	2	75.8%	2.175	43.5%	100.0%	0.0%

Cells Plated per Dish = 500,000

Survival Index = Average Viable Cells per Dish x 100

Cells Plated per Dish

Relative Survival = Survival Index x 100

Survival Index of Control

Relative Toxicity = 100 - Relative Survival

WME = Untreated Control

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 MICROBIOLOGICAL
ASSOCIATES INC.

Study No. T4656-380

TABLE 2
PARALLEL CYTOTOXICITY ASSAY
UNSCHEDULED DNA SYNTHESIS

TREATMENT	DISHES COUNTED	% VIABLE CELLS	VIABLE CELLS/DISH (X10 ⁵)	SURVIVAL INDEX	RELATIVE SURVIVAL	RELATIVE TOXICITY
BD941-01						
1.0 ul/ml	3	59.4%	1.030	20.6%	45.4%	54.6%
0.5 ul/ml	3	66.3%	0.940	18.8%	41.4%	58.6%
0.1 ul/ml	3	80.4%	1.480	25.6%	65.2%	34.8%
0.05 ul/ml	3	77.7%	1.930	38.6%	85.0%	15.0%
0.01 ul/ml	3	78.3%	1.820	36.4%	80.2%	19.8%
0.005 ul/ml	3	82.7%	1.840	36.8%	81.1%	18.9%
0.001 ul/ml	3	82.4%	1.780	35.6%	78.4%	21.6%
0.0005 ul/ml	3	80.4%	1.780	35.6%	78.4%	21.6%
DNBA 10 ug/ml						
10 ug/ml	3	46.7%	0.680	13.6%	40.5%	59.5%
3.0 ug/ml	3	61.7%	0.810	16.2%	48.2%	51.8%
DMSO (Solvent Control for DNBA)						
10 ul/ml	3	75.9%	1.680	33.6%	100.0%	0.0%
DNE (Solvent Control for Test Article)						
	3	85.4%	2.270	45.4%	100.0%	0.0%

Cells Plated per Dish : 500,000

Survival Index = Average Viable Cells per Dish X 100

Cells Plated per Dish

Relative Survival = Survival Index X 100

Survival Index of Control

Relative Toxicity = 100% - Relative Survival

DNE = Untreated Control

 MICROBIOLOGICAL
ASSOCIATES INC.

Study No. T4636.380 B2

TABLE 3
SUMMARY OF SOS ASSAY
WITH 80941-01

TREATMENT	RELATIVE SURVIVAL	SLIDE DESIGNATION	NO. OF NUCLEI COUNTED	AVERAGE NET GRAINS PER NUCLEUS	S.D.	GRAND MEAN	S.D.	PERCENT CELLS WITH 5 OR MORE NET NUCLEAR GRAINS
80941-01								
1.0 ul/ml	43.4%	37A	25	-0.3 +/-	1.7	-0.5 +/-	1.7	0.0%
		37B	25	-0.6 +/-	1.7			
0.5 ul/ml	41.4%	34A	25	0.3 +/-	2.1	-0.8 +/-	2.8	0.0%
		34B	25	-1.9 +/-	3.0			
0.1 ul/ml	65.2%	31A	25	0.7 +/-	2.0	0.4 +/-	2.2	0.0%
		31B	25	0.0 +/-	2.3			
0.05 ul/ml	85.0%	36A	25	-1.9 +/-	2.0	-1.6 +/-	2.0	0.0%
		36B	25	-1.3 +/-	2.0			
0.01 ul/ml	80.2%	32A	25	-0.4 +/-	3.5	-0.2 +/-	3.0	0.0%
		32B	25	0.1 +/-	2.5			
0.005 ul/ml	81.1%	35A	0	NOT COUNTED				
		35B	0					
		35C	0					
0.001 ul/ml	78.4%	33A	0	NOT COUNTED				
		33B	0					
		33C	0					
0.0005 ul/ml	78.4%	30A	0	NOT COUNTED				
		30B	0					
		30C	0					
DMBA								
10 ug/ml	40.5%	48A	25	25.2 +/-	9.7	25.0 +/-	7.3	100.0%
		48B	25	26.9 +/-	6.0			
3.0 ug/ml	48.2%	49A	25	36.7 +/-	7.4	33.6 +/-	6.9	100.0%
		49B	25	32.5 +/-	6.0			
DMSO (Solvent Control For DMBA)								
10 ul/ml	100.0%	50A	25	1.1 +/-	1.9	-1.3 +/-	4.1	2.0%
		50B	25	-3.8 +/-	6.2			
WME (Solvent Control for 80941-01)								
	100.0%	47A	25	+1.7 +/-	3.0	-1.2 +/-	3.2	4.0%
		47B	25	-0.6 +/-	3.3			

WME = Untreated Control
S.D. = Standard Deviation

Study No. T4636.390

Conclusion

The Procter and Gamble Company's test article B0941-01, was tested in the Rat Hepatocyte Unscheduled DNA Synthesis Assay. The test article was tested at eight dose levels ranging from 0.0005 to 1.0 ul/ml. Five doses were fully evaluated for Unscheduled DNA Synthesis.

The results of the UDS assay indicate that under the test conditions, the test article did not cause a significant increase in the Unscheduled DNA Synthesis as measured by the mean number of net nuclear grain counts (i.e., an increase of at least 5 counts over the control), at any dose level. In this study the positive control, DMSA, induced significant increases in the mean number of net nuclear grain counts over that in the solvent control.

STATEMENT OF COMPLIANCE

To the best of my knowledge, T4636.380, Test for Chemical Induction of Unscheduled DNA Synthesis in Primary Cultures of Rat Hepatocytes (By Autoradiography), 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)).

 7/30/86
Roger D. Curren, Ph.D. Date
Study Director

 MICROBIOLOGICAL
ASSOCIATES INC.

Study No. T4636.380

APPENDIX

THE PROCTER & GAMBLE COMPANY

Received by RA/QA ELH/

PROTOCOL C30B

Test for Chemical Induction of
Unscheduled DNA Synthesis in
Primary Cultures of Rat Hepatocytes
(By Autoradiography)

APPROVED

Issue Date: March 14, 1985
Supersedes Issue Dated: December 14, 1982

Test Substance Identification Number (TSIN) # B0941-01

Divisional Request Document Number (DRD) # ⁴⁵⁵⁵ 4555

Sponsor: The Procter & Gamble Company
Cincinnati, Ohio

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

Microbiological Associates, Inc.
5221 River Road
Bethesda, MD

Study # 74636.380
(To be filled in by
Testing Facility)

Purpose: To determine whether a test substance elicits Unschad-
uled DNA Synthesis (UDS) in primary cultures of rat
hepatocytes. (IN VITRO)

Justification for
Selection of Test
System:

Primary rat hepatocytes serve as the system of choice
due to the amount of background data available, and
their endogenous metabolic activation capacity.

Route of Administration
of Test Substance and
Reason for Choice:

IN VITRO. Route specified by test procedure.

Records to be
Maintained:

Preparation of primary rat hepatocyte cultures.
Documentation of test substance preparation, prepara-
tion of cells, dosing, and grain counts. Include any
other records that would be required to reconstruct the
study and demonstrate adherence to protocol.

PROTOCOL 6308 (Cont'd)Test for Chemical Induction of
Unscheduled DNA Synthesis in
Primary Cultures of Rat Hepatocytes
(By Autoradiography)

Issue Date: March 14, 1985

Test Substance(s)

<u>TSIN #</u>	<u>DSD Number</u>	<u>Description</u>		<u>Expiration Date</u>
		<u>Color</u>	<u>Physical Form</u>	
30941-01	ASOS 9555	colorless	liquid	12/6/86

Storage Conditions: (Check one)

Room temperature Refrigerator Freezer
 Other _____

Hazards: (Check one)

None known. Take ordinary precautions in handling.
 As follows: Severe eye irritant

Special Instructions: (Check one)

None
 As follows:

Dose Preparation:

Vehicles in order of preference

Water soluble to 0.4%
 DMSO
 EtOH soluble
 Acetone
 Other

Solubility (See above)

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.

PROTOCOL C30B (Cont'd)

Test for Chemical Induction of
Unscheduled DNA Synthesis in
Primary Cultures of Rat Hepatocytes
(By Autoradiography)

Issue Date: March 14, 1985

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.

