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JAN 26 1993

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FYI-0193-0866

Note to: Michele Stewart
Re: Assignment of FYI numbers

All the attached documents are studies on 1,2-ethanediyl tetrakis(?-chloro-1-methylethylene) phosphate (CAS No. 34621-99-3). They were received from the Olin Corporation on June 27, 1986. Each document should receive a separate FYI number and reflect the trade name product that was tested, *viz.*, Thermolin 102.

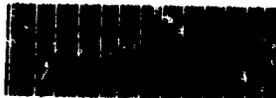
Please assign FYI numbers to each of the attached documents and then return the attached documents to me for shipment to Syracuse Research Corporation.

Thanks.

John D. Walker



FYI-93-0866
INIT 01/26/93



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93 JAN 26 PM 12:00
OIS DOCUMENT RECEIPT OFC

REPORT ON ORAL LD₅₀ IN RATS

For Olin

Project number: **77-1540**
 Sample number: **Therolin 102**
 Description: **yellow liquid**
 Vehicle: **none**

Stamberg and vents road
 post office box 703
 Spinnerstown, Pennsylvania 18968
 717-336-4110

Date received: **1/3/77**
 Date started: **1/23/77**
 Date ended: **2/16/77**

Male albino Wistar strain rats, weighing 200 to 300 g, were fasted for approximately 18 hours prior to administration of the test material. The material was given orally by intubation on a g/kg basis. Following treatment the rats were returned to their cages and food and water were freely available. The rats were observed for mortality, toxicity and pharmacological effects for 14 days. All rats were necropsied. The LD₅₀ and 95% Confidence Limits were calculated by the method of Litchfield and Wilcoxon (J. Pharm. & Exp. Therap. 96:99, 1949).

RESULTS: LD₅₀ (95% Confidence Limits) g/kg: 1.58 (1.08 - 2.29)

Doses g/kg	Deaths/ No. of rats	Mortality														
		Observation Day: Number of Dead Animals														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0.6	1/10															1
1.22	2/10	0	2													
2.47	9/10	0	9													
5.0	10/10	0	10													

Toxicity: 0.6, 1.22 g/kg - none; 2.47 g/kg - convulsions, blood around nose and mouth, piloerection, diarrhea, choriorrhoea
 5.0 g/kg - piloerection

Dose g/kg	0.6	1.22	2.47	5.0
Lungs dark	1	1	9	10
Dark areas on stomach	0	2	0	0
Portions of intestines red	1	1	7	7
Blood around nose and mouth	0	2	9	10
Necropsy Observations: Liver mottled	0	1	0	0
Kidney mottled	0	1	3	0
Lining of peritoneal cavity red	0	1	0	0
Spleen hardened, engorged with blood	1	0	0	0
Free blood in pleural cavity	0	0	1	3
Portions of stomach and intestine yellow			1	0

Respectfully submitted,

Oscar M. Moreno
 Oscar M. Moreno, Ph.D.
 President
 March 4, 1977

Therolin 102
FB 77-1540

Necropsy Observations

Sign	Number with sign	
	Deaths	Sacrificed
Lungs dark	2	
Areas of intestines red	1	
Liver pale	2	
Brown exudate around nose, mouth and anogenital region	2	
Areas of stomach red	1	
Kidneys pale		
Clotted blood around pancreas		7
Spleen enlarged, dried blood around nose, lung tissue on one side was brittle with spots of gray and beige		1

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MBR Research Laboratories, Inc.

ACUTE DERMAL TOXICITY IN RABBITS

For Olin

Project number MB 77-1540
 Sample number: ThermoLin 102
 Description: yellow liquid

Steinberg and vents roads
 post office box 203
 Springtown, Pennsylvania 18068
 717-236-4100

Material received: 1/3/77
 Test started: 2/9/77
 Test ended: 2/23/77

Ten New Zealand White rabbits were clipped free of abdominal hair. Epidermal abrasions were made longitudinally every 2 to 3 cm over the exposed area in rabbits 6 - 10. The abrasions were sufficiently deep to penetrate the stratum corneum but not deep enough to produce bleeding. A single dose of 2.0 g/kg was applied to the exposed area. The area was covered with gauze and the trunk wrapped with impervious material for 24 hours. At 24 hours the rabbits were cleansed and dermal reactions evaluated by the Draize technique. The animals were observed daily for 14 days. Necropsies were performed on all rabbits.

RESULTS: Mortality 0/10

Toxicity None

Necropsy Observations Liver blotchy - 4
 Yellow cheese-like area in liver - 1
 Intestines bloated - 1

Rabbit number	Body Weights - kg		Day 1 Dermal Reactions	
	Day 0	Day 14	Erythema	Edema
1	2.6	2.9	1	1
2	2.4	2.8	2	1
3	2.1	2.1	1	1
4	2.3	2.3	2	1
5	2.3	2.5	1	1
6	2.4	2.9	1	1
7	2.5	2.5	1	1
8	2.4	2.8	1	1
9	2.1	2.2	1	1
10	2.2	2.4	1	1

CONCLUSION Not toxic dermally

Respectfully submitted,

Cesar M. Moreno
 Cesar M. Moreno, Ph.D.
 President
 March 4, 1977

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MOR Research Laboratories, Inc.

INHALATION TOXICITY IN RATS

Project number MB 76-1540
For Olin

Steinberg and Gents roads
Post office box 703
Spinnertown, Pennsylvania 18068
717-336-4110

Sample: Therzolin 102
Description: yellow viscous liquid
Nominal Concentration: 200 mg/l
diluted to 50% with squala oil

Material received: 1/3/77
Test started: 2/17/77
Test ended: 3/13/77

Ten male albino Wistar rats were placed in a chamber and exposed to a nominal concentration of 200 mg/liter of the test material for a period of one hour. The rats were removed from the chamber at the end of the exposure, returned to their cages and observed daily for 14 days for signs of toxicity. Body weights were recorded prior to and 14 days after treatment. Necropsies were performed in all rats.

RESULTS: Mortality 3/10, Days 3, 4, 7

Toxicity Diarrhea and tremors in 1 prior to death
Chromorrhinorrhea in 1 prior to death

Necropsy Observations Attached

Rat No.	Initial Weight - g	Final Weight - g
1	230	321
2	227	272
3	220	238
4	200	Dead Day 4
5	222	Dead Day 7
6	216	312
7	225	305
8	200	Dead Day 3
9	226	316
10	225	305

CONCLUSION Not toxic by this route

Respectfully submitted,

Oscar M. Moreno
Oscar M. Moreno, Ph.D.
President

March 4, 1977

6000 5

M B Research Laboratories, Inc.

PRIMARY DERMAL IRRITATION IN RABBITS

For: Olin

Steinberg and West roads
post office box 703
Pinnerstown, Pennsylvania 18668
717-536-4110

Material received: 1/3/77
Test started: 1/10/77
Test ended: 1/13/77

Project number MB 77-1540
Sample: Thernolin 102
Description: yellow liquid

Six New Zealand White rabbits were closely clipped over the back and sides with an electric clipper. A site to the left of the spinal column was abraded and a site on the right was left intact. The abrasions were minor incisions through the stratum corneum, not sufficiently deep to disturb the derma or produce bleeding. 0.5 g (solids dissolved in appropriate solvent) or 0.5 ml (liquids) of the test material was applied to surgical gauze, one inch square, two layers thick. The patches were placed on test sites and secured with adhesive tape. The trunk was wrapped with impervious material. After 24 hours the patches were removed. Dermal reactions were evaluated at 24 and 72 hours. The primary irritation score was calculated. By definition a primary irritant is a substance which is not corrosive and has an empirical score of 5 or more. (16 CFR 1500.3 (c)(4) and 1500.41).

RESULTS

	Rabbit number						Mean Score
	1	2	3	4	5	6	
Erythema & Eschar Formation							
Intact skin.....24 hrs	1	1	2	0	1	0	0.83
Intact skin.....72 hrs	0	1	1	0	0	0	0.33
Abraded skin.....24 hrs	1	1	2	0	1	0	0.83
Abraded skin.....72 hrs	0	0	1	0	0	0	0.17
Edema							
Intact skin.....24 hrs	1	2	2	0	0	0	0.83
Intact skin.....72 hrs	0	0	1	0	0	0	0.17
Abraded skin.....24 hrs	1	2	2	0	0	0	0.83
Abraded skin.....72 hrs	0	0	1	0	0	0	0.17
Sum of Mean Scores =							4.16
Primary Irritation Score = Sum of Mean Scores/4							1.04

Non-irritant.

Respectfully submitted,

Oscar M. Moreno
Oscar M. Moreno, Ph.D.
President

March 4, 1977

MBR Research Laboratories, Inc.

Steinburg and
port office box
Spinnertown, I
713-536-4110

REPORT ON RABBIT EYE IRRITATION

Project number MB 77.

For Olin

Material: Sample number:
Description:

Thermolin 102
yellow liquid

Date material received:

1/3/77

Date test started:

1/10/77

Date test ended:

1/13/77

Six prescreened New Zealand White rabbits were used for this study. One-tenth of a milliliter of the test material was instilled into the conjunctival sac of one eye of each rabbit on Day 0. In all cases the contralateral eye served as a control. The ocular reactions were graded in accordance with the Consumer Product Safety Act, Title 16 CFR 1500.42 at 1, 2, and 3 days after instillation of the test material. A copy of the scale for scoring ocular lesions is attached.

The individual daily scores are presented on the following pages.

Irritant.

Respectfully submitted,

Oscar M. Moreno
Oscar M. Moreno, Ph.D.
President

February 25, 1977

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**Rabbit Eye Irritation Study
Individual Daily Scores**

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Material: Thernolin 102

Rabbit

Rabbit No.	Item	Tissue	Reading	Day 1	Day 2	Day 3
1	A	Cornea	Opacity	0	0	0
	B	Cornea	Area			
(1) Cornea Total = (AxB)x5						
1	C	Iris		0	0	0
	(2) Iris Total = (C) x 5					
1	D	Conjunctiva	Redness	1	1	0
	E	Conjunctiva	Chemosis	0	0	0
	F	Conjunctiva	Discharge	1	0	0
(3) Conjunctiva Total = (D+E+F)x2				2	1	0
Totals Added = (1 + 2 + 3)				2	1	0
2	A	Cornea	Opacity	0	0	0
	B	Cornea	Area			
(1) Cornea Total = (AxB)x5						
2	C	Iris		0	0	0
	(2) Iris Total = (C) x 5					
2	D	Conjunctiva	Redness	2	1	1
	E	Conjunctiva	Chemosis	2	1	0
	F	Conjunctiva	Discharge	2	1	1
(3) Conjunctiva Total = (D+E+F)x2				12	6	4
Totals Added = (1 + 2 + 3)				12	6	4
3	A	Cornea	Opacity	0	0	0
	B	Cornea	Area			
(1) Cornea Total = (AxB)x5						
3	C	Iris		0	0	0
	(2) Iris Total = (C) x 5					
3	D	Conjunctiva	Redness	2	1	0
	E	Conjunctiva	Chemosis	2	1	0
	F	Conjunctiva	Discharge	2	0	0
(3) Conjunctiva Total = (D+E+F)x2				12	4	0
Totals Added = (1 + 2 + 3)				12	4	0

See enclosed sheet for "Scale for Scoring Ocular Lesions".

