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

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

CONTAIN NO CBI

Dear Sir or Madam:

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

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

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

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

Sincerely,
THE PROCTER & GAMBLE COMPANY

Richard J. Hackman
Associate Director
Regulatory & Technical External Relations
(513) 983-0534
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Index of Health & Safety Studies submitted by Procter & Gamble
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Chemical Name	CAS #	Lab Study #	Title	Company Acc #
1,3-Hexanediol, 2-ethyl-	94-96-2	85-029	Semi-Continuous Activated Sludge (SCAS) Removability Test on B0859.01	31559
1,3-Hexanediol, 2-ethyl-	94-96-2	BW-86-1-1928	Acute Toxicity of B0859.01 to Bluegill (<i>Lepomis macrochirus</i>)	32348
1,3-Hexanediol, 2-ethyl-	94-96-2	165-09-1100-1	Toxicity of B0859.01 to <i>Microcystis aeruginosa</i> .	32358
1,3-Hexanediol, 2-ethyl-	94-96-2	85-030	CO ₂ Production Test on B0859.01	32091
1,3-Hexanediol, 2-ethyl-	94-96-2	BW-85-11-1884	Acute Toxicity of B0859.01 to <i>Daphnia magna</i> .	32066
1,3-Hexanediol, 2-ethyl-	94-96-2	MVS1482	Testing 2-ethyl-1,3-hexanediol in the mouse <i>in vivo</i> skin micronucleus model	MVS1482
1,3-Hexanediol, 2-ethyl-	94-96-2	191-1215	Rabbit Eye Irritation (Low Volume Procedure)	31928
1,3-Hexanediol, 2-ethyl-	94-96-2	T4636.380	Test for Chemical Induction of Unscheduled DNA Synthesis in Primary Cultures of Rat Hepatocytes (by Autoradiography)	33081
1,3-Hexanediol, 2-ethyl-	94-96-2	851013	Repeated Insult Patch Test	44564
1,3-Hexanediol, 2-ethyl-	94-96-2	LSR 69	Human Repeat Insult Patch Test LSR 69 ECM BTS 1083, E2751.01	35365
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	4708-126/69	Delayed Contact Hypersensitivity Study in the Guinea Pig. (Buehler Test) Test Article RO 163	42321
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	2-5-253-85	Guinea Pig Sensitization Testing modified by Ritz and Buehler on R 0163	42322
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	78-368-21	Delayed Contact Hypersensitivity Study in Guinea Pigs of R0060-01	21114
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	WIL-1179-78	Delayed Hypersensitivity Study in Guinea Pigs of R0060-02.	20749
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	78-086-21	Delayed Contact Hypersensitivity Study in Guinea Pigs of R0060	19932
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	L08321-SNO9	Performance of the Murine Local Lymph Node Assay	36754
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	930/040	Magnusson & Kligman Maximisation Study in the Guinea Pig.	100345
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	V 92.392/352063	Sensitization study with xxx in guinea pigs (maximization test).	103104
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	TES810036	Guinea Pig Sensitization Study – Magnusson-Kligman Maximization Method – Positive Control	44906
Benzene, 1-chloro-2,4,-dinitro-	97-00-7	50931	Delayed Contact Hypersensitivity Study in Guinea Pigs (Buehler Sensitization Test) of xxx	103074

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

Test Substance Description

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not available
Comments: Test substance was included as a "known sensitizer" as part of laboratory qualification for this endpoint. Test material was coded as "RO 163"

Method

Method / Guideline: Buehler method
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1985
GLP Compliant: Yes
Species: Guinea pig – Dunkin-Hartley albino strain
Number of Animals: 30 (15 male / 15 female)
Study Design: 20 (10 M/10 F) in test group and 10 (5 M/5 F) in control
Screening study: 0.4 mL of test substance at concentrations of 3.0, 1.0, 0.3 and 0.1 % (w/v) in 80% ethanol was applied for 6 hours on the shaven back of each of 4 animals. The skin sites were evaluated 24 hours after treatment to determine the highest non-irritating concentration that could be used at induction; 0.3% (w/v) test substance in 80% ethanol was selected.
Induction: The upper left quadrant of the back was clipped with electric clippers. The following day, a 25 mm Hill Top Chamber moistened with 0.4 ml of 0.3% (w/v) test substance in 80% ethanol was placed on the clipped areas of 20 test animals, for 6 hours. Patches were reapplied weekly to the same, freshly clipped areas, for 3 consecutive weeks.
Challenge: After a two-week rest period, test and control animals were challenged with a 0.2% (w/v) test substance in acetone on a fresh application site for 6 hours. Depilated animals were scored for erythema severity using a 0-3 scale, 24 and 48 h post-challenge.

Results

Result: The test material induced delayed contact hypersensitivity.
Comments: Study was conducted as part of new laboratory qualification for this study.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Report Number: 4708-126/69
Reference: Procter & Gamble, 1985. Delayed Contact Hypersensitivity Study in the Guinea Pig, Test Article RO 163. Accession #42321

ACC #42321



CONFIDENTIAL

DNLF5

PROJECT ECM B'S 1044

TEST ARTICLE RO 163:

DELAYED CONTACT HYPERSENSITIVITY STUDY

IN THE GUINEA PIG (BUEHLER TEST)

RECEIVED	15-10-1985
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Report for:

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Prepared by:

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Report No:

4706-126/69

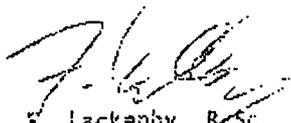
Date:

September 1985

AUTHENTICATION

REPORT NO. 4708-126/69

I, the undersigned, hereby declare that the work described in this report was performed under my supervision, as Study Director, in accordance with the agreed protocol, and Hazleton Standard Operating Procedures unless otherwise stated, and that the report provides a true and accurate record of the results obtained.



F. Lackenby, B.Sc.
Study Director, Acute Toxicology

The following scientific and supervisory personnel were involved in the study under the overall supervision of the Study Director.

D. Breckon,
Study Supervisor

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1. SUMMARY

1.1 A study was performed to determine the skin sensitisation potential of RO 163 in the albino guinea pig based on the Buehler Test.

1.2 The incidence and severity of the skin reactions observed in both test and control animals were:

Experimental group	Observation time (hour)	Skin responses (scale 0-3)					Incidence (N)	Severity (A)
		0	0.5	1	2	3		
Test	24	7	7	4	2	0	6/20	0.58
	48	3	15	2	0	0	2/20	0.48
Control	24	10	0	0	0	0	0/10	0
	48	10	0	0	0	0	0/10	0

N = Number of animals showing skin responses ≥ 1 .

A = Sum of skin responses divided by the number of animals tested.

These results indicated that RO 163 does cause delayed contact hypersensitivity in the albino guinea pig using the Buehler Test.

1.3 The study was performed during April, May and June 1985.

2. INTRODUCTION

The study was performed to determine the skin sensitisation potential of RO 163 in the albino guinea pig based on the Buehler Test.*

The study was performed in the Acute Studies Unit at Hazleton Laboratories Europe Ltd., Otley Road, Harrogate, North Yorkshire, England.

3. EXPERIMENTAL PROCEDURES

3.1 Adherence to protocol

The study was performed in accordance with the agreed protocol OECD 5, March 1985. The following amendment was made at the request of the study sponsor.

Protocol section 3 Test system

The group size in the main study was 20 Test (10 male, 10 female) and 10 Control (5 male, 5 female).

3.2 Test article

3.2.1 Description, identification and storage conditions

The test article was supplied by the study sponsor as follows:

Identification:	ECM BTS 1044 RO 163.
Description:	Pale yellow powder.
Container:	Brown glass screw capped bottle.
Quantity:	Approximately 2 g.
Date of arrival:	17 April 1985.
Storage conditions:	Room temperature.

3.2.2 Route and method of administration

The test article was administered during the induction and challenge applications by topical application from a disposable 1 ml syringe to sites on the shaved flank or back of each guinea pig.

* Buehler E.V. Delayed Contact Hypersensitivity in the Guinea Pig. Arch Dermatol 91, 171, 1965.

A sensitisation study using the topical route of application is recommended for materials which are likely to come into repeated contact with the skin during their manufacture, handling and use.

3.2.3 Method of test article preparation

The test article was applied for the 3 inductions as a colourless 0.3% w/v solution in 80% ethanol. The test article was applied as a colourless 0.2% w/v solution in acetone for the challenge.

3.2.4 Frequency of administration

The test article was applied on days 1, 8 and 15 during the induction period, followed by a single challenge application 2 weeks after the final induction application. Each application remained in contact with the skin for 6 hours.

3.2.5 Analysis of test article formulation

Analysis of the test article formulation was not required by the study sponsor.

3.2.6 Determination of the degree of absorption of the test article

The determination of the degree of absorption of the test article was not performed as it was considered not necessary to meet the objectives of the study.

3.3 Test system (animals)

3.3.1 Species, strain and supplier

Thirty four (17 male, 17 female) albino Dunkin-Hartley guinea pigs were obtained from S.F. Animal Supply Ltd., Henfield.

3.3.2 Justification for the selection of the test system

The albino guinea pig was the species of choice because it is readily available, is of relatively low cost and is easy to handle, house and dose. The absence of pigmentation in the skin facilitates evaluation of induced skin reactions, and

there is a considerable amount of published information available concerning sensitisation reactions in guinea pigs to assist in the assessment of the significance to man of any treatment-induced changes. Sensitised guinea pigs clearly exhibit a local erythematous reaction at the site of challenge with test substances characteristic of delayed hypersensitivity.

3.3.3 Specification

At the start of the study the animals weighed between 384 and 584 g. The animals were acclimatised to the laboratory environment for at least 5 days and were examined for signs of ill health or injury shortly before the study commenced. No animals were rejected.

3.3.4 Husbandry

The animals were housed in a single air-conditioned room maintained at a temperature between 19 and 25°C and relative humidity between 40 and 70%. The animals were exposed to a constant daily photoperiod of 12 hours artificial light and 12 hours darkness. The animals were caged in groups of 2 or 3 by sex in grid floor metal cages. Clean water bottles were provided once each week.

3.3.5 Diet and drinking water

The animals had free access to mains drinking water and food (Guinea Pig diet, Standard with Vitamin C, Special Diets Services Ltd, Witham) throughout the study. A certificate relating to the quality of the drinking water was issued by the Yorkshire Water Authority. The diet and drinking water were considered not to contain any contaminant at a level that might have affected the purpose or integrity of the study.

3.3.6 Randomisation

No formal randomisation procedure was adopted. The animals were transferred, as they came to hand from the delivery

crates, to holding cages one at a time working along the battery from left to right and from top to bottom until each cage contained one animal. This procedure was repeated for each sex until each cage contained 2 or 3 animals.

3.3.7 Identification

The animals were individually identified using a system of indelible ink markings on the ears. The numbers used were as follows:

Group number	Colour code	Group description	Animal numbers	
			Male	Female
1	Pink	Test	5-14	15-24
2	Buff	Control	25-29	30-34

Each cage displayed a group-related, coloured card giving details of the test article, dosage, project number, sex, animal number, date of first treatment, route of administration and the name of the Home Office licensee responsible for the study.

3.4 Treatment procedures

The day before treatment the backs of the guinea pigs were clipped free of hair using electric clippers.

3.4.1 Screening study

The test article (0.4 ml) at concentrations of 3.0, 1.0, 0.3 and 0.1% w/v in 80% ethanol was applied for 6 hours under 25 mm Hill Top Chambers to test sites on the shaven back of each of 4 guinea pigs. The skin sites were evaluated 24 hours after treatment.

3.4.2 Main study

The experimental design was represented diagrammatically as follows:

	INDUCTION	CHALLENGE
DAY	1, 9, 5, 8, 10, 12, 15, 17, 18	33
	occlusive 6 h 0.4 ml sol	occlusive 6 h 0.4 ml sol
1. (TEST)		
2. (CONTROL)		

3.4.2.1 Induction

A 25 mm Hill Top Chamber moistened with a 0.3% w/v preparation of test article (0.4 ml) in 80% ethanol was applied to the left flank of each guinea pig for a contact period of 6 hours. The Hill Top Chambers were held in place with occlusive tape wound round the torso of the animal. The treatment was repeated on days 8 and 15 on the same site, which was shaved the day before each application.

3.4.2.2 Challenge

3.4.2.2.1 Treatment group (group 1)

The test animals were challenged by application of a Hill Top Chamber moistened with a 0.2% w/v preparation of test article (0.4 ml) in acetone on the right flank of each animal. After 6 hours the patches were removed. The following day all animals were treated with depilatory cream on the challenge sites, rinsed thoroughly and dried with a disposable paper towel.

3.4.2.2.2 Negative control (group 2)

Ten previously untreated controls each received an identical application of the

test solution using the same procedure as for the treatment group.

3.5 Evaluation of effects

Three hours after depilation the challenge sites from both groups were evaluated (24 hour reading). The evaluation was repeated 24 hours later (48 hour reading).

Reactions were scored on a 5 point scale:

No reaction	0
Slightly patchy erythema	0.5
Slight, but confluent or moderate patchy erythema	1
Moderate erythema	2
Severe erythema, with or without oedema	3

3.6 Evaluation of data

A comparison of the intensities and durations of reactions between groups permitted identification of sensitisation.

Incidence score = The number of animals showing responses of 1 or greater at each evaluation related to the number of animals evaluated.

Severity score = The sum of the test grades at each evaluation divided by the number of animals evaluated.

Grades of 1 or greater in animals in the test group provide clear indication of sensitisation provided grades of less than 1 are seen in the negative control animals. If grades of 1 or greater are noted in negative control animals, then reactions of test animals that exceed the more severe reaction in controls are presumed to be due to sensitisation. The assessment of sensitisation took into account both incidence and severity scores.

4. RESULTS

4.1 Screening study (Table 1)

Severe erythema was noted in all test sites exposed to test article at a concentration of 3.0% w/v in 80% ethanol and in 1 of the 4 test sites exposed to test article at a concentration of 1.0% w/v in 80% ethanol.

Slight to moderate erythema was noted in the test sites at 0.3% and slight, patchy erythema at 0.1%. A concentration of 0.3% w/v in 80% ethanol was selected for the induction applications and 0.2% w/v in acetone for the challenge.

4.2 Main study (Tables 2 and 3)

Slight erythema was noted in 11 test animals (8 male, 3 female) at the 24 hour observation and in 17 test animals (10 male, 7 female) at the 48 hour observation. Moderate erythema was observed in 2 test animals at the 24 hour reading, the other animals appearing normal.

The control animals showed no skin reactions at either observation.

Experimental group	Observation time (hour)	Skin responses (scale 0-3)					Incidence (N)	Severity (A)
		0	0.5	1	2	3		
Test	24	7	7	4	2	0	6/20	0.58
	48	3	15	2	0	0	2/20	0.48
Control	24	10	0	0	0	0	0/10	0
	48	10	0	0	0	0	0/10	0

N = Number of animals showing skin responses >1

A = Sum of skin responses divided by the number of animals tested.

These results indicated that RD 163 does cause delayed contact hypersensitivity in the guinea pig using the Buehler test.

5. ARCHIVE

All primary data, or copies thereof, will be retained in the HLE archive for 10 years after submission of the final report. At the end of this period we will discuss with the sponsors whether they require storage for a longer period, either at HLE or in the sponsor's own archives.

Primary data will be taken to include laboratory data sheets, records, memoranda, notes, photographs, microfilm and computer records that are a result of the original observations and activities of the study and which are necessary for the reconstruction and evaluation of the report of the study.

TABLE 1

Skin reactions in the screening study

Test article: 40 163
Vehicle: 80% ethanol

Solution Concentration (% w/v)	Number of animals showing skin response (scale 0 - 3)				
	0	0.5	1	2	3
3.0	0	0	0	0	4
1.0	0	0	1	2	1
0.3	0	2	1	1	0
0.1	0	4	0	0	0

TABLE 2

Skin reactions in test animals at
challenge application

Test article: RO 163
Concentration: 0.2% w/v in acetone

Animal		Weight (g)		Skin reactions (hrs. after end of challenge application)	
Number	Sex	Initial	Terminal	24	48
5		575	833	0.5	0.5
6		463	654	0.5	0.5
7		507	756	1	0.5
8		546	733	1	0.5
9	M	445	640	0.5	0.5
10		528	770	0.5	0.5
11		496	730	1	0.5
12		509	691	0.5	0.5
13		544	702	2	0.5
14		520	727	0	0.5
15		420	585	0	0.5
16		384	531	0	0.5
17		424	587	1	1
18	F	451	626	0	0.5
19		448	584	0	0
20		396	503	2	1
21		409	550	0.5	0.5
22		453	630	0.5	0.5
23		394	546	0	0
24		425	587	0	0
Sum of test grades (A)				11.5	9.5
Number showing response ≥ 1 (B)				6	2
Incidence N/20				6/20	2/20
Severity Σ /20				0.50	0.40
Highest grade observed				2	1

TABLE 3

Skin reactions in control animals at
challenge application

Test article: RO 163
Concentration: 0.2% w/v in acetone

Animal		Weight (g)		Skin reactions (hrs. after end of challenge application)	
Number	Sex	Initial	Terminal	24	48
25		584	840	0	0
26		447	564	0	0
27	M	569	785	0	0
28		492	623	0	0
29		528	696	0	0
30		405	580	0	0
31		411	573	0	0
32	F	446	595	0	0
33		420	605	0	0
34		414	576	0	0
Sum of test grades (A)				0	0
Number showing response ≥ 1 (N)				0	0
Incidence N/10				0/10	0/10
Severity $\Sigma A \approx 10$				0	0
Highest grade observed				0	0

RESULTS AND CONCLUSIONS

BCM STS 1044

1. Skin Sensitization (Guinea pig) (YBR 2-5-253-85)

<u>Test Material</u>	<u>Concentration</u>		<u>Challenge Results</u>				
	<u>Induction</u>	<u>Challenge</u>	<u>Grade</u>	<u>Test</u>		<u>Control</u>	
				<u>24h</u>	<u>48h</u>	<u>24h</u>	<u>48h</u>
Dinitrochloro- benzene (DNCB) RO 163	0.3% in 80% ethanol	0.2% in 80% ethanol	0 + 1 2 3	- - - 15 5	- - + 6 14	- 2 8 - -	- 1 7 2 -

Comments : DNCB induced distinct reactions compared to the control group.

2. Skin Sensitization (Guinea pig) (HLE No 47DB- 126/9)

<u>Test Material</u>	<u>Concentration</u>		<u>Challenge Results</u>				
	<u>Induction</u>	<u>Challenge</u>	<u>Grade</u>	<u>Test</u>		<u>Control</u>	
				<u>24h</u>	<u>48h</u>	<u>24h</u>	<u>48h</u>
Dinitrochloro- benzene (DNCB) RO 163	0.3% in 80% ethanol	0.2% in acetone	0 + 1 2	7 7 4 2	3 15 2 -	10 - - -	10 - - -

Comments : under the conditions of the test DNCB (RO 163) does cause delayed contact hypersensitivity in the guinea pig using the Buehler test.

Test Substance

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not stated
Comments: Test material was coded as "R0060-02"

Method

Method / Guideline: Buehler method
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1978
GLP Compliant: Not stated.
Species: Guinea pig – Hartley albino strain
Number of Animals: 30 (15 male / 15 female)
20 (10 M/10 F) in test group and 10 (5 M/5 F) in control
Study Design: Induction: The upper left quadrant of the back was clipped with electric clippers. The following day, a 20x20 Webril swatch moistened with 0.4 ml of 0.3% (w/v) test substance in 80% ethanol was placed on the clipped areas of 20 test animals, for 6 hours. Patches were reapplied weekly to the same, freshly clipped areas, for 3 consecutive weeks.
Challenge: After a two-week rest period, test and control animals were challenged with a 0.2% and 0.02(w/v) test substance in acetone.
Depilated animals were scored for erythema severity using a 0-3 scale, 24 and 48 h post-challenge.

Results

Result: When tested at 0.2%, the test material produced 17 grades of 3 and 2 grades of 2 at the 24-hour reading and 17 grades of 3 and 2 grades of 2, and 1 grade of 1 at the 48-hour reading. When tested at 0.02%, the test material produced 1 grade of 3, 3 grades of 2, and 5 grades of 1 at the 24-hour reading and 1 grade of 3, 3 grades of 2, and six grades of 1 at the 48-hour reading the 48-hour reading.

Comments: No positive responses were seen in any of the control animals.

Data Quality

Reliability (Klimisch): 2

Reference

Laboratory Report Number: WIL-1179-78
Reference: Will Research Laboratories, 1978. Delayed Hypersensitivity Study in Guinea Pigs of R0060-02. Accession# 20749

ACC # 20749



RECEIVED BY
JUN 21 1978
OPERATIONS SECTION

Project Number: WL-1179-78

Client: The Procter & Gamble Company

Delayed Hypersensitivity Study In Guinea Pigs
of R0060-02

Purpose:

To evaluate the potential of ROO60-02 to induce delayed contact hypersensitivity in guinea pigs.

Test Materials

The sample used in this study was received from Procter & Gamble on April 3, 1978. ROO60-02 is a yellow, crystalline material with a slight odor.

Procedure:

The procedure used was based on that of Bashler.¹

The animals used in this study were received from Sweetwater Farm, Inc., Hillsboro, Ohio, (Hartley albino strain). The animals were maintained on medicated water containing 4% of a sulfathiazopyridazine (A-25% S.E.Z.,² American Cyanamid) for four days. At the end of this period they were furnished with non-medicated water and Purina Guinea Pig Chow was available ad libitum throughout the study. The animals were of a size that would easily fit into restraining used (see below), and they were individually housed in wire-mesh cages suspended above the droppings throughout the study. The animals were acclimated for 12 days before initiation of the study.

For the sample tested, a group of 30 guinea pigs was used. The group of 30 was divided into 20 guinea pigs which served as test animals throughout the study and 10 guinea pigs which served as controls. The latter animals were maintained without treatment until primary challenge application.

The study was initiated on May 2, 1978.

All test and control animals were sexed the day before the first induction application. An equal ratio of male and female guinea pigs were rounded for the study.

The upper left quadrant of the backs of the test guinea pigs was clipped using electric clippers.

On the following day the patches were applied using a Parks-Davis Road Bandage covered with a 20 x 20 mm Whatl swatch moistened with 0.4 ml of ROQ60-02 as a 0.3% w/v solution in 80% ethanol. The guinea pigs were placed in restrainers and rubber dental damming was placed over the animals' backs and secured to the restrainers with clips.

After an exposure period of six hours, the patches were removed and the animals were returned to their cages.

The patches were reapplied to the same site once each week for a total of three applications.

The same site was shaved the day before each application was made.

After a two week rest period fresh application sites for primary challenge were prepared by clipping the lower left quadrant of the backs of the test and control guinea pigs. On the following day challenge patches were applied to the sites using ROQ60-02 as 0.2% and 0.3% w/v solution in ethanol and the technique described previously. The patches were rotated to prevent bias due to site to site variation.

On the day following application, the clipped areas were depilated with Nect cream hair remover (Whitehall Laboratories, Inc., New York, N.Y.). The depilatory was allowed to remain on the sites for 15 to 30 minutes and was then washed off with warm (37°C) tap water. The patch sites were scored for irritation three to five hours later (24-hour reading). The sites were scored again for a 48-hour reading without any additional depilation.

The scoring scale is shown below:

- 0 No reaction
- 1 Slight patchy erythema
- 2 Moderate or moderate patchy erythema
- 3 Severe erythema
- 4 Erythema, edema and cracking of skin

The results following the primary challenge application of ROO60-02 are shown in Table 1.