Preparation of Dosing
Solutions:

Using the Standard Operating Procedures of the Test Facility, immediately prior to each assay, test articles will be diluted in the appropriate solvent to form a series of concentrations that when diluted into culture medium will yield the appropriate set of test concentrations. The final concentration of solvent will be maintained at 1% or less to minimize the possibility of a cytotoxic effect in response to the solvent.

Both solvent and positive controls will be used in every UDS assay. The positive control will be 7,12-dimethylbenzanthracene, a chemical known to induce UDS in this system (1).

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 _____.

PROTOCOL C30B (Cont'd)

Test for Chemical Induction of
Unscheduled DNA Synthesis in
Primary Cultures of Rat Hepatocytes
(By Autoradiography)

Issue Date: March 14, 1985

Test System
Identification:

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

Test System:

Primary cultures of Fisher 344 or Sprague-Dawley rat hepatocytes prepared by in situ perfusion with collagenase according to the method of Williams et al (2).

Methods:

Primary Cell Culture

Primary rat hepatocytes isolated by in situ collagenase perfusion of adult, male Fisher 344 or Sprague-Dawley rats will be used for the UDS assay. The Standard Operating Procedures of the Test Facility for culture preparation are based on the procedure of Williams et al (1, 2). Cultures will be initiated on coverslips in a suitable tissue culture vessel in Williams Medium E supplemented with 10% (v/v) fetal bovine serum and antibiotics. After a period of 1.5-2 hours at $37 \pm 1.0^\circ\text{C}$, the attached cells will be washed to remove floating (nonviable) cells. Cells will then be refed and incubated at $37 \pm 1.0^\circ\text{C}$ in an atmosphere of 5% CO_2 in air unless used immediately for a UDS or toxicity assay. Because the ability of the hepatocytes to metabolically activate promutagens drops quickly in culture, only cultures freshly prepared on the same day will be used for the UDS assay.

Preliminary Toxicity Test

A preliminary cytotoxicity test will be performed according to the Standard Operating Procedures of the Test Facility to establish the appropriate dose range for the UDS assay. The index of viability that will be used following exposure to test chemical will be exclusion of trypan blue (0.04-0.08%). The maximum dose chosen for the UDS assay, if possible, will be one that induces at least a 50% reduction in cell viability relative to the solvent control. Subsequent doses will

PROTOCOL C30B (Cont'd)Test for Chemical Induction of
Unscheduled DNA Synthesis in
Primary Cultures of Rat Hepatocytes
(By Autoradiography)

Issue Date: March 14, 1985

Methods (Cont'd):Preliminary Toxicity Test (Cont'd)

be chosen to span a range down to no apparent relative toxic effect. If no relative toxic effect is observed at any dose, the doses chosen for the UDS assay will be based on the solubility of the test substance. In this case, the highest dose tested should exceed the solubility of the test substance or be 10 mg/ml, whichever is smaller. Test liquids may be tested on the basis of volume with 10 μ l/ml being the highest dose tested in the absence of toxicity.

UDS Test

For each UDS assay, three or four freshly prepared hepatocyte cultures will be used for each dose of the test substance. At least five doses chosen on the basis of a preliminary toxicity assay or a previous UDS assay will be used. Using the Standard Operating Procedures of the Test Facility, cultures will be exposed to both test substance and 10 μ Ci/ml 3 H-thymidine (specific activity 20-50 Ci/mole) for 18-20 hours at $37 \pm 1.0^\circ$ C under an atmosphere of 5% CO_2 in air. Exposures will be done in serum-free Williams Medium E.

Following the exposure period, the cultures will be scored for toxicity or washed with a buffered, balanced salt solution and then processed for autoradiography according to the Standard Operating Procedures of the Test Facility.

Incorporation of 3 H-thymidine into nuclear DNA will then be determined by counting darkened grains localized over nuclei in at least 50 randomly chosen but normal appearing cells per dose group. (Cells visibly suffering from toxic effects of treatment will not be scored, i.e. constricted cells, irregularly shaped, vary darkly stained, etc.) The 50 cells will be chosen from at least two coverslips per dose group. The counts for both nuclei of binucleated cells will be recorded separately. Background incorporation will be determined by counting at least two nucleus-sized areas of cytoplasm adjacent to each nucleus. Net nuclear grain counts will be determined by subtracting the appropriate background. All grain counts will be done using an electronic colony counter (such as an

PROTOCOL C30B (Cont'd)Test for Chemical Induction of
Unscheduled DNA Synthesis in
Primary Cultures of Rat Hepatocytes
(By Autoradiography)

Issue Date: March 14, 1985

Methods (Cont'd):

Artek 880 or 980) equipped with a microscope-mounted auxiliary T.V. camera. Grain counts may be done either directly by using the "count" mode of the colony counter or indirectly by determining the relative area covered by darkened grains by using the "area" mode of the counter. Area counts may then, in turn, be related to minimum numbers of grains by comparison to the average grain size. Data for each coverslip scored will be reported separately.

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 recorded for each negative and positive control and each dose of test substance. Raw data consists of individual grain counts and dose preparation information.

The mean net grain count \pm the standard deviation or error is reported for each coverslip culture scored.

Because the primary hepatocyte cultures employed in this assay may be composed of heterogeneous populations of cells with varying metabolic and repair capacities, the observed net nuclear grain counts may be a complex distribution. As a result, before reaching conclusions based on the data, statistical analysis employing "t" or ANOVA tests may be applied to the mean or median grain counts for each coverslip. Such analysis will be done at the direction of the Sponsor or by the Sponsor or his/her representative.

Results of each test will be considered independently, but in order to be considered a valid test, solvent and positive control mean net nuclear grain counts should fall in a proper historical range. Repeat testing may sometimes be required in some cases such as sporadic or apparent single dose responses.

PROTOCOL C30B (Cont'd)

Test for Chemical Induction of
Unscheduled DNA Synthesis in
Primary Cultures of Rat Hepatocytes
(By Autoradiography)

Issue Date: March 10, 1985

Report:

Final Report

A report of the results will be prepared for this study by the contract facility. 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.
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.

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

PROTOCOL C308 (Cont'd)

Test for Chemical Induction of
Unscheduled DNA Synthesis in
Primary Cultures of Rat Hepatocytes
(By Autoradiography)

Issue Date: March 14, 1985

Sponsor: W. Bruce Gibson W. Bruce Gibson
Divisional Toxicologist

Date Approved by Sponsor's Divisional Toxicologist: 12/20/85

Proposed Starting Date: February 13, 1986 } ^{to compare to other protocol} start date
as of 7/2/86

Defined as Preliminary Cytotoxicity }

Proposed Completion Date: April 3, 1986 } ^{17 ENTRY FROM R-2/2/3/86}

Defined as Final Report Date } To be completed
by the Test
Facility

Study Director: Rodger D. Lamm

Date: 2/3/86

Study Cost: \$5,200.00

References:

1. G. H. Williams. The detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res. 37: 1845-1851 (1977).
2. G. H. Williams, E. Bermudez, and D. Scaramuzzino. Rat hepatocyte primary cell cultures. III. Improved dissociation and attachment techniques and the enhancement of survival by culture medium. In Vitro 13: 809-817 (1977).

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 1 of 2
 PARS SECT. HD.
 C.M. Bergtholz *cmB*
 ORIG. SECT. HD.
 P.C. Pfaff *pf*

Test Substance Identification Number (TSIN): B0941-01
 Safety Test Request Number: DBTS- 955, 955S
 Principle Investigator: W. Bruce Gibson

Product or Ingredient: 2-Ethyl-1,3-Hexanediol Brand Notebook Ref: TR 1229-16
 Physical Description: clear liquid Solubility: SS/H₂O pH: 5.52
 Recommended Storage Conditions: room temperature Expiration Date: 12/6/85
 Hazards (i.e. flammability, toxic gases): combustible (N.D. 2007)
 Dept. of Transportation Hazard Classification: non-hazardous CAS No. (a): 94-96-2

Formulated Composition (b)

<u>Component (c)</u>	<u>Mol. Wt.</u>	<u>Nominal Level (% by Wt.)</u>	<u>Acceptable Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Number (NB-Ref.)</u>
<u>ETHD (1)</u>	<u>146</u>	<u>100%</u>	<u>N.K.</u>	<u>N.K.</u>	<u>Union Carbide</u>	<u>025884</u>

(1) 2-Ethyl-1,3-Hexanediol

- (a) Includes CAS number(s) for the three most major components of a formulation or for single chemical products. Footnote to the material with which the respective number is associated.
- (b) If information requested is not known, then the symbol NK will be entered.
- (c) Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-chemical names, such as Tergitol 15-S-0 or Yellow Dye #10, may not be acceptable but should be previewed with the responsible toxicologist. Nondefinitive identification (e.g. Arquad, BC-base) is not acceptable.