**Rabbit Eye Irritation Study
Individual Daily Scores**

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Material: ThermoLin 102

Rabbit No.	Item	Tissue	Reading	Day 1	Day 2	Day 3
4	A	Cornea	Opacity	0	0	0
	B	Cornea	Area			
(1) Cornea Total = (AxB)x5						
4	C	Iris		0	0	0
	(2) Iris Total = (C) x 5					
4	D	Conjunctiva	Redness	1	0	0
	E	Conjunctiva	Chemosis	1	1	0
	F	Conjunctiva	Discharge	1	0	0
(3) Conjunctiva Total=(D+E+F)x2				6	2	0
Totals Added - (1 + 2 + 3)				6	2	0
	A	Cornea	Opacity	0	0	0
	B	Cornea	Area			
(1) Cornea Total = (AxB)x5						
5	C	Iris		0	0	0
	(2) Iris Total = (C) x 5					
5	D	Conjunctiva	Redness	2	2	2
	E	Conjunctiva	Chemosis	1	0	0
	F	Conjunctiva	Discharge	1	1	1
(3) Conjunctiva Total=(D+E+F)x2				8	6	6
Totals Added = (1 + 2 + 3)				8	6	6
	A	Cornea	Opacity	0	0	0
	B	Cornea	Area			
(1) Cornea Total = (AxB)x5						
6	C	Iris		0	0	0
	(2) Iris Total = (C) x 5					
6	D	Conjunctiva	Redness	2	2	1
	E	Conjunctiva	Chemosis	2	1	0
	F	Conjunctiva	Discharge	1	0	0
(3) Conjunctiva Total=(D+E+F)x2				10	6	2
Totals Added = (1 + 2 + 3)				10	6	2

Scale for Scoring Conjunctival Lesions

Scale for Scoring Ocular Lesions**

(1) Cornea

(A) Opacity-degree of density (area most dense taken for reading)	
No opacity	0
Scattered or diffuse area, details of iris clearly visible	1°
Easily discernible translucent areas, details of iris slightly obscured	2°
Opaque areas, no details of iris visible, size of pupil barely discernible	3°
Opaque, iris invisible	4°
(B) Area of cornea involved	
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4
Score equals $A + B \times 5$	Total maximum = 50

(2) Iris

(A) Values	
Normal	0
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof) iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, hemorrhage, gross destruction (any or all of these)	2
Score equals $A \times 5$	Total maximum = 10

(3) Conjunctivae

(A) Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)	
Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2°
Diffuse beefy red	3°
(B) Chemosis	
No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
(C) Discharge	
No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3
Score equals $(A + B + C) \times 2$	Total maximum = 20

The maximum total score is the sum of all scores obtained for the cornea, iris, and conjunctivae. Total maximum score possible = 110.

*An animal shall be considered as exhibiting a positive reaction.

**Draize, J.H. et al. J. Pharm. Exp. Ther. 82:377-390, 1954

CONCLUSIONS

Non-irritant	0 or 1 rabbit with positive score
Indeterminate	2 or 3 rabbits with positive scores
Irritant	4 to 6 rabbits with positive scores

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National Science Foundation
Occupational Safety and Health Administration

TSCA INTERAGENCY TESTING COMMITTEE
U.S. EPA/OTF (20-302)
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Washington, DC 20460

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JAN 26 1993

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Mr. Thomas G. L. Williams
Executive Assistant
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Note to: Michele Stewart
Re: Assignment of FYI numbers

All the attached documents are studies on 1,2-ethanedithyl tetrakis(2-chloroethyl) phosphate (CAS No. 33125-86-9). They were received from the Olin Corporation on June 27, 1986. Each document should receive a separate FYI number and reflect the trade name product that was tested, e.g., the first group of clipped studies tested Thermolin 101B 13934A which equals Thermolin 101 plus 1% hydroquinone, the second group of clipped studies tested Thermolin 101B 13934B which equals Thermolin 101 plus 1% Weston 600.

Please assign FYI numbers to each of the attached documents and then return the attached documents to me for shipment to Syracuse Research Corporation.

Thanks.

John D. Walker

OTS DOCUMENT PROJECT 090
93 JAN 26 PM 12:00

CC

Contains NO GHS

MB Research Laboratories, Inc.

Steinsburg and Wentz roads
post office box 703
Spinnerstown, Pennsylvania 18068
717-533-4110

REPORT ON INHALATION TOXICITY IN RATS

Project number MB 76-1149
For Olin

Date material received: 3/23/76
Date test started: 3/31/76
Date test ended: 4/14/76

Material: Sample number: Thermo In 101 B 13934 (A) = Tharmolin 101
Description: light yellow liquid plus 1% hydrogen peroxide

Ten albino Wistar rats, 200 to 250 g, were placed in a 50 liter chamber and exposed to a nominal concentration of 200 mg/liter of the test material for a period of one hour. The rats were removed from the chamber at the end of the exposure, returned to their cages and observed daily for 14 days for signs of toxicity. Body weights were recorded prior to and 14 days after treatment.

RESULTS

Mortality - Number of Deaths Day of Death
0/10

Signs of Toxicity - None

Necropsy Observations - Liver pale in 1/10

Body weights - g

Rat No.	Day 0	Day 14	Rat No.	Day 0	Day 14
1	222	289	6	210	251
2	236	304	7	295	329
3	235	319	8	296	325
4	224	298	9	285	310
5	224	327	10	278	288

By the criteria of this test, this compound is not considered to be toxic.

Respectfully submitted,

Oscar M. Moreno
Oscar M. Moreno, Ph.D.
President
April 16, 1976



12

file

13

Stansbury and Gents roads
post office box 303
Spangertown, Pennsylvania 18068
717-336-4110

REPORT ON ORAL LD₅₀ IN RATS

Project number MB 75-1149 For Olin
Date material received: 3/23/76
Date test started: 3/24/76
Date test ended: 4/15/76
Material: Sample number: Thermolin 101 B 13934 A
Description: light yellow liquid

Male albino rats, Wistar strain, weighing 200 to 250 grams were fasted for approximately 18 hours prior to administration of the test material. The test material was given orally by intubation on a g/kg basis. Following treatment the animals were returned to their cages and food and water was freely available. The rats were observed for signs of toxicity and pharmacological effects for 14 days following treatment. The oral LD₅₀ and 95% Confidence Limits were determined by the method of Litchfield and Wilcoxon (J. Pharm. & Exp. Therap. 96:99, 1949).

RESULTS: LD₅₀ - g/kg: estimated 1.08
95% Confidence Limits - g/kg: 0.78 - 1.49
Distribution of Mortality:

Doses g/kg	Deaths/ No. of rats	Observation Day: Number of dead animals													
		0	1	2	3	4	5	6	7	8	9	10	11	12	13
0.34	0/10														
0.67	0/10														
1.31	9/10	0	9												
2.56	10/10	0	8	1	0	1									
5.0	10/10	1	9												

Signs of Toxicity: 0.34 g/kg - none, 0.67 g/kg - slight lethargy, 1.31 g/kg - lethargy, loss of coordination in 3, 2.56 g/kg - tremors, heavy breathing, ptosis, lethargy, coma. 5.0 g/kg tremors, piloerection, ptosis.

Necropsy observations	0.34	0.67	1.31	2.56	5.0
liver dark or mottled			8	8	9
kidney dark or mottled	1	1		2	4
lung hemorrhagic			9	7	10
small intestine very red			9	9	0

Respectfully submitted,

Oscar H. Moreno
Oscar H. Moreno, Ph.D.
President
April 28, 1976

MB

MB Research Laboratories, Inc.

steinsburg and vents roads
post office box 703
spinnerstown, pennsylvania 18068
715-536-4110

REPORT ON ACUTE DERMAL TOXICITY IN RABBITS

Project number MB 76-1149 Date material received: 3/23/76
For Olin Date test started: 3/31/76
 Date test ended: 4/14/76

Material: Sample number: Thermolin 101 B13934A
 Description: light yellow liquid

Ten New Zealand White rabbits, weighing 2.0 to 3.2 kg, were prepared by clipping the abdominal area free of hair. Epidermal abrasions were made longitudinally every 2 to 3 cm over the exposed area. The abrasions were sufficiently deep to penetrate the stratum corneum but not deep enough to produce bleeding. A single dose, 2.0 g/kg, was applied to the exposed area. The area was covered with gauze and the trunk wrapped with impervious material for 24 hours. After this period the impervious material was removed and the animals were cleansed. The animals were observed for signs of toxicity, daily, for 14 days.

RESULTS: Mortality - 0/10

Number of Deaths	Day of Death
------------------	--------------

Signs of Toxicity - None

Necropsy Observations - Normal

Respectfully submitted,

Oscar M. Moreno

Oscar M. Moreno, Ph.D.
President

MB

14

M B Research Laboratories, Inc.

PRIMARY DERMAL IRRITATION IN RABBITS

For: Olin Corporation

Stamberg and West, Road

Post Office Box 203

Spinnerstown, Pennsylvania 19066

713-336-4110

Material received: 3/23/76
 Test started: 3/30/76
 Test ended: 4/2/76

Project number MB 76-1149
 Sample: Thiomelin 101 219934A
 Description: light yellow liquid

Six New Zealand White rabbits were closely clipped over the back and sides with an electric clipper. A site to the left of the spinal column was abraded and a site on the right was left intact. The abrasions were minor incisions through the stratum corneum, not sufficiently deep to disturb the derma or produce bleeding. 0.5 g (solids dissolved in appropriate solvent) or 0.5 ml (liquids) of the test material was applied to surgical gauze, one inch square, two layers thick. The patches were placed on test sites and secured with adhesive tape. The trunk was wrapped with impervious material. After 24 hours the patches were removed. Dermal reactions were evaluated at 24 and 72 hours. The primary irritation score was calculated. By definition a primary irritant is a substance which is not corrosive and has an empirical score of 5 or more. (16 CFR 1500.3 (c)(4) and 1500.41).

RESULTS

	Rabbit number						Mean Score
	1	2	3	4	5	6	
Erythema & Eschar Formation							
Intact skin.....24 hrs	2	1	2	1	0	1	1.17
Intact skin.....72 hrs	2	1	2	1	0	0	1.0
Abraded skin.....24 hrs	2	1	2	1	0	0	1.0
Abraded skin.....72 hrs	1	1	2	1	0	0	0.83
Edema							
Intact skin.....24 hrs	0	1	1	0	0	0	0.33
Intact skin.....72 hrs	0	0	1	0	0	0	0.17
Abraded skin.....24 hrs	0	1	1	0	0	0	0.33
Abraded skin.....72 hrs	0	0	1	0	0	0	0.17

Sum of Mean Scores = 5.0

Primary Irritation Score = Sum of Mean Scores/4 = 1.25

By the criteria of this test, this compound is not considered to be an irritant.

Respectfully submitted,

Oscar H. Morse

Oscar H. Morse, Ph.D.

President

April 16, 1976

MD

PRIMARY DERMAL IRRITATION SCORING CODE IN RABBITS

Skin reaction	Value
Erythema and eschar formation	
None	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (best redness) to slight eschar formations (injuries in depth)	4
Edema formation	
None	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

The mean values (6 rabbits) for erythema/eschar and edema formation on intact and abraded skin at 24 and 72 hours (a total of 8 values) are added and divided by 4 to give the primary irritation score.

By the criteria of this test primary irritant means a substance that results in an empirical score of 5 or more.

15 CFR 1500.41 and 16 CFR 1500.3

MB Research Laboratories, Inc.

Stenberg and vents roads
post office box 703
Springtown, Pennsylvania 18088
717-336-4110

REPORT ON RABBIT EYE IRRITATION

Project number MB 76-1109

For Olin Corporation

Material: Sample number: ThermoLin 101 B 1995A A
Description: light yellow liquid
Date material received: 3/23/76
Date test started: 3/23/76
Date test ended: 4/1/76

Six prescreened New Zealand White rabbits were used for this study. One-tenth of a milliliter of the test material was instilled into the conjunctival sac of one eye of each rabbit on Day 0. In all cases the contralateral eye served as a control. The ocular reactions were graded in accordance with the Consumer Product Safety Act, Title 16 CFR 1500.42 at 1, 2, and 3 days after instillation of the test material. A copy of the scale for scoring ocular lesions is attached.

The individual daily scores are presented on the following pages.

All rabbits had negative scores. By the criteria of this test, this compound is not considered to be an irritant.