During the primary challenge of ROO60-02 as a 0.2% w/v solution in acetone, reactions noted in the test animals at the 24-hour reading included seventeen grades of 3, two grades of 2 and one grade of 1. At the 48-hour reading seventeen grades of 3, two grades of 2 and one grade of 1 were noted in the test animals. For the control animals three grades of 1, five grades of 1 and two grades of 0 were noted at the 24-hour reading and one grade of 1, six grades of 1 and three grades of 0 were noted at the 48-hour reading. These same animals received a simultaneous primary challenge application of ROO60-02 as a 0.02% w/v solution in acetone. Results for the test animals included one grade of 3, three grades of 2, five grades of 1, nine grades of 1 and two grades of 0 at the 24-hour reading and one grade of 3, three grades of 2, six grades of 1, nine grades of 1 and one grade of 0 at the 48-hour reading. For the control animals two grades of 1 and eight grades of 0 were noted at both the 24 and 48-hour readings.

The study was completed on June 1, 1978, and the animals were terminated on June 12, 1978.

The severity indices were calculated to be as follows:

Sample	Conc.	Animals	Incidence	Severity	
				24-hour	48-hour
ROO60-02	0.2%	Test	20/20	2.8	2.8
	0.2%	Control	3/10	0.6	0.4
ROO60-02	0.02%	Test	10/20	0.9	1.0
	0.02%	Control	0/10	0.1	0.1

Summary:

When tested according to the method of Bushier, ROO60-02 as a 0.2% w/v solution in acetone produced seventeen grades of 3 and two grades of 2 at the 24-hour reading and seventeen grades of 3, two grades of 2 and one grade of 1 at the 48-hour reading for the test animals and three

grades of 1 at the 24-hour reading and two grades of 1 at the 48-hour reading for the control animals. No other positive reactions were noted in the control animals. The simultaneous primary challenge application of ROC60-02 as a 0.02% w/v solution produced one grade of 3, three grades of 2 and five grades of 1 at the 24-hour reading and one grade of 3, three grades of 2 and six grades of 1 at the 48-hour reading among ten of the test animals. No positive reactions were noted in the control animals.

Susan M. Young 6/16/78
Susan Young, Technician Date

Ralph Anderson 6/16/78
Ralph Anderson, Project Leader Date

Emil R. Adamik 6/14/78
Emil R. Adamik, Study Director Date

... primary challenge application for the test animals which received...
 ... 0.02% w/v solution in acetone...
 ... 0.2 and 0.02% w/v solutions in acetone. Also...
 ... the control for the control animals which received a single primary challenge application of
 ... 0.2 and 0.02% w/v solutions in acetone.

Primary Challenge Readings

Cotton Top Number & Sex	Site Injection	A = 0.2%		B = 0.02%	
		24 Hours	48 Hours	24 Hours	48 Hours
100	A,B	3	3	+	0
101	B,A	+	1	+	+
102	A,B	3	3	0	+
103	B,A	2	2	1	1
104	A,B	3	3	+	+
105	B,A	3	3	+	+
106	A,B	3	3	+	+
107	B,A	3	3	+	1
108	A,B	3	3	+	1
109	B,A	3	3	2	2
110	A,B	3	3	1	1
111	B,A	3	3	2	1
112	A,B	3	3	+	+
113	B,A	3	3	+	+
114	A,B	3	3	2	2
115	B,A	2	2	2	2
116	A,B	3	3	+	+
117	B,A	3	3	3	3
118	A,B	3	3	0	+
119	B,A	3	3	+	+
Control Group					
200	A,B	1	1	0	0
201	B,A	1	+	0	0
202	A,B	+	+	+	+
203	B,A	0	0	0	0
204	A,B	1	+	0	0
205	B,A	0	0	0	0
206	A,B	+	0	0	0
207	B,A	+	+	+	+
208	A,B	+	+	0	0
209	B,A	+	+	0	0

A = 1 site

B = 2 sites

H. L. Rice
(Name)

PHYS. ASOC. DIR.
PCAS ASSOC. DIR.

Name of Product or Ingredient (or code designation) 1-chloro-2,4-dinitrobenzene
Brand Notebook Ref. (including Production Code if available) Eastman Kodak Co. Lot R2A
Physical Form Crystals Color Yellow Density NA
Solubility NA Sample Expiration Date not determined
Recommended Storage Conditions Room temperature
Hazards (i.e. flammability, toxic gases) Potentially potent sensitizer

Formulated Composition

<u>Component (a)</u>	<u>Nominal Level (X by Wt.)</u>	<u>Acceptable (b) Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Number (b) (NB-Ref.)</u>
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Commercial sample - Eastman Kodak Co. Lot No. R2A

(a) Ingredients will be listed by chemical name; Non-chemical names such as Tergitol 15-S-9 or Yellow Dye B&C #10 may be acceptable but should be provided with the responsible toxicologist. Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-definitive identifications (e.g. Arquad, DC-benz) are not acceptable.

(b) If information requested is not known then the symbol NA will be entered.

The above information provided by:

H. L. Rice
(Name)

(Signature)

3/27/78
(Date)

Test Substance

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not stated
Comments: Test material was coded as "R0060-01"

Method

Method / Guideline: Buehler method
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1978
GLP Compliant: Not Stated
Species: Guinea pig – Hartley albino strain
Number of Animals: 30 (15 male / 15 female)
20 (10 M/10 F) in test group and 10 (5 M/5 F) in control
Study Design: Induction: The upper left quadrant of the back was clipped with electric clippers. The following day, a 20x20 Webril swatch moistened with 0.4 ml of 0.3% (w/v) test substance in 80% ethanol was placed on the clipped areas of 20 test animals, for 6 hours. Patches were reapplied weekly to the same, freshly clipped areas, for 3 consecutive weeks.
Challenge: After a two-week rest period, test and control animals were challenged with a 0.2% and 0.02(w/v) test substance in acetone.
Depilated animals were scored for erythema severity using a 0-3 scale, 24 and 48 h post-challenge.

Results

Result: When tested at 0.2% the test material produced 7 grades of 2 and 9 grades of 1 at the 24-hour reading and 15 grades of 1 at the 48-hour reading. When tested at 0.02%, the test material produced 5 grades of 1 at the 24-hour read and 1 grade of 1 at the 48-hour reading.

Comments: No positive responses were seen in any of the control animals.

Data Quality

Reliability (Klimisch): 2

Reference

Laboratory Report Number: 78-368-21
Reference: Hill Top Study. Delayed Contact Hypersensitivity Study in Guinea Pigs of R0060-01. Accession# 21114

Acc # 21114

C



Hill Top-Toxicology

Miamiville, Ohio 45147 (513) 831-3114

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JUN 26 1978

N. E. GILMAN

June 16, 1978

Ref: 78-368-21
(P&G Ref. No. PRSSE-81)

DELAYED CONTACT HYPERSENSITIVITY STUDY IN GUINEA PIGS OF R0060-01

For The Procter & Gamble Company

PURPOSE

To evaluate the potential of R0060-01 to induce delayed contact hypersensitivity in guinea pigs.

TEST MATERIAL

The sample used in this study was received from The Procter & Gamble Company on April 3, 1978. R0060-01 is a pale yellow powder.

PROCEDURE

The procedure used was based on that of Buehler.¹

The animals used in this study were received from Murphy Breeding Laboratories, Inc. (Hartley albino strain). The animals were maintained on medicated water containing 4% of sulfaethoxypyridazine (6.25% S.E.Z.[®], American Cyanamid) for four days. At the end of this period they were furnished with non-medicated water ad libitum; Purina Guinea Pig Chow was available ad libitum throughout the study. The animals were housed singly in wire mesh cages suspended above the droppings throughout the study. The animals were of a size that would fit easily into the restrainers used (see below) throughout the study.

The animals were acclimated to the laboratory for at least four days before they were used.

For the sample tested, a group of 30 guinea pigs was used. The group of 30 was divided into 20 guinea pigs which served as test animals throughout the study and 10 guinea pigs which served as controls. The latter animals were maintained without treatment until primary challenge application.

¹Buehler, E. V., "Delayed Contact Hypersensitivity in the Guinea Pig," Arch. Dermat. 91, 171-175, 1965).



All test and control animals were sexed the day before the first induction application. The ratio of males to females was 50:50.

The upper left quadrant of the backs of the test guinea pigs was clipped using electric clippers. On the following day the patches were applied using a Parke-Davis Read Bandage coverlet with a 20 x 20 mm Webril swatch moistened with 0.4 ml of R0060-01 as a 0.3% w/v solution in 80% ethanol. The guinea pigs were placed in restrainers and rubber dental damming was placed over the animals' backs and secured to the restrainers with clips.

After an exposure period of six hours, the patches were removed and the animals were returned to their cages.

The patches were reapplied to the same site once each week for a total of three applications. The same site was shaved the day before each application was made.

After a two-week rest period a fresh application site for primary challenge was prepared by clipping the lower left quadrant of the backs of the test and control guinea pigs. On the following day two challenge patches were applied to the sites in anterior to posterior positions using a 0.2% w/v solution of R0060-01 in acetone and a 0.02% w/v solution of R0060-01 in acetone and the technique previously described. The patch sites were rotated to avoid bias due to site-to-site variation.

On the day following application, the clipped areas were depilated with Nect Cream Hair Remover (Whitehall Laboratories, Inc., New York, N.Y. 10017). The depilatory was allowed to remain on the sites for 15 to 30 minutes and was then washed off with warm (ca. 37°C) tap water.

The patch sites were scored for irritation two to three hours later. The sites were scored again for a 48-hour reading without additional depilation. The scoring scale is shown below:

0	No reaction
±	Slight patchy erythema
1	Slight confluent or moderate patchy erythema
2	Moderate erythema
3	Severe erythema, with or without edema

RESULTS

The results following the simultaneous primary challenge application of R0060-01 as 0.2% and 0.02% w/v solutions in acetone are shown in Table 1.

During the primary challenge of R0060-01 as a 0.2% w/v solution in acetone, reactions noted in the test animals at the 24-hour reading included seven grades of 2, nine grades of 1, three grades of ± and one grade of 0. At the 48-hour reading fifteen grades of 1, four grades of ± and one grade of 0 were noted in the test animals. For the control animals, one grade of ± and nine grades of 0 were noted at the 24-hour reading and ten grades of 0 were noted at the 48-hour reading.

During the primary challenge of R0060-01 as a 0.02% w/v solution in acetone, reactions noted in the test animals at the 24-hour reading included five grades

June 16, 1978

of 1, ten grades of \pm and five grades of 0. At the 48-hour reading one grade of 1, twelve grades of \pm and seven grades of 0 were noted in the test animals. For the control animals, ten grades of 0 were noted at the 24-hour reading and ten grades of 0 were noted at the 48-hour reading.

The Incidence and Severity Indices were calculated to be as follows:

<u>Sample</u>	<u>Conc.</u>	<u>Animals</u>	<u>Incidence</u>	<u>Severity</u>	
				<u>24-hr.</u>	<u>48-hr.</u>
R0060-01	0.2%	Test	17/20	1.2	0.9
		Control	0/10	0.1	0
R0060-01	0.02%	Test	5/20	0.5	0.4
		Control	0/10	0	0

SUMMARY

When tested according to the method of Buehler, R0060-01 produced seven grades of 2 and nine grades of 1 at the 24-hour reading and fifteen grades of 1 at the 48-hour reading of the primary challenge of R0060-01 as a 0.2% w/v solution in acetone among 17 of the test animals. No positive reactions were noted in the remaining test and the control animals.

When tested as a 0.02% w/v solution in acetone, R0060-01 produced five grades of 1 at the 24-hour reading and one grade of 1 at the 48-hour reading of the primary challenge among five of the test animals. No positive reactions were noted in the remaining test and the control animals.

Submitted by:

Jeffrey D. Wyatt
 Jeffrey D. Wyatt
 Junior Technician, Toxicology

Approved by:

Marian B. Vinegar
 Marian B. Vinegar, Ph.D.
 Director, Toxicology

Scores recorded at primary challenge for test albino guinea pigs which received three weekly induction applications of R0060-01 as a 0.3% w/v solution in 80% ethanol and one simultaneous primary challenge application of R0060-01 as 0.2% w/v and 0.02% w/v solutions in acetone. Also shown are the scores for the control animals which received a single primary challenge application of R0060-01 as 0.2% w/v and 0.02% w/v solutions in acetone.

Guinea Pig No.	Sex	*	Reading (0.2%)		Reading (0.02%)	
			24-hour	48-hour	24-hour	48-hour
1	M	A,B	±	±	0	0
2	M	B,A	2	1	1	±
3	M	A,B	1	±	±	±
4	M	B,A	1	1	1	1
5	M	A,B	2	1	1	±
6	M	B,A	1	1	±	±
7	M	A,B	2	1	0	0
8	M	B,A	1	1	±	±
9	M	B,A	2	1	±	±
10	M	A,B	2	1	±	±
11	F	B,A	1	1	±	±
12	F	A,B	±	±	0	0
13	F	B,A	0	0	0	0
14	F	A,B	1	1	0	0
15	F	B,A	1	1	±	0
16	F	A,B	1	1	±	±
17	F	B,A	1	±	±	0
18	F	A,B	2	1	1	±
19	F	B,A	2	1	1	±
20	F	A,B	±	1	±	±
<u>Control</u>						
21	M	B,A	0	0	0	0
22	M	A,B	0	0	0	0
23	M	B,A	0	0	0	0
24	M	A,B	0	0	0	0
25	M	B,A	0	0	0	0
26	F	A,B	0	0	0	0
27	F	B,A	0	0	0	0
28	F	A,B	0	0	0	0
29	F	B,A	0	0	0	0
30	F	A,B	±	0	0	0

M - Male

F - Female

* - Site Order

A - R0060-01 as a 0.2% w/v solution in acetone

B - R0060-01 as a 0.02% w/v solution in acetone



Hill Top-Toxicology

Miamiville, Ohio 45147 (513) 831-3114

IMPORTANT NOTICE

Hill Top - Toxicology submits this report with the understanding that no portion of it will be used for advertising or promotion without obtaining our prior written consent to the specific proposed use. When such use is desired, we will be glad to assist in the preparation of mutually acceptable excerpts or summaries.

SAMPLE DISPOSAL PROCEDURE

At the conclusion of a test program, each sample will be stored for three months. At that time the sample will be returned to the client.



TEST SUBSTANCE CHARACTERIZATION REPORT (TSR)

Side 1 of 2

Test Substance Identification Number R0060
 Safety Test Request Number PRSSK 77
 Principal Investigator H. L. Ritz
 (Name)

PARS SECT. NO.	8313
ORIG. SECT. HD.	8313
ORIG. ASSOC. DIR.	<i>[Signature]</i>
PARS ASSOC. DIR.	<i>[Signature]</i>

Name of Product or Ingredient (or code designation) 1-chloro-2,4-dinitrobenzene
 Brand Notebook Ref. (including Production Code if available) Eastman Kodak Co. Lot #B2A
 Physical Form Crystals Color Yellow Density N/A
 Solubility N/A Sample Expiration Date Not Determined
 Recommended Storage Conditions Room temperature
 Hazards (i.e. flammability, toxic gases) Potentially potent sensitizer

Formulated Composition

Component (a)	Nominal Level (X by Wt.)	Acceptable (b) Range	Stock Code No.	Supplier	Lot Number (b) (NB-Ref.)
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Commercial sample - Eastman Kodak Co. Lot No. B2A

(a) Ingredients will be listed by chemical name. Non-chemical names such as Tergitol 15-S-9 or Yellow Dye MC 220 may be acceptable but should be provided with the responsible toxicologist. Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in subsequent footnotes. Non-definitive identifications (e.g. Acqua, 20-benz) are not acceptable.

(b) If information requested is not known then the symbol UK will be entered.

The above information provided by:

H. L. Ritz *1/3/78*

ANALYZED CHARACTERIZATION

Test Substance Identification Number R0060

Safety Test Request Number FRSSE 70

Analyzed Composition
(if available)

<u>Date Submitted</u>	<u>Submitter Code No.</u>	<u>Component or Property</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
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Commercial sample Lot #B2A from Eastman Kodak Co.

Analytical Information Verified By:

(Name) (Signature) (Date)

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

Project Leader _____ (Signature) _____ (Date)

Principal Investigator Harold King (Signature) _____ (Date) 1/3/78

Test Substance

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not stated
Comments: Test material was coded as "R 0163"

Method

Method / Guideline: Buehler method
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1985
GLP Compliant: Not stated, but reviewed by Lab's Quality Assurance Unit
Species: Guinea pig – derived from Hartley albino strain
Number of Animals: 30 (15 male / 15 female)
Study Design: 20 (10 M/10 F) in test group and 10 (5 M/5 F) in control
Induction: The upper left quadrant of the back was clipped with electric clippers. The following day, Hill Top Chambers were applied with 0.3 ml of 0.3% (w/v) test substance in 80% ethanol and were placed on the clipped areas of 20 test animals, for 6 hours. Patches were reapplied weekly to the same, freshly clipped areas, for 3 consecutive weeks.
Challenge: After a two-week rest period, test and control animals were challenged with a 0.2% (w/v) test substance in acetone. Depilated animals were scored for erythema severity using a 0-3 scale, 2, 24 and 48 h post-challenge.

Results

Result: The control group showed slight erythema during the entire observation period. The test material produced mainly severe erythema and edema.
Comments: All animals showed a normal weight gain.

Data Quality

Reliability (Klimisch): 2

Reference

Laboratory Report Number: 2-5-253-85
Reference: IBR Forschungs GmbH. Guinea Pig Sensitization Testing modified by Ritz and Buehler on R 0163. Accession# 42322

Acc # 42322



Forschungs GmbH

Südkampen Nr. 31
3030 Walsrode 2

Telefon: (05166) 1366

Telex: 924342 ibr d

Vormals in Hannover/
Krumme Straße 7

June 1985 / ct

Project-No.: 2-5-253-85

Guinea Pig Sensitization Testing

modified by Ritz, H. L.

and Buehler, E. V. on

R 0163 (ECM BTS '044)

according to P. & G. Protocol-No. C 4 A, April 1985

D.N.C.E.

RECEIVED : 26-06-1985	
CHECKED BY:	ASPTZS P
	Dr 29/7/85 R

Sponsor:
Procter & Gamble
European Technical Center
Tenzelstr 100
D - 1820 Grimbergen (Stronbeek-Zever)

C O N T E N T S

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1. INTRODUCTION

It was the objective of this study to determine whether "0163"
(EOM BTB 100) causes an increased reaction in the skin of guinea pigs
when compared to appropriate controls.

For the performance of this study the Procter & Gamble protocol no. C 4 2
(issue date April 18, 1965) was followed.

II. SUMMARY

- a) For this investigation male and female guinea pigs were divided in two groups (test- and control group).

All signs of erythema and edema were recorded after a 3-week induction period and a primary challenge two weeks later.

- b) During the induction period the sample induced slight to moderate erythema.
After the challenge the controlgroup showed mainly slight erythema.
The animals of the testgroup showed moderate to severe erythema.

According to the results described above, the sample "R 0163" (EQM BTS 1044) induced distinct reactions compared to the controlgroup.

III. MATERIAL AND METHOD

1. Animals
 - 1.1 Species: Guinea-pigs
 - 1.2 Strain: Pirbright
 - 1.3 Substrain: Hoe: DHPK (SPF-LAC.) /Hoe.
 - 1.4 Source: Lippische Versuchstierzucht
Hagemann GmbH & Co. KG
Hamelner Straße 3
4923 Extertal 1
 - 1.5 Colour: white
 - 1.6 Background of strain: Originally bred at Duncan Hartley's,
England.
Erection of a SPF-strain after hysterectomy at the LAC (Laboratory Animal Center, GB). Hoechst, Frankfurt, continued breeding animals that were descended from LAC after hysterectomy.
1976 the breeder received animals from Hoechst, with which he continued breeding under SPF-conditions.
 - 1.7 Date of receipt: 12.4.85
 - 1.8 Acclimatization time: 7 days at least
 - 1.9 Randomization: by the way of lottery drawing
 - 1.10 Animal identification: earmark and/or colour identification
Cage card with the following information: dosage, sex, (ear-mark), test, day of the beginning of the study.
 - 1.11 Weight range at the beginning of the study: 6 320 g

2. Husbandry

- 2.1 Caging: max. 5 animals in one cage
- 2.2 Cagetype: Macrolon Plastic cages IV
20 cm high, 33 cm width, 55 cm length
- 2.3 Lighting: Fluorescent light, 4000 K, 120 Lux
- 2.4 Lighting periods: 12 hours daily, from 7.00 a.m. to 7.00 p.m.
- 2.5 Temperature: $18^{\circ} \text{C} \pm 2^{\circ} \text{C}$
- 2.6 Relative humidity: 50 - 85 %
- 2.7 Registration: by thermohygrometer
- 2.8 Timing: in the morning and in the afternoon

3. Food and feeding

- 3.1 Producer: Saniff Spezialfutter GmbH
4770 Soest/Westfalen
- 3.2 Name: Saniff-G (Alleindiät für Meerschweinchen)
- 3.3 Type: pellets, 1.0 cm large, 0.5 cm diameter
- 3.4 Composition:

crude nutritive substance:	crude protein	21,0 %
	crude fat	3,5 %
	crude fibre	15,0 %
	crude ash	8,2 %
	humidity	12,0 %
	N-free extract agent	39,0 %

metabolisable energy:	Kcal/kg	2680
	kJ/kg	11218

Amino acid:	Lysin	1,20 %
	Methionin	0,30 %

Vitamins:	24.000 I.U. Vitamin A	
	2.000 I.U. Vitamin D ₃	
	2.000 mg Vitamin C	
	45 mg Vitamin E	

Minerals and Trace elements:	Ca	1400 mg
	P	900 mg
	Na	200 mg
	Mg	200 mg
	K	13200 mg
	Cl	8400 mg
	Fe	230 mg
	Mn	58 mg
	Cu	16 mg
	Zn	35 mg
	S	4600 mg
	Co	300 mg
	J	350 mg

4. Bedding

- 4.1 Producer: Seniff Spezialfutter GmbH
4770 Soest/Westfalen
- 4.2 Name: Seniff - Bedding
- 4.3 Production: from pure spruce-, fir- and pine-wood,
dried and disdusted
- 4.4 Sterilization: 180° C
- 4.5 Water binding capacity
(% of bedding used): 276,5

5. Water

- 5.1 Administration: ad libitum
- 5.2 System: Macrolon drinking bottles,
Fa. Becker & Co., 4620 Castrop-Raukel
- 5.3 Quality: aqua fontana as for human consumption
- 5.4 Quality control: half-yearly analytical and bacteriological
controls

6. Test material

The test substance "R 0163" (ECM BIS 1044) was supplied by Frocter & Gamble European Technical Center, Grimbergen (Strombeek-Bever), Belgium.

6.1 DRD-Number: ECM BIS 1044

6.2 General characteristics: "R 0163" (ECM BIS 1044) is a light yellow, crystalline powder.

6.3 Storage: room temperature

7. Experimental design

7.1 Preparation of the animals

Following an acclimatization period of at least 7 days to accustom the guinea-pigs to the environmental conditions existing in our laboratories, the test was initiated.

The animals were allocated in two groups (1 testgroup and 1 controlgroup), the testgroup contained 20 animals and the controlgroup 10 animals. Equal numbers of male and female guinea-pigs were used. They were marked by colour identification.

Prior to treatment the left shoulder of each animal was clipped with a small animal clipper.

7.2 Preliminary study

In the course of a preliminary test, the highest non-irritating concentration was determined.

Therefore the entire back and both sides of 4 animals were clipped one day prior to application.

The following day the animals were exposed for one 6-hour period to various concentrations of the test substance. In accordance with the sponsor the following concentrations were used: 3 %, 1 %, 0,3 % and 0,1 % in 80 % ethanol (g/g).

The different concentrations were tested on alternating test sites localized bilateral to the spine. Hereby the lowest concentration was used on the left anterior quadrant of the left side.