The above information provided by: Lisa A. DeHoyos Jim A. DeHoyos 12/6/85
 (Name) (Signature) (Date)

The above information reviewed and accepted by:

Principle Investigator: W. Bruce Gibson W. Bruce Gibson 12/6/85
 (Name) (Signature) (Date)

TSCRLD:ja

TEST SUBSTANCE CHARACTERIZATION REPORT (TSCR)

Side 2 of 2

Test Substance Identification Number (TSIN): B0961-01

Analyzed Composition

<u>Date Submitted</u>	<u>Submitter Code</u>	<u>Analysis Code/Analysis</u>	<u>Estimated Value</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
12/26/85	YTB 3059 (955)	4199/Special GC (Solvent verification & purity)	100%		ES&HCFD

Analytical need not be completed prior to start of test.

12.16/85	YTB3060 (9555)	3299/Special request: /Hydrogen peroxide /Formaldehyde /Zinc /Cadmium /Lead /Arsenic /Mercury /Aluminum /Chromium	< 50ppm < 10ppm < 200ppm < 20ppm < 5000ppm < 20ppm < 50ppm < 5000ppm < 20ppm		
		Purity will be determined at NVL 4117/Methanol 2137/pS, 0.5%	D 6.4 - 8.0		ES&HCFD

Analytical Information Verified by: G. McCabe

(Signature)

Date:

This test substance is suitable for animal (non-clinical) safety testing.

Principle Investigator:

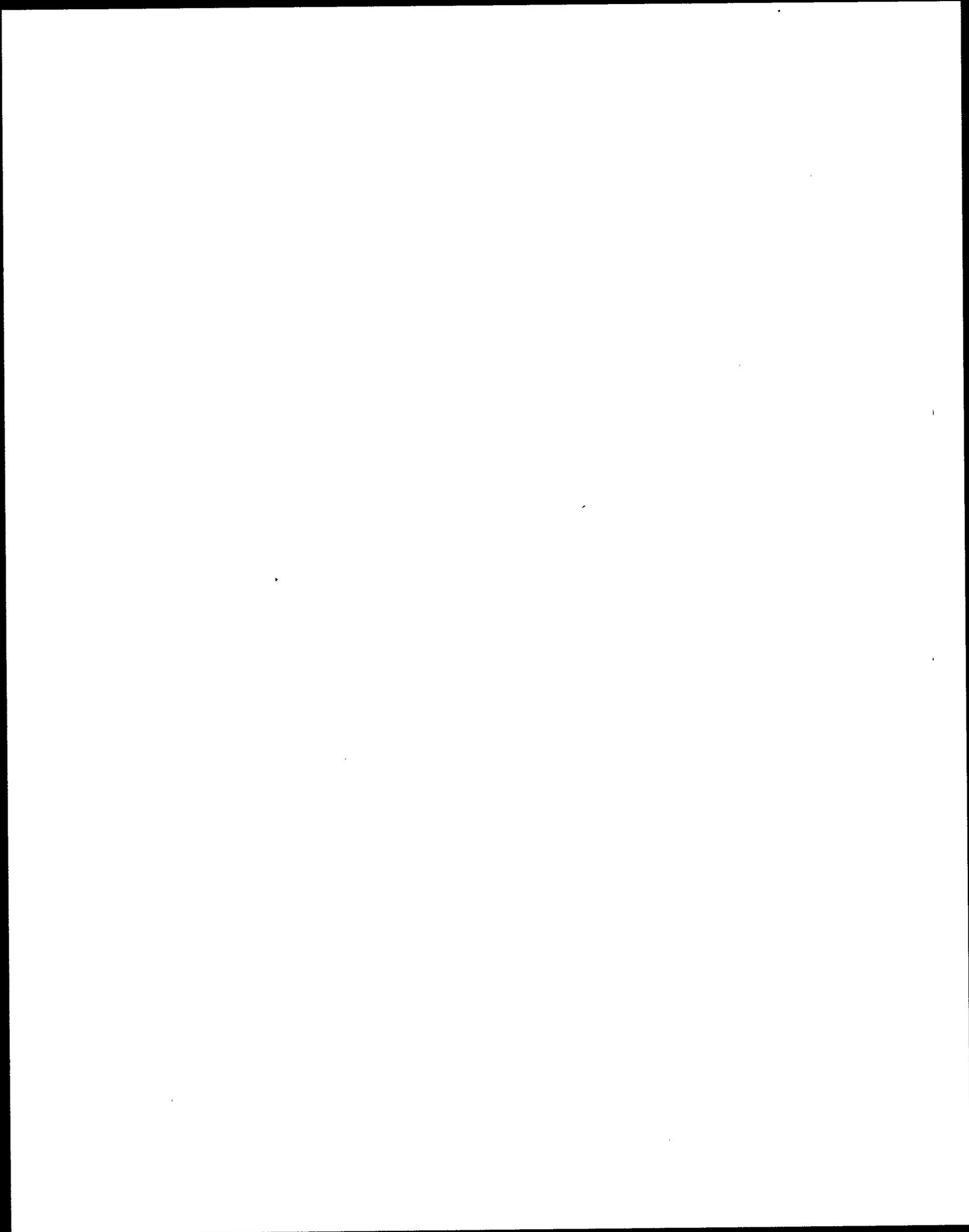
(Signature)

Date:

This test substance is suitable for human (clinical) safety testing.

Principle Investigator: W. Bruce Gibson

Date:



Test Substance

CAS Number: 94-96-2
Identity: 2-Ethyl Hexane Diol-1,3
Purity: 100%
Remarks: Portions of the report were redacted prior to receipt by Procter & Gamble.

Method

GLP: Not specified.
Report/Study Year: 1985
Method/Guideline Followed: Human Repeat Insult Patch Test
Test Type: Human Repeat Insult Patch Test
Species: Humans (215 volunteers started; 203 completed the study)
Sex: Males and females
Route of Administration: Occlusive patch
Exposure Period: Three week induction period during which an occlusive patch was applied to the infrascapular area of the back, either to the right or left of the midline, each Monday, Wednesday, and Friday. Each patch was left in place for 24 hours and then removed by the subject. 14 days after the last induction application, patches were applied to sites previously unexposed for 24 hours.
Doses: 0.2 mL of 100% solution under occlusive patch

Results

Result: Two subjects developed reactions on challenge to the test substance and were rechallenged. Subject #5453 had no evidence of sensitization on rechallenge testing. Subject #5493 had results on rechallenge indicative of probable irritation although sensitization could not be entirely excluded.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Study Number: 851013
Reference: TKL Research, Inc., 1985. Repeated Insult Patch Test. Accession # 44564



Acc # 44564

REPEATED INSULT PATCH TEST

WCC.

STUDY #851013

CONDUCTED FOR:

Union Carbide Corporation
Old Saw Mill River Road
Tarrytown, N.Y. 10591

Attn: Dr. B. Ballantyne

DATE OF REPORT:

October 25, 1985

TITLE:

Repeated Insult Patch Test

STUDY NO :

851013

SPONSOR:

Union Carbide Corporation
Old Saw Mill River Road
Tarrytown, N.Y. 10591

Attn: Dr. B. Ballantyne

TEST MATERIAL(S):.

100% 2-Ethyl-1,3-Hexanediol

DATE INITIATED:

August 26, 1985

DATE COMPLETED:

October 3, 1985

INVESTIGATIVE PERSONNEL:

Michael L. Reed, M.D.
Consulting Dermatologist

Robert C. Reardon, Ph.D.
Project Director

Diana Napoli, R.N.
Clinical Study Manager

Joanne Mruczek, R.N.
Clinical Research Associate

Marilyn Smith
Clinical Research Assistant

CONDUCTED BY:

TKL RESEARCH, INC.
133 East 58th Street
New York, N.Y. 10022

CLINICAL SITE(S)

TKL Research, Inc.
578 Driggs Avenue
Brooklyn, N.Y. 11211

TKL Research, Inc.
360 Main Street
Hackensack, N.J. 07601

SUMMARY

Four samples (100% 2-Ethyl-1,3-Hexanediol) were tested to determine their ability to irritate and/or sensitize the skin of normal volunteer subjects using an occlusive repeated insult patch test. Two hundred and three (203) subjects completed the study.