Respectfully submitted,

Oscar R. Moreno
Oscar R. Moreno, Ph.D.
President

April 16, 1976

1

**Rabbit Eye Irritation Study
Individual Daily Scores**

Material: Thernolin 101 B 13934 A

Rabbit No.	Item	Tissue	Reading	Day 1	Day 2	Day 3	
1	A	Cornea	Opacity	0	0	0	
	B	Cornea	Area	0	0	0	
	(1) Cornea Total = (AxB)x5				0	0	0
	C	Iris		0	0	0	
	(2) Iris Total = (C) x 5				0	0	0
	D	Conjunctiva	Redness	1	0	0	
	E	Conjunctiva	Chemosis	0	0	0	
	F	Conjunctiva	Discharge	0	0	0	
	(3) Conjunctiva Total=(D+E+F)x2				2	0	0
	Total: Added - (1 + 2 + 3)				2	0	0
2	A	Cornea	Opacity	0	0	0	
	B	Cornea	Area	0	0	0	
	(1) Cornea Total = (AxB)x5				0	0	0
	C	Iris		0	0	0	
	(2) Iris Total = (C) x 5				0	0	0
	D	Conjunctiva	Redness	0	0	0	
	E	Conjunctiva	Chemosis	0	0	0	
	F	Conjunctiva	Discharge	0	0	0	
	(3) Conjunctiva Total=(D+E+F)x2				0	0	0
	Totals Added = (1 + 2 + 3)				0	0	0
3	A	Cornea	Opacity	0	0	0	
	B	Cornea	Area	0	0	0	
	(1) Cornea Total = (AxB)x5				0	0	0
	C	Iris		0	0	0	
	(2) Iris Total = (C) x 5				0	0	0
	D	Conjunctiva	Redness	1	0	0	
	E	Conjunctiva	Chemosis	0	0	0	
	F	Conjunctiva	Discharge	0	0	0	
	(3) Conjunctiva Total=(D+E+F)x2				2	0	0
	Totals Added = (1 + 2 + 3)				2	0	0

See attached sheet for "Scale for Scoring Ocular Lesions".

Rabbit Eye Irritation Study
Individual Daily Scores
Material: Thermo 13 13754 A

Rabbit No.	Item	Tissue	Reading	Day 1	Day 2	Day 3
4	A	Cornea	Opacity	0	0	0
	B	Cornea	Area	0	0	0
	(1) Cornea Total = (AxB)x5			0	0	0
	C	Iris		0	0	0
	(2) Iris Total = (C) x 5			0	0	0
	D	Conjunctiva	Redness	0	0	0
	E	Conjunctiva	Chemosis	0	0	0
	F	Conjunctiva	Discharge	0	0	0
	(3) Conjunctiva Total=(D+E+F)x2			0	0	0
	Totals Added - (1 + 2 + 3)			0	0	0
5	A	Cornea	Opacity	0	0	0
	B	Cornea	Area	0	0	0
	(1) Cornea Total = (AxB)x5			0	0	0
	C	Iris		0	0	0
	(2) Iris Total = (C) x 5			0	0	0
	D	Conjunctiva	Redness	0	0	0
	E	Conjunctiva	Chemosis	0	0	0
	F	Conjunctiva	Discharge	0	0	0
	(3) Conjunctiva Total=(D+E+F)x2			0	0	0
	Totals Added - (1 + 2 + 3)			0	0	0
6	A	Cornea	Opacity	0	0	0
	B	Cornea	Area	0	0	0
	(1) Cornea Total = (AxB)x5			0	0	0
	C	Iris		0	0	0
	(2) Iris Total = (C) x 5			0	0	0
	D	Conjunctiva	Redness	1	1	1
	E	Conjunctiva	Chemosis	0	0	0
	F	Conjunctiva	Discharge	0	0	0
	(3) Conjunctiva Total=(D+E+F)x2			2	2	2
	Totals Added - (1 + 2 + 3)			2	2	2

See attached sheet for "Scale for Scoring Ocular Lesions".

Scale for Scoring Ocular Lesions **

(1) Cornea

(A) Opacity-degree of density (area most dense taken for reading)

- No opacity 0
- Scattered or diffuse area, details of iris clearly visible 1
- Easily discernible translucent areas, details of iris slightly obscured 2
- Opaque areas, no details of iris visible, size of pupil barely discernible 3
- Opaque, iris invisible 4

(B) Area of cornea involved

- One quarter (or less) but not zero 1
- Greater than one quarter, but less than half 2
- Greater than half, but less than three quarters 3
- Greater than three quarters, up to whole area 4

Score equals A + B

Total maximum = 80

(2) Iris

(A) Values

- Normal 0
- Folds above normal, congestion, swelling, circumferential injection (any or all of these or combination of any thereof) iris still reacting to light (sluggish reaction is positive) 1
- No reaction to light, hemorrhage, gross destruction (any or all of these) 2

Score equals A + B

Total maximum = 10

(3) Conjunctivae

(A) Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)

- Vessels normal 0
- Vessels definitely injected above normal 1
- Mild diffuse, deeper crimson red, individual vessels not easily discernible 2
- Diffuse beefy red 3

(B) Chemosis

- No swelling 0
- Any swelling above normal (includes protruding membrane) 1
- Oblvious swelling with partial closure of lids 2
- Swelling with lids about half closed 3
- Swelling with lids about half closed to completely closed 4

(C) Discharge

- No discharge 0
- Any amount different from normal (does not include small amounts observed in inner canthus of normal animals) 1
- Discharge with moistening of the lids and hairs just adjacent to lids 2
- Discharge with moistening of the lids and hairs, and considerable area around the eye 3

Score equals (A + B + C)

Total maximum = 20

The maximum total score is the sum of all scores obtained for the cornea, iris, and conjunctivae. Total maximum score possible = 110.

*An animal shall be considered as exhibiting a positive reaction.

**Draize, J.H. et al. J. Pharm. Exp. Ther. 82:377-390, 1944

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MB Research Laboratories, Inc.

Stamberg and cents road
post office box 703
Spencerstown, Pennsylvania 18008
717-336-4110

REPORT ON INHALATION TOXICITY IN RATS

Project number MB 76-1150
For Olin

Date material received: 3/23/76
Date test started: 3/31/76
Date test ended: 4/14/76

Material: Sample number: Tbermolin 101 B 139348
Description: light yellow liquid *Thermolin 101 plus 12 Wistar 600*

Ten albino Wistar rats, 200 to 250 g, were placed in a 50 liter chamber and exposed to a nominal concentration of 200 mg/liter of the test material for a period of one hour. The rats were removed from the chamber at the end of the exposure, returned to their cages and observed daily for 14 days for signs of toxicity. Body weights were recorded prior to and 14 days after treatment.

RESULTS

Mortality - Number of Deaths Day of Death
0/10

Signs of Toxicity - none

Necropsy Observations - Normal

Body weights - g					
Rat No.	Day 0	Day 14	Rat No.	Day 0	Day 14
1	247	358	6	257	338
2	228	317	7	263	337
3	269	370	8	259	366
4	243	338	9	261	347
5	241	325	10	262	330

By the criteria of this test, this compound is not considered to be an irritant.

Respectfully submitted,

Oscar M. Moreno
Oscar M. Moreno, Ph.D.
President

April 16, 1976

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Steinburg and cents roads
 post office box 203
 Spinnerstown, Pennsylvania 18968
 715-536-4110

REPORT ON ORAL LD₅₀ IN RATS

Project number MB 76-1150 Date material received: 3/23/76
 For Olin Date test started: 3/30/76
 Material: Sample number: Thermolin 101 B 13934 B Date test ended: 4/19/76
 Description: light yellow liquid

Male albino rats, Wistar strain, weighing 200 to 250 grams were fasted for approximately 18 hours prior to administration of the test material. The test material was given orally by intubation on a g/kg basis. Following treatment the animals were returned to their cages and food and water was freely available. The rats were observed for signs of toxicity and pharmacological effects for 14 days following treatment. The oral LD₅₀ and 95% Confidence Limits were determined by the method of Litchfield and Wilcoxon (J. Pharm. & Exp. Therap. 96:99, 1949).

RESULTS: LD₅₀ - g/kg: 1.10
 95% Confidence Limits - g/kg: 0.77 - 1.57
 Distribution of Mortality:

Doses g/kg	Deaths/ No. of rats	Observation Day: Number of dead animals														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0.34	0/10															
0.67	1/10															
1.31	7/10	0	6	0	0	1				1						
2.56	10/10	5	5													

Signs of Toxicity: 0.34 and 0.67 g/kg - none, 1.31 g/kg - slight lethargy, loss of righting reflex in 2, piloerection, 2.56 g/kg - tremors, flaccid muscle tone.

Necropsy Observations	0.34	0.67	1.31	2.56
nottled kidney	3			
liver dark		1	7	10
small intestine very red			6	5
free blood in abdomen				2
stomach partially filled with clear fluid				6
fluid around nostrils				

Respectfully submitted,
Oscar M. Moreno
 Oscar M. Moreno, Ph.D.
 President
 April 28, 1976

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MB Research Laboratories, Inc.

Stamberg and Sons, Inc.
Post Office Box 703
Springtown, Pennsylvania 18008
717-336-4110

REPORT ON ACUTE DERMAL TOXICITY IN RABBITS

Project number MB 76-1150 Date material received: 3/23/76
For Olin Date test started: 3/31/76
Date test ended: 4/14/76

Material: Sample number: ThermoLin 101 B 1394B
Description: light yellow liquid

Ten New Zealand White rabbits, weighing 1.8 to 2.9 kg, were prepared by clipping the abdominal area free of hair. Epidermal abrasions were made longitudinally every 2 to 3 cm over the exposed area. The abrasions were sufficiently deep to penetrate the stratum corneum but not deep enough to produce bleeding. A single dose, 2.0 g/kg, was applied to the exposed area. The area was covered with gauze and the trunk wrapped with impervious material for 2nd hours. After this period the impervious material was removed and the animals were cleansed. The animals were observed for signs of toxicity, daily, for 14 days.

RESULTS: Mortality - 1/10
Number of Deaths Day of Death
1 8

Signs of Toxicity - none

Necropsy Observations - kidney bottled in 1
kidney bottled, enlarged and
had pockets of white fluid on surface

Respectfully submitted,

Quar M. Moran

Quar M. Moran, Ph.D.
President

April 16, 1976

23

M B Research Laboratories, Inc.

PRIMARY DERMAL IRRITATION IN RABBITS

For: Olin Corporation

Steinsburg and vents: roads
 post office box 203
 Spinnerstown, Pennsylvania 18968
 715-536-4110

Material received: 3/23/76
 Test started: 3/30/76
 Test ended: 4/2/76

Project number MB 76-1150
 Sample: Thermolin 101 B13934 B
 Description: light yellow liquid

Six New Zealand White rabbits were closely clipped over the back and sides with an electric clipper. A site to the left of the spinal column was abraded and a site on the right was left intact. The abrasions were minor incisions through the stratum corneum, not sufficiently deep to disturb the derma or produce bleeding. 0.5 g (solids dissolved in appropriate solvent) or 0.5 ml (liquids) of the test material was applied to surgical gauze, one inch square, two layers thick. The patches were placed on test sites and secured with adhesive tape. The trunk was wrapped with impervious material. After 24 hours the patches were removed. Dermal reactions were evaluated at 24 and 72 hours. The primary irritation score was calculated. By definition a primary irritant is a substance which is not corrosive and has an empirical score of 5 or more. (16 CFR 1500.3 (c)(4) and 1500.41).

RESULTS

	Rabbit number						Mean Score
	1	2	3	4	5	6	
Erythema & Eschar Formation							
Intact skin.....24 hrs	2	1	2	1	0	1	1.17
Intact skin.....72 hrs	2	1	1	2	0	0	1.0
Abraded skin.....24 hrs	2	1	2	1	0	0	1.0
Abraded skin.....72 hrs	2	1	1	1	0	0	0.83
Edema							
Intact skin.....24 hrs	0	1	1	0	0	0	0.33
Intact skin.....72 hrs	1	0	0	1	0	0	0.33
Abraded skin.....24 hrs	0	1	1	0	0	0	0.33
Abraded skin.....72 hrs	1	0	0	0	0	0	0.17

Sum of Mean Scores = 5.16

Primary Irritation Score = Sum of Mean Scores/4 = 1.54

By the criteria of this test, this compound is not considered to be an irritant.

Respectfully submitted,

Oscar M. Moreno
 Oscar M. Moreno, Ph.D.
 President

April 16, 1976

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PRIMARY DERMAL IRRITATION SCORING CODE IN RABBITS

Skin reaction	Value
Erythema and eschar formation	
None	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formations (injuries in depth)	4
Edema formation	
None	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

The mean values (6 rabbits) for erythema/eschar and edema formation on intact and abraded skin at 24 and 72 hours (a total of 8 values) are added and divided by 4 to give the primary irritation score.

By the criteria of this test primary irritant means a substance that results in an empirical score of 5 or more.

16 CFR 1500.41 and 16 CFR 1500.3

MB Research Laboratories, Inc.