The next higher was tested on the left posterior quadrant, then the right anterior quadrant was used a.s.o..

The responses were graded at 24 hours and at 48 hours according to the procedure described below (refer to 7.6).

7.3 Preparation of the test substance

a) Preliminary study

The sample was applied as a 3 %, 1 %, 0,3 % and 0,1 % dilution in 80 % ethanol (g/g).

b) Main study

Referring to the preliminary study and according to an agreement with the sponsor the test sample "R 0163" (EOM BTS 1044) was applied as a 0,3 % dilution in 80 % ethanol (challenge: 0,2 %).

7.4 Treatment

Induction of Sensitization

The day before exposure the left shoulder of each test animal was clipped with a small animal clipper.

0.3 ml of the freshly prepared test substance were applied to the "Hill Top Chambers" using a calibrated (monthly) "Eppendorf-Pipet".

The patches were placed on the clipped surface of each animal and secured with several wrappings of plastic material.

The animals were immobilized in restrainers for 6 hours. After that time the patches were taken off and the test substance was removed with a gentle rinse of warm water (about 37° C) before returning the animals to their cages.

This procedure was repeated at the same site once a week for the next two weeks for a total of three 6-hour exposures. After the last induction exposure the animals were left untreated for 2 weeks before primary challenge.

Primary challenge

The animals previously exposed during the induction period as well as the previously untreated animals were treated following the same patching procedure with the "Hill Top Chambers" as for the induction. However the patches were applied to a freshly clipped naive skin site (left posterior quadrant of the side and back of the animal), underneath the induction area.

7.5 Observations

24 hours after the primary challenge all animals were depilated with "Nest" (used for cosmetical depilation, produced by Whitehall Laboratories N. Y.). The depilatory was used according to the instructions of the producer. The test sides were graded 2, 24 and 48 hours after the depilation. Grading of all animals was done by positioning (distance max. 1 m) the animal under a four tube fluorescent type light of 160 watts (Osram, 36 watt L 40 W/20 light white). The control animals were graded before the test animals.

7.6 Scores

No reaction	0
Slightly patchy erythema	± (= 0,5)
Slight, but confluent or moderate patchy erythema	1
Moderate erythema	2
Severe erythema with or without edema	3

7.7 Mean response

The sums of the test grades were divided by the total number of animals tested in a given group determined for 2 h, 24 h, 48 h after depilation.

7.8 Body weights

The body weights are recorded at day 0 (beginning of the experiment) and at the end of the study (1st day of the challenge).

IV. RESULTS

Under the described conditions the following was recorded:

a) Preliminary study

3 %: On the treated areas the sample induced partly obvious erythema (edema).

1 % and 0,3 %: On the treated areas mainly slight erythema were observed.

0,1 %: No signs of erythema and/or edema were observed.

b) Main study

Induction (0,3 %)

The sample induced after every treatment slight to obvious erythema (partly eschar).

Challenge (0,2 %)

Controlgroup

The controlgroup showed during the entire observation period mainly slight erythema (all animals).

Testgroup

The sample induced during this observation period mainly severe erythema (edema).

Body weights

All animals showed a normal weight gain.

c) Conclusion

According to the results described above, the sample "R 0163" (EG: ETS 1043) induced distinct reactions compared to the controlgroup.

V. PROTOCOLS

Table 1

Individual val:

Preliminary study

Animal- No.	sex	body- weight (g)	Primary irritation							
			24 h				48 h			
			3 %	1 %	0,3 %	0,1 %	3 %	1 %	0,3 %	0,1 %
1	♂	323	1 ^a	± ^a	0	0	1 ^a	1 ^a	±	0
2	♂	290	2 ^a	± ^a	1	0	3 ^a	± ^a	1	0
3	♀	303	2 ^a	1 ^a	±	0	3 ^a	± ^a	0	0
4	♀	302	2 ^a	2 ^a	±	0	3 ^a	1 ^a	0	0

^a treatment areas
discharging wounds

^b escher

Table 2

Individual values

Main study

Animal- No.	sex	body- weight (g), start	Induction 0,3 % i. 80 % ethanol			Challenge 0,2 % i. 80 % ethanol			body- weight (g), end	
			after the	1.	2.	3.	2 h	24 h		48 h
						24 h	48 h			
1	♂	334	0	1	0,5	1	2	2	1	542
2	♂	315	0,5	1-2	1	1	2	3	3	555
3	♂	337	0	2	1	0,5	2	3	3	524
4	♂	338	0	2	1	1	3	3	3	574
5	♂	316	0,5	2	2	2*	2	3	3	511
6	♂	312	1	1	1	0,5*	2	2	2	564
7	♂	345	0,5	2	1	1*	2	2	2	577
8	♂	320	0,5	1	1	1*	2	3	3	571
9	♂	325	0	2	1	1	2	3	3	553
10	♂	319	0	2	2	2	2	2	2	558
11	♀	315	0	2	1	2*	3	3	3	497
12	♀	360	0	1	1	2	2	3	2	529
13	♀	312	0,5	2	1	1	3	3	2	488
14	♀	329	0,5	2	1	1*	2	3	3	529
15	♀	341	0,5	2	1	0,5	2	3	2	504
16	♀	350	1	1	1	1*	2	2	2	570
17	♀	322	0	2	1	2	3	3	3	556
18	♀	344	1	2	2	2*	2	2	3	538
19	♀	303	1	2	1	1*	2	3	3	482
20	♀	338	0,5	2	1	1	3	3	3	548
							mean response	2,25	2,65	1,85

* each

Table 3

Individual values after challenge

Main study

Controlgroup

Animal- No.	Sex	Body- weight (g), start	0,2 % i. 20 % ethanol			body weight (g), end
			2 h	24 h	48 h	
21	♂	316	1	1	0,5	578
22	♂	336	0,5	0,5	0	511
23	♂	300	0,5	2	1	585
24	♂	327	1	1	0,5	547
25	♂	228	1	1	0,5	575
26	♀	313	1	1	0,5	500
27	♀	305	1	1	1	489
28	♀	332	1	1	1	570
29	♀	301	1	1	0,5	570
30	♀	349	1	2	1	580
mean response			0,96	1,15	0,65	

VI. GENERAL INFORMATION:

Sponsor: Procter & Gamble
European Technical Center
Fensholtan 100
1520 Grimbergen (Stroubbaek-Bever)

Study performed by: TBR Forschungs GmbH
Südkampen Nr. 31
3090 Walsrode 7

Scientific Director and Management: Dr. Dr. W. Starnar
Fachtierarzt für
Pharmakologie u. Toxikologie
Expert agrée
pharmacologue, toxicologue
Fachtierarzt für
klin. Laboratoriumsdiagnostik
Fachtierarzt für
Versuchstierkunde

Study director: Dr. med. vet. G. Chibanguza
Fachtierarzt für
Pharmakologie u. Toxikologie

Project leader: Frau M. Fürst

Technical assistants: Fri. H. Händchenke,
Rarr H. Queren, Herr K. Kühne

Quality assurance: P. Vollmann

Time of study: 22.4. - 26.4.85

Archives and documents: All raw data and a copy of the final
report will be stored in the archives
of ISF.
The test substance was returned to
the sponsor.

DECLARATION

The conditions for the performance of all studies, e.g. animal care, rooms, technical equipment and personnel are regularly controlled by the Quality Assurance Unit.

This report provides a correct and faithful record of the results obtained.

P. Volkmann
QAU


.....

Südampfen, 11.06.05.....

Test Substance

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not stated
Comments: Test material was coded as "RO060"

Method

Method / Guideline: Buehler method
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1978
GLP Compliant: Not stated.
Species: Guinea pig – Hartley albino strain
Number of Animals: 30 (15 male / 15 female)
20 (10 M/10 F) in test group and 10 (5 M/5 F) in control
Study Design: Induction: The upper left quadrant of the back was clipped with electric clippers. The following day, a 20x20 Webril swatch moistened with 0.4 ml of 0.3% (w/v) test substance in 80% ethanol was placed on the clipped areas of 20 test animals, for 6 hours. Patches were reapplied weekly to the same, freshly clipped areas, for 3 consecutive weeks.
Challenge: After a two-week rest period, test and control animals were challenged with a 0.2% and 0.02(w/v) test substance in acetone.
Depilated animals were scored for erythema severity using a 0-3 scale, 24 and 48 h post-challenge.

Results

Result: When tested at 0.2%, the test material produced five grades of 2 and 14 grades of 1 at the 24-hour reading and three grades of 2 and 14 grades of 1 at the 48-hour reading. When tested at 0.02%, the test material produced 1 grade of 1 at the 48-hour reading.

Comments: No positive responses were seen in any of the control animals.

Data Quality

Reliability (Klimisch): 2

Reference

Laboratory Report Number: 78-086-21
Reference: Hill Top Toxicology, 1978. Delayed Contact Hypersensitivity Study in Guinea Pigs of R0060. Accession# 19932

ACC # 19932

MAR 13 1978

2 copies sent to H.L. R.S.
3/13/78



Hill Top - Toxicology

Miamiville, Ohio 45147 (513) 831-3114

Project No. 78-086-21
P&G Ref. No. PRSSE-77

March 7, 1978

DELAYED CONTACT HYPERSENSITIVITY STUDY IN GUINEA PIGS OF R0060

For The Procter & Gamble Company

PURPOSE

To evaluate the potential of R0060 to induce delayed contact hypersensitivity in guinea pigs.

TEST MATERIAL

The sample used in this study was received from The Procter & Gamble Company on January 4, 1978. Sample R0060 consists of various-sized pale yellow granules.

PROCEDURE

The procedure used was based on that of Buehler.¹

The animals used in this study were received from Sweetwater Farm, Inc. (Hartley albino strain). The animals were maintained on medicated water containing 4% of sulfaethoxypyridazine (6.25% S.E.Z.^R, American Cyanamid) for four days. At the end of this period they were furnished with non-medicated water ad libitum; Purina Guinea Pig Chow was available ad libitum throughout the study. The animals were housed singly in wire mesh cages suspended above the droppings throughout the study. The animals were of a size that would easily fit into the restrainers used (see below) throughout the study.

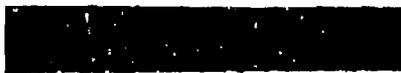
The animals were acclimated to the laboratory for at least four days before they were used.

¹E. V. Buehler, "Delayed Contact Hypersensitivity in the Guinea Pig," (Arch. Dermat. 91, 171-175, 1965).



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Hill Top Research / International Bio-Research, Inc. / Woodson-ferent Laboratories



For the sample tested, a group of 30 guinea pigs was used. The group of 30 was divided into 20 guinea pigs which served as test animals throughout the study and 10 guinea pigs which served as controls. The latter animals were maintained without treatment until primary challenge application.

All test and control animals were sexed the day before the first induction application. The ratio of males to females was 50:50.

The upper left quadrant of the backs of the test guinea pigs was clipped using electric clippers. On the following day the patches were applied using a Parke-Davis Readi-Bandage coverlet with a 20 x 20 mm Webril swatch moistened with 0.4 ml of a 0.3% w/v solution of R0060 in 80% ethanol. The guinea pigs were placed in restrainers and rubber dental damming was placed over the animals' backs and secured to the restrainers with clips.

After an exposure period of six hours, the patches were removed and the animals were returned to their cages.

The patches were reapplied to the same site once each week for a total of three applications. The same site was shaved the day before each application was made.

After a two-week rest period, two fresh application sites for primary challenge were prepared by clipping the lower left quadrant of the backs of the test and control guinea pigs. On the following day, challenge patches were applied to the sites in anterior to posterior positions, using 0.2% and 0.02% w/v solutions in acetone and the technique described previously. The patch site order was rotated to prevent bias due to site-to-site variation.

On the day following application, the clipped areas were depilated with Nect Cream Hair Remover (Whitehall Laboratories, Inc., New York, N. Y. 10017). The depilatory was allowed to remain on the sites for 15 to 30 minutes and was then washed off with warm (ca. 37°C) tap water.

The patch sites were scored for irritation three to five hours later.

The scoring scale is shown below:

0	No reaction
+	Slight patchy erythema
1	Slight confluent or moderate patchy erythema
2	Moderate erythema
3	Severe erythema, with or without edema

RESULTS

The results following the primary challenge application of R0060 are shown in Table 1.

One test animal died prior to the third induction. Gross necropsy revealed excessive salivation stains and diarrhea stains externally, and advanced autolytic alterations internally. One control animal was found to be pregnant.

During the primary challenge of R0060 as a 0.2% solution in acetone, reactions noted in the test animals at the 24-hour reading included five grades of 2, and fourteen grades of 1.

At the 48-hour reading, three grades of 2, 14 grades of 1, and two grades of + were noted in the test animals at the sites exposed to 0.2% R0060 in acetone. For the control animals, four grades of +, and six grades of 0 were noted at the 24-hour and 48-hour readings at the sites exposed to 0.2% R0060 in acetone.

At the sites exposed to 0.02% R0060 in acetone (w/v), seventeen grades of +, and two grades of 0 were noted in the test animals at the 24-hour reading. At the 48-hour reading, one grade of 1, sixteen grades of +, and two grades of 0 were noted at these sites. For the control animals, one grade of + and nine grades of 0 were noted at both the 24-hour and 48-hour readings at the sites exposed to 0.02% R0060 in acetone.

The Incidence and Severity Indices were calculated to be as follows:

<u>SAMPLE</u>	<u>CONC.</u>	<u>ANIMALS</u>	<u>INCIDENCE</u>	<u>Severity</u>	
				<u>24-hr</u>	<u>48-hr</u>
R0060	0.2%	Test	19/19	1.3	1.1
		Control	0/10	0.2	0.2
	0.02%	Test	1/19	0.4	0.5
		Control	0/10	0.1	0.1

Amended by: Marian B. Vinegar
Marian B. Vinegar, Ph.D.
Director, Toxicology

Date: April 11, 1978

RESULTS

The results following the primary challenge application of R0060 are shown in Table 1.

One test animal died prior to the third induction. Gross necropsy revealed excessive salivation stains and diarrhea stains externally, and advanced autolytic alterations internally. One control animal was found to be pregnant.

During the primary challenge of R0060 as a 0.2% solution in acetone, reactions noted in the test animals at the 24-hour reading included five grades of 2, and fourteen grades of 1.

At the 48-hour reading, three grades of 2, 14 grades of 1, and two grades of + were noted in the test animals at the sites exposed to 0.2% R0060 in acetone. For the control animals, four grades of +, and six grades of 0 were noted at the 24-hour and 48-hour readings at the sites exposed to 0.2% R0060 in acetone.

At the sites exposed to 0.02% R0060 in acetone (w/v), seventeen grades of +, and two grades of 0 were noted in the test animals at the 24-hour reading. At the 48-hour reading, one grade of 1, sixteen grades of +, and two grades of 0 were noted at these sites. For the control animals, one grade of + and nine grades of 0 were noted at both the 24-hour and 48-hour readings at the sites exposed to 0.02% R0060 in acetone.

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<u>SAMPLE</u>	<u>CONC.</u>	<u>ANIMALS</u>	<u>INCIDENCE</u>	<u>SEVERITY</u>	
				<u>24-hr</u>	<u>48-hr</u>
R0060	0.2%	Test	19/19	1.3	1.1
		Control	0/10	0.2	0.2
	0.02%	Test	1/19	0.4	0.5
		Control	0/10	0.1	0.1

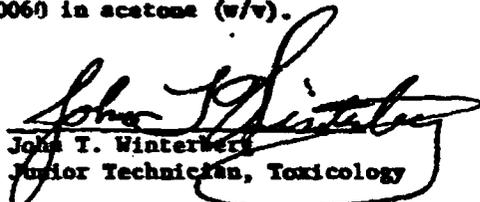
Summary

When tested according to the method of Boehler, R0060 produced five grades of 2 and 14 grades of 1 at the 24-hour reading, and three grades of 2 and fourteen grades of 1 at the 48-hour reading on patch sites exposed to a challenge concentration of R0060 as a 0.2% w/v solution in acetone among 19 guinea pigs.

R0060 produced one grade of 1 in one test animal at the 48-hour reading on patch sites exposed to a challenge concentration of R0060 as a 0.02% w/v solution in acetone.

No positive responses were observed in any of the control animals at sites exposed to either 0.2% or 0.02% R0060 in acetone (w/v).

Submitted by


John T. Winterberg
Junior Technician, Toxicology

Approved by


Marian B. Vinegar, Ph.D.
Director, Toxicology

Project No. 10-476-22

TABLE 1. Scores recorded at primary challenge for test albino guinea pigs which received three weekly induction applications of R0060 as a 0.5% w/v solution in 80% ethanol and one simultaneous primary challenge application of R0060 as 0.2% and 0.02% w/v solutions in acetone.

Also shown are the scores for the control animals which received a single simultaneous primary challenge application of R0060 as 0.2% and 0.02% w/v solutions in acetone.

Guinea Pig No.	Sex	Site Order*	Reading			
			24-hour		48-hour	
			A 0.2%	B 0.02%	A 0.2%	B 0.02%
1	M	A,B	1	+	+	0
2	M	A,B	2	+	2	+
3	M	A,B	2	+	2	+
4	M	A,B	1	+	1	+
5	M	A,B	1	0	+	0
6	M	B,A	2	+	1	+
7	M	B,A	1	+	1	+
8	M	B,A	1	+	1	+
9	M	B,A	1	+	1	+
10	M	B,A	1	+	1	+
11**	F	-	-	-	-	-
12	F	A,B	1	+	1	+
13	F	A,B	1	+	1	+
14	F	A,B	2	+	2	+
15	F	A,B	1	+	1	+
16	F	B,A	1	+	1	+
17	F	B,A	1	+	1	+
18	F	B,A	1	+	1	+
19	F	B,A	2	+	1	+
20	F	B,A	1	0	1	+
<u>Control</u>						
21	M	B,A	+	+	0	0
22	M	B,A	0	0	0	0
23	M	B,A	0	0	+	0
24	M	A,B	0	0	0	+
25	M	A,B	+	0	0	0
26	F	B,A	0	0	0	0
27	F	B,A	0	0	0	0
28	F	A,B	+	0	+	0
29	F	A,B	0	0	+	0
30 F	F	A,B	+	0	+	0

* anterior, posterior

** died prior to third induction

P = pregnant

M = male

F = female



Hill Top-Toxicology

Miamhville, Ohio 45147 (513) 831-3114

IMPORTANT NOTICE

Hill Top - Toxicology submits this report with the understanding that no portion of it will be used for advertising or promotion without obtaining our prior written consent to the specific proposed use. When such use is desired, we will be glad to assist in the preparation of mutually acceptable excerpts or summaries.

SAMPLE DISPOSAL PROCEDURE

At the conclusion of a test program, each sample will be stored for three months. At that time the sample will be returned to the client.



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Test Substance Identification Number R0060
 Safety Test Request Number PRSS 77
 Principal Investigator H. L. Ritz
 (Name)

PARS SECT. NO.	831/3
CRIG. SECT. NO.	831/3
ORIG. ASSOC. DIR.	<i>[Signature]</i>
PARS ASSOC. DIR.	<i>[Signature]</i>

Name of Product or Ingredient (or code designation) 1-chloro-2,4-dinitrobenzene
 Brand Notebook Ref. (including Production Code if available) Eastman Kodak Co. Lot #B2A
 Physical Form Crystals Color Yellow Density N/A
 Solubility N/A Sample Expiration Date Not Determined
 Recommended Storage Conditions Room temperature
 Hazards (i.e. flammability, toxic gases) Potentially potent sensitizer

Formulated Composition

<u>Component (a)</u>	<u>Nominal Level (X by Wt.)</u>	<u>Acceptable (b) Range</u>	<u>Stock Code No.</u>	<u>Supplier</u>	<u>Lot Number (b) (NB-Ref.)</u>
----------------------	---------------------------------	-----------------------------	-----------------------	-----------------	---------------------------------

Commercial sample - Eastman Kodak Co. Lot No. B2A

(a) Ingredients will be listed by chemical name; Non-chemical names such as Terptol 15-S-9 or Yellow Dye D&C #10 may be acceptable but should be provided with the responsible toxicologist. Chemical names which are inconveniently long may be abbreviated in tables but should be listed in full in referenced footnotes. Non-definitive identifications (e.g. Arquad, BC-base) are not acceptable.

(b) If information requested is not known then the symbol NK will be entered.

The above information provided by:

[Signature] (Signature) _____ 1/3/78
 (Name) (Date)

Test Substance Identification Number R0060

Safety Test Request Number PHSK 70

Analyzed Composition
(if available)

<u>Date Submitted</u>	<u>Submitter Code No.</u>	<u>Component or Property</u>	<u>Measured Value</u>	<u>Testing Laboratory</u>
-----------------------	---------------------------	------------------------------	-----------------------	---------------------------

Commercial sample Lot #B2A from Eastman Kodak Co.

Analytical Information Verified By:

(Name) (Signature) (Date)

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

Project Leader _____ (Signature) _____ (Date)

Principal Investigator Harold P. King (Signature) _____ (Date)
(Name) 1/3/78

IAN 8/24/77

Test Substance

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: 99%
Comments: Dinitrochlorobenzene (DNCB) data were included as a positive control for sensitization studies conducted at the laboratory. DNCB was also identified as SI0074.01. The other test materials of the report were unrelated to the 8(d) rule.

Method

Method / Guideline: Murine Local Lymph Node Assay
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1992
GLP Compliant: Yes
Species: CBA/J Mouse (female)
Number of Animals: 45
Study Design: 5 groups of animals received 12.5 µl of 0.1% test substance to the ventral and dorsal surface of both pinnae (total of 25 µl per ear, 50 ul per mouse) for 4 consecutive days. On day 5, each mouse was injected i.v. with 20 µCi of titrated thymidine (specific activity of 6.7 Ci/mmol) in a total volume of 0.25 ml of phosphate buffered saline. Lymph nodes were removed five hours later and dissociated. The radioactivity was measured by liquid scintillation spectrometry.

Results

Result: The test substance (positive control) had significant effect on auricular lymph node cellularity and thymidine incorporation by the constituent lymph node cells demonstrating an appropriate response as a positive for contact allergenic potential.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Report Number: L08321-SNO9
Reference: Procter & Gamble, 1992. Performance of the Murine Local Lymph Node Assay. Accession #36754.

ACC # 36754

PERFORMANCE OF THE MURINE LOCAL LYMPH NODE ASSAY

FINAL REPORT

**IITRI PROJECT NO L08321
STUDY NO. SN09**

Contractor:

**IIT Research Institute
Life Sciences Research
10 West 35th Street
Chicago, IL 60616-3799**

Sponsor:

**The Procter & Gamble Company
5299 Spring Grove Avenue - Room 3W82
Cincinnati, OH 45217-1087**

January, 1992

IITRI

since 1936

COMMITMENT TO EXCELLENCE

Performance of the Murine Local Lymph Node Assay

FOREWORD

This report describes a study conducted by the Life Sciences Department, IIT Research Institute, for the Procter & Gamble Company during the period of September 26, 1991 to October 16, 1991. The study was performed under IITRI Project No. L08321, Study No. SN09. Tim S. Elliott served as representative of the Procter & Gamble Company.

Robert V. House was Principal Investigator and served as Study Director. Peter W. Barbera, Martin J. Byrne, Shelagh Cofer, J. Brooks Harder, and Bill Stevens also participated in the study. Experimental data are recorded in a ring binder labeled L08321. All raw data with the exception of chemical analyses of the test substances, and a copy of the final report, will be archived at IITRI according to standard operating procedures. All chemical analyses of the test substances and attendant documentation are the responsibility of the Sponsor.