Two subjects developed reactions on challenge to 2-Ethyl 1,3-Hexanediol which required rechallenge testing. Subject #5453 had no evidence of sensitization on rechallenge testing. Subject #5493 had results on rechallenge indicative of probable irritation although sensitization could not be entirely excluded. However, sensitization, if present, is very mild and not clinically significant since it could only be elicited under occlusive conditions.

1.0 INTRODUCTION

1.1 OBJECTIVE

The purpose of this study was to determine whether the test material is capable of sensitizing the skin of humans under controlled patch test conditions, and if so, to classify the test material as a sensitizer on the basis of the observed clinical responses.

1.2 RATIONALE

Substances intended for topical application to human skin need to be tested for their propensity to irritate and/or sensitize. Once appropriate animal studies have been performed, a reproducible, standardized, quantitative patch testing procedure must be used to demonstrate that a particular product can be applied safely to human skin without significant risk of adverse reactions. The method herein employed is generally accepted for such a purpose.

Repeated Insult Patch testing is a modified predictive patch test that can detect weak sensitizers that require multiple applications to induce a cell-mediated (Type IV) immune response sufficient to cause an allergic reaction. In addition, repeated applications of a given material to the same site (at 48 hour intervals) may elicit a "fatigue reaction". This is an irritant reaction seen only after one site is repeatedly exposed to a substance in contrast to the classical "primary" irritant reaction which often occurs on first exposure. In a fatigue reaction, each successive exposure increases the probability of a positive reaction. Results are interpreted as per the working criteria, which are based upon published works as well as the clinical experience of TKL Research, Inc. These working criteria are periodically reviewed and amended subject to new information which becomes available.

1.3 BACKGROUND

Four (4) samples[†]

2-Ethyl,3-Hexanediol)
were submitted to conduct a repeated insult patch test using an exclusive panel of two hundred (200) volunteer subjects. On the basis of information provided by the sponsor and based upon previous testing at this facility (see data on file #852001, #853000 & #853001), these samples were considered reasonably safe for testing on human subjects.

2.0 TEST MATERIALS

2.1 HANDLING OF TEST MATERIALS

Upon arrival at TKL Research, Inc., the material used in this study was entered into a general log book. This serves as a permanent record of the receipt and disposition of all test material. Prior to initiating the study, the material was transferred into 20 ml bottles from which all applications were made. The bottles were labelled in black ink with all pertinent information relating to the study. At the conclusion of the test program, a sample of each product was reserved to be stored for a period of five (5) years. The remainder of the test material was discarded. All information regarding the handling of test material has been recorded on a Clinical Material Record form (see Appendix IV).

2.2 NATURE AND PREPARATION OF TEST MATERIAL

Identification No.	:	100% 2-Ethyl-1,3-Hexanediol
Appearance	:	Clear Liquid
Quantity Provided	:	One (1) Liter
Amount Tested	:	0.2 ml

2.3 SITE DEFINITION

The patches were applied to the infrascapular area of the back, either to the right or left of the midline.

2.4 PATCH DEFINITION

Occlusive: Non-porous, plastic film adhesive bandage with a 2cm² Webril pad. The patch was affixed with Scanpor tape as needed.

3.0 EXPERIMENTAL DESIGN

3.1 PANEL SELECTION (See Demographics - Appendix III)

Number of subjects enrolled	215
Number of completed cases*	203
Age Range	18-79
Sex	Male & Female
Race	Not Designated

*To be considered a completed case, a subject must have had six or more applicatons during induction and at least one reading during challenge. Only completed cases are used to assess sensitization.

NOTE: Subjects who withdrew from the study did so for reasons unrelated to the product.

3.11 INCLUSION CRITERIA

1. Individuals eighteen (18) years of age or older.
2. Individuals free of any systemic or dermatolgic disorder which, in the opinion of the investigative personnel, would have interfered with the results.
3. Individuals who had read, understood and signed an informed consent document.

3.12 EXCLUSION CRITERIA

1. Individuals with any visible skin disease at the test site which might have interfered with the evaluation.
2. Individuals taking medication which, in the opinion of the investigative personnel, would have interfered with the test results.
3. Individuals with active atopic dermatitis.
4. Individuals with psoriasis.
5. Individuals who are currently being tested for asthma.
6. Females who are pregnant or planning a pregnancy.

3.13 INFORMED CONSENT

The consent forms which were signed by each subject in this study are kept on file at TKL Research, Inc., as required by FDA regulations. A sample of the informed consent agreement is included as Appendix V.

3.2 PROCEDURE

The entire study extended over a six (6) week period. It involved three phases: (1) Induction, (2) Rest, and (3) Challenge. Prior to study entry, the subjects were screened to assure that they met the inclusion/exclusion criteria. Informed consent was obtained. Each subject was provided with a schedule of the study activities. They were told to avoid wetting the test sites and were asked not to engage in activities that caused excessive perspiration. They were instructed to notify the staff if they experienced any discomfort beyond mild itching and/or observed any adverse changes at the test sites, while on the study or within two (2) weeks of completing the study.

APPENDIX I

Computer Tabulated Data

UNION CARBIDE
 Product #2-ETHYL 1,3-HEXANEDIOL (100%)

Study #851013
 TKL Research, Inc.

REACTIONS TO APPLICATION #
 =====

Subject # =====	INDUCTION PHASE									CHALLENGE	
	1	2	3	4	5	6	7	8	9	48h72h =====	
53) 5172	-	-	-	-	-	-	-	-	-	-	-
54) 5175	-	-	-	-	-	-	-	-	-	-	-
55) 5178	-	-	-	-	-	-	-	-	-	-	-
56) 5201	-	-	-	-	-	-	-	-	-	-	-
57) 5202	-	-	-	-	-	-	-	-	-	-	-
58) 5206	-	-	-	-	-	-	-	-	-	-	-
59) 5223	-	-	-	-	-	-	-	-	-	-	-
60) 5226	-	-	-	-	-	-	-	-	-	-	-
61) 5227	-	-	-	-	-	-	-	-	-	-	-
62) 5232	-	-	-	-	-	-	-	-	-	-	-
63) 5241	-	-	-	-	-	-	-	-	-	-	-
64) 5258	-	-	-	-	-	-	-	-	-	-	-
65) 5262	-	-	-	-	-	-	-	-	-	-	-
66) 5264	-	-	-	x	-	-	-	-	-	-	-
67) 5270	-	-	-	-	-	-	-	-	-	-	-
68) 5275	-	-	-	-	-	-	-	-	-	-	-
69) 5277	-	-	-	-	-	-	-	-	-	-	-
70) 5279	-	-	-	-	-	-	-	-	-	-	-
71) 5294	-	-	-	-	-	-	-	-	-	-	-
72) 5300	-	x	-	-	-	-	-	?	-	-	-
73) 5301	-	-	-	-	-	-	-	-	-	-	-
74) 5302	-	-	-	-	-	-	-	-	-	-	-
75) 5307	-	-	-	-	-	-	-	-	-	-	-
76) 5309	-	-	-	-	-	-	-	-	-	-	-
77) 5313	-	-	-	-	-	-	-	-	-	-	-
78) 5315	-	-	-	-	-	-	-	-	-	-	-
79) 5316	-	-	-	-	-	-	-	-	-	-	-
80) 5317	-	-	-	-	-	-	-	-	-	-	-
81) 5319	-	-	x	x	x	x	x	x	x	x	x
82) 5322	-	-	-	?	?	?	?	?	?	-	-
83) 5324	-	-	-	-	-	-	-	-	-	-	-
84) 5326	-	-	-	-	-	-	-	-	-	-	-
85) 5328	-	-	-	-	-	-	-	-	-	-	-
86) 5329	-	?	-	?	?	?	?	?	?	?	?
87) 5336	-	-	-	-	-	-	-	-	-	-	-
88) 5341	-	-	-	-	-	-	-	-	-	-	-
89) 5343	-	-	-	-	-	-	-	?	?	-	-
90) 5345	-	-	-	-	-	-	-	-	-	-	-
91) 5356	-	-	-	-	-	-	-	-	-	-	-
92) 5359	-	x	-	-	-	-	-	-	-	-	-
93) 5363	-	-	-	?	?	?	?	?	?	-	-
94) 5367	-	-	-	-	-	-	-	-	-	-	-
95) 5370	-	-	-	-	-	-	-	-	-	-	x
96) 5371	-	-	-	-	-	-	-	-	-	x	x
97) 5372	-	-	-	-	-	-	-	?	?	-	-
98) 5376	-	-	-	-	-	-	-	-	-	-	-
99) 5377	-	-	-	-	-	-	-	-	-	-	-
100) 5381	-	-	-	-	-	-	-	-	-	-	-
101) 5382	x	-	-	-	-	-	-	-	-	-	-
102) 5387	-	-	-	-	-	-	-	-	-	-	-
103) 5391	-	-	-	-	-	-	-	-	-	-	-
104) 5395	-	-	-	-	-	?	?	?	?	-	-