Steinsburg and vents road:
post office box 703
Spinnerstown, Pennsylvania 18068
715-536-4110

REPORT ON RABBIT EYE IRRITATION

Project number MB 76-1150

For Olin Corporation

Material: Sample number: Thermolin¹⁰¹ B 13934 B
Description: light yellow liquid

Date material received: 3/23/76
Date test started: 3/29/76
Date test ended: 4/1/76

Six prescreened New Zealand White rabbits were used for this study. One-tenth of a milliliter of the test material was instilled into the conjunctival sac of one eye of each rabbit on Day 0. In all cases the contralateral eye served as a control. The ocular reactions were graded in accordance with the Consumer Product Safety Act, Title 16 CFR 1500.42 at 1, 2, and 3 days after instillation of the test material. A copy of the scale for scoring ocular lesions is attached.

The individual daily scores are presented on the following pages.

All rabbits had negative scores. By the criteria of this test, this compound is not considered to be an irritant.

Respectfully submitted,

G. M. Moreno
G. M. Moreno, Ph.D.
President

April 16, 1976

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NO COPY AVAILABLE

Rabbit Eye Irritation Study
Individual Daily Scores

Material: Thermolin 101 B 13934 B

Rabbit No.	Item	Tissue	Reading	Day 1	Day 2	Day 3
4	A	Cornea	Opacity	0	0	0
	B	Cornea	Area	0	0	0
	(1) Cornea Total = (AxB)x5			0	0	0
	C	Iris		0	0	0
	(2) Iris Total = (C) x 5			0	0	0
	D	Conjunctiva	Redness	1	1	0
	E	Conjunctiva	Chemosis	0	0	0
	F	Conjunctiva	Discharge	1	0	0
	(3) Conjunctiva Total=(D+E+F)x2			4	2	0
	Totals Added - (1 + 2 + 3)			4	2	0
5	A	Cornea	Opacity	0	0	0
	B	Cornea	Area	0	0	0
	(1) Cornea Total = (AxB)x5			0	0	0
	C	Iris		0	0	0
	(2) Iris Total = (C) x 5			0	0	0
	D	Conjunctiva	Redness	1	1	1
	E	Conjunctiva	Chemosis	0	0	0
	F	Conjunctiva	Discharge	1	0	0
	(3) Conjunctiva Total=(D+E+F)x2			4	2	2
	Totals Added = (1 + 2 + 3)			4	2	2
6	A	Cornea	Opacity	0	0	0
	B	Cornea	Area	0	0	0
	(1) Cornea Total = (AxB)x5			0	0	0
	C	Iris		0	0	0
	(2) Iris Total = (C) x 5			0	0	0
	D	Conjunctiva	Redness	1	0	0
	E	Conjunctiva	Chemosis	0	0	0
	F	Conjunctiva	Discharge	1	0	0
	(3) Conjunctiva Total=(D+E+F)x2			4	0	0
	Totals Added = (1 + 2 + 3)			4	0	0

0 0 3 0

Scale for Scoring Ocular Lesions**

(1) Cornea

- (A) Opacity degree of density taken most dense taken for reading:
- No opacity 0
 - Scattered or diffuse area, details of iris clearly visible 1
 - Large discernible translucent areas, details of iris slightly obscured 2
 - Opaque areas, no details of iris visible, size of pupil barely discernible 3
 - Opaque, iris invisible 4
- (B) Area of cornea involved:
- One quarter or less but not zero 1
 - Greater than one quarter, but less than half 2
 - Greater than half, but less than three quarters 3
 - Greater than three quarters, up to whole area 4
- Score equals A + B Total maximum = 80

(2) Iris

- (A) Values
- Normal 0
 - Folds above normal, congestion, swelling, circumferential injection (any or all of these or combination of any thereof) iris still reacting to light (sluggish reaction is positive) 1
 - No reaction to light, hemorrhage, gross destruction (any or all of these) 2
- Score equals A Total maximum 10

(3) Conjunctivae

- (A) Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)
- Vessels normal 0
 - Vessels definitely injected above normal 1
 - Mild diffuse deeper crimson red, individual vessels not easily discernible 2
 - Diffuse beefy red 3
- (B) Chemosis
- No swelling 0
 - Any swelling above normal includes the nating membrane 1
 - Obvious swelling with partial closure of lids 2
 - Swelling with lids about half closed 3
 - Swelling with lids about half closed or completely closed 4
- (C) Discharge
- No discharge 0
 - Any amount different from normal (does not include small amounts observed in inner canthus of normal animals) 1
 - Discharge with moistening of the lids and hairs just adjacent to lids 2
 - Discharge with moistening of the lids and hairs and considerable area around the eye 3
- Score equals A + B + C Total maximum = 20

The maximum total score is the sum of all scores obtained for the cornea, iris and conjunctivae. Total maximum score possible = 110

*An animal shall be considered as exhibiting a positive reaction.

**Braize, J.H. et al. J. Pharm. Exp. Ther. 82:377-390, 1944

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TSCA INTERAGENCY TESTING COMMITTEE

U.S. EPA/CPTT (TS-702)
401 M Street, SW
Washington, DC 20460

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Dr. John D. Walker
Executive Director
Telephone (202) 390-1800
Ms. Norma S. L. Williams
Executive Assistant
Telephone (202) 390-1825
Fax (202) 290-1784

Note to: Michele Stewart

Re: Assignment of FYI numbers

All the attached documents are studies on 1,2-ethanediyl tetrakis(2-chloroethyl) phosphate (CAS No. 33125-86-9). They were received from the Olin Corporation on June 27, 1986. Each document should receive a separate FYI number and reflect the trade name product that was tested, Thermolin 101.

Please assign FYI numbers to each of the attached documents and then return the attached documents to me for shipment to Syracuse Research Corporation.

Thanks.

John D. Walker

93 JAN 26 PM 12:01
OIS DOCUMENT RECEIPT OFF

3 September 1976

Final Report

**IN VITRO MICROBIOLOGICAL VIRULENCE STUDIES
OF TWO OLEIN COMPANY COMPONDS**

*file
Thermodur 101*

Prepared for:

**OLEIN COMPANY
Research Center
275 Winchester Avenue
New Haven, Connecticut 06504**

Attention: Dr. John H. Wedig

Submitted by:

**Vincent F. Sinner, Ph.D.
Manager, Microbial Genetics Program**

**Sharon Eckford
Microbiologist**

SRI Project LSC 3086

Approved:



**G. W. Newell, Director
Department of Toxicology**



**W. A. Skinner, Executive Director
Sciences Division**

SUMMARY

101
NI examined Olin Company compounds Thionin and 3-methyl-1-phenanthroline (MPP) for mutagenic activity with five strains of the bacteria Salmonella typhimurium--TA98, TA100, TA1535, TA1537, and TA1538--and with the yeast Saccharomyces cerevisiae D3. Each assay was performed in the presence and in the absence of a metabolic activation system. Neither compound was mutagenic in the bacterial assay with S. typhimurium, either in the presence or in the absence of the liver metabolic activation system, and neither increased mitotic recombination in the S. cerevisiae D3 assay.

INTRODUCTION

NIH examined 614 compounds Thionin and SIF for mutagenicity by in vitro microbiological assays with five strains of S. typhimurium and with S. cerevisiae 53. An Areolar 1204-stimulated, rat-liver-homogenate metabolic activation system was included in the assay procedure to provide metabolic steps that the bacteria either are incapable of conducting or do not carry out under the assay conditions. The purpose of this study was to determine whether the compounds elicited a mutagenic response in microorganisms. The assay procedure with S. typhimurium has been proven to be approximately 85 to 90% accurate in detecting carcinogens as mutagens, and it has about the same accuracy in identifying chemicals that are not carcinogenic.¹

The assay procedure with S. cerevisiae is about 60% accurate in detecting carcinogens as agents that increase mitotic recombination.² The combination of the two assay procedures significantly enhances the probability of detecting potentially hazardous chemicals. However, because the test systems are not 100% accurate, neither a positive nor negative response proves that a chemical is hazardous or non-hazardous to man.

METHODS

Salmonella typhimurium strains TA1538, TA1537, TA1535, TA100 and TA100

The *S. typhimurium* strains used at SRI were obtained from Dr. Bruce Ames of the University of California at Berkeley.³⁻⁵ All are histidine auxotrophs (*his*⁻) by virtue of mutations in the histidine operon. In addition to the mutations in the histidine operon, five of the indicator strains have mutations in the lipopolysaccharide coat (*rfa*⁻) and deletions that cover a gene involved in the re. ir of uv damage (*uvrB*⁻). The *rfa*⁻ mutation makes the strains more permeable to large molecules, thereby increasing their sensitivity to these molecules. The *uvrB*⁻ mutation decreases repair of some types of chemically damaged DNA and thereby enhances sensitivity to some mutagenic chemicals. Strain TA1535 is reverted to histidine prototrophy (*his*⁺) by many mutagens that cause base-pair substitutions. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. TA1537 is more sensitive than TA1538 to mutation by some acridians and benzantracenes, but the difference is quantitative rather than qualitative. TA100 is derived from TA1535 by the introduction of the R factor plasmid pKM101.⁶ The introduction of this plasmid, which confers ampicillin resistance to the strain, greatly enhances the sensitivity of the strain to some base-pair substitution mutagens. We have shown that mutagens such as benz. 1 chloride and 2-(2-furyl-3-5-nitro-2-furyl) acrylamide (known as AF2) can be detected in plate assays by TA100 but not by TA1535. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens--e.g., 104-141, benzo a pyrene, aflatoxin B₁, and 7,12-dimethylbenz. a anthracene. TA100 is derived from TA1538 by the addition of the same plasmid, and the plasmid makes this strain more sensitive to some mutagens.

All the indicator strains are stored at -80°C. For each experiment, a 10⁸ inoculum from frozen stock cultures is grown overnight at 37°C in a minimal broth consisting of 1% tryptone and 0.5% yeast extract.

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stationary overnight growth, the cultures are shaken for 3 to 4 hours to ensure optimal growth. Each culture is checked for sensitivity to crystal violet. The presence of the rfa^- mutation makes the indicator strains sensitive to this dye, whereas the parent strain, rfa^+ , is not sensitive to the dye. However, the mutation is reversible, leading to the accumulation of rfa^+ cells in the culture. Therefore, the cells must be tested routinely to ensure their sensitivity to crystal violet. Each culture also is tested by specific mutagens known to revert each test strain (positive controls).

To a sterile 13 x 100 mm test tube placed in a 43°C heating block, we add in the following order:

Assays in agar

- (1) 2 ml of 0.6% agar*
- (2) 0.1 ml of indicator organisms
- (3) 0.20 ml of metabolic activation mixture (optional)
- (4) Up to 100 μ l of a solution of the test chemical.

For negative controls, we use steps (1), (2), and (3) (optional) and 100 μ l of the solvent used for the test chemical.

This mixture is stirred gently and then poured onto minimal agar plates.** After the soft agar has set, the plates are incubated at 37°C for 2 days. The number of his^+ revertants (colonies that grow on plates lacking a sufficient amount of histidine to support colony formation) are counted and recorded. Some of the revertants are routinely tested to confirm that they are his^+ , require biotin, and are sensitive to crystal violet rfa^- .

* 0.6% agar contains 0.05 mM histidine and 0.05 mM biotin.

** Minimal agar plates consist of 15 g of agar, 20 g of glucose, 0.2 g of $MgSO_4 \cdot 7 H_2O$, 2 g of citric acid monohydrate, 10 g of K_2HPO_4 , and 3.5 g of $NaH_2PO_4 \cdot H_2O$ per liter.

Saccharomyces cerevisiae 20

The yeast S. cerevisiae 20 is a diploid heterozygous for a mutation in an adenine-metabolizing enzyme.⁷ Cells homozygous for this mutation produce a red dye when grown on medium containing adenine. Adenine-requiring homozygotes can be generated from the heterozygotes by mitotic recombination. Many mutagens increase the frequency of mitotic recombination. Mitotic recombination is indicated by the development of colonies with red pigmentation, and the degree of conversion to this pigmented colony indicates the mutagenicity of a compound or its metabolite.⁸

The Saccharomyces test strain from the liquid nitrogen is grown overnight at 30°C with aeration of 1.0% tryptone and 0.5% yeast extract. The cells are washed twice in 0.067M PO_4 buffer (pH 7.4) and resuspended in the same buffer at a concentration of 10^8 cells/ml.