Respectfully submitted,

R. V. House 01-02-92
Robert V. House, Ph.D. Date
Research Immunologist
Life Sciences Research

Approved by:

Peter T. Thomas 1-2-92
Peter T. Thomas, Ph.D. Date
Program Manager, Immunology/Microbiology
Life Sciences Research

IIT RESEARCH INSTITUTE

Page 2 of 28

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I. OBJECTIVE

The objective of this study was to examine the potential of test substances to induce contact hypersensitivity using the murine local lymph node assay. The assays used for end point determination were total auricular lymph node cellularity and radiolabeled thymidine incorporation into the auricular node cells.

II. SUMMARY

Mice were treated daily for four consecutive days with either nothing (naive control), acetone (vehicle control), or one of seven test substances on each pinna. Approximately 24 hours following final test substance application the animals were injected intravenously with radiolabeled thymidine to label proliferating cells. Five hours after this injection, the animals were euthanized by CO₂ asphyxiation, the auricular (i.e., draining) lymph nodes were removed in toto, and single-cell suspensions were prepared. These suspensions were standardized by volume and total auricular lymph node cellularity per mouse was quantitated by Coulter counting. The cells were washed with saline to remove unbound radiolabel and subsequently precipitated with trichloroacetic acid. The total radiolabel incorporation in these precipitates was subsequently quantitated by liquid scintillation spectrometry.

Treatment of mice with all test substances except SI0074.01 had no significant effect on either auricular lymph node cellularity or thymidine incorporation by the constituent lymph node cells. Based upon previously-published criteria, only test substance SI0074.01 may be classified as a contact sensitizer following exposure by the regimen utilized in this study.

III. MATERIALS AND METHODS

A. TEST SUBSTANCES

The test substances examined in this study were SI0062.01 (0.1%, 1%, and 5%), SI0073.01 (0.1%, 1%, and 5%), and SI0074.01 (0.1%). All test substances were received on 10-07-91 as clear liquids, and were stored refrigerated. All test substances were dosed directly from the solutions provided by the sponsor, and the solutions remaining at the end of the study were returned to the sponsor. All test substances were assumed to be skin, eye, and respiratory irritants/sensitizers, and appropriate precautions were observed to prevent accidental exposure. No analyses of the

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concentration, stability, or homogeneity of the test substance-vehicle mixtures were required. Documentation of the strength, purity, and composition of the test substances (including the stability and homogeneity of the test substance-carrier mixtures) were the responsibility of the Sponsor.

B. EXPERIMENTAL ANIMALS

Experimental animals used in this study were 56 female CBA/J mice, obtained from Jackson Laboratories, Bar Harbor, ME. The mice were born on 8-12-91, and were received at IITRI on 9-26-91. Following quarantine, animals were randomized, housed individually, and provided Certified Rodent Chow (Purina 5002) and water ad libitum. Each animal received an identification mark by indelible ink on the tail, with the number of marks corresponding to the number of the animal within its respective dosage group. Individual cage cards were provided bearing the animal number, project number, and study number. The animal room was air-conditioned with a 100% fresh air supply and was provided with adjustable diurnal light cycle. Room temperature ranged between 23°C and 25°C, and relative humidity ranged between 25% and 55% during the course of the study. Overall animal health status was reviewed by the IITRI Staff Clinical Veterinarian before clearing animals for use in the study. Animals were observed daily for morbidity and mortality.

The IITRI animal facilities have been fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) since 1975. The animal facilities are also registered with the U.S. Department of Agriculture in compliance with appropriate laws and are regularly inspected by USDA veterinarians. The animal facilities were operated under the overall supervision of the IITRI Staff Clinical Veterinarian, Dr. J. Brooks Harder.

C. EXPERIMENTAL DESIGN

Five mice per group were dosed once daily for four consecutive days beginning on 10-07-91. Animals were dosed with nothing (control), acetone, or one of the seven test substances. On day 5 of the assay, mice were radiolabeled by an iv injection of tritiated thymidine, and five hours later the auricular nodes were excised and processed for experimental end points.

D. MURINE LOCAL LYMPH NODE ASSAY

For dosing, the animals were restrained by hand, and 12.5 μ l of vehicle or test substance was applied to the ventral and dorsal surfaces of both pinnae (a total of 25 μ l per ear) daily for four consecutive days. On Day 5 of the study, each mouse was injected intravenously with 20 μ Ci of $[^3H]TdR$ (specific activity of 6.7 Ci/mole) in a total volume of 0.250 ml saline. Five hours later the animals were euthanized by CO_2 asphyxiation, and the auricular lymph nodes were removed in toto. The nodes were dissociated by rubbing them through nylon macromesh, and the cells were washed once in saline. Total auricular lymph node cellularity per mouse was subsequently quantitated with a Coulter counter Model 2M. The remaining cells were centrifuged and resuspended in 5% trichloroacetic acid (TCA). Approximately 18 hours later the cells were washed a final time, resuspended in fresh TCA and added to liquid scintillation cocktail fluid. The acid-precipitable counts were then measured by liquid scintillation spectrometry.

E. STATISTICAL ANALYSIS AND DATA PRESENTATION

Analysis of variance (ANOVA) and Dunnett's multicomparison and analysis of variance were used to evaluate the statistical significance of experimental treatment. Experimental data were considered significantly different from their respective control values at $P \leq 0.01$.

Data are presented graphically as comparisons of total auricular lymph node cellularity, as well as total thymidine incorporation. In addition, the stimulation indices (SI) were calculated to allow classification of the compounds as sensitizers or non-sensitizers.

IV. RESULTS AND DISCUSSION

A. INDIVIDUAL BODY WEIGHTS

In accordance with the experimental Protocol, individual body weights were quantitated and recorded at the initiation of the study, which is here defined as randomization prior to test compound exposure. These weights are presented in Table 1.

B. AURICULAR LYMPH NODE CELLULARITIES

Cellularities are reported as the total auricular node cellularity per animal (Table 2). Exposure to all test substances except SI0074.01 resulted in a slight degree of cellularity increase, although this increase was neither dose-related nor statistically significant (Figure 1). In comparison, treatment with 0.1% SI0074.01 resulted in an almost two-fold increase in cellularity; this increase was not significantly different from the acetone control. The mean (\pm SEM) cellularity of naive mice in this study was $3.18 \pm 0.6 \times 10^6$ cells.

C. RADIOLABEL INCORPORATION

Individual radiolabel incorporation data are shown in Table 3. Exposure to test substances examined in this study had little effect on radiolabel incorporation except for test substance SI0074.01, which resulted in a pronounced increase in thymidine incorporation in lymph node cells. This increase was statistically different from the acetone control ($P < 0.01$ by Dunnett's). The mean (\pm SEM) thymidine incorporation for naive animals in this study was 497.4 ± 89.0 DPM (disintegrations per minute).

D. STIMULATION INDICES

The stimulation index (SI) is determined by dividing the value of the experimental parameter by the value of the vehicle control. Historically, a SI of 3.0 or greater is considered to represent a positive contact sensitizer. Based upon this criterion, the only test substance examined in this study that may be classified as a contact sensitizer was SI0074.01 (Table 4).

V. **QUALITY ASSURANCE STATEMENT**

Study Title: Performance of the Murine Local Lymph Node Assay
Project No.: L08321
Study Number: SN09
Study Director: Robert V. House, Ph.D.
Report Audit Date: October 28, 1991

This study was not intended to be submitted to a regulatory agency and as such was not listed on the Master Schedule for GLP regulated studies. The study has been subjected to inspection and the report has been audited by the IITRI Quality Assurance Unit. The report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study. Analyses of stability, homogeneity and concentration of the Test Article/Carrier mixtures were the Sponsor's responsibility. There were no significant deviations from the FDA Good Laboratory Practice Regulations.

The following are the inspection dates and the dates inspection reports were submitted:

<u>Dates of Inspection</u>	<u>Inspection Reports Submitted to: Study Director</u>	<u>Management</u>
9/18/91	9/18/91	9/18/91
10/ 8/91	10/ 8/91	10/11/91


Ronald A. Boyne, B.S. Date
Manager, Quality Assurance

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VI. TABLES

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TABLE 1. BODY WEIGHTS OF INDIVIDUAL MICE IN STUDY NO. 8809

Treatment	Body Weight in Grams				
<u>Naive Control</u>	22.7	19.5	19.0	21.5	22.2
<u>Acetone Control</u>	21.5	18.9	20.1	21.4	22.6
<u>SI0062.01 0.1%</u>	23.0	20.0	21.1	22.5	17.2
<u>SI0062.01 1.0%</u>	21.9	21.2	18.9	23.3	19.7
<u>SI0062.01 5.0%</u>	23.1	19.1	18.6	21.9	20.5
<u>SI0073.01 0.1%</u>	19.9	19.0	22.8	22.2	21.0
<u>SI0073.01 1.0%</u>	19.1	21.4	24.4	22.4	17.4
<u>SI0073.01 5.0%</u>	17.6	19.8	21.4	23.1	22.3
<u>SI0074.01 0.1%</u>	22.7	22.2	20.0	21.4	19.0

TABLE 2. LYMPH NODE CELLULARITY DATA FOR INDIVIDUAL ANIMALS

	Mouse Number Within Group				
	1	2	3	4	5
<u>Naive Control</u>	5.219*	1.877	2.963	2.710	3.148
<u>Acetone Control</u>	3.949	3.329	2.529	4.013	10.272
<u>SI0062.01 0.1%</u>	3.587	4.195	9.250	4.509	2.638
<u>SI0062.01 1.0%</u>	3.583	3.928	5.020	2.436	4.673
<u>SI0062.01 5.0%</u>	6.927	5.321	4.223	15.471	3.755
<u>SI0073.01 0.1%</u>	11.645	4.881	9.129	5.082	2.435
<u>SI0073.01 1.0%</u>	6.622	2.960	3.827	3.872	7.655
<u>SI0073.01 5.0%</u>	2.505	4.820	4.177	3.818	3.699
<u>SI0074.01 0.1%</u>	6.097	15.224	8.832	6.151	8.319

* All values expressed as total auricular lymph node cells per animal X 10⁶.

TABLE 3. RADIOLABEL INCORPORATION DATA FOR INDIVIDUAL ANIMALS

	Mouse Number Within Group				
	1	2	3	4	5
<u>Naive Control</u>	630*	317	769	317	454
<u>Acetone Control</u>	587	462	352	304	1442
<u>SI0062.01 0.1%</u>	114	322	1019	778	408
<u>SI0062.01 1.0%</u>	411	419	570	551	411
<u>SI0062.01 5.0%</u>	818	861	634	3084	489
<u>SI0073.01 0.1%</u>	2070	1000	2174	593	442
<u>SI0073.01 1.0%</u>	986	491	433	578	2485
<u>SI0073.01 5.0%</u>	370	417	544	556	451
<u>SI0074.01 0.1%</u>	6346	10212	4089	5454	1004?

* All values expressed as disintegrations per minute (DPM).

TABLE 4. STIMULATION INDICES FOR LYMPH NODE CELLULARITY AND THYMIDINE INCORPORATION FOLLOWING EXPOSURE TO TEST SUBSTANCES

Test Substance	PARAMETER TESTED	
	CELLULARITY	RADIOLABEL
<u>Naive Control</u>	0.660	0.790
<u>Acetone Control</u>	-	-
<u>SI0062.01 0.1%</u>	1.004	0.839
<u>SI0062.01 1.0%</u>	0.815	0.751
<u>SI0062.01 5.0%</u>	1.482	1.870
<u>SI0073.01 0.1%</u>	1.377	1.995
<u>SI0073.01 1.0%</u>	1.035	1.580
<u>SI0073.01 5.0%</u>	0.789	0.743
<u>SI0074.01 0.1%</u>	1.852	11.485

VII. FIGURES

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FIGURE 1. AURICULAR LYMPH NODE CELLULARITY OF MICE TREATED FOR FOUR DAYS WITH TEST SUBSTANCES (SN03)

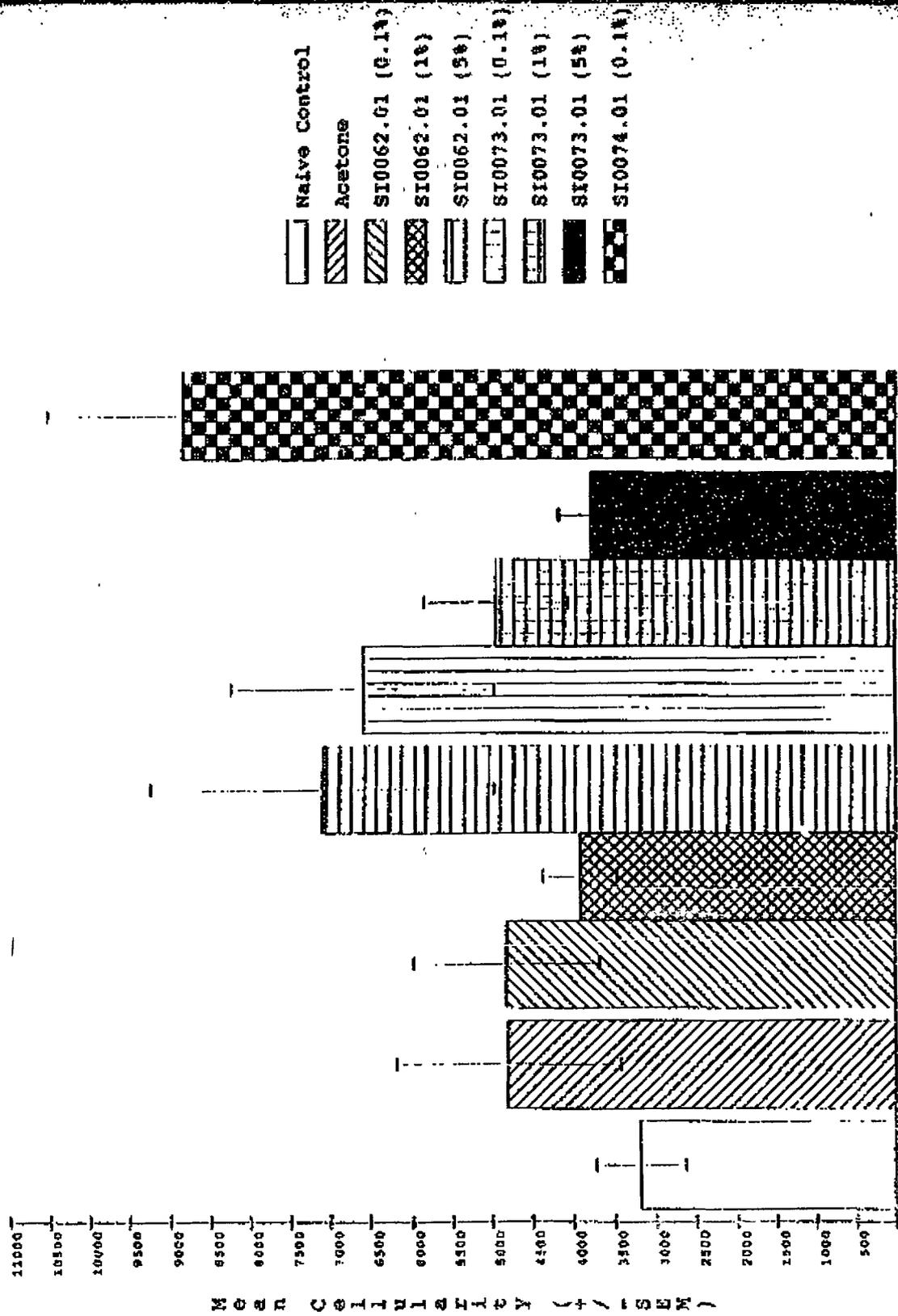
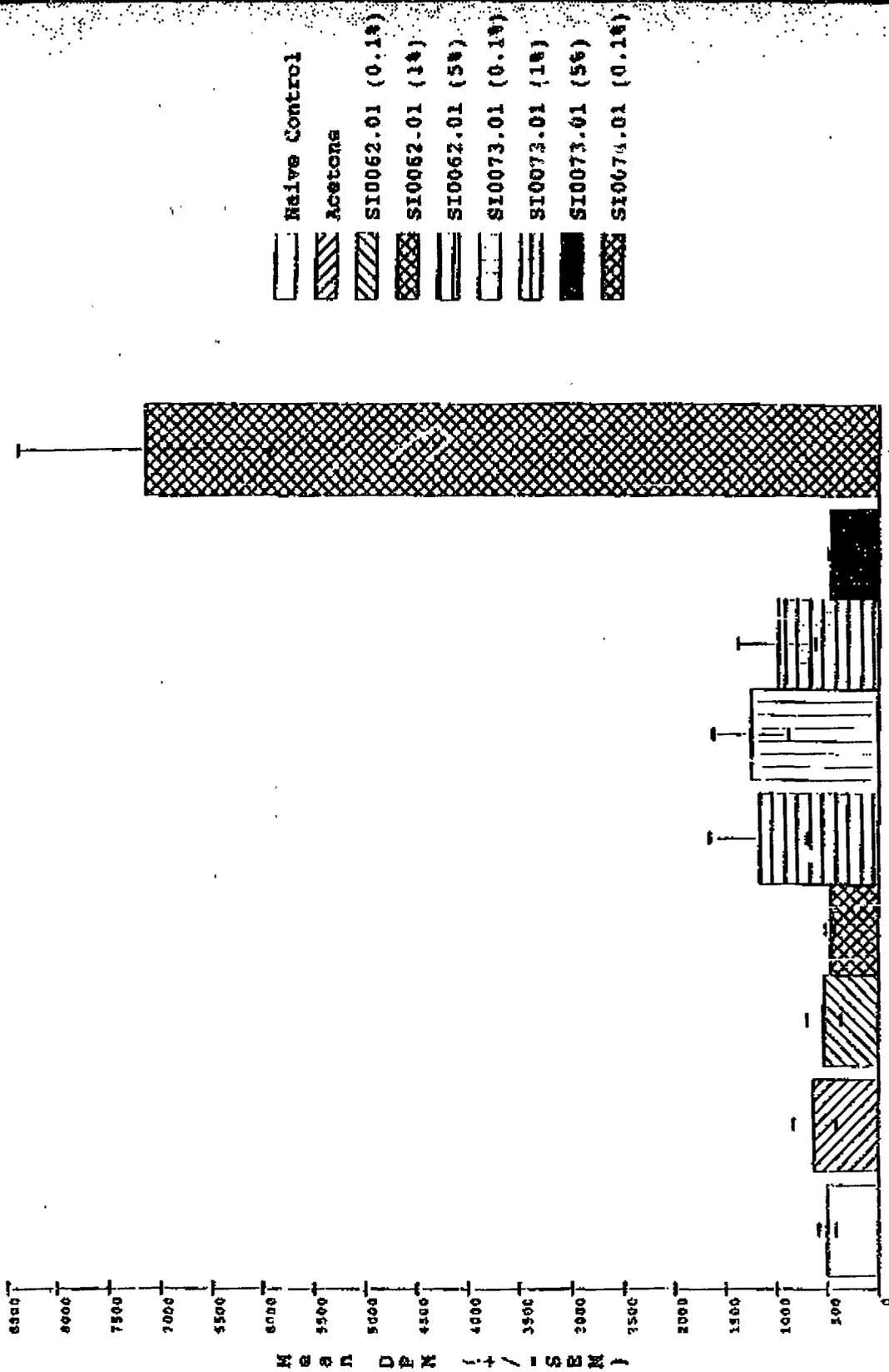


FIGURE 2. THYMIDINE INCORPORATION INTO AURICULAR LYMPH NODES OF MICE TREATED FOR FOUR DAYS WITH TEST SUBSTANCES (SM09)



* Significantly different from vehicle control at $P < 0.01$ by Dunnett's

VIII. APPENDIX

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Quality Assurance:

This study and final report will be audited by the Quality Assurance Unit in accordance with IITRI's standard operating procedures.

Diet and/or Water Analysis Required:

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

Test Substance:

CAS Number	Description		Expiration Date
	Color	Physical Form	
* See Attached Sheet		LIQUID	8/92

Storage Conditions: (Check one)

Room Temperature Refrigerate Freezer
 Other: _____

Hazards: (Check one)

None known. Take ordinary precautions in handling.
 Unknown (not evaluated).
 As follows: *Avoid eye contact; skin irritant*

Test Substance Characterization:

Information on the methods of formulation and data on the composition or other characteristics that define the test and control substances (including the stability and homogeneity of the test substance-vehicle mixture) will be the responsibility of the Sponsor.

Test Substance Retention:

Any unused test substance will be returned to the Sponsor at the end of the study, or if instructed, disposed of in accordance with IITRI's standard operating procedures.

Note:

Analysis of concentration/stability/homogeneity of the test substance-vehicle mixture(s) will ; will not be required.

If an analysis is required, a sufficient quantity of the test substance-vehicle mixture(s) will be prepared so that a portion can be returned to the Sponsor's divisional toxicologist. The solution/mixture will be stored at room temperature; refrigerator; freezer; other _____.

Shipping Instructions:

Send approximately _____ ml. Send frozen; under ambient conditions; other overnight delivery on wet ice.

Special Instructions:

None.
 As follows:

Animals:

Female CBA/J mice will be obtained from Jackson Laboratories, Bar Harbor, ME. Mice will weigh approximately 15 to 20 grams and be 6-8 weeks of age at initiation of the study. Control and test groups will consist of five mice each.

Animal Care and Diet:

The acclimation period will be a minimum of seven days before animals are placed on test. Mice will be provided access to tap water and Purina 5002 rodent chow (or equivalent) ad libitum.

Note: The animals will be observed daily during treatment for morbidity and mortality. Any animals that exhibit signs of undue stress or discomfort, as judged by the study director or the test facility veterinarian, will be euthanized immediately for humane reasons following AAALAC approved guidelines and/or test facility Institutional Animal Care and Use Committee (IACUC) approved procedures. The Sponsor's Divisional Toxicologist will be notified within 24 hours of the animal's euthanasia. Animals which die or are euthanized prior to study termination will be grossly examined. All findings will be recorded.

Environmental Conditions:

Environmental conditions for the animal room will be observed and recorded daily.

Animal Identification:

Mice will be housed individually in plastic shoe box type cages for the duration of the study. Control of bias will be addressed by randomization of mice with a computer randomization program prior to initiation of the study, with each animal assigned a unique number. Animals will be weighed only during the randomization procedure. Each cage will be labeled with the study number, animal number and group number or letter. Each individual animal will be identified by a color code using a

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water proof felt-tipped pen mark to identify the group and dots of the same color ink on the under side of the tail to identify individual animals within the groups. One dot being animal number 1, two dots indicating animal 2, etc.

Experimental Design:

Five groups of five animals will be used for each test substance. The induction period consists of treating the animals once a day for four consecutive days. Approximately 24 hours between applications of test substance will be maintained (22 to 26 hours). On day five the animals will be given i.v. injections of [³H]-thymidine (18 to 24 hours after the last application of test substance to the ears). Five hours after the i.v. injections, the animals will be euthanized and the auricular nodes removed. Single cell suspensions of the node cells will be prepared and then counted on a liquid scintillation counter to quantitate [³H]-thymidine incorporation.

Dose Preparation and Test Concentrations:

All test substance/vehicle solutions will be prepared on the day of use as indicated below: (Check one).

- Three concentrations of the test substance, a vehicle control group and a naive control group.
- Four concentrations of test substance and a vehicle control.
- Other: See attached sheet for instructions.

<u>Test Substance</u>	<u>Concentrations</u>	<u>Vehicle</u>
-----------------------	-----------------------	----------------

* All test samples will be dosed as received from the Sponsor.

Procedures:

Note: All animal procedures will follow applicable animal welfare procedures, e.g., AAALAC approved guidelines and/or test facility Institutional Animal Care and Use Committee (IACUC) approved procedures. This study will be conducted in accordance with the Testing Facility's Standard Operating Procedures.