Table 1RECHALLENGE DATA

Product: 100% 2-Ethyl-1,3-Hexanediol

<u>Subject No.</u>		<u>Occlusive</u>		<u>Semi-Occlusive</u>	
		<u>48hr</u>	<u>72hr</u>	<u>48hr</u>	<u>72hr</u>
5493	Arm	+	++	?	?
	Back	?	+	-	?
5453	Arm	?	+	-	-
	Back	?	?	-	-

Study #851013

TKL Research, Inc.

Sponsor: UNION CARBIDE

Test Material: 2-ETHYL 1,3-HEXANEDIOL (100%)

READING	REACTIONS TO APPLICATION #									CHALLENGE	
	INDUCTION PHASE									48H	72H
	1	2	3	4	5	6	7	8	9		
Total # of Readings	209	204	203	204	201	201	202	202	199	203	202
Total # of Patches Dislodged	0	0	0	0	0	0	0	0	0	0	0
GRADE											
-	200	195	185	189	186	189	189	179	177	198	197
?	8	7	16	14	15	12	13	21	20	5	3
+	1	2	2	1	0	0	0	2	1	0	2
+	0	0	0	0	0	0	0	0	0	0	0
++	0	0	0	0	0	0	0	0	1	0	0
+++	0	0	0	0	0	0	0	0	0	0	0
Total # of Panelists Absent	6	11	12	11	14	14	13	13	16	12	13

Test Substance

CAS Number: 94-96-2
Identity: 2-Ethyl Hexane Diol-1,3
Purity: 100%
Remarks: Portions of the report were redacted prior to receipt by Procter & Gamble.

Method

GLP: Not specified.
Report/Study Year: 1985
Method/Guideline Followed: Human Repeat Insult Patch Test
Test Type: Human Repeat Insult Patch Test
Species: Humans (215 volunteers started; 203 completed the study)
Sex: Males and females
Route of Administration: Occlusive patch
Exposure Period: Three week induction period during which an occlusive patch was applied to the infrascapular area of the back, either to the right or left of the midline, each Monday, Wednesday, and Friday. Each patch was left in place for 24 hours and then removed by the subject. 14 days after the last induction application, patches were applied to sites previously unexposed for 24 hours.
Doses: 0.2 mL of 100% solution under occlusive patch

Results

Result: Two subjects developed reactions on challenge to the test substance and were rechallenged. Subject #5453 had no evidence of sensitization on rechallenge testing. Subject #5493 had results on rechallenge indicative of probable irritation although sensitization could not be entirely excluded.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Study Number: 851013
Reference: TKL Research, Inc., 1985. Repeated Insult Patch Test. Accession # 44564

APPENDIX II

Working Criteria

REPEATED INSULT PATCH TEST WORKING
CRITERIA FOR INTERPRETATION OF SENSITIZATION OF DATA

SENSITIZATION

1. A distinction between irritation and sensitization is primarily made using the pattern of reaction, although in certain instances, the physical characteristics of the reactions (e.g., reaction spreading beyond the site of application) may be useful.
2. Sensitization is generally presumed when a numerical equivalent score on challenge is greater than or equal to 1.5 greater than the greatest score obtained on induction. This is confirmed whenever possible by rechallenging the individual at a later date. However, sensitization may also occur when a numeral equivalent score on challenge is less than 1.5 greater than the greatest score obtained on induction. These circumstances are explained below (Sections 3, 4, and 5).
3. Sensitization reactions may occur during the induction phase. This is suggested by the sudden appearance of a ++ or +++ reaction (rather than a gradual increase in severity). If a +++ reaction occurs, a new site is used for a continuation of the induction phase. If a strongly positive reaction (greater than or equal to +++) occurs within 48 hours at the new site, sensitization is very likely and the material is no longer reapplied until the challenge phase.
4. Subjects with reaction patterns that cannot be interpreted as definite sensitization are rechallenged to clarify the results.
5. If a subject with an ambiguous reaction pattern cannot be rechallenged or if rechallenge still gives inconclusive data, the following procedure will be used to determine sensitization potential.

The numerical equivalent of the strongest reaction obtained during challenge will be compared to the numerical equivalent of the strongest reaction obtained during induction (reactions felt to be indicative of sensitization during induction will be excluded) for each panelist. If greater than or equal to 3% of panelists have a "numerical equivalent" difference greater than or equal to 1 point (challenge score greater than induction score), this result will be considered to be evidence for sensitization according to the following scale:

No evidence of sensitization when x is less than 3%

Interpretation may vary as discussed below* when

$3\% \leq x < 5\%$

(x is greater than or equal to 3%, but less than 5%)

Evidence of definite sensitization when $x \geq 5\%$

(x is greater than or equal to 5%)

*Interpretation of values obtained in this range may vary depending upon the nature of the product, the types of reactions obtained and the clinical judgement of the principal investigators.

NOTE: The preceding "working" criteria will be re-evaluated periodically and modified as needed to make interpretation of data more accurate.

APPENDIX III

Demographics

Key:

F = Female
M = Male

C = Caucasoid
N = Negroid
H = Hispanic
M = Mongoloid
O = Other

UNION CARBIDE
Study #851013

TKL Research, Inc.

DEMOGRAPHICS

SUBJECT # =====	SEX ===	RACE =====	AGE ===
3006	F	C	79
3008	F	C	74
3026	F	C	72
3063	F	C	72
3072	F	C	53
3079	F	C	66
3080	M	C	70
3081	F	C	71
3085	F	C	73
3127	F	C	59
3137	F	C	75
3816	F	C	53
3822	F	C	63
3824	F	C	56
3834	F	C	45
3863	F	C	25
3873	F	C	67
3878	F	C	75
3879	F	C	42
3994	F	N	42
3997	F	C	53
4033	F	C	61
4040	F	C	50
5001	F	C	77
5007	F	C	70
5020	F	C	66
5023	F	O	45
5026	F	N	42
5038	F	C	60
5039	F	C	59
5049	F	O	38
5058	F	C	61
5062	F	C	64
5064	F	C	45
5072	F	C	61
5073	M	C	55
5080	F	C	49
5083	F	C	65
5087	F	C	35
5099	F	C	56
5105	F	O	23
5106	M	C	31
5114	F	C	59
5115	M	C	30
5116	F	C	35
5117	F	C	40
5120	F	C	38
5133	F	O	32
5142	F	C	68
5148	F	C	38
5164	M	C	35
5166	M	C	70
5172	F	C	29

UNION CARBIDE
Study #851013

TKL Research, Inc.