The in vitro yeast mitotic recombination assay in suspension consists of 5×10^7 washed, stationary-phase yeast cells in 1 ml of 0.067 M PO_4 buffer (pH 7.4) and 50 mg/ml of the test chemical (or a fraction of the concentration required to give 50% killing). The suspension is incubated at 30° for 4 hours. After incubation, the sample is diluted serially in sterile saline and plated on tryptone-yeast agar plates. Plates of a 10^{-3} dilution are incubated for 2 days at 30°C, followed by 2 days at 4°C to enhance the development of the red pigment indicative of adenine-negative homozygosity. To detect red colonies or red sectors, we scan the plates with a dissecting microscope at 10 \times magnification. Plates of a 10^{-5} dilution are incubated for 2 days at 30°C for determination of the total number of colony-forming units.

The in vitro yeast mitotic recombination assay in suspension with metabolic activation is conducted as above with the addition of the metabolic activation system to the incubation mixture.

Aroclor 1234-Stimulated Metabolic Activation System

Some carcinogenic mutagens (e.g., dimethylnitrosamine) are inactive unless they are converted to their active form by being metabolized. Amer

has described the metabolic activation systems we use.⁹⁻¹⁰ Adult male mice are given a single 500- μ g/kg intraperitoneal injection of a poly-chlorinated biphenyl (Aroclor 1254).¹⁰ Four days after the injection, the animals' food is removed. On the fifth day, the mice are killed.

The livers are removed aseptically and placed in preweighed, sterile glass beakers. The organ weight is determined, and all subsequent operations to the metabolic activation step are conducted in an ice bath. The organ is washed in an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 5000 \times g, and the supernate is removed and stored in liquid nitrogen. To the postmitochondrial supernate are added $MgCl_2$, KCl, glucose-6-phosphate, TPN, and sodium phosphate (pH 7.4).

RESULTS AND DISCUSSION

Thermolin and SMP were not antagonic in assays with five strains of S. typhimurium or with S. cerevisiae D3.

Table 1 presents the results of the microbiological assays with S. typhimurium. The compounds were tested over a wide range of doses, from 1 to 2000 μ g. Thermolin was neither antagonic nor toxic in these assays. SMP was toxic at doses of 200 μ g/plate and higher but it was not antagonic. The results are an average of at least two experiments conducted on separate days.

Table 2 presents the results of the microbiological assays for mitotic recombination in S. cerevisiae D3. The compounds were tested once over a broad range of doses to determine the maximum tolerable dose to the microorganism. They were tested a second time at a narrow range of doses to determine their effect on mitotic recombination. A positive response is indicated by a more than threefold increase in the absolute number of mitotic recombinants per milliliter as well as in the relative number of mitotic recombinants per 10^5 survivors.

Neither compound was antagonic in any of the assays with S. typhimurium and neither increased mitotic recombination in the assays with S. cerevisiae; therefore, we do not believe they are antagones by these procedures.

Table 1
MICROBIOLOGICAL ASSAYS OF
3-METHYL-1,10-PHENANTHROLINE AND THIOCOLIN
IN FIVE STRAINS OF SALMONELLA TYPHIMURUM

Compound	Metabolic Activation	Dose (microgram/plate)	S. typhimurium Strain				
			TA1538	TA1537	TA1535	TA98	TA104
Negative control	-		46	13	14	104	120
	+		38	18	23	71	147
Positive controls							
5-Propylthiouracil	-	80	800				
9-Amino acridine	-	100		800			
2-Nitrofluorene	-	80			448		
AP-2	-	0.1				280	620
2-Anthranic acid	+	25	370	800	1800	1100	1700
3MP	-	1	83	11	22		150
	-	5	32	12	16		120
	-	10	30	19	9	97	110
	-	50	42	17	11	87	120
	-	100	49	15	11	52	120
	-	800	0	0	0	0	0
	+	1	31	19	22		
	+	5	39	14	35		
	+	10	29	18	31	104	
	+	50	25	18	26	142	
+	100	24	13	19	84		
+	800	0	0	0			

Table 1 (concluded)

Compound	Metabolic Activation	Dose (microgram/plate)	<i>S. typhisarium</i> Strain				
			TA1835	TA1837	TA1838	TA98	TA100
negative control	-		32	13	14	47	137
	+		40	18	23	61	164
positive controls							
γ-Propiolactone	-	80	695				
9-Amino acridine	-	100		590			
2-Nitrofluorene	-	80			443		
AF-2	-	0.1				440	1150
2-Anthracene	+	20	387	580	1800	1600	1700
Thermolin	-	10	38	14	19	38	118
	-	80	37	12	19	39	129
	-	100	46	11	18	49	136
	-	500	20	7	17	39	143
	-	1000	44	12	19	46	131
	-	5000	35	10	15	36	141
	+	10	38	13	28	41	139
	+	80	39	14	27	47	126
	+	100	36	16	26	42	134
	+	500	36	15	28	42	111
	+	1000	35	15	18	51	113
	+	5000	37	13	36	45	131

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Table 2

IN VITRO ASSAYS OF 8-METHYL-1, 20-PHENANTHROLINE AND VERMICIDE IN SACCHAROMYCES CEREVISIAE 20

Compound	Mutabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per Milliliter ($\times 10^{-7}$)	Percent Survivors	Recountants per Milliliter ($\times 10^{-3}$)	Recountants per 10^6 Survivors
negative control	-	0.2	100	6.0	10	
		3.3	100	6.3	12	
M	-	0.2	66	Taste	4.0	14
		0.1	68		2.0	3.0
		0.05	71		6.0	16
		0.01	84		7.3	16
		0.005	81		11	22
		0.3	Taste		4.1	9.0
		0.27			77	
		0.17			74	
		0.05			78	
		0.01			91	
0.005	91	7.0	15			
negative control	-	0.2	100	6.0	10	
		3.3	100	6.3	12	
in	-	5	84	11	21	
		1	84	12	21	
		0.5	79	3.0	1.1	
		0.1	100	4.0	1.4	
		0.05	87	4.0	1.4	
		5	100	6.0	1.0	
		1	100	3.0	1.0	
		0.5	106	7.5	1.0	
		0.1	92	4.0	1.0	
		0.05	113	6.0	1.0	

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**EFFECTS OF THIOCELLIN^(R) 101
ON THE IN VITRO INDUCTION OF SISTER
CHROMATID EXCHANGES IN CHINESE
HNEYER Ovary CELLS**

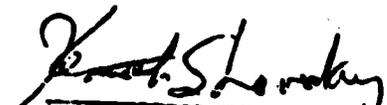
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CONTENTS

Page No.

1. INTRODUCTION	1
1.1 Objective of the study	1
1.2 Principles of the assay	1
2. MATERIALS	
2.1 Test substance	
2.1.1 Description	2
2.1.2 Sample preparation	2
2.1.3 Formulation	2
2.2 Negative control substance	
2.2.1 Description	2
2.3 Positive control substance	
2.3.1 Description	3
2.3.2 Formulation	4
2.4 CHO cells culture	4
2.5 Microsomal activation system	4
3. METHODS	
3.1 Toxicity and dose determination	5
3.2 Cells treatment	5
3.2.1 Non-activated system	
3.2.2 Activated system	
3.3 SCEs analysis	6
3.4 Statistical analysis	6
4. RESULTS	
4.1 Preliminary toxicity screening assay	7
4.2 SCE induction	7
4.2.1 Non-activated assay	
4.2.2 Activated assay	
5. CONCLUSION	10
6. TABLES AND GRAPHS	11
Appendix A: Verification Sheet	14
Appendix B: Protocol Amendments	
Quality Assurance Report	15

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1. INTRODUCTION

1.1 Objective of the study

The objective of this *in vitro* assay is to evaluate the ability of a test substance to induce SCE in CHO cells, with and without metabolic activation.

1.2 Principles of the assay

Numerous studies have shown that the exposure of Chinese hamster cells (CHO) or human lymphocytes to certain chemical agents results in an increase of the frequency of sister chromatid exchanges (SCEs). These SCEs are the result of an exchange of DNA-replication products and template that can be visualized in metaphase chromosomes as a symmetrical exchange between sister chromatids at an homologous locus. Although the molecular mechanism of the exchange is unknown, the ability to observe changes at the chromosomal level presents a biological system with great potential for detecting mutagens.

In the more recent procedures for SCE assay, cells are grown in the presence of BrdU, a thymidine analog, for two replication cycles, and metaphase-chromosome preparations are made. When resulting second-division chromosomes are treated with a combination of a fluorescent dye and Giemsa (Lett, 1973), the substituted chromatids stain differentially; one is dark and the other is pale.

The use of rat-liver microsomal-activating system (S9) has made possible the application of the *in vitro* SCE test to compounds that require metabolic activation, such as the procarcinogens.

Numerous studies have shown that known or suspected mutagens, clastogens or carcinogens, can induce SCEs, and therefore, the assay can be used for semi-quantitative assessment of genetic risk. In addition, Lett et al (1980) have reported that increased yields of SCEs using CHO cells occur at such lower concentrations than those required to produce chromosome aberrations.

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2. MATERIALS

2.1 Test Substance

2.1.1 Description: The following information on the test substance was supplied by the sponsor:

Name: Thiomolin (TM) 101; Tetrakis (2-chloroethyl) ethylene diphosphate
CAS No.: 33125-86-9
Composition: $C_{10}H_{16}Cl_4O_4P_2$
Physical state: liquid at room temperature
Purity: Not available
Stability: Unstable at $>100^{\circ}C$
Density: 1.45 g/ml at $25^{\circ}C$
Solubility: Soluble in H_2O , 3% at $24^{\circ}C$
Storage conditions: Stored in BSC's sample repository, at room temperature, in darkness, OSHA cabinet
Safety precautions: Avoid contact with skin, eyes, clothing. Avoid breathing mist or vapor. Do not take internally
Supplier: Olin Corporation
Lot/Batch No.: ACF-1018
Expiration date: not given

2.1.2 Sample Preparation

No preliminary sample preparation nor processing was necessary nor required before formulation of the test material.

2.1.3 Formulation

The test material was assayed in solution, using DMSO as a vehicle. A 10% (v/v) solution was prepared immediately prior to use, and aliquots added to the exposure medium; the final concentration (8 v/v) in the incubation flask is given for each assay, in the results section.

2.2 Negative Control Substance

2.2.1 Description

Name: Dimethylsulfoxide; DMSO
CAS No.: 000067685
Composition: C_2H_6SO
Physical state: liquid at room temperature
Purity: reagent grade
Stability: indefinite
Density: 1.095 g/ml

Storage conditions: at 25°C, original container
Safety precautions: avoid topical and respiratory exposure
Supplier: Fisher Scientific Co.
Lot/Batch No.: 70006
Expiration Date: 2/85

2.3 Positive Control Substances

2.3.1 Description

- a. Name: Nitroquin C
CAS No.: 00000077
Composition: $H_2O, C_{15}H_{18}$
Physical state: solid
Color: brown-gold
Purity: not known
Stability: at least 4 years at 4°C, unopened original container
Solubility: soluble in H_2O
Storage conditions: at 4°C
Safety precautions: avoid topical and respiratory contact
Supplier: Sigma Chemical Co.
Lot/Batch No.: 49C-0411
Expiration Date: 6/85
Stability of formulation: not known
- b. Name: Cyclophosphamide, CP
CAS No.: 00000100
Composition: $H_2O, PCl_2, C_2H_7N_5$
Physical state: powder
Color: white
Purity: ~~not available~~
Stability: at least 4 years at 4°C
Solubility: soluble in H_2O at 40 mg/ml
Storage conditions: at 4°C
Safety precautions: avoid topical and respiratory contact
Supplier: Sigma Chemical Co.
Lot/Batch No.: 69C-0034 and 69C-0510
Expiration Date: 11/85
Stability of formulation: undetermined, prepared fresh with each use

2.3.2 Formulation

Stock solutions of the positive control agents were prepared, and aliquots added to the medium:

Chemical	Solvent	Stock Solution	Concentration per flask (ug/ml)
Mitomycin C	H ₂ O	50 ug/ml	0.01, 0.03, 0.06
Cyclophosphamide	DMSO	5 mg/ml	10, 15, 20, 25

Stock solutions of Mitomycin C are stored at 4°C and used for a period of up to 6 months; stock solutions of Cyclophosphamide are prepared fresh prior to use.