A. Application of Test Substance:

The animals will be restrained in such a manner as to allow free access to the dorsal and ventral sides of both ears. Using an adjustable push button pipet (Rainin Pipetman[®] or equivalent), 12.5 µl of test substance will be applied to the dorsal and ventral sides of each ear, for a total of 25 µl of test substance per ear. Care will be taken to ensure that the test substance does not run off the ear during application. Pipets are to be calibrated at least every three months (90 days) to assure accurate delivery of the 0.0125 ml volume.

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B. Intravenous Injection of [³H]-Thymidine:

Each animal will receive 0.250 ml of phosphate buffered saline (PBS) containing 20 μ Ci of [³H]-thymidine (specific activity of 6.7 Ci/mmol). A heat lamp may be used to dilate the tail veins for easier i.v. injections. The animal will be restrained in such a manner as to allow complete access to the tail. A 1 cc disposable syringe and 27 gauge needle may be used for i.v. injections. An animal will be excluded from the study if the full 0.250 ml of [³H]-thymidine is not properly i.v. injected. Statistical analysis will not be performed on a group when more than one mouse is excluded from the group.

C. Lymph Node Removal:

Five hours after the [³H]-thymidine injections, the animals will be euthanized with CO₂ and the auricular lymph nodes removed. Care will be taken to assure that the intact lymph nodes are removed. Once the lymph nodes are removed, they will be placed in a 12x75 mm capped tissue culture tube (approximately 4.5 ml capacity) containing 4 ml of PBS.

D. Single Cell Suspension:

The lymph nodes will be transferred to a 60mm tissue culture dish by pouring the PBS from the tubes containing the lymph nodes. Both the top and bottom of the tissue culture dish may be used for preparing single cell suspensions for each individual animal. An approximately 1 inch square section of nylon macromesh (mesh opening 100 microns, 85 microns thick) will be placed in the inverted lid of a tissue culture dish. The lymph nodes will be placed on the nylon mesh using a small pair of forceps and the capsule snipped with a small pair of pointed surgical scissors. Using a transfer pipet (Pasteur pipet) a small amount of PBS (approximately 1-1.5 ml) will be placed on the nodes. The nodes will be gently rubbed through the nylon screen using the flat surface of a plunger from a 1 cc disposable syringe. A Pasteur pipet and a small pair of forceps will be used to rinse the screen with PBS in the bottom portion of the tissue culture dish. The nylon filter will be discarded after rinsing and the tissue culture dish rinsed with the PBS. The PBS will be placed back into the 12x75 mm round bottom tube to allow the capsule debris to settle to the bottom. After approximately 5 minutes the PBS will be carefully drawn off with a Pasteur pipet and placed in a 15 ml conical bottom centrifuge tube. Six ml of PBS will be added to each tube (approximately 10 ml total tube volume) and the cell suspensions centrifuged at 200xg for 10 minutes. After the first wash in PBS the cells will be resuspended in 1 ml of PBS. A 20 μ l sample will be removed and added to 10 ml of Isoton II and the samples counted on a Coulter Counter Model ZM. After removal of the 20 μ l sample the remaining suspension will be added to the tubes and the second wash will be completed. After completion of the second wash, the cells will be suspended in 3 ml of 5% trichloroacetic acid (TCA) (w/v, distilled H₂O) and left at approximately 4°C for 18 to 60 hours.

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Preparation for Scintillation Counting:

The cell suspensions will be centrifuged at 200g for 10 minutes and resuspended in 3 ml of 95% TCA. Scintillation vials (borosilicate, 20 ml volume) containing 10 ml of scintillation cocktail (Zcoluon or equivalent) will be appropriately labeled with the individual animal numbers. The individual cell suspensions will be transferred into the appropriate vials along with an additional 1 ml of TCA which has been used to rinse the bottom portion of the tube. The TCA and scintillation fluid will be thoroughly mixed by gently swirling the contents of the vial until the solution becomes clear.

Scintillation Counting:

A Tracor Analytic Mark II Liquid Scintillation System will be used for counting. The samples will be counted for 5-10 minutes and the counts recorded in disintegrations per minute (DPM).

Report:

The report will include how the study was conducted, including any deviations from the protocol, and the dates of study initiation and completion. Data for each animal will be included in the final report. Also, calculation of the group mean DPM \pm standard error of the mean (SE) for each group and a graphic representation of the results using the group mean and SE (standard error is to be calculated using $n-1$) will be included. In addition, the fold increase in the mean of isotope incorporation relative to the vehicle control group will be determined and included in the final report. A Dunnett's test will be used to evaluate statistical analysis. Statistical analysis of the data will compare all test groups to the vehicle and, if appropriate, to the naive control group (level of significance is 0.01). The report shall conform to all requirements outlined in CFR Title 40, Part 792, Good Laboratory Practice Regulations.

Maintenance of Raw Data and Records:

Original data or copies thereof, will be available at the testing facility to facilitate auditing of the study during its progress and before acceptance of the final report. When the final report is completed, all original paper data, magnetically encoded records generated by the testing facility, and a copy of the final report will be retained in the archive of the testing facility.

Sponsor: T. S. ELLIOTT 513-627-6952
Telephone No.

Alternative Contact: J. D. INNIS 513-629-5779
Telephone No.

Date approved by sponsor: 9/6/91

Proposed Study Starting Date: 10-7-91

Defined as: First day of dosing

Proposed Completion Date: 10-14-91

Defined as: Samples in scintillation counter

Study Director: R. V. HARRIS

Date: 9-18-91

Quality Assurance Representative: K. A. BOYRE

Date: 9-20-91

PROTOCOL ATTACHMENT
L8832-SN09

ELISA Test Samples

ALL TEST SAMPLES ARE TO BE DONE AS RECEIVED FROM SPONSOR

Study 1

Test Sample	Concentration	Bottle Number	DOB
S10062.01	5%	A1	S1875 0030-82
S10062.02	1%	B1	--
S10062.03	0.5%	C1	--
S10074.01	0.1%	D1	--
Acetone	nest	E1	--

Study 2

Test Sample	Concentration	Bottle Number	DOB
S10075.01	5%	A2	S1875 0030-82
S10075.02	1%	B2	--
S10075.03	0.5%	C2	--
S10073.01	0.5%CMC+0.01%LAS	D2	--
Naive Control	-----	E2	--

NOTE: These test samples will be sent to IITRI (overnight delivery) upon notification to Tim Elliott of the starting date.

The above samples, supplied by the Sponsor, will be tested as per instructions in Protocol L88321-SN09.

Study Director: R. V. Gamm 7-19-82
Date

PROTOCOL DEVIATION NO. 1

Effective Date: October 7, 1991

Project No.: L08321

Study No.: SN-02

Nature of Deviation:

1. The Protocol Attachment for L08321/SN02 states that the test substances were to include:

SI0062.01	5%	SI0075.01	5%
SI0062.02	1%	SI0075.02	1%
SI0062.03	0.5%	SI0075.03	0.5%
SI0074.01	0.1%	SI0073.01	0.5% CMC+0.01% LAS
Acetone	Heat	Naive Control	

When the samples arrived from the Sponsor on 10/07/91 the designations were found to be:

P2666.01 in SI0062.01	5%
P2666.01 in SI0062.01	1%
P2666.01 in SI0062.01	0.1%
SI0074.01 in SI0062.01	0.1%
Acetone	Heat
P2666.01 in SI0073.01	5%
P2666.01 in SI0073.01	1%
P2666.01 in SI0073.01	0.1%

The vehicle control (SI0073.01) was not received. The Sponsor indicated by telephone conversation that the above designations were correct and to proceed with the study as originally planned.

Reason for Deviation:

1. Test substance designations changed by the Sponsor.

Potential Effect on Study:

1. No effect since all test substances were unknowns.

Study Director: R.V. Ham

Date: 12-17-91

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PROTOCOL DEVIATION NO. 2

Effective Date: October 1, 1991

Project No.: 108121

Study No.: SN-09

Nature of Deviation:

1. Page 3, "Animals" states that mice will weigh approximately 15 to 20 grams. . . at the initiation of the study". Many of the mice were in fact out of this weight range;

Reason for Deviation:

1. Mice were of correct age when ordered; however, the word "approximately" was interpreted to indicate a certain degree of flexibility on the part of the Study Director. It was felt that this slight deviation in weight range, considering the age of the animals, would not result in a significant alteration of the results;

Potential Effect on Study:

1. None expected or noted.

Study Director:

R. V. How

Date:

11-18-91

U.S. Soap Sector
TEST SUBSTANCE CHARACTERIZATION
REPORT (TSCR)

Page 1 of 2

Extension List
Non-Decoratory

Section I: Unique Identifiers (to be completed by P&RS)

TEST SUBSTANCE IDENTIFICATION NUMBER (TSIN) 25074.01

DIVISIONAL REQUEST DOCUMENT(S) SBITS 0030-32

P&RS Sample Log Notebook Reference _____

Section II: Product Description (to be completed by Originator, PD Project person. No spaces can be left blank. Use 'NK' for 'not known' and 'NA' when 'not applicable'. NK and NA cannot be used for Notebook references, hazards, or expiration dates.

Name of Product or Ingredient (for code designation): DNCS

Brand Notebook Reference: MP-3451-74

Physical Form powder Color yellow pH unknown

Density/Sp. Gravity unknown Mol. Wt. NA Solubility soluble in acetone

Section III: Storage and Stability (to be completed by Originator)

Storage Conditions room temperature

Expiration Date (mo/day/yr) 8/92 Stability Notebook Ref. MP-3451-74

Section IV: Safety and Handling Information (to be completed by Originator)

P&G Hazard Ratings: Health 2 Fire 0 Instability 0

DOT Hazard Classification Non-hazardous

Hazards: None known. Use normal handling precautions.

As follows: skin sensitizer

Instructions for Section V & VI: Compositional and Analysis Information located on page 2 (to be completed by Originator and verified by Analytical. Originator must check with Divisional Toxicologist to see what analyses are necessary for the type of testing to be performed. If no analytical work is to be performed, justification must be provided by the Divisional Toxicologist.

Component: Use chemical name which uniquely identifies component. Common names may be indicated in parentheses following the chemical name. 'Misc.' or 'balance' is not permitted.

Stock Code Number: Raw material identification code.

CAS# : List the Chemical Abstracts Services number for all ingredients.

Nominal % by Weight: Enter the target level of each component. Total nominal composition must equal 100%. 'Balance' for Gases or water is not permitted.

Acceptable Range: Enter the range for all key components which will be analyzed. Measured levels outside this range will render the material unsuitable for testing.

Measured Component Level: Enter the level as determined by the analyzed value with any conversion factors. Calculations to convert the 'analyzed value' to the 'measured level' must be documented in the brand notebook. If conversion factors are not necessary, the 'measured level' is equal to the 'analyzed value'.

Submitter's Code: Unique identifier for the sample in the analytical system.

Analysis Code: Code from Analytical Lab to identify procedure used to determine analyzed value.

Analyzed Value: Values must be entered exactly as reported by analytical. Rounding is not permitted.

Analytical Reference: Notebook or file number where the original analytical data is maintained.

Please note: Any changes made after TSCR is signed must be made on yellow original only and must be initialed and dated, and a reason for the change stated (GLP requirement).

TSIN: SID074.01
Page 2 of 2

Section V: Compositional Information (Instructions on Page 1)

Stock Code	Case	Normal Level % In. Vol.	Acceptable Range	Measured Component Level	Submitter's Code	Analyte Code	Analyzed Value	Analytical Reference
1000000	1000000							

DMOB (0.1%) dissolved in acetone. Material supplied by Sigma Chemical Company, St. Louis, Mo. (99% pure) from G.F. Gerberlet

Section VI: Analytical Information (Instructions on Page 1)

Submitter's Code	Analyte Code	Analyzed Value	Analytical Reference

Compositional Information Provided by: (Organism or material)

(Signature verifies integrity of all data provided in Section II-VI)

Section VII: Test Substance Acceptability and Documentation (Signatures should be placed on yellow original only)

This test substance characterization has been reviewed and the test material is acceptable for the safety testing indicated by the Divisional Request Document(s).

Project Leader E. E. Gattly Signature E. E. Gattly Date 9/30/91

Divisional Toxicologist J. E. Halliwell Signature J. E. Halliwell Date 9/30/91

AFIS Section Head Signature J. E. Halliwell Date 9/30/91

PURS Section Head J. E. Halliwell Signature J. E. Halliwell Date 9/30/91

Form revised 1/28/91

Analytical Verification by: (Signature/Date)

MA
(Signature verifies only Analyzed Value and Reference)

Test Substance

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not available
Comments: Dinitrochlorobenzene (DNCB) data were included as a positive control for sensitization studies conducted at the laboratory (Appendix XI of the report) from July 1995-November 1997. The actual test material of the report was unrelated to the 8(d) rule and has been redacted.

Method

Method / Guideline: OECD 406 "Skin Sensitization" Magnusson & Kligman Maximization Study
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1998
GLP Compliant: Yes
Species: Guinea pig – Dunkin-Hartley albino strain
Number of Animals: Positive control animals: 10 males and 10 females (in separate studies)
Study Design: Study 1: Intradermal Induction – 0.1% in arachis oil BP
Topical Induction – 0.75% in absolute ethanol
Challenge – 0.25% and 0.1% in absolute ethanol
Study 2: Intradermal Induction – 0.1% in arachis oil BP
Topical Induction – 0.75% in 80% ethanol
Challenge – 0.25% and 0.1% in 80% ethanol

Results

Result: DNCB, as the positive control for delayed contact hypersensitivity studies, elicited the appropriate response - 100% sensitization.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Report Number: 930/040
Reference: Safepharm Laboratories Limited, 1998. Magnusson & Kligman Maximisation Study in the Guinea Pig. Accession #100345

ACC # 100345

PAGE 1 OF 31 PAGES

CONFIDENTIAL

MAGNUSSON & KLIGMAN MAXIMISATION

STUDY IN THE GUINEA PIG

SPL PROJECT NUMBER: 930/040

AUTHOR: D J Allen

STUDY SPONSOR:

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GEDAR00338

QUALITY ASSURANCE REPORT

The routine inspection of short term studies at Safepharma is carried out as a continuous process designed to encompass all major phases of each study type once per month. Inspection findings are reported to Management/Study Directors on the day of inspection in each case. Dates of relevant monthly inspections are as follows:

06, 14, 21 May 1998

This report has been audited by Safepharma Quality Assurance Unit. It is considered to be an accurate account of the data generated and of the procedures followed.

Date of Report Audit:

12 June 1998

..... DATE: **14 JUL 1998**.....

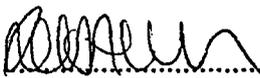
J M Crowther MIScT
For Safepharma Quality Assurance Unit

GLP COMPLIANCE STATEMENT

I, the undersigned, hereby declare that the objectives laid down in the protocol were achieved and as nothing occurred to adversely affect the quality or integrity of the study, I consider the data generated to be valid. This report fully and accurately reflects the procedures used and data generated.

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1997 (SI 1997/654)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 87/18/EEC and 88/320/EEC.

These international standards are acceptable to the United States Environmental Protection Agency and Food and Drug Administration, and fulfil the requirements of 40 CFR Part 160, 40 CFR Part 792 and 21 CFR Part 58 (as amended).

.....  DATE: 13 JUL 1998

D J Allen BSc (Hons)
Study Director
for Safepharm Laboratories

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SUMMARY

STUDY SPONSOR : MITSUBISHI GAS CHEMICAL
COMPANY, INC.

STUDY TITLE : MAGNUSSON & KLIGMAN
MAXIMISATION STUDY IN THE
GUINEA PIG

TEST MATERIAL :

1. A study was performed to assess the contact sensitisation potential of the test material in the albino guinea pig. The study was performed in compliance with the OECD Guidelines for Testing of Chemicals No. 406 "Skin Sensitisation" (adopted 17 July 1992) and Method B6 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

The results may be used as a basis for classification and labelling under Annex VI of Council Directive 67/548/EEC (as adapted to technical progress by Commission Directive 93/21/EEC).

2. Ten test and five control animals were used for the main study.

Based on the results of sighting tests, the concentrations of test material for the induction and challenge phases were selected as follows:

Intradermal Induction	:	0.05% w/v in distilled water
Topical Induction	:	5% v/v in distilled water
Topical Challenge	:	2% and 1% v/v in distilled water

3. The test material produced a 0% (0/10) sensitisation rate and was classified as a non-sensitiser to guinea pig skin. The test material did not meet the criteria for classification as a sensitiser according to EU labelling regulations. No risk phrase is required.

MAGNUSSON & KLIGMAN MAXIMISATION
STUDY IN THE GUINEA PIG

1. INTRODUCTION

The study was performed to assess the contact sensitisation potential of the test material (Safepharm Standard Method Number 576.02). The study was performed in compliance with the recommendations of the OECD Guidelines for the Testing of Chemicals No. 406 "Skin Sensitisation" (adopted 17 July 1992) and Method B6 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC).

The results may be used as a basis for classification and labelling under Annex VI of Council Directive 67/548/EEC (as adapted to technical progress by Commission Directive 93/21/EEC).

The test system was chosen because the guinea pig has been shown to be a suitable species for this type of study and is recommended in the test method. The strain used in these laboratories has been shown to produce satisfactory sensitisation responses using known positive sensitisers (see Appendix XI). The results of the study are believed to be of value in predicting the likely contact sensitisation potential of the test material to man.

The study was performed between 16 March 1998 and 14 May 1998.

2. TEST MATERIAL AND EXPERIMENTAL PREPARATION

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	
Batch number	:	60902
Date received	:	22 May 1997
Description	:	colourless liquid
Storage conditions	:	room temperature in darkness

Data relating to the identity, purity and stability of the test material are the responsibility of the sponsor.

2.2 Experimental Preparation

For the purpose of this study the test material was freshly prepared as follows:

Intradermal Induction	:	0.05% w/v in distilled water 0.05% w/v in a mixture of Freund's Complete Adjuvant plus distilled water (1:1)
Topical Induction	:	5% v/v in distilled water
Topical Challenge	:	2% and 1% v/v in distilled water

Determination by analysis of the concentration, homogeneity and stability of the test material preparations was not appropriate because it was not specified in the Study Plan and is not a requirement of the Test Guideline.

3. METHODS

3.1 Animals and Animal Husbandry

Nineteen female and six male albino Dunkin Hartley guinea pigs supplied by David Hall Limited, Burton-on-Trent, Staffordshire, UK were used. At the start of the main study the animals weighed 328 to 405g, and were approximately eight to twelve weeks old. After an acclimatisation period of at least five days, each animal was selected at random and given a number unique within the study which was written on a small area of clipped rump using a black indelible marker-pen.

The animals were housed singly or in pairs in solid-floor polypropylene cages furnished with woodflakes. Free access to mains tap water and food (Guinea Pig FD1 Diet, Special Diets Services Limited, Witham, Essex, UK) was allowed throughout the study.

The animal room was maintained at a temperature of 18 to 21 °C and relative humidity of 46 to 60%. The rate of air exchange was approximately fifteen changes per hour and the lighting was controlled by a time switch to give twelve hours continuous light and twelve hours darkness.

3.2 Procedure

The method used for assessing the sensitising properties of the test material was based on the Guinea Pig Maximisation test of Magnusson B & Kligman A M, J. Invest. Dermatol. (1969) 52: 268 - 276.

3.2.1 Selection of Concentrations for Main Study (Sighting Tests)

The concentrations of test material to be used at each stage of the main study were determined by 'sighting tests' in which groups of guinea pigs were treated with various concentrations of test material. The procedures were as follows:

3.2.1.1 Selection of Concentration for Intradermal Induction

Four concentrations of test material were investigated (5%, 1%, 0.5% and 0.1% w/v in distilled water). A total of four guinea pigs were used, each guinea pig receiving four 0.1 ml injections of only one concentration of test material. The degree of erythema at the injection sites was assessed approximately 24, 48 and 72 hours and 7 days after injection according to the Draize scale shown in Appendix X. The degree of oedema was not evaluated. Any evidence of systemic toxicity was also recorded. The highest concentration that caused only mild to moderate skin irritation, and which was well tolerated systemically, was selected for the intradermal induction stage of the main study.

3.2.1.2 Selection of Concentration for Topical Induction

Two guinea pigs were treated with undiluted test material and three preparations of the test material (75%, 50% and 25% v/v in distilled water). An additional two guinea pigs were treated with four preparations of the test material (25%, 10%, 5% and 2% v/v in distilled water).

Applications were made to the clipped flanks under occlusive dressings for an exposure period of 48 hours. The degree of erythema and oedema was evaluated 1, 24 and 48 hours after dressing removal. The highest concentration producing only mild to moderate dermal irritation was selected for the topical induction stage of the main study.

3.2.1.3 Selection of Concentration for Topical Challenge

Four preparations of the test material (10%, 5%, 2% and 1% v/v in distilled water) were applied to the clipped flanks of two guinea pigs under occlusive dressings for an exposure period of 24 hours. These guinea pigs did not form part of the main study but had been

treated identically to the control animals of the main study, up to Day 15. The degree of erythema and oedema was evaluated approximately 1, 24 and 48 hours after dressing removal. The highest non-irritant concentration of the test material and one lower concentration were selected for the topical challenge stage of the main study.

3.2.2 Main Study

A group of fifteen guinea pigs was used for the main study, ten test and five control. The bodyweight of each animal was recorded at the start and end of the study.

Two main phases were involved in the main study; (a) an induction of a response and (b) a challenge of that response.

3.2.2.1 Induction

Induction of the Test Animals: Shortly before treatment on Day 0 the hair was removed from an area approximately 40 mm x 60 mm on the shoulder region of each animal with veterinary clippers. A row of three injections (0.1 ml each) was made on each side of the mid-line. The injections were:

- a) Freund's Complete Adjuvant plus distilled water in the ratio 1:1
- b) a 0.05% w/v emulsion of the test material in distilled water
- c) a 0.05% w/v emulsion of the test material in a 1:1 preparation of Freund's Complete Adjuvant plus distilled water.

Approximately 24 and 48 hours after intradermal injection the degree of erythema at the test material injection sites (ie. injection site b) was evaluated according to the scale shown in Appendix X.

One week later (Day 7), the same area on the shoulder region used previously for intradermal injections was clipped again and treated with a topical application of the test material formulation. A filter paper patch (WHATMAN No.4: approximate size 40 mm x 20 mm), saturated with the test material formulation (5% v/v in distilled

water) was applied to the prepared skin and held in place with a strip of surgical adhesive tape (BLENDERM: approximate size 50 mm x 30 mm) covered with an overlapping length of aluminium foil. The patch and foil were further secured with a strip of elastic adhesive bandage (ELASTOPLAST: approximate size 250 mm x 35 mm) wound in a double layer around the torso of each animal. This occlusive dressing was kept in place for 48 hours.

The degree of erythema and oedema was quantified one and twenty-four hours following removal of the patches using the scale shown in Appendix X.

Any other reactions were also recorded.

Induction of the Control Animals: Intradermal injections were administered using an identical procedure to that used for the test animals, except that the injections were:

- a) Freund's Complete Adjuvant plus distilled water in the ratio 1:1
- b) distilled water
- c) a 50% w/v formulation of distilled water in Freund's Complete Adjuvant/distilled water 1:1

Approximately 24 and 48 hours after intradermal injection the degree of erythema at the vehicle injection sites (ie injection site b) was evaluated according to the scale shown in Appendix X.

The topical applications followed the same procedure as for the test animals except that the vehicle alone was applied to the filter paper. Skin reactions were quantified as for the test animals.

3.2.2.2 Challenge

Shortly before treatment on Day 21, an area of approximately 50 mm x 70 mm on both flanks of each animal, was clipped free of hair with veterinary clippers.