DEMOGRAPHICS

SUBJECT # =====	SEX ===	RACE =====	AGE ===
5178	M	C	33
5201	F	C	53
5202	F	C	71
5206	F	C	65
5223	M	C	67
5226	F	C	30
5227	F	C	34
5232	F	C	35
5241	F	C	19
5258	F	C	54
5262	M	C	43
5264	M	C	55
5270	F	C	47
5275	M	N	35
5277	F	C	23
5279	F	C	64
5294	F	C	25
5300	M	C	34
5301	F	C	74
5302	F	C	69
5307	F	C	61
5309	F	C	57
5313	F	C	19
5315	F	C	58
5316	F	C	68
5317	F	C	57
5319	F	M	26
5322	F	C	39
5324	F	C	65
5326	F	C	50
5328	F	C	66
5329	M	C	64
5336	F	C	32
5341	F	H	24
5343	F	H	33
5345	F	H	25
5356	F	H	25
5359	F	C	65
5363	F	C	45
5367	F	C	38
5370	M	C	68
5371	M	C	57
5372	F	H	52
5376	F	C	37
5377	F	C	43
5381	F	H	37
5382	F	H	29
5387	F	C	36
5391	F	H	43
5395	F	H	32
5396	F	H	73
5400	F	C	68
5401	F	C	39
5402	F	C	68

DEMOGRAPHICS

SUBJECT # =====	SEX ===	RACE =====	AGE ===
5404	F	C	27
5405	F	H	63
5406	M	H	60
5408	M	H	22
5412	F	O	39
5414	F	H	30
5415	F	C	25
5417	F	H	18
5419	F	C	32
5427	F	C	33
5430	F	C	57
5431	M	H	57
5434	F	C	31
5437	F	C	55
5438	F	C	45
5447	F	C	55
5453	F	H	47
5454	F	H	51
5457	F	C	38
5459	F	H	40
5460	F	H	54
5462	F	H	37
5465	F	H	44
5472	M	H	69
5473	F	H	28
5483	M	C	47
5484	F	H	75
5486	M	H	50
5487	F	H	72
5493	F	H	32
5494	F	H	29
5505	F	H	32
5517	F	H	23
5520	F	H	35
5521	M	C	68
5523	M	H	33
5524	F	C	27
5525	F	C	42
5526	M	C	26
5527	M	C	18
5528	F	C	42
5529	F	C	39
5530	F	C	62
5531	F	C	24
5532	F	C	45
5533	F	C	35
5534	F	C	57
5535	F	C	41
5536	M	C	43
5537	M	N	52
5538	M	H	25
5539	F	H	30
5540	F	H	26
5541	M	H	18

DEMOGRAPHICS

SUBJECT # =====	SEX ===	RACE ====	AGE ===
5542	M	H	29
7006	F	C	30
7007	F	C	29
7009	F	C	55
7010	F	C	39
7011	M	C	51
7013	F	C	35
7016	F	N	28
7020	F	C	24
7029	F	C	42
7030	M	C	29
7034	F	C	55
7039	F	C	27
7040	F	C	35
7045	F	C	49
7048	F	C	53
7050	F	C	48
7051	F	C	49
7053	F	C	66
7054	F	C	30
7059	F	C	35
7060	F	C	55
7070	F	C	70
7076	F	C	46
7078	F	C	40
7080	F	C	31
7082	F	C	42
7086	F	N	53
7092	F	C	29
7099	F	C	52
7100	F	C	52
7105	F	C	45
7106	F	C	37
7107	F	C	30
7108	F	C	44
7109	F	C	47
7110	F	C	25
7111	F	C	36
7112	M	C	73
7113	F	C	35
7114	F	C	29
7115	F	C	41
7116	F	C	37
7117	F	C	32
7118	F	C	43
7119	M	C	65
7120	F	C	54
7121	F	C	30
7122	F	C	54
8413	F	C	28
8414	F	C	31
8415	F	C	32
9570	F	C	30

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APPENDIX IV

Clinical Material Record

CLINICAL MATERIAL RECORD

SPONSOR UNION CARBIDE STUDY # 851013

NAME OF CLINICAL MATERIAL 2-ethyl 1,3 hexanediol

IDENTIFICATION # _____ LOT # _____ EXP. DATE _____

DESCRIPTION OF MATERIAL _____

DATE OF RECEIPT 5/22/85 7/3/85

QUANTITY RCVD 1 liter (5%) 1 liter (100%)

SAMPLE STORAGE 20 ml (100%) (AMOUNT) 10/4/85 DA (INITIAL AND DATE)

MATERIAL DISCARDED remainder (AMOUNT) 10/25/85 DN (INITIAL AND DATE)

MATERIAL RETURNED TO SPONSOR _____ (CARRIER)

_____ (DATE)

_____ (INITIALS)

APPENDIX V

Informed Consent

I N F O R M E D C O N S E N T

PANEL #: _____

1. PURPOSE

To determine the ability of one or more test materials to cause irritation and/or sensitization (i.e. allergy).

2. STUDY REQUIREMENTS

An individual participating in this study:

- (a) must be eighteen years of age or older.
- (b) must inform the staff and/or investigator of all medications he or she is now taking or uses at any time during the study. An individual may not be taking any medication which, in the opinion of the investigator, would interfere with the test results.
- (c) must inform the staff of any skin disorders.
- (d) must not be pregnant or planning a pregnancy.
- (e) must not be on asthma therapy.

3. PROCEDURE

This study involves research to see if the test material can be put on human skin without causing some degree of irritation or sensitization (i.e. allergy). The study will extend over a _____ period and will involve a minimum of _____ participants.

The test samples will contain materials which are intended for or may come into contact with human skin. Some of these materials may be irritating under certain conditions, but the degree of irritation is not expected to be greater than described below.

The test material will be put on your back or arm with a "patch" (i.e. a small adhesive square with a cotton pad). The patch will remain on your back (or arm) for 24, 48 or 72 hours. You will return to TKL Research at specified times to have the materials reapplied or the patches removed. At each of these visits a technician will examine your back to see if you are reacting. If you have a strong reaction at the test site, the test material will not be applied to that site, but may be applied to another site(s).

4. POTENTIAL RISKS

Individuals participating in this study may develop a localized reaction characterized by redness, swelling, itching, cracking, peeling, or in rare cases, small blisters or sores. Reactions usually occur only where the patch pad touches the skin. On rare occasions, the reactions may spread a few inches beyond the patch. A reaction may result in localized lightening or darkening of the skin, which may persist in an occasional individual. Reactions may be due to either skin irritation or allergy. It may be necessary to do additional patch testing (rechallenge) to determine if the reaction is allergic. If it should prove to be allergic, you can expect to react to this material if you encounter it at a later

continues

TKL RESEARCH, INC.

133 EAST 58 STREET

NEW YORK, N.Y. 10022

Panel #: _____

I N F O R M E D C O N S E N T
(RIPT/PROPHETIC/PRIMARY IRRITATION)

Page -2-

date. Whenever possible, you will be informed as to the identity of the causative agent, in order that you may avoid contact with it in the future.

In the event of a reaction, no additional financial compensation will be provided. However, dermatologic care for any severe skin reaction resulting from the above procedure is available from Dr. Michael Reed. Dr. Reed can be contacted at TKL Research (759-7969) or at his office at 338 East 30th Street, New York, N.Y. 10016 (889-0470).

5. POTENTIAL BENEFITS

You will be paid a predetermined amount upon completion of this study. If you drop out on your own accord for personal reasons, or are dismissed for refusal to obey rules or follow instructions, you will not be paid. If, in the judgment of the investigating personnel, it is best to discontinue your participation in the experiment for other reasons, you will be paid for that portion of the test already completed.

Participation in the study is voluntary and you may withdraw at any time without obligation or prejudice to you, and without loss of benefits to which you are otherwise entitled, except as stated above.

Reports prepared by TKL Research will utilize statistical information only and at no time will your name be used. However, the Company(s) whose product is being tested, the Food & Drug Administration, and others in certain legal action, may inspect the records of this study which may include access to names of, and information relating to, test participants.

The foregoing does not constitute a waiver or a release of the investigator, the sponsoring company, TKL Research, Inc. or its agents from liability or negligence.

I HAVE READ AND FULLY UNDERSTAND THIS PROCEDURE, THE RISKS AND BENEFITS. I HAVE BEEN GIVEN THE OPPORTUNITY TO ASK QUESTIONS. I UNDERSTAND THAT ADDITIONAL INFORMATION REGARDING THIS RESEARCH IS AVAILABLE EITHER BEFORE OR DURING THE COURSE OF THE STUDY, I HAVE NO QUESTIONS AT THIS TIME. I AGREE TO PARTICIPATE IN THE STUDY AS OUTLINED ABOVE.

A COPY OF THIS CONSENT FORM HAS BEEN GIVEN TO ME.