2.4 CHO Cells Culture

Cells used in this assay were obtained from Dr. Latt at the Children's Hospital Medical School, Boston, MA. Dr. Latt originally obtained the line from Dr. Pardee at the Sydney Farber Cancer Center, Boston, MA. This is a permanent cell line with an average cycle time of 16-18 hours.

2.5 Microsomal Activation System

An S9 mixture prepared from rat livers, was used in the activated assay. The S9 mix consisted of one part of a rat liver S9 fraction prepared from Aroclor 1254-treated rats, and nine parts of a cofactor mixture consisting of 8 mM Mg Cl₂, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, and 100 mM Na₂HPO₄ buffer pH 7.4. The S9 mixture was prepared fresh and maintained at 4°C during use, for a maximum of up to 5 hours.

The following S9 fraction was used in the study:

Source: Litton Bionetics, Kensington, MD
Lot/Batch No.: FDL 127
Expiration Date: 6/86
Storage Conditions: -80°C

3. METHODS

3.1 Toxicity and Dose Determination

The effect of the test substance on the survival of the indicator cells was determined by exposing the cells to a range of the test material concentrations in complete growth medium.

In the non-activated system, the toxicity of the test substance on CHO cells was measured by the absence of scored division cells induced by a 30-32 hour exposure in presence of BrdU and different concentrations of the test substance. In the activated system, the toxicity of the test substance was assessed by growing cells for 4 hours in serum free culture medium in presence of a S9 metabolic activation system and different concentrations of the test substance; cells were then cultured for another 30 hours in a medium containing BrdU.

After incubation, cells were harvested, slides made and stained, and the cells observed to determine the concentrations that prevented two cycles of cell growth. Details of these procedures are given in Section 3.3.

3.2 Cells Treatment

CHO cells in T-75 plastic flasks were cultured at 37°C in 10 ml of Ham's F10 medium supplemented with 10% (v/v) Newborn calf serum, 100 units/ml penicillin, and 100 u/ml streptomycin (normal medium). The flasks were seeded with $1-1.5 \times 10^5$ cells and cultured for approximately 24 hours, at the end of which time the cells were growing exponentially.

3.2.1 Non-activated System

To determine if the test substance could induce SCEs in the absence of S9 mix, the medium in the flasks with cells that had been cultured for 24 hours was replaced with medium containing 2×10^{-5} M BrdU and 10.0% (v/v) Newborn calf serum. Various concentrations of the test substance in DMSO were added to each flask. The treated cells were allowed to grow for 27-28 hours (two doubling times). Vinblastine was added at a final concentration of 0.25 u/ml for the final 2-3 hours of BrdU incubation.

3.2.2 Activated System

To determine if the test substance could induce SCEs in the presence of S9 mix, the medium in the flasks with cells that had been cultured overnight was replaced with serum free media. Various concentrations of the test substance dissolved in DMSO were added to each flask, together with S9 mix and the incubation continued at 37°C. After 4 hours the medium with the test substance and S9 mix was replaced with normal medium containing BrdU, and the cells were cultured for an additional 30 hours.

As controls for the 4-hour exposure, some cells were treated identically except that the 100 μ M mix was omitted.

3.3 SCEs Analysis

At the end of the incubation period, metaphase cells were collected by treatment with trypsin and concentrated by centrifugation with a table top centrifuge for 5 minutes. The cells were suspended in hypotonic 0.03 M KCl and 0.01 M sodium citrate for 2 minutes to spread the metaphase chromosomes and then were fixed in 3:1 methanol: acetic acid. After three washings in the fixative, drops of the concentrated cell suspension were placed on glass slides, and air dried. Slides were stained in a solution of 50 μ g/ml 33258 Hoechst in 1/3 phosphate buffered saline. A #1 cover slip was placed over the slide and the cells were irradiated with a UV lamp for 30 minutes. The cover slips were removed, the slides were rinsed with deionized water and incubated in 2X SSC for 15 minutes at 65°C (SSC: 0.015 M sodium citrate, 0.15 M NaCl). The slides were rinsed with H₂O and stained with SA Gieson for 5 minutes at room temperature.

Approximately equal number of metaphase cells from each flask were photographed through a microscope using a 100 X objective and a 35 mm camera with automatic exposure, and using high contrast film and processing. Photographic prints (5 x 7 in) were made from these negatives, and second-division cells were scored for the number of SCEs per cell.

3.4 Statistical Analysis

Results were analyzed for the effects of the test substance concentrations, by using a model 1 fixed treatment single classification analysis of variance (one-way ANOVA), according to procedures described by Sokal and Rohlf (Biometry, Freeman and Co., 1969), and Snedecor and Cochran (Statistical Methods, Iowa State University Press, 1980). In all cases, a level of significance $\alpha = 0.05$ was used to define a statistically significant effect.

The trend lines in Figure 1 were constructed using the method of linear regression to obtain the best-fitting straight line with the point of origin fixed on the pooled negative control SCE value.

4.0 RESULTS

4.1 Preliminary Toxicity Screening Assay

Results of the effect of different concentrations of Thiamolin 101 on the survival of CHO cells growing in medium containing DMSO, are shown in Table 1.

In the non-activated assay, concentrations of the test substance in the flask ranging from 0.002 to 0.008 (v/v) were used. Results indicate that Thiamolin 101 was toxic to cells at concentrations equal or greater than 0.004, and that second division metaphase cells were only observed in those cells treated with 0.002 Thiamolin 101 or lower concentrations. In view of these results, a concentration of 0.002 was used as the upper limit for the range of concentrations of Thiamolin 101 used in the non-activated assay.

In the activated assay, concentrations of the test substance in the flask ranging from 0.002 up to 0.1000 (v/v) were assayed for solubility; only those concentrations below 0.0004 were totally miscible with the medium. In the cytotoxic analysis, no metaphase cells were found at or above 0.0004. Few metaphase cells were found among those cells treated with 0.050 Thiamolin, although all of these were differentially stained. Cells treated with lower concentrations were in second division. These results indicated the use of 0.0004 as the upper limit for the range of concentrations of Thiamolin 101 in the activated assay.

4.2 SCE Induction

4.2.1 Non-activated assay

Results on the induction of SCEs in CHO cells by exposure to various concentrations of Thiamolin 101 in absence of metabolic activation system, are shown in Table 2.

In a first assay (assay 24), Thiamolin was tested at concentrations of 0.001, 0.003, 0.005, 0.006 and 0.00750 (v/v). The background frequencies of SCEs/cell were 12.0 for DMSO and 11.5 for sodium only. Mitomycin C resulted in 88.1 and 94.8 SCEs/cell. The statistical analysis of the frequency of SCEs, including those from the negative controls, indicated a significant effect of the test substance on the induction of SCEs. Significance of the results was attributed to the higher frequencies observed in cells treated with greater than 0.0030, as compared to the negative controls and lower concentrations.

In a second assay (assay 25), duplicate flasks at 0.004, 0.006, and 0.0075% Thiomol in DMSO were assayed. The background frequencies of SCEs/cell were 9.4 and 10.0 for DMSO and 8.3 for medium only. The analysis of variance indicated a significant effect of the test substance on SCE frequencies. Most of the significance was attributed to the SCE frequency for 0.006% Thiomol (9.3) compared to that from 0.0075% (11.3). The negative control data apparently contributed little to the significant result suggesting that the statistical significance was not biologically meaningful.

Finally, results from assays 24 and 25 were pooled and analyzed for the effects of the test substance. The one-way ANOVA indicated a significant effect, which was attributed to the differences between the 0.003% and 0.005% concentrations against the DMSO control group.

4.2.2 Activated assay

Results on the induction of SCEs in CHO cells by exposure to various concentrations of Thiomol 101 in presence of metabolic activation system, are shown in Table 3.

In a first assay (assay 22), Thiomol was tested at concentrations of 0.004, 0.008, 0.010, 0.020 and 0.040% (v/v). The statistical analysis of the SCEs scores including those from the DMSO + S9 group indicated a highly significant effect of the test substance on SCE frequencies. The frequency of SCEs/cell for those treated with DMSO and S9 mix was 15.1. Cells treated with 0.004, 0.020, and 0.040% Thiomol had SCE frequencies of 21.1, 21.0, and 21.6 respectively. The significance of the results was attributed to the higher frequencies found at these concentrations, as compared against frequencies found in the DMSO + S9 group. In the same assay, cyclophosphamide induced a significant number of SCEs both at 15 and 25 $\mu\text{g}/\text{ml}$, as compared against the DMSO + S9 group; the number of SCEs induced by cyclophosphamide in absence of the S9 fraction were similar to those found in the DMSO + S9 or medium - S9 groups.

In the second assay (assay No. 26), duplicate flasks at 0.020, 0.030 and 0.040% Thiomol in DMSO were assayed. The background frequencies of SCEs/cell were 17.4 and 22.1 for DMSO + S9 and 13.6 for medium only. Cells treated with Thiomol had frequencies of SCEs/cell between 25.7 and 27.8. An analysis of variance indicated a significant effect of the test substance on the frequency of SCEs. In this analysis, pooled negative control data from cells exposed to DMSO and S9, were used. The data from cells exposed only to medium (no S9) was not used since the SCE frequency for those cells, 13.6, was lower than cells exposed to DMSO & S9, 17.4 and 22.1. A negative control using cells exposed to S9 was preferred since all the experimental flasks had both

Thiamin, DMSO, and SO. The reason for the variation in SCF frequencies for cells exposed to medium only or to DMSO + SO is not known. In general, SO does not significantly raise the SCF frequency, as evidenced by assay 22 (Table 3). Cyclophosphamide had a significant effect only in presence of the activation fraction.

Finally, results from assays 27 and 28 were pooled and analyzed for the effects of the test substances. The one-way ANOVA indicated a highly significant effect, which was attributed to the differences between the scores found at 0.02, 0.03 and 0.04% Thiamin in DMSO, as compared against the DMSO + SO negative controls.

5. CONCLUSION

The results of thernolin-101 in the non-activated sister chromatid exchange assay are inconclusive. Although one assay seemed to indicate that the compound was positive, the second assay was negative and there is no evidence that the compound induced SCEs consistently and reproducibly.

The results of the activated assay indicate that thernolin-101 is a weak inducer of SCEs. In the first assay, a statistically significant result was seen along with a slightly positive slope to the trend line. In the repeat experiment there were statistically significant differences between control populations of cells and cells exposed to thernolin-101. There is again a slight positive slope to the trend line, although the data may be better fitted by a non-linear curve with a plateau at approximately 0.02%.

TABLE 1

Preliminary Toxicity Screening Assay of Thiamin 101

Assay	Concentration^a (%, w/v)	Result
Non-activated	0.000	No viable cells
	0.000	No viable cells
	0.000	No viable cells
	0.020	No second division metaphases
	0.010	No second division metaphases
	0.008	No second division metaphases
	0.006	Second division metaphases
	0.004	Second division metaphases
	0.002	Second division metaphases
	Activated (104 39)	0.100
0.080		No metaphase cells
0.060		Few metaphase cells but in second division
0.040		Second division metaphases
0.020		Second division metaphases
0.010		Second division metaphases
0.008		Second division metaphases
0.006		Second division metaphases
0.004		Second division metaphases
0.002		Second division metaphases

^aFinal concentration in the flask

TABLE 2

Induction of SCEs by exposure of CHO cells to Thapsalgin 101
in absence of metabolic activation system^a

Assay	Compound	Concentration	SCEs/cell (mean ± SD)	No. cells counted	Statistical analysis ^{b,c,d}
24	Medium	---	11.5 ± 3.4	31	F = 5.22 n ₁ = 5 n ₂ = 197 FC = 2.21
	DMSO	0.5% (v/v)	12.0 ± 4.7	29	
	Thapsalgin 101	0.001%	11.6 ± 4.4	29	
	"	0.003%	14.2 ± 3.9	28	
	"	0.005%	15.1 ± 5.5	31	
	"	0.006%	15.7 ± 6.4	27	
	"	0.0075%	15.6 ± 5.9	28	
	Mitomycin	0.03 ug/ml	94.8 ± 14.5	13	
"	0.06 ug/ml	89.1 ± 9.3	8		
25	Medium	---	8.3 ± 2.6	26	F = 2.27 n ₁ = 6 n ₂ = 236 FC = 2.10
	DMSO	0.5%	10.0 ± 3.7	30	
	"	0.5%	9.4 ± 2.8	24	
	Thapsalgin 101	0.004%	8.7 ± 4.1	22	
	"	0.004%	10.2 ± 3.9	31	
	"	0.006%	8.3 ± 3.5	26	
	"	0.006%	10.2 ± 3.4	25	
	"	0.0075%	9.8 ± 3.8	29	
	"	0.0075%	11.3 ± 4.0	30	
	Mitomycin C	0.01 ug/ml	66.5 ± 14.2	15	

^a Results of two separate assays are given, and results of scores obtained from different flasks

^b Results of one-way ANOVA; F-values, their respective degrees of freedom, and tail probabilities are given.