A square filter paper patch (WHATMAN No.4: approximate size 20 mm x 20 mm), saturated with the test material formulation at the maximum non-irritant concentration (2% v/v in distilled water) was applied to the shorn right flank of each animal and was held in place with a strip of surgical adhesive tape (BLENDERM: approximate size 40 mm x 50 mm). To ensure that the maximum non-irritant concentration was used at challenge, the test material at a concentration of 1% v/v in distilled water was similarly applied to a skin site on the left shorn flank. The patches were occluded with an overlapping length of aluminium foil and secured with a strip of elastic adhesive bandage (ELASTOPLAST: approximate size 250 mm x 75 mm) wound in a double layer around the torso of each animal.

After 24 hours, the dressing was carefully cut using blunt-tipped scissors, removed and discarded. The challenge sites were swabbed with cotton wool soaked in distilled water to remove residual material. The position of the treatment sites was identified by using a black indelible marker-pen.

Prior to the 24-hour observation the flanks were clipped using veterinary clippers to remove regrown hair.

3.2.2.3 Evaluation of Skin Reactions

Approximately 24 and 48 hours after challenge dressing removal, the degree of erythema and oedema was quantified using the scale shown in Appendix X.

Any other reactions were also recorded.

3.3 Interpretation of Results

The percentage of test animals that showed a more severe reaction at the test material challenge site than the most severe reaction seen in the control animals, was compared with the following scale:

Percentage of animals sensitised	Classification of sensitisation potential
0	non-sensitiser
> 0 - 8	weak sensitiser
> 8 - 28	mild sensitiser
> 28 - 64	moderate sensitiser
> 64 - 80	strong sensitiser
> 80 - 100	extreme sensitiser

The data obtained may be used to classify the test material according to Commission Directive 93/21/EEC adapting Council Directive 67/548/EEC on the classification, packaging and labelling of dangerous substances.

The test material will be classified as sensitising and assigned the symbol "Xi", the indication of danger 'irritant' and the risk phrase R 43 "MAY CAUSE SENSITISATION BY SKIN CONTACT" if 30% or more of the test animals show a sensitisation response.

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for a period of five years. After this period, the Sponsor's instructions will be sought.

5. RESULTS

5.1 Intradermal and Topical Sighting Tests

A summary of the results of the intradermal sighting test and the individual skin reactions observed in the topical sighting tests are given in Appendices I to III.

Based on these results, the following concentrations were selected for the main study:

Intradermal Induction	:	0.05% w/v in distilled water
Topical Induction	:	5% v/v in distilled water
Topical Challenge	:	2% and 1% v/v in distilled water

5.2 Main Study

5.2.1 Skin Reactions Observed After Intradermal Induction

Individual reactions observed at the test material intradermal injection sites of the test group animals and vehicle intradermal injection sites of the control group animals are given in Appendices IV and V respectively.

Well-defined or moderate to severe erythema was noted at the intradermal induction sites of all test group animals at the 24 and 48-hour observations.

Very slight erythema was noted at the intradermal induction sites of two control group animals at the 24-hour observation.

5.2.2 Skin Reactions Observed After Topical Induction

Individual skin reactions observed at the topical induction sites of the test and control group animals are given in Appendices VI and VII.

Very slight to well-defined erythema and incidents of slight oedema were noted at the induction sites of all test group animals at the 1-hour observation and eight test group animals at the 24-hour observation.

No signs of irritation were noted at the induction sites of control group animals at the 1 and 24-hour observations.

Incidents of bleeding from the intradermal injection sites were noted in seven test group animals at the 1-hour observation.

5.2.3 Skin Reactions Observed After Topical Challenge

Individual skin reactions at the challenge sites of the test and control group animals are given in Tables 1 and 2.

No skin reactions were noted at the challenge sites of the test or control group animals at the 24 or 48-hour observations.

5.3 Bodyweight

Individual bodyweights and bodyweight gains of the test and control group animals are given in Appendices VIII and IX.

Bodyweight gains of guinea pigs in the test group, between Day 0 and Day 24, were comparable to those observed in the control group animals over the same period.

6. CONCLUSION

The test material, . produced a 0% (0/10) sensitisation rate and was classified as a NON-SENSITISER to guinea pig skin. The test material did not meet the criteria for classification as a sensitiser according to EU labelling regulations. No risk phrase is required.

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
 TABLE 1
 INDIVIDUAL SKIN REACTIONS IN TEST ANIMALS AT CHALLENGE

CHALLENGE CONCENTRATIONS: 2% AND 1% v/v VEHICLE: DISTILLED WATER

Animal Number	Skin Reactions (Hours After Removal of Dressing)											
	24 Hours				48 Hours				1%			
	Er	Oe	Other	Other	Er	Oe	Other	Other	Er	Oe	Other	Other
1	0	0	-	-	0	0	-	-	0	0	-	-
2	0	0	-	-	0	0	-	-	0	0	-	-
3	0	0	-	-	0	0	-	-	0	0	-	-
4	0	0	-	-	0	0	-	-	0	0	-	-
5	0	0	-	-	0	0	-	-	0	0	-	-
6	0	0	-	-	0	0	-	-	0	0	-	-
7	0	0	-	-	0	0	-	-	0	0	-	-
8	0	0	-	-	0	0	-	-	0	0	-	-
9	0	0	-	-	0	0	-	-	0	0	-	-
10	0	0	-	-	0	0	-	-	0	0	-	-

Er = erythema Oe = oedema - = no other reactions noted

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
 TABLE 2
 INDIVIDUAL SKIN REACTIONS IN CONTROL ANIMALS AT CHALLENGE

CHALLENGE CONCENTRATIONS: 2% AND 1% v/v VEHICLE: DISTILLED WATER

Animal Number	Skin Reactions (Hours After Removal of Dressing)											
	24 Hours				48 Hours							
	2%		1%		2%		1%		2%		1%	
Er	Oe	Er	Other	Er	Oe	Er	Other	Er	Oe	Er	Other	
11	0	0	-	-	0	0	-	-	0	0	-	-
12	0	0	-	-	0	0	-	-	0	0	-	-
13	0	0	-	-	0	0	-	-	0	0	-	-
14	0	0	-	-	0	0	-	-	0	0	-	-
15	0	0	-	-	0	0	-	-	0	0	-	-

Er = erythema Oe = oedema - = no other reactions noted

APPENDICES

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
 A P P E N D I X I
 INTRADERMAL SIGHTING TEST - SUMMARY OF RESULTS

VEHICLE: DISTILLED WATER

Animal Identification	Time of Observation	Concentration of Test Material (% w/v)	Grade of Erythema at Injection Sites	Evidence of Systemic Toxicity
A	24 Hours	1	4 E Animal humanely killed	None
	48 Hours			
	72 Hours			
	7 Days			
B	24 Hours	5	4 E Animal humanely killed	None
	48 Hours			
	72 Hours			
	7 Days			
C	24 Hours	0.1	3 3 2-3 Animal found dead	None None None None
	48 Hours			
	72 Hours			
	7 Days			
D	24 Hours	0.5	4 N 4 E 4 E 4 E	None None None None
	48 Hours			
	72 Hours			
	7 Days			

E = eschar
 N = green necrosis

The concentration of the test material selected for the intradermal induction stage of the main study was 0.05% w/v in distilled water

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
 APPENDIX II
 TOPICAL SIGHTING TEST FOR INDUCTION APPLICATION
 (48-HOUR EXPOSURE) - INDIVIDUAL SKIN REACTIONS

VEHICLE: DISTILLED WATER

Animal Identification	Concentration of Test Material (% v/v)	Skin Reactions (Hours After Removal of Patches)											
		1			24			48					
		Er	Oe	Other	Er	Oe	Other	Er	Oe	Other			
E	100	4N	?Od	-	4N	?Od	-	?e	?Od	-	Su		
	75	4N	?Od	-	4N	?Od	-	?e	?Od	-	Su		
	50	4N	2	-	4N	2	SsSTA	?e	?Od	-	Su		
	25	2	2	-	2	2	DSTA	2	1	-	DSTA		
F	100	4N	?Od	-	4N	?Od	-	?e	?Od	-	Su		
	75	4N	?Od	-	4N	?Od	-	?e	?Od	-	Su		
	50	3	2	-	2	2	DSTA	?e	2	-	DLeLf		
	25	2	2	-	1	0	STA	1	0	-	DSTA		

Er = erythema
 Oe = oedema
 - = no other reactions noted
 D = desquamation
 Le = loss of skin elasticity
 Lf = loss of skin flexibility

N = green necrosis
 Ss = small superficial scattered scabs
 STA = yellow-coloured staining
 Su = sunken, hardened, dark brown/black coloured scab resembling a crater
 ?e = adverse reactions prevented accurate evaluation of erythema
 ?Od = adverse reactions prevented accurate evaluation of oedema

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
 A P P E N D I X 11 (continued)
 TOPICAL SIGHTING TEST FOR INDUCTION APPLICATION
 (48-HOUR EXPOSURE) - INDIVIDUAL SKIN REACTIONS

VEHICLE: DISTILLED WATER

Animal Identification	Concentration of Test Material (% v/v)	Skin Reactions (Hours After Removal of Patches)													
		1				24				48					
		Er	Oe	Other	Er	Oe	Other	Er	Oe	Other	Er	Oe	Other		
I	25	4N	2	-	4N	?Od	-	?e	-	?Od	-	?e	-	?Od	St
	10	2	2	STA	2	1	-	2	1	-	2	1	-	1	D
	5	1	0	-	0	0	-	0	0	-	0	0	-	0	-
	2	1	0	-	0	0	-	0	0	-	0	0	-	0	-
J	25	4N	2	-	4N	?Od	-	?e	-	?Od	-	?e	-	?Od	St
	10	2	2	STA	2	1	-	2	1	-	2	1	-	1	D
	5	2	0	-	1	0	-	0	0	-	0	0	-	0	-
	2	1	0	-	0	0	-	0	0	-	0	0	-	0	-

Er = erythema
 Oe = oedema
 - = no other reactions noted
 ?Od = adverse reactions prevented accurate evaluation of oedema
 D = desquamation

N = green necrosis
 St = hardened dark brown/black-coloured scab
 STA = yellow-coloured staining
 ?e = adverse reactions prevented accurate evaluation of erythema

The concentration of the test material selected for the main study topical induction was 5% v/v in distilled water

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
 APPENDIX III
 TOPICAL SIGHTING TEST FOR CHALLENGE APPLICATION
 (24-HOUR EXPOSURE) - INDIVIDUAL SKIN REACTIONS

VEHICLE: DISTILLED WATER

Animal Identification	Concentration of Test Material (% v/v)	Skin Reactions (Hours After Removal of Patches)												
		1			24			48						
		Er	Oe	Other	Er	Oe	Other	Er	Oe	Other				
K	10	2	1	-	1	1	-	1	0	-	1	0	-	D
	5	1	0	-	0	0	-	0	0	-	0	0	-	-
	2	1	0	-	0	0	-	0	0	-	0	0	-	-
	1	0	0	-	0	0	-	0	0	-	0	0	-	-
L	10	2	2	-	2	1	-	2	1	-	1	1	-	-
	5	1	0	-	1	0	-	1	0	-	0	0	-	-
	2	1	0	-	0	0	-	0	0	-	0	0	-	-
	1	0	0	-	0	0	-	0	0	-	0	0	-	-

Er = erythema
 Oe = oedema

D = desquamation
 - = no other reactions noted

The concentrations of the test material selected for the main study topical challenge were 2% and 1% v/v in distilled water

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
APPENDIX IV
INTRADERMAL INDUCTION - INDIVIDUAL SKIN REACTIONS IN TEST ANIMALS

INDUCTION CONCENTRATION: 0.05% w/v

VEHICLE: DISTILLED WATER

Animal Number	Grade of Erythema at Observation Time					
	24 Hours		48 Hours		48 Hours	
	Left Side	Right Side	Left Side	Right Side	Left Side	Right Side
1	3	3	2	2	2	2
2	2	2	2	2	2	2
3	3	3	2	2	2	2
4	3	3	2	2	2	2
5	3	3	3	3	3	3
6	3	2	2	2	2	2
7	3	3	2	2	2	2
8	3	3	2	2	2	2
9	2	2	2	2	2	2
10	3	3	3	3	3	2

MAGNUSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
A P P E N D I X V
INTRADERMAL INDUCTION - INDIVIDUAL SKIN REACTIONS IN CONTROL ANIMALS

VEHICLE: DISTILLED WATER

Animal Number	Grade of Erythema at Observation Time			
	24 Hours		48 Hours	
	Left Side	Right Side	Left Side	Right Side
11	0	0	0	0
12	0	0	0	0
13	1	0	0	0
14	0	0	0	0
15	1	1	0	0

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
 A P P E N D I X V I
 TOPICAL INDUCTION - INDIVIDUAL SKIN REACTIONS IN TEST ANIMALS

INDUCTION CONCENTRATION: 5% v/v VEHICLE: DISTILLED WATER

Animal Number	Skin Reactions (Hours After Removal of Dressing)									
	1 Hour					24 Hours				
	Er	Oe	Other	Er	Other	Er	Oe	Other	Er	Other
1	2	1	Bs	1		1	0			
2	2	1	Bs	1		1	0			
3	2	0	-	0		0	0			
4	2	1	Bs	1		1	1			
5	2	1	Bs	2		2	0			
6	2	1	Bs	1		1	0			
7	2	0	Bs	0		0	0			
8	2	0	-	1		1	0			
9	2	0	-	1		1	0			
10	2	1	Bs	1		1	0			

Er = erythema
 Oe = oedema

Bs = bleeding from intradermal injection sites
 - = no other reactions noted

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
A P P E N D I X V I I
TOPICAL INDUCTION - INDIVIDUAL SKIN REACTIONS IN CONTROL ANIMALS

VEHICLE: DISTILLED WATER

Animal Number	Skin Reactions (Hours After Removal of Dressing)					
	1 Hour			24 Hours		
	Er	Oe	Other	Er	Oe	Other
11	0	0	-	0	0	-
12	0	0	-	0	0	-
13	0	0	-	0	0	-
14	0	0	-	0	0	-
15	0	0	-	0	0	-

Er = erythema Oe = oedema - = no other reactions noted

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
APPENDIX VIII
INDIVIDUAL BODYWEIGHTS AND BODYWEIGHT GAINS OF TEST ANIMALS

Animal Number	Bodyweight (g)		Bodyweight (g) Increase
	Day 0	Day 24	
1	328	539	211
2	358	563	205
3	373	545	172
4	394	613	219
5	380	570	190
6	392	566	174
7	338	549	211
8	337	545	208
9	329	549	220
10	341	517	176

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
A P P E N D I X I X
INDIVIDUAL BODYWEIGHTS AND BODYWEIGHT GAINS OF CONTROL ANIMALS

Animal Number	Bodyweight (g)		Bodyweight (g) Increase
	Day 0.	Day 24	
11	405	607	202
12	391	574	183
13	377	520	143
14	403	564	161
15	358	553	195

MAGNUSSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG

A P P E N D I X X

EVALUATION OF SKIN REACTIONS*

Erythema and Eschar Formation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Oedema Formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 millimetre)	3
Severe oedema (raised more than 1 millimetre and extending beyond the area of exposure)	4

* Draize J.H., (1977) "Dermal and Eye Toxicity Tests" In: Principles and Procedures for Evaluating the Toxicity of Household Substances, National Academy of Sciences, Washington D.C. p.31

MAGNUSON & KLIGMAN MAXIMISATION STUDY IN THE GUINEA PIG
 A P P E N D I X X I
 SUMMARY OF POSITIVE CONTROL DATA FOR THE MAGNUSON AND KLIGMAN MAXIMISATION STUDY
 (JULY 1995 TO NOVEMBER 1997)

Project Number	Date Start	Date End	Number of Animals and Sex*		Positive Control Material	Concentration		Incidence of Sensitisation
			Test	Control		Intradermal	Topical	
544/002	13/06/95	07/07/95	10 Male	10 Male	2,4-Dinitrochlorobenzene	0.1% in arachis oil BP	0.75% in absolute ethanol	100% (10/10)
039/143	25/07/95	19/08/95	20 Male	10 Male	Neomycin Sulphate	10% in distilled water	75% in distilled water	60% (12/20)
039/163	31/01/96	24/02/96	10 Female	5 Female	2-Mercaptobenzothiazole	10% in arachis oil BP	50% in acetone: PEG 400 (70:30)	70% (7/10)
413/26	19/08/96	21/09/96	10 Female	10 Female	2,4-Dinitrochlorobenzene	0.1% in arachis oil BP	0.75% in 80% aqueous ethanol	100% (10/10)
039/239	11/11/96	06/12/96	10 Female	5 Female	2-Mercaptobenzothiazole	10% in arachis oil BP	50% in acetone: PEG 400 (70:30)	90% (9/10)
039/249	22/05/97	15/06/97	10 Female	5 Female	2-Mercaptobenzothiazole	10% in arachis oil BP	50% in acetone: PEG 400 (70:30)	70% (7/10)
039/258	17/10/97	10/11/97	10 Female	5 Female	2-Mercaptobenzothiazole	10% in arachis oil BP	50% in acetone: PEG 400 (70:30)	90% (9/10)

* All animals supplied by David Hall Ltd., Burton-on-Trent, Staffordshire, UK

APPENDIX XII



THE DEPARTMENT OF HEALTH OF THE GOVERNMENT
OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 88/020 EEC

LABORATORY

TEST TYPE

SafePharm Laboratories Ltd.
P.O. Box No. 45
Derby DE1 2BT

Analytical Chemistry
Environmental Tox.
Environmental Fate
Mutagenicity
Phys/Chem. tests
Toxicology

DATE OF INSPECTION

22 January 1996

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

27/2/96

D.F. Moore
Director
UK GLP Monitoring Authority

Test Substance

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not available
Comments: Dinitrochlorobenzene (DNCB) data were included as a positive control for sensitization studies conducted at the laboratory. The actual test material of the report was unrelated to the 8(d) rule and has been redacted.

Method

GLP: Yes
Method / Guideline: OECD 406 "Skin Sensitization" Magnusson & Kligman Maximization Study
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1992
Species: Guinea pig
Strain: SPF-bred albino strain
Number of Animals: Positive control animals: 5 males and 5 females (in separate studies)
Study Design: Intradermal Induction – 2 injections with Freund's Complete Adjuvant (FCA), 2 injections with 0.1% test substance in maize oil, 2 injections with 0.1% test substance in mixture of FCA and maize oil
Topical Induction – 0.1% in vaseline
Challenge – 0.05% in vaseline

Results

Result: DNCB, as the positive control for delayed contact hypersensitivity studies, elicited the appropriate response - 100% sensitization.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Report Number: V 92.392/352063
Reference: TNO Nutrition and Food Research. 1992. Sensitization study with xxx in guinea pigs (maximization test). Accession # 103104.

TNO Nutrition and Food Research

Acc # 103104

P.O. Box 360
3700 AJ Zeist
Utrechtseweg 48
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Fax +31 3404 5 72 24
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TNO-report
V 92.392/352063
Sensitization study with
(maximization test)

in guinea pigs

Author:
M.K. Prinsen

At the request of:

Project number:
352063/09

Date:
November 1992

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Number of pages:
24

Number of tables:
1

Number of figures:
1

Number of appendices:
3

Number of annexes:
3

Netherlands organisation for
applied scientific research

TNO Nutrition and Food Research conducts technological, biotechnological, analytical, nutritional and toxicological research on foods and allied products, including feedstuffs. Institutes and departments are found in Zeist, Wageningen, Muiden and Leiden.



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SUMMARY

1. The test substance _____ was examined for possible sensitizing properties by a maximization test in guinea pigs using 20 test animals and 10 controls.

2. The test comprised:
 - test animals, induction treatment by intradermal injections of Freund's Complete Adjuvant (FCA), a 1% dilution of the test substance in demineralized water (demi-water), and a 1% dilution of the test substance in a 1:1 mixture of FCA and demi-water, followed one week later by topical application of a 50% dilution in vaseline,
 - challenge treatment, 14 days after the last induction, by topical application of a 50% and a 30% dilution in vaseline, and of vaseline alone,
 - controls, induction treatment by intradermal injections of FCA, demi-water, and a 1:1 mixture of FCA and demi-water, followed one week later by topical application of vaseline, and
 - challenge treatment, 14 days after the last induction, by topical application of a 50% and a 30% dilution of the test substance in vaseline, and of vaseline alone.

3. The challenge treatment with _____) did not induce signs of sensitization in the test animals. On the basis of the results, it was concluded that under the conditions of this study and according to the EEC-standards (mentioned in EEC-Directive 91/325/EC and published in the Official Journal of the European Communities, L 180, Volume 34, 8 July 1991), the test substance _____ is not a sensitizer.

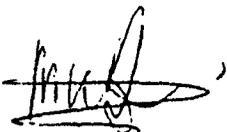
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STATEMENT OF GLP-COMPLIANCE

We, the undersigned, hereby declare that the report following constitutes a true and faithful account of the procedures adopted and the results obtained in the performance of this study. The study, that was conducted by the Department of Biological Toxicology and the Department of Experimental Toxicology of the TNO Toxicology and Nutrition Institute, was performed in accordance with the current OECD Good Laboratory Practice Principles.

We, the undersigned, fulfilled the responsibilities required by these regulations.

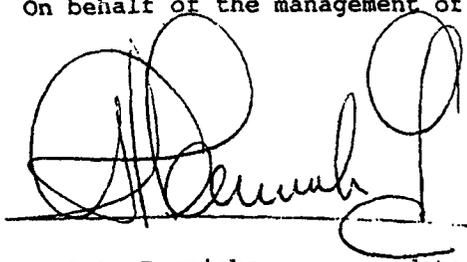
Submitted by:



M.K. Prinsen
Study director

date: November 24, 1992

On behalf of the management of the Department of Biological Toxicology:



Dr A.H. Penninks
Head, General Toxicology section

date: November 25, 1992.

QUALITY ASSURANCE UNIT
P.O. Box 360
3700 AJ ZEIST, the Netherlands

QUALITY ASSURANCE STATEMENT

On : Sensitization study with
in guinea pigs (Maximization test)
Report no. : V 92.392/352063
Date : November 1992

The execution of this type of short-term study is not always individually inspected. The processes involved are inspected at intervals according to a pre-determined schedule.

This report has been audited according to the appropriate Standard Operating Procedure and is considered to be an accurate presentation of the methods and procedures employed and an accurate presentation of the findings.



Ing. P.A. de Lang
Quality Assurance Officer

date: *November 25, 1992*

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1 INTRODUCTION

At the request of _____, a sample of the title substance was examined for possible sensitization potential in guinea pigs by means of the maximization test at the testing facilities of the Department of Biological Toxicology and the Department of Experimental Biology, TNO Toxicology and Nutrition Institute, Utrechtseweg 48, 3704 HE Zeist, the Netherlands. The study was carried out according to protocol no. P 352063/09, dated July 1992.

2 MATERIALS AND METHODS

2.1 Test substance

Date of receipt : August 3, 1992
Quantity : 785.95 g (gross weight)
Designation :
16.07.92
Name test substance :
Trade name :
Batch no. :
Chemical name :
Purity : 95.1% (see annex 3)
CAS Reg. no. : 128446-36-6
General appearance :
Storage conditions : at room temperature (circa 20 °C); small
containers with portions of the test substance
were kept in a bigger container on silica.

Test dilutions were prepared just prior to use.

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2.2 Animals and maintenance

Species : SPF-bred albino guinea pigs (Cr1:(HA)BR)
Supplier : Charles River Wiga GmbH, F.R. Germany
Sex and age : males and females, young adult
Identification : earmarking: males even nos. 724-752; females
odd nos. 609-637
Date of arrival : August 18, 1992
Start date of study : August 25, 1992
Termination date of study : September 18, 1992
Body weight range prior
to start of study : males 259-296 g; females 255-301 g
Caging : individually in suspended, stainless steel
cages, fitted with wire mesh floor and front
Lighting : 12 hours light/12 hours dark cycle
Temperature : 22 ± 3°C
Humidity : 45%-92.5% (upper limit higher than the
intended 70%, because of meteorological
circumstances or because of wet cleaning of
the animal room)
Ventilation : ca 10 air changes/hour
Diet : pelleted, natural ingredient diet for guinea
pigs (Hope Farms, Woerden, The Netherlands)
and tap water, ad libitum

2.3 Experimental design

The experiment was conducted essentially according to the guinea pig maximization test method as described by Magnusson and Kligman (1969 and 1970). It fully covered the requirements of:

- OECD guideline no. 406, Skin Sensitization, adopted May 12, 1981, and
- EEC-Directive 84/449, Annex V, Part B: Methods for the determination of toxicity, B.6. Acute toxicity, skin sensitization, dated September 1984.