SIGNATURE OF PARTICIPANTS:

DATE: _____

SIGNATURE OF INVESTIGATOR: _____ Date: _____

SIGNATURE OF WITNESS: _____ Date: _____

The Induction Phase consisted of nine (9) consecutive applications of the test material and subsequent evaluations of the test sites. The subjects were required to remove the patches approximately twenty-four (24) hours after application. They returned to the facility at forty-eight (48) hour intervals to have the sites evaluated, and identical patches reapplied. Prior to application of the patches, the sites were outlined using a gentian violet skin marker. Those patches applied on Friday were removed on Saturday. Of necessity, evaluation of the test sites were made on Monday, i.e., 72 hours after application. Following the ninth evaluation, the subjects were dismissed for a fourteen (14) day rest period.

The Challenge Phase was initiated during the sixth week of the study, with identical patches applied to sites previously unexposed to the test material. These patches were removed after twenty-four (24) hours. The sites were graded twenty-four hours and forty-eight (48) after removal, i.e., 48 and 72 hours after application.

3.3 DEFINITIONS USED FOR GRADING RESPONSES

The symbols found in the computer data accompanying this report are used to express the response observed at the time of examination:

- No reaction
- ? Doubtful response, barely perceptible erythema, only slightly different from surrounding skin
- + Definite erythema
Minimal or doubtful edema
- +* Definite edema
Minimal or doubtful edema
- ++ Definite erythema
Definite edema
- +++ Definite erythema
Definite edema & vesiculation
- X Panelist Absent

4.0 DATA SUMMARY

See Computer Tabulated Data - Appendix I.

5.0 INTERPRETATION

See Working Criteria - Appendix II.

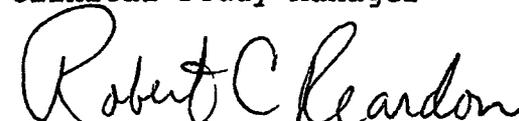
6.0 RESULTS & DISCUSSION

Two subjects (#5453 and #5493) developed reactions on challenge to the 2-Ethyl 1,3-Hexanediol, which required additional testing to differentiate between irritation and sensitization (i.e., allergy). Subject #5453 had no evidence of sensitization on rechallenge testing. Subject #5493 had results on rechallenge indicative of probable irritation although sensitization could not be entirely excluded. However, sensitization, if present, is very mild and not clinically significant since it could only be elicited under occlusive conditions. The results are shown on Table 1 - Appendix I.

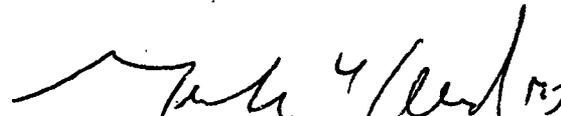
6.0 SIGNATURE PAGE

Prepared and Reviewed By:


Diana Napoli, R.N.
Clinical Study Manager


Robert C. Reardon, Ph.D.
Project Director

Approved By:


Michael L. Reed, M.D.
Consulting Dermatologist

NOTE: Copies of all reports, including raw data, will be kept on file at TKL Research, Inc., for a period of five (5) years after completion of the study.

Test Substance

CAS Number: 94-96-2
Identity: 2-Ethyl Hexane Diol-1,3
Purity: Not specified
Remarks: Test substance was identified as E2751.01

Method

GLP: Clinical study conducted under the SOP's of the Clinical Research Organization and was reviewed by the CRO's Quality Assurance Unit.
Report/Study Year: 1986
Method/Guideline Followed: Human Repeat Insult Patch Test
Test Type: Human Repeat Insult Patch Test
Species: Humans (83 volunteers started; 79 completed)
Sex: Males and females
Route of Administration: Occlusive patch
Exposure Period: Three week induction period during which an occlusive patch was applied to the upper arm each Monday, Wednesday, and Friday. Each patch was left in place for 24 hours and then removed by the subject. Seventeen days after the last induction application, duplicate challenge patches were applied for 24 hours.
Doses: 5% w/v in liquid paraffin

Results

Result: There was no evidence of sensitization in any of the volunteer panelists.

Data Quality

Reliability (Klimisch): 2

Reference

Laboratory Study Number: LSR 69
Reference: Procter and Gamble, 1985. Human Repeat Insult Patch Test LSR 69 ECM BTS 1083, E2751.01. Accession # 35365

Acc# 35365

14 APR 1986 ✓

CONFIDENTIAL

25/PGH574D/164

HUMAN REPEAT INSULT PATCH TEST LSR 69

ETHD

ECK BT5 1083, E2751.01

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20 March 1986



LIFE SCIENCE RESEARCH

HUMAN REPEAT INSULT PATCH TEST LSR 69

ECM BTS 1083, E2751.01

LSR Report No. : 86/PGH574D/164

QUALITY ASSURANCE INSPECTIONS

Inspection	Dates (Day.Month.Year)	
	Report to Study Director	Report to Management
PROTOCOL Inspection of protocol was made in accordance with LSR Standard Operating Procedure QAU/020. Dates for inspection of protocol amendments in accordance with this S.O.P. are not quoted	20.1.86	20.1.86
DATA Inspection of data generated on this type of study was made in accordance with LSR Standard Operating Procedure QAU/050	19.2.86	27.2.86
PROCEDURES Inspection of procedures on this type of study was made in accordance with LSR Standard Operating Procedure QAU/040	11.2.86 11.2.86 11.2.86	- - 12.2.86

Other routine procedures used in this type of study, and facilities were inspected regularly and reports made in accordance with LSR Standard Operating Procedure QAU/040.

This report has been reviewed by the LSR Quality Assurance Unit employing methods laid down in LSR Standard Operating Procedure QAU/060. The reported methods and procedures were found to describe those used and the results to constitute an accurate representation of the data recorded.

This review was completed on 9 April 1986.

B.J. Ford, B.Sc., Ph.D.
(Head of Quality Assurance Unit)


Date 9 April 1986



LIFE SCIENCE RESEARCH

SUMMARY

1. Eighty-three subjects received occlusive patches containing test materials, generally according to the LSR Standard Protocol No. HUM/917 dated 13 May 1985. Seventy-nine subjects (18 of whom received only seven induction patches) completed the trial.
2. Throughout the test E2751.01 was used at 5.0% (w/v) in liquid paraffin.
3. No evidence of skin sensitization was seen.
4. Irritation scores were distributed as follows:

	<u>U</u>	<u>1</u>	<u>H7G</u>	<u>Not recorded</u>
ECM BTS 1083, E2751.01	98 ⁰	0	1	1

A. MacLennan
.....

A. MacLennan, Ph.D.
(Study Director)

Date *20/12/86*

R. Davies
.....

R. Davies, B.Sc.

Date *20/3/86*

METHODS

In order to assess the sensitization potential of the test material in human volunteers, the LSR Standard Protocol No. HUM/917 dated 13 May 1985 for a human repeat insult patch test was generally followed.

This consists of a three week induction period during which an occlusive patch was applied to the upper arm each Monday, Wednesday and Friday. E2751.01 was not available for use until the third test day therefore the pilot panel (Subject Nos. 1-24) received only seven induction patches. One of these subjects (No. 8) received eight induction patches. Each patch was left in place for 24 hours and then removed by the subject. Seventeen days after the last induction application, duplicate challenge patches were applied for 24 hours. This rest period for those subjects receiving a make-up patch was 14 days.

Patches consist of a 5 cm wide strip of Bienderm tape, to which three Webril discs (ca 24 mm diameter) and one 2 x 2 cm square of test fabric (E2810.01) were fixed along the midline at equal intervals. E2751.01 was applied in 0.4 ml samples to one disc of each patch. Three other test materials: ECM BTS 1090, E2760.01; ECM BTS 1118, E2794.01; and ECM BTS 1100, E2810.01 were tested simultaneously and are reported separately under report numbers 86/PGN574A/161, 86/PGN574B/162 and 86/PGN574C/163 respectively. The test materials were applied to the patch strips in different orders at random among the panel except for the pilot panel who all received E2751.01 on the bottom disc.

Scoring of test sites was performed after a ca 24 hour rest period (48 hours over a weekend) and immediately preceding the next patch application. Challenge sites were scored 48 and 98 hours after application. Skin reactions were assessed by R. Hill (14 and 17 February) and N. Vert (all remaining dates) according to the scoring procedure reproduced in Appendix 1.