^c Negative control data used in the analysis of variance was pooled from the DMSO and medium controls. The values used in Assay 24 were: n = 60, weighted average = 11.7, and pooled S.D. = 4.1. The values used in Assay 25 were: n = 80, weighted average = 9.3, and pooled S.D. = 2.1.

^d Critical value (t) are for α = .05

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TABLE 3

Induction of SCNs by exposure of CHO cells to Thapsolin 101 in presence of metabolic activation system^a

Assay	Compound	Concentration	SCNs/cell (mean ± SD)	No. cells counted	Statistical analysis ^{b,c,d}	S.E.	
22	Medium (-S9)	—	16.1 ± 5.4	29		1	
	DMSO (+S9)	0.4%	15.1 ± 4.6	30		0.62	
	Thapsolin 101	0.0001	21.1 ± 8.2	30	F = 4.57 D.F. = 5 D.F. = 164 FC = 2.21	1.49	
	"	0.0004	17.3 ± 6.0	24		1.22	
	"	0.0100	17.4 ± 6.4	29		1.18	
	"	0.0200	21.0 ± 6.5	30		1.18	
	"	0.0400	21.6 ± 8.0	27		1.53	
	Cyclophosphamide (+S9)	15 ug/ml	69.4 ± 11.5	20		2.12	
	"	25 ug/ml	62.9 ± 10.7	20		4.12	
	Cyclophosphamide (-S9)	25 ug/ml	15.4 ± 6.5	30		1.18	
	26	Medium (-S9)	—	13.6 ± 3.8	29		0.70
		DMSO (+S9)	0.4%	17.4 ± 5.4	19		1.46
"		0.4%	22.1 ± 6.5	29		1.20	
Thapsolin 101		0.0200	25.9 ± 8.7	31	F = 3.05 D.F. = 6 D.F. = 189 FC = 2.10	1.50	
"		0.0201	26.9 ± 11.5	28		2.12	
"		0.0300	26.7 ± 9.4	23		1.50	
"		0.0300	27.8 ± 9.3	25		1.50	
"		0.0400	26.5 ± 11.3	13		1.50	
"		0.0400	25.7 ± 9.6	27	1.50		
Cyclophosphamide (+S9)		10 ug/ml	41.6 ± 10.6	28		1.50	
Cyclophosphamide (-S9)		20 ug/ml	15.6 ± 5.0	28		1.50	

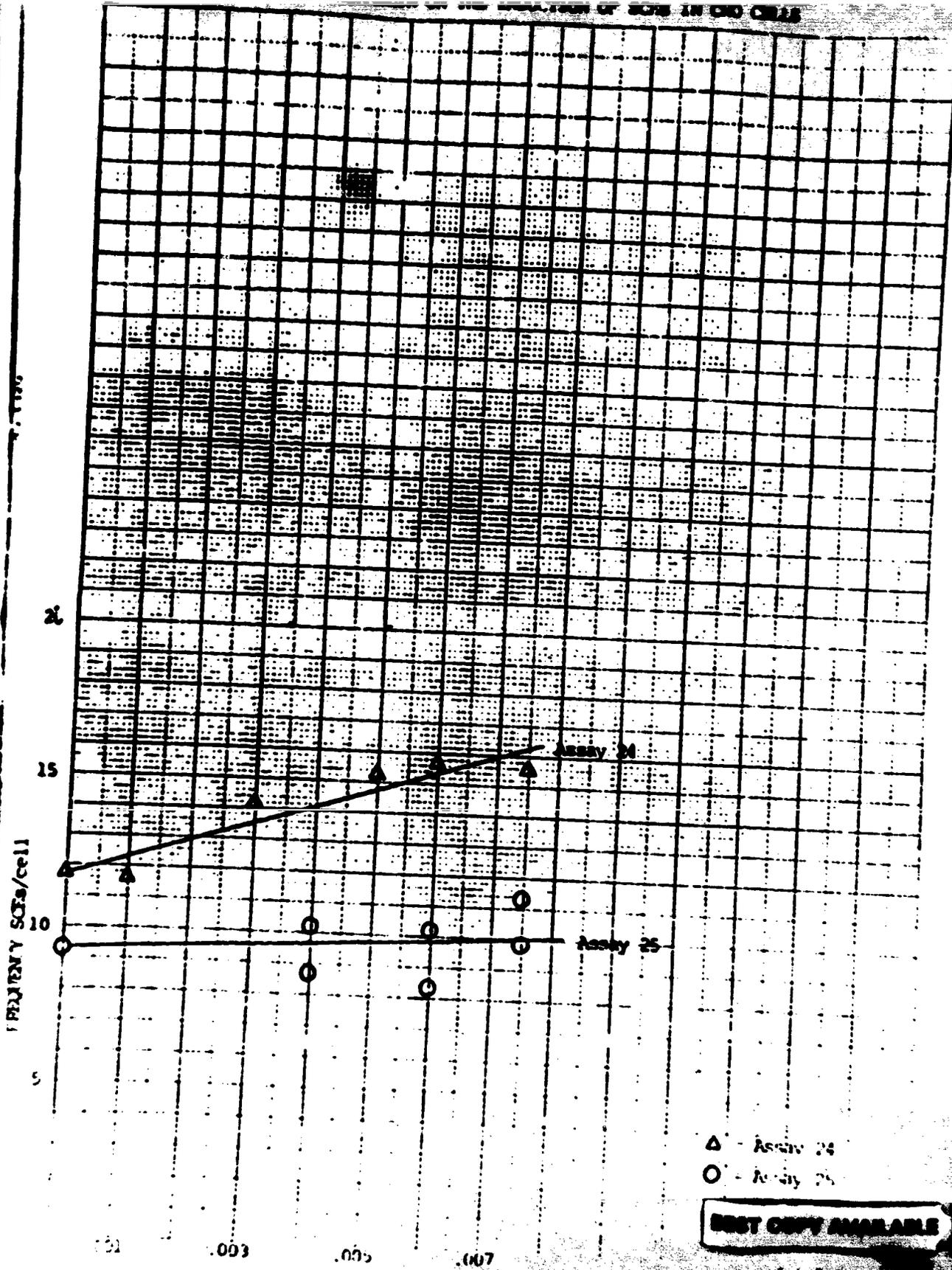
^aResults of two separate assays are given, and results of scores obtained from different flasks

^bResults of one-way ANOVA; F-values, their D.F., and tail probabilities are given.

^cResults of two-way ANOVA; F-values, their D.F., and tail probabilities are given.

^dResults of two-way ANOVA; F-values, their D.F., and tail probabilities are given.

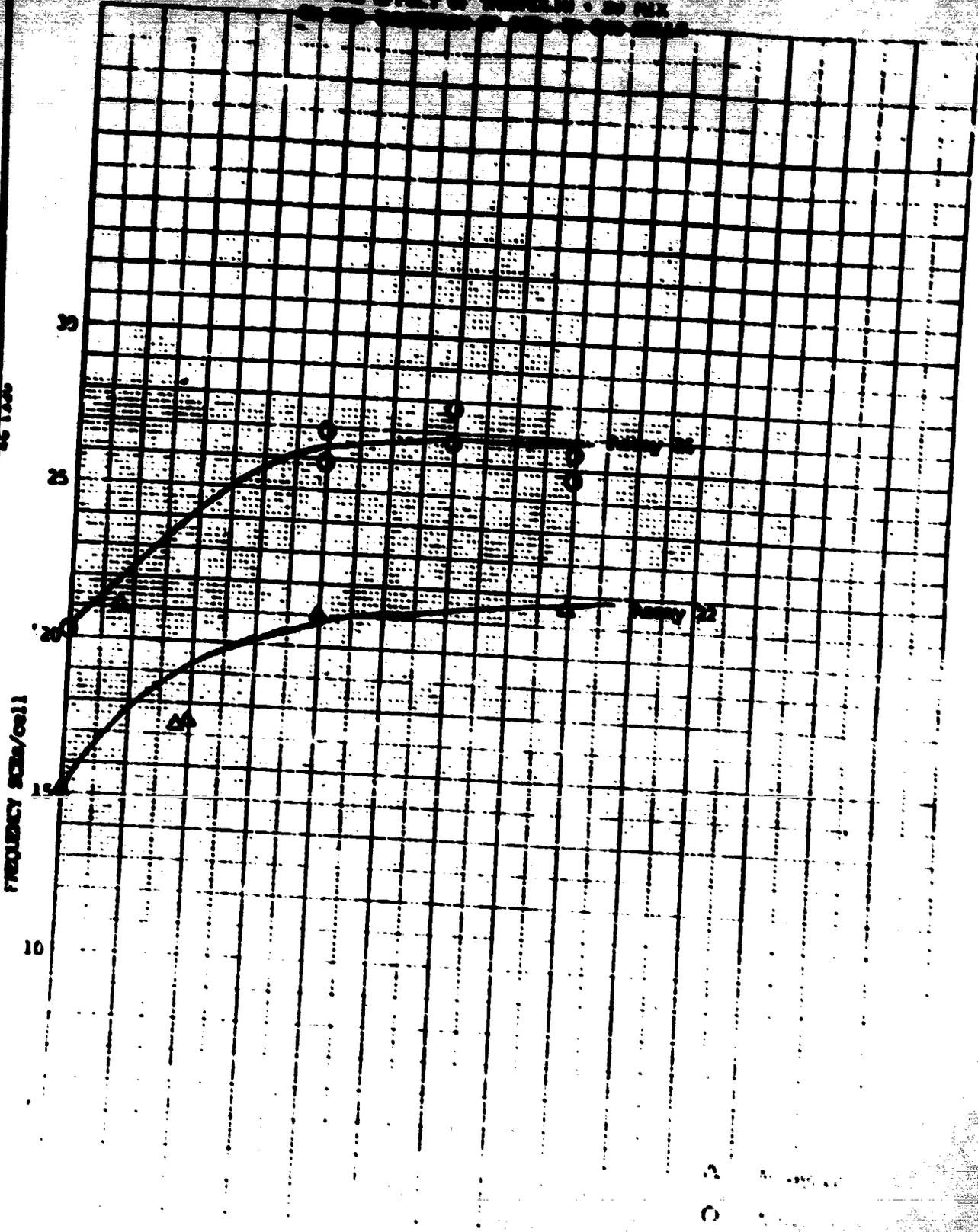
THE EFFECT OF BARRIER ON THE DEGRADATION OF SOCs IN CRD CELLS



△ - Assay 24
○ - Assay 25

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THE EFFECT OF TEMPERATURE ON THE
FREQUENCY CAPACITY OF THE CELL



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APPENDIX A
VERIFICATION FORM

1. **Supervisory Personnel**

Kenneth S. Lovelady, Ph.D., Study Director

Justine Gorodacki, M.S., Technical Supervisor

Peter J. Mione, M.S., Manager, Quality Assurance

2. **Dates**

Test Material: Received: 12/14/81

Technical Work: Initiated: 1/20/82

Completed: 3/10/82

3. **Storage Location Information**

Test material: Sample Storage Cabinet, Room 177, BSC

Raw data: Bioassay Systems Corporation Archives

Final report: Bioassay Systems Corporation Archives

BIOSSEY SYSTEMS CORPORATION
225 Wilkes Road Avenue
Warren, MI 48090

QUALITY ASSURANCE REPORT

SPONSOR: Olin Corporation

TEST SUBSTANCE: ThermoLin[®] 101 (NDC 010304-1)

FINDINGS: The report on the effects of ThermoLin[®] on the in vitro induction of SCEs in CHO cells was reviewed by Biosassy Systems Corporation Quality Assurance Unit personnel. All reported results were inspected and found to accurately reflect the original data. This study was performed in compliance with Good Laboratory Practice Regulations. However, dosage analysis was not performed on any of the dose formulations. Laboratory audits were not conducted for this study, however, dose prep and administration were audited for other SCE assays conducted during the same time period.