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The study consisted of an induction treatment, followed by a resting period of 14 days, which preceded the challenge treatment.

Preliminary observations were made to establish the concentrations of the test substance for intradermal injection and for topical application in the main study.

2.3.1 Preliminary tests

The irritation response upon intradermal injection of various concentrations were examined in 3 guinea pigs.

A sufficiently large area of the flanks was clipped free of hair with electric clippers. Amounts of 0.1 ml of a 50%, a 30%, a 10%, a 3%, and a 1% dilution of the test substance in demi-water were applied by intradermal injection. A concentration causing slight to moderate irritation but otherwise well-tolerated by the animals, is usually taken for intradermal injection of the test substance in the induction phase of the main study.

The irritation response to topical treatment of a 50%, a 30%, a 10%, and a 3% dilution of the test substance in vaseline was examined. For this purpose the flanks of each of three animals was clipped free from hair with electric clippers. Patches (Silverpatch, v.d. Bend B.V., Brielle, the Netherlands) were loaded with the various test dilutions. The patches, each loaded with a different concentration, were placed separately on an intact area of the clipped skin of each animal, and covered with a piece of hypoallergenic paper bandage (Leukopor) that was secured by elastic adhesive bandage (Tensoplast), 7.5 cm in width, wound around the torso of the animal. The dressing was left in place for 24 hours. After removal of the dressing and 24 hours later, the animals were examined for signs of skin irritation. A concentration causing slight to moderate skin irritation is usually chosen for topical induction and a non-irritant concentration for topical challenge.

2.3.2 Main study

Fifteen male and 15 female guinea pigs were randomly divided into two groups, viz. a test group of 10 males and 10 females and a control group of 5 males and 5 females. The animals were weighed one day before the study was initiated and at the completion of the study.

2.3.2.1 Induction

Induction was effected in two different ways, firstly by intradermal injections and secondly, one week later, by topical application over the injection sites.

a. Intradermal injections

For this purpose an area of about 24 cm² of dorsal skin in the scapular region was clipped free from hair with electric clippers. Pairs of intradermal injections (0.1 ml each) were made simultaneously in the clipped area as shown in Fig. 1. The following preparations were injected:

test animals

- two injections with Freund's Complete Adjuvant (FCA)
- two injections with a 1% dilution of the test substance in demi-water
- two injections with a 1% dilution of the test substance in demi-water and FCA (1:1)

control animals

- two injections with FCA
- two injections with demi-water
- two injections with FCA and demi-water (1:1)

Skin readings were made at 24 hours after the treatment.

b. Topical application

One week after the intradermal injections, the dorsal skin in the scapular region of the test and control animals was closely shaved again. The induction by topical application was also made in this region. The test animals were treated as follows:

A circa 2 x 4 cm patch of Whatman No. 3 MM filter paper was loaded with

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a 50% dilution of the test substance in vaseline. The loaded patch was placed over the sites of the intradermal injections and was secured as described in section 2.3.1. The dressing was left in place for 48 hours. The control animals were similarly treated with empty patches. Skin readings were made directly after removal of the patches.

2.3.2.2 Challenge

The topical challenge was carried out two weeks after the topical induction as follows:

An area of circa 5 x 5 cm on both flanks of each test and control animal was clipped free from hair. Patches were loaded with a 50% or a 30% test dilution in vaseline, or with vaseline only. One patch loaded with the 50% test dilution was placed on the clipped area of the left flank of each test and control animal. The right flank was treated with the 30% test dilution in vaseline and with vaseline alone. The patches were covered with Leukopor bandage, and held in place by Tensoplast for 24 hours. Skin readings were made at 24 and 48 hours after removal of the patches.

2.3.3 Scoring and evaluation of the results

The skin reactions were scored by the scale as given in appendix 1. The results were evaluated according to the EEC-standards (EEC-Directive 91/325/EC as published in the Official Journal of the European Communities, L 180, Volume 34, 8 July 1991), which state that a substance is considered a sensitizer if 30% or more of the test animals show a positive reaction.

2.4 Retention of records

All raw data and the master copy of this report is filed in the archives of the TNO Toxicology and Nutrition Institute under the reference "352063/09, sensitization, guinea pig" and will be retained in the archives for a period of at least fifteen years after reporting of the study. Unless otherwise agreed, remaining test substance will be retained for at least six months after submission of the report.

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2.5 Responsible personnel

Study director : M.K. Prinsen
Biotechnicians : J.Y.C. de Smit
 M.R. van Kooten-van Someren
 D.C. Veldhuysen
 T. Romijn
Archives : Mrs R. Dekker
Head of Quality Assurance : P.B. Davis BA
Management : Dr A.H. Penninks

2.6 Deviation from the protocol

On some occasions, the relative humidity exceeded the top limit of 70%.

3 RESULTS

3.1 Preliminary tests

After intradermal treatment with the test dilutions in demi-water, abscesses or necrosis were observed on the 50%, 30% and 3% test sites of the 3 animals. In addition, slight to very severe erythema was observed. The 1% dilution caused slight erythema in 2 out of the 3 animals. No signs of systemic toxicity were observed in any of the 3 animals. Since the degree of irritation observed with the 1% dilution was considered acceptable, it was decided to use this concentration of the test substance for intradermal treatment during the induction phase.

Topical treatment with the 50% dilution in vaseline induced well-defined erythema in 2 animals. The 30% and 3% dilution did not induce skin effects, whereas the 10% dilution caused very slight or well-defined erythema in 2 out of the 3 animals. The next day, very slight erythema was still observed on one 50% test site.

On the basis of these results, it was decided to use a 50% dilution in vaseline for topical treatment during the induction phase and both a 50% and a 30% dilution in vaseline during the challenge phase of the study.

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3.2 Main study

All animals remained in good health during the experimental period.

3.2.1 Induction

The individual scores of the skin reactions made during the induction phase of the study are given in Appendices 2 and 3.

The intradermal injections generally caused the following skin reactions:

test animals

- with FCA: severe erythema,
- with the 1% dilution of the test substance in demi-water: slight or moderate erythema in 12 animals,
- with the 1% dilution of the test substance in a mixture of FCA and demi-water (1:1): slight, moderate or severe erythema and abscesses,

control animals

- with FCA: severe erythema,
- with demi-water alone: slight erythema in 1 animal,
- with the mixture of FCA and demi-water: slight or moderate erythema and abscesses.

After topical application of the patches loaded with vaseline only, very slight erythema with or without very slight or slight oedema was observed in the controls. Topical application of the 50% dilution in vaseline induced very slight or well-defined erythema with or without very slight oedema in the test animals.

3.2.2 Challenge

The results of the challenge treatment are given in Table 1.

The challenge treatment with the 50% dilution in vaseline induced very slight erythema in 6 out of the 20 test animals and in 2 out of the 10 controls. The 30% dilution in vaseline or vaseline alone did not induce skin reactions in either the 20 test animals or the 10 controls.

4 DISCUSSION AND CONCLUSION

The challenge treatment with the 50% test dilution produced a similar skin response in the test animals and controls. In the preliminary irritation experiment, this concentration also showed some skin irritating properties. Therefore, the skin effects observed after the challenge treatment with the 50% dilution were attributed to skin irritation, rather than sensitization. With the 30% dilution none of the animals showed skin reactions. Because none of the test animals showed signs of sensitization, it was concluded that under the conditions of this study and according to the EEC-standards (mentioned in EEC-Directive 91/325/EC and published in the Official Journal of the European Communities, L 180, Volume 34, 8 July 1991), is not a sensitizer.

5 SENSITIVITY OF THE TEST SYSTEM

The sensitivity of this test system is checked at intervals by means of a positive control study with 2,4-dinitrochlorobenzene (DNCB). The results of such a positive control study, performed in August/September 1991 are given in annex 1 and 2 at the end of this report. The challenge treatment with a 0.05% dilution of DNCB in demi-water induced distinct skin reactions (positive reactions) in all 10 test animals, whereas no skin reactions were observed in any of the 5 control animals. These results clearly show sensitization by DNCB. Therefore, it can be concluded that the experimental design and the strain of guinea pigs used are suitable to detect possible sensitizing potential of test materials.

6 LITERATURE

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Table 1.1 - Dermal reactions elicited by the challenge application of vaseline alone

Scores at 24 and 48 h after removal of the dressing					
Animal number	Males		Animal number	Females	
	24 h A-B	48 h A-B		24 h A-B	48 h A-B
CONTROL GROUP					
744	0-0	0-0	629	0-0	0-0
746	0-0	0-0	631	0-0	0-0
748	0-0	0-0	633	0-0	0-0
750	0-0	0-0	635	0-0	0-0
752	0-0	0-0	637	0-0	0-0
TEST GROUP					
724	0-0	0-0	609	0-0	0-0
726	0-0	0-0	611	0-0	0-0
728	0-0	0-0	613	0-0	0-0
730	0-0	0-0	615	0-0	0-0
732	0-0	0-0	617	0-0	0-0
734	0-0	0-0	619	0-0	0-0
736	0-0	0-0	621	0-0	0-0
738	0-0	0-0	623	0-0	0-0
740	0-0	0-0	625	0-0	0-0
742	0-0	0-0	627	0-0	0-0

A = erythema (including eschar formation: ischemia, haemorrhages,
and incrustation)

B = oedema

The grading system is explained in Appendix 1.

Table 1.2 - Dermal reactions elicited by the challenge application of a
30% dilution of _____ in vaseline

Scores at 24 and 48 h after removal of the dressing					
Animal number	Males		Animal number	Females	
	24 h A-B	48 h A-B		24 h A-B	48 h A-B
CONTROL GROUP					
744	0-0	0-0	629	0-0	0-0
746	0-0	0-0	631	0-0	0-0
748	0-0	0-0	633	0-0	0-0
750	0-0	0-0	635	0-0	0-0
752	0-0	0-0	637	0-0	0-0
TEST GROUP					
724	0-0	0-0	609	0-0	0-0
726	0-0	0-0	611	0-0	0-0
728	0-0	0-0	613	0-0	0-0
730	0-0	0-0	615	0-0	0-0
732	0-0	0-0	617	0-0	0-0
734	0-0	0-0	619	0-0	0-0
736	0-0	0-0	621	0-0	0-0
738	0-0	0-0	623	0-0	0-0
740	0-0	0-0	625	0-0	0-0
742	0-0	0-0	627	0-0	0-0

A = erythema (including eschar formation: ischemia, haemorrhages,
and incrustation)

B = oedema

The grading system is explained in Appendix 1.

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Table 1.3 - Dermal reactions elicited by the challenge application of
50% dilution of in vaseline

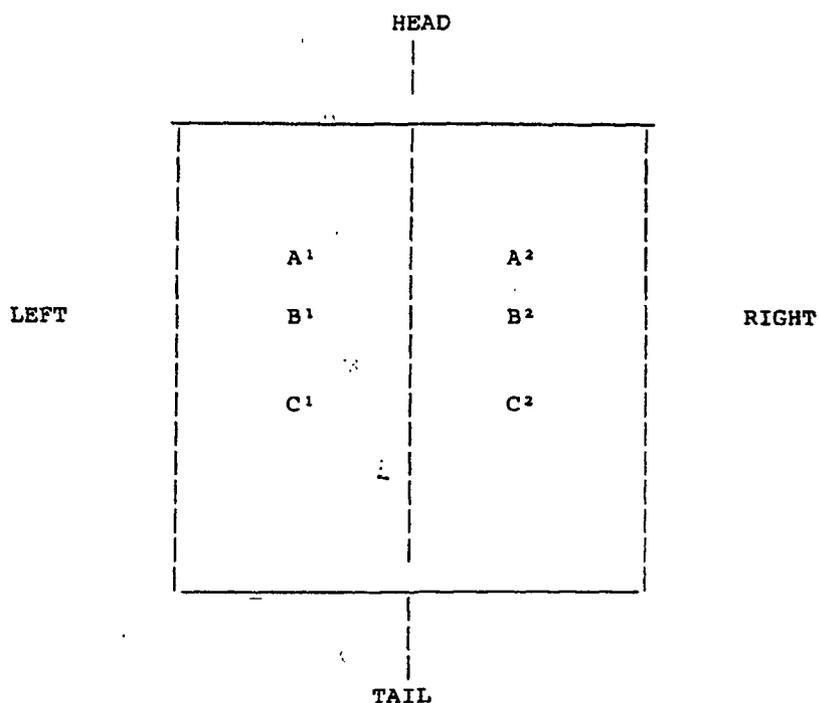
Scores at 24 and 48 h after removal of the dressing					
Animal number	Males		Animal number	Females	
	24 h A-B	48 h A-B		24 h A-B	48 h A-B
CONTROL GROUP					
744	0-0	0-0	629	0-0	0-0
746	0-0	0-0	631	0-0	0-0
748	0-0	0-0	633	1-0	1-0
750	1-0	0-0	635	0-0	0-0
752	0-0	0-0	637	0-0	0-0
TEST GROUP					
724	0-0	0-0	609	0-0	0-0
726	0-0	0-0	611	1-0	1-0
728	0-0	0-0	613	0-0	0-0
730	0-0	0-0	615	1-0	0-0
732	1-0	0-0	617	0-0	0-0
734	0-0	0-0	619	1-0	1-0
736	0-0	0-0	621	0-0	0-0
738	1-0	1-0	623	0-0	0-0
740	1-0	0-0	625	0-0	0-0
742	0-0	0-0	627	0-0	0-0

A = erythema (including eschar formation: ischemia, haemorrhages,
and incrustation)

B = oedema

The grading system is explained in Appendix 1.

Figure 1 - Position of intradermal injections in the shoulder region of the guinea pig made in the induction phase of the study



—+ = shaved area (4 cm x 6 cm)

A¹+A² = 0.1 ml Freund's Complete Adjuvant (FCA), control and test animals

B¹+B² = 0.1 ml of the appropriate dilution of the test substance in a suitable carrier (test animals), or the carrier alone (controls)

C¹+C² = 0.1 ml of the appropriate dilution of the test substance in a mixture (1:1) of FCA and the carrier (test animals), or the mixture alone (controls)

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Appendix 1 - Reading of skin reactions

1. Reading of skin reactions after intradermal injection.

0 = no visible changes

1 = slight erythema

2 = moderate erythema

3 = severe erythema; eschar formation (injuries in depth)

a = abscess; n = necrosis

2. Reading of skin reactions after topical application, according to Draize et al. (J. Pharmacol. Exp. Therap. 84 (1944) 377-390).A. Erythema and eschar formation

value

No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness); eschar formation (injuries in depth: ischemia, haemorrhages, and incrustation)	4

B. Oedema formation

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 millimeter)	3
Severe oedema (raised more than 1 millimeter, extending beyond the area of exposure)	4

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Appendix 2 - Individual skin reactions observed in controls during the induction phase of the study

Animal number	body weight (g) init. term.		scores during the induction period							
			intradermal						topical	
			at 24 hr after inj. no.:						after removal	
		1	2	3	4	5	6	A	B	
MALES										
744	296	507	3	3	0	0	2a	2	1-2	
746	287	462	3	3	0	0	2	2	0-1	
748	259	387	3	3	0	0	1	1	0-1	
750	269	469	3	3	0	0	2a	2	0-0	
752	287	439	3	3	0	1	2	2	1-1	
FEMALES										
629	276	466	3	3	0	0	1	1a	0-0	
631	279	415	3	0	0	0	2a	2a	1-2	
633	283	436	3	3	0	0	2	2	1-1	
635	277	424	3	3	0	0	2	2	0-0	
637	263	425	3	3	0	0	2a	2a	0-0	

init. = initial; term. = terminal; inj. = injection

A = erythema; B = oedema; a = abscesses

The grading system is explained in Appendix 1.

injection no. 1 and 2: FCA

injection no. 3 and 4: demi-water

injection no. 5 and 6: FCA and demi-water (1:1)

topical applications : patches loaded with vaseline.

Appendix 3 - Individual skin reactions observed in the test animals during the induction phase of the study

Animal number	body weight		scores during the induction period						topical	
	init.	term.	intradermal						after removal	
			at 24 hr after inj. no.:						A-B	
			1	2	3	4	5	6		
MALES										
724	272	526	3	3	0	0	2a	2	2-0	
726	292	491	3	3	1	0	2a	2	0-0	
728	278	463	3	3	2	0	3a	3a	1-0	
730	266	476	3	3	2	0	3a	3a	0-0	
732	278	504	3	3	2	1	3a	2a	0-0	
734	267	471	3	3	0	0	2a	2	1-0	
736	288	494	3	3	1	0	2a	1	1-0	
738	293	531	3	3	2	1	3a	2a	1-1	
740	277	500	3	3	2	1	2a	2a	0-0	
742	281	466	3	3	1	1	2a	2a	0-0	
FEMALES										
609	281	435	3	3	0	0	2	3a	1-1	
611	255	386	3	3	0	0	2a	2a	0-1	
613	281	467	3	3	0	0	1a	1a	0-0	
615	301	485	3	3	0	0	2a	2a	0-1	
617	297	424	3	3	0	0	2a	2a	0-0	
619	287	442	3	3	0	1	2a	2a	0-0	
621	265	411	3	3	0	0	3	3	1-1	
623	256	387	3	3	1	0	1	1a	0-1	
625	272	442	3	3	1	0	1a	2a	0-0	
627	274	437	3	0	0	1	2a	2a	0-0	

init. = initial; term. = terminal; inj. = injection
A = erythema; B = oedema; a = abscesses

The grading system is explained in Appendix 1.

injection no. 1 and 2: FCA

injection no. 3 and 4: 1% dilution in demi-water

injection no. 5 and 6: 1% dilution in demi-water and FCA (1:1)

topical application : 50% in vaseline.

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ANNEX 1 - INDIVIDUAL SKIN REACTIONS OBSERVED IN MALE AND FEMALE ANIMALS
DURING THE INDUCTION PHASE OF A POSITIVE CONTROL STUDY WITH DNCB
(STUDY PERFORMED IN AUGUST/SEPTEMBER 1991, ACCORDING TO PROTOCOL
P 210063/03)

Animal number	body weight (g)		scores during the induction period						
			intradermal				topical		
			at 24 hr after inj. no.:						
init.	term.	1	2	3	4	5	6	A - B	
CONTROL GROUP									
162	303	470	3a/w	3	0	0	2	2	0 - 0
164	327	517	3	3	0	0	3	3	0 - 0
166	298	488	3	3	0	0	3	2	0 - 0
161	318	477	3	3	0	0	2	2	0 - 0
163	336	470	3	3	0	0	2	3	0 - 0
TEST GROUP									
152	336	509	3	3	1	1	2	2	2 - 0
154	311	489	3	3	1	1	2	2	2 - 0
156	316	500	3	3	1	1	2	2	0 - 0
158	353	562	3	3	1	1	2	2	1 - 0
160	349	518	3	3	1	1	2	2	1 - 0
151	317	465	3	3	0	0	2	2	1 - 0
153	306	452	3	3	1	0	2	2	2 - 0
155	325	446	3	3	1	1	2	2	2 - 0
157	320	438	3	3	1	1	2	2	1 - 0
159	309	441	3	3	1	0	0	2	2 - 0

init. = initial; term. = terminal; inj. = injection
A = erythema; B = oedema; Males = even numbers; Females = odd numbers
a = abscesses; w = wound
The grading system is explained in Appendix 1.

Control animals:

injection site no. 1 and 2: FCA
injection site no. 3 and 4: maize oil
injection site no. 5 and 6: FCA and maize oil (1:1)
topical application : vaseline

test animals:

injection site no. 1 and 2: FCA
injection site no. 3 and 4: a 0.1% dilution of the test substance in maize oil
injection site no. 5 and 6: a 0.1% dilution of the test substance in a mixture of FCA and maize oil (1:1)
topical application : a 0.1% dilution (w/w) of the test substance in vaseline

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ANNEX 2 - DERMAL REACTONS ELICITED BY THE CHALLENGE APPLICATION
OF A 0.05% DILUTION OF DNCB IN VASELINE (POSITIVE
CONTROL STUDY, AUGUST/SEPTEMBER 1991, ACCORDING TO PROTOCOL
P 210063/03)

Scores at 24 and 48 h after removal of the dressing

Animal number	Males		Animal number	Females	
	24 h A-B	48 h A-B		24 h A-B	48 h A-B
CONTROL GROUP					
162	0-0	0-0	161	0-0	0-0
164	0-0	0-0	163	0-0	0-0
166	0-0	0-0			
TEST GROUP					
152	2-2	2-2 ¹	151	3-2	2-2 ¹
154	2-1	2-1 ¹	153	3-3 ²	3-3 ^{1,2}
156	1-0	1-1 ¹	155	2-2	2-2 ¹
158	2-2	2-2 ¹	157	2-2 ³	2-2 ^{1,3}
160	2-3	2-3 ¹	159	2-1	2-2 ¹

A = erythema; B = oedema; Males = even numbers; Females = odd numbers

The grading system is explained in Appendix 1.

¹ = scaliness

² = small wound

³ = incrustation

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1992

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ANNEX 3

CONSORTIUM FÜR ELEKTROCHEMISCHE INDUSTRIE GMBH

Certificate of Analysis

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Test Substance

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not available

Method

Method / Guideline: Guinea Pig Sensitization Study - Magnusson & Kligman
Maximization Method
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1982
GLP Compliant: Not available
Species: Guinea pig
Strain: Dunkin-Hartley albino strain
Number of Animals: 10 males
Study Design: Intradermal Induction – 0.1% in physiological saline
Topical Induction – 2 x 4 cm filter paper saturated with material
and applied to injection sites area under occlusion
Challenge – 2 x 2 cm filter paper saturated with material and
applied to left side under occlusion

Results

Result: DNCB elicited the appropriate sensitization response in the albino guinea pig.

Data Quality

Reliability (Klimisch): 2

Reference

Report #: TES810036
Reference: Biosearch/Calgon Corporation. 1982. Guinea Pig Sensitization Study – Magnusson-Kligman Maximization Method – Positive Control. Accession # 44906

ACC # 44906



BIOSEARCH INCORPORATED
P.O. BOX 8598, PHILADELPHIA, PENNSYLVANIA 19101
TELEPHONE: (215) 739-4499

Project Number: 81-2824A
Calgon Project Number: TES810036

Submitted to: Calgon Corporation
Box 1346
Pittsburgh, Pennsylvania
15230

Material: Positive Control - 1-chloro-2,4-dinitrobenzene
(Eastman Kodak Co., Rochester, N.Y.)

Sample Received: Supplied by Biosearch, Incorporated

Study Initiated: 12/2/81 Study Completed: 12/24/81

Date of Report: 1/26/82

Test: Guinea Pig Sensitization Study - Magnusson-Kligman
Maximization Method - Positive Control

Object of Test: To assess the dermal sensitization potential of the subject
material in guinea pigs.

Method of Test: A group of ten (10) male albino guinea pigs of the Dunkin-
Hartley Strain, weighing between 300 and 400 grams each, was
employed in this study. The animals were housed and
maintained in compliance with the Animal Welfare Act (Pub. L-
94-279) 9 CFR Part 3.