RESULTS

Eighty-three volunteers were recruited for the study which took place at Highcliffe Community Centre, Rettendon View, Wickford, Essex, England from 13 January 1986 to 7 March 1986.

Four subjects dropped out of the study, for reasons unrelated to the test. The age and sex of the 79 subjects who completed the study are tabulated in Appendix 2.

Skin reactions observed in the study are presented in Appendix 3.

The test material showed no evidence of skin sensitization.

APPENDIX I
SCORING AND DEFINITION OF
SYMBOLS USED IN TABULATING DATA

- 0 - No visible reaction. This score would include superficial skin responses such as glazing, peeling, cracking.
 - 1 - Mild erythematous reaction. Faint pink to definite pink.
 - 1E - Mild erythematous reaction with papules and/or oedema.
 - 2 - Moderate erythematous reaction. Definite pink to red erythema (similar to sunburn).
 - 2E - Moderate erythematous reaction with oedema and/or papules.
 - 3 - Strong erythematous reaction. Beet red.
 - 3E - Strong erythematous reaction with marked oedema, papules and/or few vesicles.
 - 4 - Severe reaction with erythema, oedema, papules and vesicles (may be evidence of weeping).
 - 5 - Bullous reaction.
 - (5) - Reaction spread beyond Webril pad area.
- Note: Erythema, papules, oedema and vesicles are judged to be present if they involve 25% or more of the patch site.
- Move
Criteria: Any grade greater than 1 (which includes the addition of any letter designation) during induction necessitates relocation of the patch.
- Double
grade - Indicates patch moved to new adjacent site.* First number is the grade for the new site; second number is the grade for the residual reaction at the old site. Generally a residual reaction is only read or reported once following a single move. If sensitization is suspected, a grade for an old site may be reported more than once.
- A - Marked reaction to adhesive (patch relocated).
 - X - Succeeding patch not applied, and succeeding grade is residual reaction**.
 - R - Right arm used during induction.
 - W - Subject started test on Wednesday therefore no grade recorded.
 - L - Patch came off (lost) during first 12 hours.
 - (-) - Subject absent.
 - N7G - No seventh grade. Subject has worn seven induction patches but was not present for scoring following seventh induction application.
 - * - The portion of the patch strip containing the material in question is cut from the remaining materials. Only this portion is moved to an adjacent site.
 - ** - Explanation given in test records and in report if patch not applied for reason other than residual reaction at intended application site.

APPENDIX 2

Composition of Panel by Age and Sex

<u>Age</u>	<u>Male</u>	<u>Female</u>
17		1
22		1
24		1
26		3
27		2
28		2
30		2
31		2
32		1
33		1
34		3
35		2
36		3
37		4
38		6
39		1
40		4
41		2
42		5
43		1
45		2
46		2
47		1
49		3
50		1
52		2
56		1
58		1
60		1
63		2
64	1	1
65	1	2
66	1	2
67	1	
68		1
70	1	
71	1	1
74	1	
76	1	
78		1
80		1
Total	8	71

APPENDIX 3

E2751.01 : individual responses to applications of 5.0% (w/v) in liquid paraffin

Subject Number	Session										Post-Challenge			
	2	3	4	5	6	7	8	9	10	MU	48 Hr.		96 Hr.	
											Or	Alt	Or	Alt
1	-	-	-	0	0	0	0	0	0	0	0	0	0	0
2	-	-	-	0	0	0	0	0	0	0	0	0	0	0
3	-	-	-	0	0	0	0	0	0	0	0	0	0	0
5R	-	-	-	0	0	0	0	0	0	0	0	0	0	0
6	-	-	-	0	0	0	0	0	0	0	0	0	0	0
7R	-	-	-	0	0	0	0	0	0	0	0	0	0	0
8R	W	-	-	0	0	0	0	0	0	0	0	0	0	0
9	-	-	-	0	0	0	0	0	0	0	0	0	0	0
10R	-	-	-	0	0	0	0	0	0	0	0	0	0	0
12	-	-	-	0	0	0	0	0	0	0	0	0	0	0
14R	-	-	-	0	0	0	0	0	0	0	0	0	0	0
15R	-	-	-	0	0	0	0	0	0	0	0	0	0	0
17R	-	-	-	0	0	0	0	0	0	0	0	0	0	0
19	-	-	-	0	0	0	0	0	0	0	0	0	0	0
20R	-	-	-	0	0	0	0	0	0	0	0	0	0	0
21	-	-	-	0	0	0	0	0	0	0	0	0	0	0
22	-	-	-	0	0	0	0	0	0	N76	0	0	0	0
23R	-	-	-	0	0	0	0	0	0	0	1	0	0	0
24	-	-	-	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28R	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31R	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0	0	0	0	0
33	0	0	0	0	0	0	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0	0	0	0	0	0	0
38	W	0	0	0	0	0	0	0	0	0	0	0	0	0
39R	0	0	0	0	0	0	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	1	1	1	0	0	0	0	0
43	0	0	0	0	0	0	0	1	1	1	0	0	0	0
45	0	0	0	0	0	0	0	0	0	0	0	0	0	0
45R	W	0	0	0	0	0	0	0	0	0	0	0	0	0

Δ Score not recorded

Continued on next page

APPENDIX 3 - continued

E2751.01 : individual responses to applications of 5.0% (w/v) in liquid paraffin

Subject Number	S e s s i o n										Post-Challenge			
	2	3	4	5	6	7	8	9	10	MU	48 Hr.		96 Hr.	
											Or	Alt	Or	Alt
47	0	0	0	0	0	0	0	0	0		0	0	0	0
48	0	0	0	0	0	0	0	0	0		0	0	0	0
50	0	0	0A	-	0	0	0	0	0		0	0	0	0
51R	0	0	0	0	0	0	0	0	0	0	0	0	0	0
52	W	0	0	0	0	0	0	0	0	0	0	0	0	0
53R	0	0	0	0	0	0	0	0	0		0	0	J	0
54R	0	0	0	0	0	0	0	0	0		0	0	0	0
55	0	0	0	0	0	0	0	0	0		0	0	0	0
56R	0	0	0	0	0	0	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0		0	0	0	0
58R	0	0	0	J	0	-	0	0	0	0	0	0	0	0
60R	0	0	0	J	0	0	0	0	0		0	0	0	0
62	0	0	0	0	0	0	0	0	0		0	0	0	0
63	0	0	0	0	0	0	0	0	0		0	0	0	0
64	0	0	0	0	0	0	0	0	0		0	0	0	0
65R	0	0	0	0	0	-	0	0	0	0	0	0	0	0
67R	0	0	0	0	0	0	0	0	0		0	0	0	0
69R	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72	0	0	0	0	0	0	0	0	0		0	0	0	0
73R	0	0	0	0	0	0	0	0	0	0	0	0	0	0
75	0	0	0	0	0	-	0	0	0		0	0	0	0
77	0	0	0	0	0	0	0	0	0		0	0	0	0
78R	0	0	0	0	0	-	0	0	0	0	0	0	0	0
79	0	0	0	0	0	0	0	0	0	0	0	0	0	0
81	0	0	0	0	0	0	0	0	0		0	0	0	0
84	0	0	0	0	0	0	0	0	0		0	0	0	0
85	0	0	0	0	0	0	0	0	0		0	0	0	0
86	0	0	0	0	-	0	0	0	0	0	0	0	0	0
87	0	0	0	0	0	0	0	0	0		0	0	0	0
90	W	0	0	0	0	0	0	0	0	0	0	0	0	0
91	0	0	0	0	0	0	0	0	0		0	0	0	0
92	0	0	0	0	0	0	0	0	0		0	0	0	0
93R	0	0	0	0	0	0	0	0	0	0	0	0	0	0
94R	0	0	0	0	0	0	0	0	0		0	0	0	0
95	0	0	0	0	0	0	0	0	0		0	0	0	0
96	0	1	0	0	1	0	0	0	0		0	0	0	0
97	0	0	0	0	0	0	0	0	0		0	0	0	0
98	0	0	0	0	0	0	0	0	0		0	0	0	0
99	0	0	0	0	0	0	0	0	0		0	0	0	0
100	0	0	0	0	0	0	0	0	0		0	0	0	0

^ Patch left on for 48 hours