Husbandry Conditions:
Temperature - 70°F ± 2°F
Relative Humidity - 45% ± 5%
Light - 12 hours light/dark cycle
Diet - Charles River Guinea Pig Formula and tap water were
provided ad libitum. Based on our current knowledge
no contaminants were known to be in this diet or
water that might be expected to interfere with the
objectives of the study.
Caging - Stainless steel with elevated wire mesh flooring
5 guinea pigs/cage
Bedding - Techboard
Shepherd Products Company
Kalamazoo, Michigan 49005

The positive control material was prepared and dosed as a 0.1%
w/v suspension in physiological saline. This was prepared
fresh for each administration.

Project Number: 81-2824A
Calgon Project Number: TES810036

Calgon Corporation

Positive Control - 1-chloro-2,4-dinitrobenzene, as a 0.1% w/v suspension in physiological saline.

Guinea Pig Sensitization Study - Magnusson-Kligman Maximization Method.

Method of Test: INDUCTION STAGE
 (continued)

1. An area of 4 x 6 cm was clipped free of hair with an electric clipper over the shoulders of the ten guinea pigs.
2. Three pairs of intradermal injections were made simultaneously using a 3/8" x 26 gauge hypodermic needle.
 - (a) 0.1 ml of Freund's Complete Adjuvant (Difco Laboratories, Detroit, Mich.) (50:50 dilution with distilled water).
 - (b) 0.1 ml of a 0.1% 1-chloro-2,4-dinitrobenzene w/v suspension in physiological saline.
 - (c) 0.1 ml of a 0.1% 1-chloro-2,4-dinitrobenzene w/v suspension in physiological saline mixed with Freund's Complete Adjuvant (1:1).
3. After seven days, the test site area was re-clipped. A 2 x 4 cm filter paper was saturated with the experimental material and applied to the injection sites area and occluded using an overlapping, impermeable plastic adhesive tape, Blenderm Adhesive Tape (3M Co.). This dressing was kept in place for 48 hours.

CHALLENGE STAGE

1. After a two week rest period, a 3 x 3 cm area of the flank was shaved on the left side of the guinea pig.
2. A 2 x 2 cm filter paper was saturated with the experimental material and applied to the left side. The areas were occluded and wrapped as described above.
3. Twenty-one hours later the bandaging was removed and the sites were wiped clean. Three hours later the sites were examined for elicited skin reactions. Twenty-four hours later the sites were re-examined. Scoring was done on the basis of 0 - 3.

<u>Score</u>	<u>Skin Reaction</u>
0	No reaction
1	Scattered mild redness
2	Moderate and diffuse redness
3	Intense redness and swelling

Project Number: 81-2824A
Calgon Project Number: TES810036

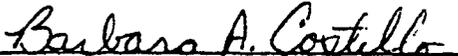
Calgon Corporation

Positive Control - 1-chloro-2,4-dinitrobenzene, as a 0.1% w/v suspension in physiological saline.

Guinea Pig Sensitization Study - Magnusson-Kligman Maximization Method.

Results: See Table 1.

Conclusion: The positive control material appears to be a sensitizer in the albino guinea pig.

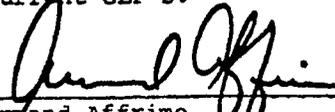

Barbara A. Costello, M.Ag.
Laboratory Supervisor

1/26/82
Date


Martin R. Gilman, Ph.D.
Study Director

1/26/82
Date

This report is in compliance with current GLP's.


Armand Affrime
Quality Assurance Officer

1/26/82
Date

Project Number: 81-2824A
Calgon Project Number: TES810036

Table 1
Guinea Pig Sensitization Study - Magnusson-Kligman Maximization Method - Males

POSITIVE CONTROL

Material: 1-chloro-2,4-dinitrobenzene, as a 0.1% w/v suspension in physiological saline.

Readings After Challenge Application

Guinea Pig No.	24 Hours	48 Hours
1	1	0
2	2	0
3	2	0
4	2	0
5	1	0
6	2	0
7	2	0
8	0	0
9	1	0
10	1	0

Test Substance Description

CAS Number: 97-00-7
Identity: Dinitrochlorobenzene
Purity: Not available
Comments: Dinitrochlorobenzene (DNCB) data were included as a positive control for sensitization studies conducted at the laboratory. The actual test material of the report was unrelated to the 8(d) rule and has been redacted.

Method

Method / Guideline: Buehler method
Test Type: Delayed contact hypersensitivity / Skin sensitization
Report Year: 1986
GLP Compliant: Not Available
Species: Guinea pig – Hartley albino strain
Number of Animals: 51
Study Design: Screening study: 0.4 mL of test substance at concentrations of 0.5, 0.1, or 0.05% (w/v) in acetone was applied for 6 hours on the shaven back of each of 4 animals. The skin sites were evaluated 24 hours after treatment to determine the highest non-irritating concentration that could be used at induction; 0.1% (w/v) test substance was selected.
Induction: The entire back was clipped with electric clippers. The following day, a 25 mm Hill Top Chamber moistened with 0.4 ml of 0.1% (w/v) test substance in 70% ethanol was placed on the clipped areas of 10 test animals, for 6 hours. Patches were reapplied weekly to the same, freshly clipped areas, for 3 consecutive weeks.
Challenge: After a 19-day rest period, test and control animals were challenged with a 0.05% (w/v) test substance in acetone on a fresh application site for 6 hours. Depilated animals were scored for erythema severity using a 0-4 scale, 24 and 48 h post-challenge.

Results

Result: The test material induced delayed contact hypersensitivity; a response expected for the positive control in the assay.

Data Quality

Reliability (Klimisch): 1

Reference

Laboratory Report Number: 50931
Reference: Mobil Environmental and Health Science Laboratory. 1986. Delayed Contact Hypersensitivity Study in Guinea Pigs (Buehler Sensitization Test).

Acc # 103074

MOBIL ENVIRONMENTAL AND HEALTH SCIENCE LABORATORY

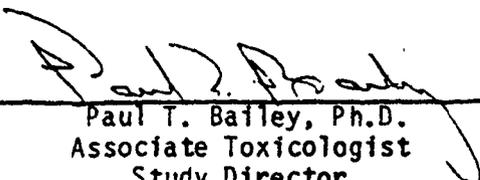
STUDY RECORD

NAME: Delayed Contact Hypersensitivity Study
in Guinea Pigs (Buehler Sensitization
Test) of

STUDY NUMBER: 50931

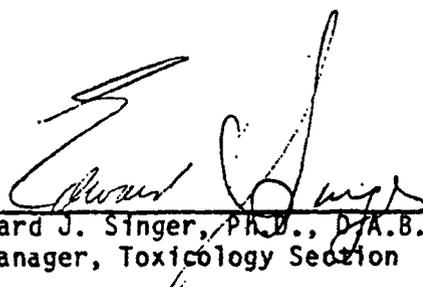
DATE: May 5, 1986

APPROVALS:



Paul T. Bailey, Ph.D.
Associate Toxicologist
Study Director

5/1/86
Date



Edward J. Singer, Ph.D., D.A.B.T.
Manager, Toxicology Section

5/9/86
Date

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ABSTRACT

was evaluated for dermal sensitization, according to the method of Ritz and Buehler, in Hartley albino guinea pigs. (neat) was applied dermally to 10 female guinea pigs one time per week for three weeks (three applications). Twenty and twenty-seven days following the third application, challenge and rechallenge doses were applied to the "induced" animals; in addition, a naive control group of 10 female guinea pigs was dosed with the test article.

The groups were observed and rated for dermal responses at 24 and 48 hours following the initial challenge; one week later the animals were rechallenged, observed, and rated after 24 and 48 hours for dermal reactions.

In this study, (neat) caused no reactions indicative of sensitization following challenge and rechallenge at a concentration of 75.0% (w/w) in Squibb Mineral Oil. Based on these data, was shown not to be a contact sensitizer in the Buehler guinea pig sensitization test.

1.0 INTRODUCTION

Delayed-type allergic contact sensitization refers to an immunologically mediated cutaneous reaction to a chemical. Such allergic sensitization develops as a result of one or more contacts with a chemical that activates the immune system. The condition generally develops no sooner than 1 to 2 weeks after the effective exposure and may require years of exposure to an agent. Subsequent exposure of the skin of the sensitized individual to a sufficient concentration of the sensitizer or related substance (cross-sensitizer) can result in an intense response. This response generally takes many hours or even days to develop, hence it is termed "delayed." Responses may be characterized by erythema, edema or induration, papules, vesicles, bullae, or combinations of these. Reactions generally subside over a period of days if there is no further contact with the sensitizer.

Allergic contact dermatitis can be induced in a variety of laboratory animals, such as mice, rats, hamsters, and chickens. The guinea pig, however, remains the animal of choice for the assay of materials which may be contact allergens in man. Chemical substances that produce a high incidence of contact sensitivity in humans (i.e., strong and moderate sensitizers) are easily identified in guinea pigs.

The objective of the present study was to evaluate the potential of (CRU #84132) to induce delayed contact hypersensitivity. The Buehler Method [1,2], which is conducted in guinea pigs, is considered an appropriate procedure for such an evaluation, because it closely mimics the mode of expected human exposure, and because of its known predictive value for man [2,4].

2.0 TEST MATERIAL

is a synthetic hydrocarbon fluid used in automotive and industrial lubricant formulations. The test article was obtained from the Chemical Products Division.

(CRU# 84132) is a clear liquid which was received and stored in a one gallon metal container at room temperature.

The positive control material, 2,4-dinitrochlorobenzene (DNCB; CRU #84097), used in this study was obtained from the Aldrich Chemical Company (Lot #7726 LK). DNCB is a pale yellow crystalline substance which was stored in an airtight amber glass container at room temperature.

3.0 METHODS

The procedure used was based on that of Ritz and Buehler [1], and is described in the protocol.

3.1 Animals

Fifty-one guinea pigs of the outbred Hartley white strain bred at Charles River Kingston (Route 209, Kingston, NY 12484), initially weighing 360-470 g and approximately 1 month old, were used.

3.2 Housing

The animals were housed individually in suspended stainless steel cages and provided with tap water ad libitum via automatic watering devices. Their individual identity was assured by ear tagging and use of cage cards. The housing room was kept at a constant temperature of $70 \pm 5^\circ\text{F}$ and a relative humidity of 40-60%; timing devices were set to provide artificial illumination 12 hours daily. The animals were fed a standard pellet diet (Purina Laboratory Guinea Pig Chow #5025) ad libitum.

3.4 Acclimation

The animals were acclimated to the laboratory for at least 14 days before they were placed on study.

3.5 Treatment

The animals for the study were randomly assigned to one of three groups for evaluation of specific parameters: 4 for range finding of primary irritation of the test article (Irritation Group), 10 test animals (Induction Group), and 10 control animals (Control Group). The control animals were maintained without treatment until primary challenge.

Twenty-four positive (DNCB) control animals for this study were also randomly assigned to one of three groups, as previously mentioned. These DNCB-treated animals were used as common positive controls for this study and for dermal sensitization Study Numbers 50941 and 50902.

3.6 Primary Irritation

To determine the highest non-irritating concentration of the test material to be used in both the challenge and rechallenge phases of the test, a group of guinea pigs was treated with various concentrations of the test article.

Hair was removed from the entire back of 4 guinea pigs using electric clippers. On the following day, patches were applied using a Hill Top Chamber System with a 25 mm Webril swatch moistened with 0.4 ml of either (neat) or 75.0, 50.0, and 25.0% (w/w) in Squibb Mineral Oil.

The positive control material (DNCB) was administered in the same manner as for the Irritation Groups. DNCB was administered at a concentration of 0.5, 0.1, or 0.05% (w/v) in acetone. These positive control irritation animals were also administered acetone via the chamber system.

The guinea pigs were wrapped with a piece of rubber dental dam (approximately 3" x 4") that was placed over the patch site and secured with Elastoplast (approximately 8" long). (Note: To reduce cost and conserve space at MEHSL, the guinea pig restraining procedure recommended by Ritz and Buehler [1] was not used. Numerous investigators [3] have established that wrapping the test site with a dental dam and Elastoplast tape is a satisfactory way of occluding the test site.) After an exposure period of approximately 6 hours, the patches were removed, the treated sites wiped with cotton gauze wet with saline, and the animals returned to their cages.

On the day following application of the test material, the clipped areas were depilated with Neet Cream Hair Remover (Whitehall Laboratories, Inc., New York, NY 10017). The depilatory was allowed to remain on the sites for 5-10 minutes and then wiped with cotton towels moistened with warm tap water.

The patch sites were scored for erythema approximately 2 hours later (24-hour reading) and approximately 24 hours later (48-hour reading).

For consistency of evaluations with other dermal studies at MEHSL, the Draize scoring scale is being used to express the data rather than the Buehler scoring scale.

The Draize scoring scale is shown below:

Erythema and Eschar Formation

- 0 No erythema
- 1 Very slight erythema (barely perceptible)
- 2 Well-defined erythema
- 3 Moderate to severe erythema
- 4 Severe erythema (beet redness) to slight eschar formation (injuries in depth)

3.7 Induction

The entire back of 10 guinea pigs was clipped using electric clippers. On the following day, the patches were applied using a Hill Top Chamber with a 25 mm Webril swatch moistened with 0.4 ml of (neat).

The Induction guinea pigs were wrapped according to the technique described above.

The patches were reapplied to the same site once each week for a total of 3 applications; 0.4 ml of (neat) was used for each application. The treatment site was clipped the day before each application was made. The exposure periods were approximately 6 hours long.

The positive control material (DNCB) was administered at a concentration of 0.1% (w/v) in 70% ethanol. The technique used for the positive control Induction animals was identical to that for the animals.

3.8 Challenge

After a 19-day rest period, a fresh application site for primary challenge was prepared by clipping the hair on the lower right quadrant of the backs of the Induction and Control guinea pigs. On the following day (first day) a challenge patch was applied to the site using 0.4 ml of (75.0% w/w in Squibb Mineral Oil) for approximately 6 hours according to the technique previously described. On the second day the sites were depilated for 5-10 minutes and scored within approximately 2 hours (24-hour reading) as described above. The sites were scored again on the third day for a 48-hour reading without additional depilation.

The positive control material (DNCB) was administered in the same manner but at a concentration of 0.05% (w/v) in acetone for the challenge phase.

3.9 Rechallenge

After a 6-day rest period a fresh application site for rechallenge was prepared by clipping the lower left quadrant of the backs of the Induction and Control guinea pigs. On the following day (first day) a rechallenge patch was applied to the site using 0.4 ml of (75.0% w/w in Squibb Mineral Oil) for approximately 6 hours as above. On the second day the sites were depilated for 5-10 minutes and scored within approximately 2 hours (24-hour reading). The sites were scored again the third day for a 48-hour reading without additional depilation.

4.0 RESULTS

Individual animal data for the Primary Irritation tests, and the Challenge and Rechallenge applications, are given in Appendices I through V, respectively. In the evaluation of the Primary Irritation potential of , the 100.0% concentration produced dermal irritant responses (Appendix I). No irritation was observed at the 75.0, 50.0, or 25.0% (w/w) concentrations of in Squibb Mineral Oil. Based on these data, the 75.0% (w/w) concentration was determined to be the highest non-irritating concentration for challenge and rechallenge.

In the evaluation of the irritation potential of DNCB in acetone, the 0.5 and 0.1% (w/v) concentrations of DNCB in acetone were found to produce significant dermal responses (see Appendix II). The 0.05% (w/v) concentration of DNCB and the acetone vehicle produced no dermal responses. Based on these data, the 0.05% (w/v) concentration was determined to be the highest non-irritating concentration for challenge.

4.2 Challenge

When the induced and naive control animals were challenged with (75.0% w/w in Squibb Mineral Oil), the following responses were obtained (see Appendix V):

		<u>Number of Animals Showing Indicated Erythema Score</u>				
		<u>0</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
24-Hour Reading	Induced	8	2	0	0	0
	Control	8	2	0	0	0
48-Hour Reading	Induced	8	2	0	0	0
	Control	7	3	0	0	0

Such a response pattern suggests that has no significant activity as a sensitizer, based on the test criterion of a minimum of a +2 response for positively-responding animals.

The hazard of allergy in the human population depends on many factors related to usage, such as the concentration of the substance, frequency and duration of exposure to skin, and whether or not the material will be applied to healthy or inflamed skin, covered or left uncovered, etc. The ideal test method for predicting the sensitization potential of substances would be under conditions which correspond to normal human circumstances (i.e., topical exposure, either with or without occlusion of the treatment site, at concentrations in the range of those to be encountered under foreseeable conditions of use). However, some materials are very irritating in test animals under conditions of human exposure, and therefore must be applied at a lower concentration in a carrier vehicle; on the other hand, if the material is applied without occlusion, the test procedure becomes rather insensitive, detecting only potent contact allergens. To balance these factors, it has become standard practice to routinely evaluate test materials under occlusive dressings, and to perform whatever dilutions are necessary to avoid any marked irritation or deterioration of the skin at the treatment site.

Numerous procedures for assessing the potential of materials to induce contact sensitization in guinea pigs have been described [4], among which is that of Buehler [1,2]. The Buehler guinea pig procedure was chosen because it employs the use of a closed patch system (occluded) which enhances the penetration of topically applied substances, resulting in a much more sensitive method for predicting the sensitization potential of test materials [1]. This procedure has been used for over 18 years and has demonstrated its ability to detect agents which can be strong, moderate, and in some cases, weak topical sensitizers in man.

The Buehler guinea pig sensitization procedure, while it may not be the most responsive to weak sensitizers, has certain features which make it preferable to other reported methods, viz. (i) there is little trauma to the guinea pigs, (ii) it is easy to evaluate positive reactions, (iii) it is less labor-intensive, and (iv) there is better simulation of human exposure.

In the present study, the potential of (neat) to produce delayed contact hypersensitivity (i.e., sensitization) in guinea pigs was evaluated using the Buehler method [1,2].

as a 75.0% (w/w) solution (in Squibb Mineral Oil) caused no reactions indicative of sensitization during challenge or at rechallenge.

The positive control group did demonstrate the sensitivity of the Charles River Hartley strain of guinea pigs to the known sensitizer DNCB (0.05% w/v in acetone).

6.0 CONCLUSION

(neat) caused no reactions indicative of sensitization following challenge and rechallenge at a concentration of 75.0% (w/w) in Squibb Mineral Oil. Based on these data, was shown not to be a contact sensitizer in the Buehler guinea pig sensitization test as performed at MEHSL.

REFERENCES

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APPENDIX I

PRIMARY IRRITATION TEST

Erythema Scores in Guinea Pigs - 24 and 48 Hours
 Following 6.0 Hour Patch Application of
 at Various Concentrations
 in Squibb Mineral Oil

Animal No.	Sex	Erythema Score (Draize Scale)							
		100%		75.0% Solution		50.0% Solution		25.0% Solution	
		24-Hrs	48-Hrs	24-Hrs	48-Hrs	24-Hrs	48-Hrs	24-Hrs	48-Hrs
034-1	F	1	1	0	0	0	0	0	0
034-7	F	1	0	0	0	0	0	0	0
034-8	F	0	0	0	0	0	0	0	0
034-19	F	1	0	0	0	0	0	0	0

APPENDIX II

PRIMARY IRRITATION TEST

Erythema Scores in Guinea Pigs - 24 and 48 Hours
 Following 6.0 Hour Patch Application of
 DNCB at Various Concentrations
 in Squibb Mineral Oil

Animal No.	Sex	Erythema Score (Draize Scale)							
		0.5% Solution		0.1% Solution		0.05% Solution		Acetone	
		24-Hrs	48-Hrs	24-Hrs	48-Hrs	24-Hrs	48-Hrs	24-Hrs	48-Hrs
034-69	F	2	0	0	0	0	0	0	0
034-70	F	3	2	2	0	0	0	0	0
034-71	F	2	2	1	1	0	0	0	0
034-75	F	2	2	1	1	0	0	0	0

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APPENDIX III
 RESPONSE DATA FOLLOWING CHALLENGE OF
 IN GUINEA PIGS

Animal No.	Sex	Erythema Score		Rating ^a
		24-Hrs	48-Hrs	
(75.0% w/w in Squibb Mineral Oil) in Induced Animals				
034-51	F	0	0	N
034-3	F	0	0	N
034-4	F	1	0	N
034-5	F	0	1	N
034-6	F	0	0	N
034-9	F	1	0	N
034-10	F	0	0	N
034-11	F	0	0	N
034-12	F	0	0	N
034-13	F	0	1	N
	Mean	0.2	0.2	0 Responders/ 10 Non-Responders
(75.0% w/w in Squibb Mineral Oil) in Control Animals				
034-14	F	0	0	N
034-15	F	0	0	N
034-16	F	0	0	N
034-17	F	0	0	N
034-18	F	1	1	N
034-20	F	1	0	N
034-21	F	0	0	N
034-22	F	0	1	N
034-23	F	0	0	N
034-24	F	0	1	N
	Mean	0.2	0.3	0 Responders/ 10 Non-Responders

^aR = Responder reaction (i.e., rating of +2 or greater); N = Non-responder reaction; rating on Draize scale.

APPENDIX IV

RESPONSE DATA FOLLOWING CHALLENGE OF
DNCB IN GUINEA PIGS

Animal No.	Sex	Erythema Score 24-Hrs	Erythema Score 48-Hrs	Rating ^a
<u>DNCB (0.05% w/v in Acetone) in Induced Animals</u>				
034-77	F	2	2	R
034-78	F	2	2	R
034-79	F	3	3	R
034-80	F	3	3	R
034-81	F	4	3	R
034-82	F	3	3	R
034-83	F	3	3	R
034-84	F	2	2	R
034-86	F	2	1	R
034-87	F	3	2	R
	Mean	2.7	2.4	10 Responders/ 0 Non-Responders
<u>DNCB (0.05% w/v in Acetone) in Control Animals</u>				
034-88	F	0	0	N
034-89	F	0	0	N
034-90	F	1	0	N
034-92	F	0	0	N
034-93	F	0	0	N
034-94	F	0	0	N
034-95	F	0	0	N
034-96	F	1	0	N
034-97	F	0	0	N
034-99	F	0	0	N
	Mean	0.2	0.0	0 Responders/ 10 Non-Responders

^aR = Responder reaction (i.e., rating of +2 or greater); N = Non-responder reaction; rating on Draize scale.

APPENDIX V

RESPONSE DATA FOLLOWING RECHALLENGE OF
IN GUINEA PIGS

Animal No.	Sex	Erythema Score		Rating ^a
		24-Hrs	48-Hrs	
<u>(75.0% w/w in Squibb Mineral Oil) in Induced Animals</u>				
034-51	F	0	0	N
034-3	F	0	0	N
034-4	F	0	0	N
034-5	F	0	0	N
034-6	F	0	0	N
034-9	F	0	0	N
034-10	F	0	0	N
034-11	F	0	0	N
034-12	F	0	0	N
034-13	F	0	0	N
	Mean	0.0	0.0	0 Responders/ 10 Non-Responders
<u>(75.0% w/w in Squibb Mineral Oil) in Control Animals</u>				
034-14	F	0	0	N
034-15	F	0	0	N
034-16	F	0	0	N
034-17	F	0	0	N
034-18	F	0	0	N
034-20	F	0	0	N
034-21	F	0	0	N
034-22	F	0	0	N
034-23	F	0	0	N
034-24	F	0	0	N
	Mean	0.0	0.0	0 Responders/ 10 Non-Responders

APPENDIX V (Continued)

Animal No.	Sex	Erythema Score		Rating ^a
		24-Hrs	48-Hrs	
				(75% w/w in Squibb Mineral Oil) in Naive Control Animals
034-111	F	0	.1	N
034-112	F	0	0	N
034-113	F	0	0	N
	Mean	0.0	0.3	0 Responders/ 3 Non-Responders

^aR = Responder reaction (i.e., rating of +2 or greater); N = Non-responder reaction; rating on Draize scale.