DATE: 5/3/82

QUALITY ASSURANCE OFFICER: Gregory H. Rival

DISCLAIMER

The results and conclusions of this study are based upon tests conducted in the laboratories of Bioassay Systems Corporation. All tests were conducted in accordance with generally accepted analytical techniques and current procedures for biological testing. Bioassay Systems disclaims, however, any responsibility for the ultimate selection of said tests. No attempt has been made to either compare the results of these tests to the results obtained from other analytical techniques or to independently evaluate the efficacy of the laboratory procedures utilized. Consequently, no representations, either expressed or implied, are made or are intended to be made regarding the validity of these tests.

0 0 6 4

In vitro gene mutation assay (SUDR locus) in
cultured Chinese hamster ovary (CHO) cells on
Thymidine - 101

Biossary Systems Corporation
Project Number 10994

Prepared for:

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Kenneth S. Lovelady, Ph.D.
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Justine Gorodecki, M.S.
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May 4, 1975
Date

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TABLE OF CONTENTS

	<u>Page No.</u>
I. INTRODUCTION	1
II. PROJECT DESCRIPTION AND METHODS	3
A. Gene Mutation Assay (CMO/REPORT)	3
1. Experimental Design	5
2. Results	6
3. Conclusions	7
4. Tables	8
III. QUALITY ASSURANCE REPORT	

1. INTRODUCTION

Objective of Study

The purpose of this study was to assess the ability of Thimulin-101 to induce mutations at the HGPRT gene locus in Chinese hamster ovary (CHO) cells.

Hymanthine-guanine phosphoribosyl transferase (HGPRT) is a purine salvage enzyme which provides a scavenger pathway for the synthesis of purine nucleotides. (Cusby and Froh, 1979).

There are several reasons for using the HGPRT system for assaying compounds for mutagenic activity in mammalian cells *in vitro*. (1) It is one of the most intensively studied genes in cultured mammalian cells. (2) It is relatively easy to select HGPRT⁻ mutants. Since the gene is located on the X chromosome, HGPRT⁻ cells are created by a single mutagenic event regardless of the origin of the cultured cells (males have one X chromosome to mutate while females have only one functional X chromosome). (3) The mutagenic effect is dose-dependent for several classes of mutagens, such as ethylmethanesulfonate (EMS), ICR-191, metallic compounds, and irradiation by X-rays and ultraviolet light. The system can also be used to detect mutagenicity of promutagens such as dimethylnitrosamine which require activation from rat liver microsomal enzymes (S9 preparation) before mutagenic activity is expressed.

Mutants deficient in HGPRT activity are selected by growing mammalian cells in medium containing 6-thioguanine (6-TG). These purine analogues are HGPRT substrates and are converted to toxic nucleotides, thus killing the wild type cells. Mutant cells, those lacking HGPRT activity, cannot metabolize the purine analogues and thus survive the selection step.

The best established HGPRT system has been developed at the Oak Ridge Laboratories under Dr. Haie, and it quantifies the frequency of mutation at the HGPRT locus in Chinese hamster cells (either lung cells, V79, or in ovary cells, CHO line) (O'Neill *et al.*, 1977a, O'Neill, *et al.*, 1977b, Shaw and Haie, 1978, O'Neill and Haie, 1979).

The system utilizes optimal selection conditions for such crucial parameters as the mutagen exposure time, phenotypic expression time (time needed to be expressed), 6-TG concentration, and the cell density which permits maximum mutant recovery. The V79/CHO HGPRT assay is a well-characterized mammalian cell culture system which measures the frequency of mutations induced at a specific gene locus. It is an excellent short term, *in vitro* assay, for evaluating the mutagenic activities of compounds, such as Thimulin-101.

Caskey, C. and Kruh, G. (1979) *Cell* 16: 1-9.

O'Neill, J., Brimer, P., Meehanoff, R., Hirsch, G., and Hais, A. (1977), *Mutation Research* 45: 91-101.

O'Neill, J., Couch, D., Meehanoff, R., San Sebastian, J., Brimer P. and Hais, A (1977) *Mutation Research* 45: 103-109.

Shaw, E., and Hais, A. (1978) *Mutation Research* 51: 237-254.

O'Neill, J., and Hais, A. (1979) *Mutation Research* 59: 109-118.

II. PROJECT DESCRIPTION AND DETAILS

Project: In Vitro Gene Mutation Assay in Cultured Chinese Hamster Ovary Cells (CHOV-1000)

Project Number: 10004

Test Substance Identification:

- A. **Name:** Thiazolin 101
- B. **SEC No.:** 10004
- C. **Physical State:** Liquid
- D. **Color:** Dark
- E. **Stability:** Not stable above 100°C, stability in DMSO not known
- F. **Solubility:** 0.3% in H₂O at 75°C; appears miscible in DMSO at concentrations up to 10%

Control Substance Identification:

Negative Control Material Characterization:

- A. **Name:** Dimethylsulfoxide (DMSO)
- B. **Supplier:** Fisher Scientific Company
- C. **Lot No.:** 705816
- D. **Physical State:** Liquid
- E. **Color:** Colorless
- F. **Purity:** Reagent grade
- G. **Melting Point:** 18°C
- H. **Specific Gravity:** 1.10
- I. **Storage Conditions:** SEC genetic toxicology laboratories at 23°C
- J. **Safety Precautions:** Avoid topical and respiratory contact

Positive Control Material Characterization (activated assay):

- A. **Name:** B(a)P
- B. **Supplier:** Sigma
- C. **Lot No.:** 18C-0378
- D. **Physical State:** Powder
- E. **Color:** Slight yellow tint
- F. **Purity:** On file at manufacturer
- G. **Stability:** Replace stocks once per year
- H. **Solubility:** DMSO medium
- I. **Storage Conditions:** 4°C
- J. **Safety Precautions:** Avoid topical and respiratory contact

101-B(a)P, DMSO-ethylmethanesulfonate

Positive Control Material Characterization (non-activated assay):

- A. Name: **MS**
- B. Supplier: **Eastman Kodak**
- C. Lot No.: **8817**
- D. Physical State: **Liquid**
- E. Color: **Slight yellow tint**
- F. Purity: **Reagent grade**
- G. Stability: **Replace stocks once per year**
- H. Specific Gravity: **1.167**
- I. Storage Conditions: **4°C**
- J. Safety Precautions: **Avoid topical and respiratory contact**

Activation Mixture:

S9 liver microsomes fraction from Aroclor induced Sprague-Dawley rats (Litton Bionetics Lot # NDL 127).

Storage Location of Data

Original Data: Bioassay System Corporation Archives

Final Report: Bioassay Systems Corporation Archives

Supervisory Personnel Involved in Study

Dr. Kenneth S. Lovelady, Study Director
Justine Gorodecki, Technical Supervisor
Peter Mione, Quality Assurance Manager

Dates:

Initiated technical work: 1/5/81
Completed technical work: 4/5/82

EXPERIMENTAL DESIGN

1. Isolation of Test Sample

The test sample was stored at room temperature. All dilutions were made in 100% dimethylsulfoxide (DMSO).

2. Experimental Procedures

a. Toxicity

The toxicity of the test substance was characterized for both activated and nonactivated conditions. From 200 to 5000 viable cells in 100 mm dishes were exposed to varying Thiamolin concentrations. For the activated assay, 0.1, 0.06, 0.03, 0.01, 0.003 and 0.001% of Thiamolin were tested. For the nonactivated, similar concentrations were tested. Exposure times for activated and nonactivated assays were 4 and 16 hours respectively. After 7 days of incubation, the cells were fixed, stained and scored. To confirm the toxicity data of the activated assay, an additional test was performed concomitantly with the mutagenicity test. Cells were seeded and exposed to Thiamolin as described below. From 16 to 19 hours after the exposure, 200 cells (total count) were seeded in petri dishes. After a week of incubation, the cells were fixed, stained and scored.

b. Mutation Induction

CNO cells were seeded at a density of approximately 5×10^5 in T25 flasks and grown for 24 hours before being exposed to the test sample. In non-activated assays the exposure time was 16 hours; in activated assays the exposure time was 4 hours in serum-free medium. Based on the toxicity data, the sample was tested at concentrations of 0.2, 0.1, 0.09, 0.08, 0.06, 0.04 and 0.02% in the activated assay, and at 0.07, 0.05, 0.03, 0.01, 0.003 and 0.0006% in the nonactivated assay. Each of the above concentrations was tested in duplicate flasks maintained independently throughout the expression time. Following removal of the test substance, the cells were replated as necessary to maintain their maximum growth rate (usually every 2-3 days). After 8-13 days following removal of the chemical (the optimum time for expression of new mutants), the cells were plated at 2×10^5 per dish in medium containing 2 ug/ml 6-thioguanine (6-TG). Usually six dishes per flask were seeded; the exact number of dishes for each case can be obtained by dividing total number of cells selected by 2×10^5 . Concomitantly, with the selection step, 200 cells from each flask were plated in medium without 6-TG to determine the cloning efficiency of the cells. Colonies were fixed with methanol, stained with Giemsa and counted after 8-9 days. The plates were scored by a trained technician. A group of cells containing a minimum of 50 cells was counted as a mutant colony. The activated part of the assay was performed by exposing cells to the test sample metabolically activated by optimal concentrations of the S9 microbial fraction mixture (see below).

c. Calibration of the Metabolic Activation System

Before testing the samples in the activated assay, the optimal conditions for metabolic activation were determined using dimethylnitrosamine (DNM). Two parameters were varied: The ratio of S9 enzymes to the S9 cofactor mix, and the volume of S9 mix added to the cells. In all cases the total volume of liquid in a 25 cm³ culture flask was 5 ml. The final amount of medium added to the flask depended both upon the amount of S9 and upon the amount of sample. For dimethylnitrosamine (DNM), the optimal activation occurred with an S9 ratio of 0.1 (0.1 ml of S9 enzymes added to 0.9 ml of cofactors) and the addition of 0.5 ml of S9 mix to the cells. Increasing the ratio of S9 to 0.3 and 0.4 did not increase the number of mutants. Based on these results, it was decided to use an S9 ratio of 0.1 (S9/S9 mix), and 0.5 ml of S9 mix per flask (Bioassay Systems Genetic Toxicology Research Book No. 1) for other promutagens and unknown samples.

RESULTS

The effects of Thiamin on the survival of CHO cells in the presence and absence of the activation mixture are presented in Table 1. Based on these results, the highest concentrations selected for the mutagenesis assays were 0.07% and 0.1% for nonactivated and activated assays respectively. The highest concentration chosen was the one allowing approximately 10% survival of the cells.

The effects of Thiamin on the induction of HGPRT⁻ mutants in CHO cells in the absence of S9 mix are presented in Table 2. Six concentrations were tested: 0.07, 0.05, 0.03, 0.01, 0.008 and 0.006%. Since the 0.07% proved to be too toxic, only the five lower ones were selected. The background mutation frequencies were obtained for F10 medium (6.1×10^{-6} and 16.0×10^{-6}) and for DMSO (12×10^{-6} and 18.6×10^{-6}) controls. Among the experimental concentrations only 0.006% (one replicate) gave a mutation frequency that approached two times the negative values. This frequency of 33.2×10^{-6} is not concentration dependent and appears in only one replicate set; therefore, it cannot be called a positive result. The positive control, EMS, induced a mutation frequency of 34.1×10^{-6} . Since the induction by EMS was relatively low, the top three concentrations were tested again (Table 2, experiment 2). The background values of media and DMSO controls were 7.5×10^{-6} , 2.7×10^{-6} and 4.1×10^{-6} , 3.1×10^{-6} respectively. None of the mutation frequencies obtained for Thiamin concentrations were significantly higher than that of the background. Two EMS controls were used in this experiment. A concentration of 360 ug/ml of EMS was used to mutagenize CHO cells, but unexpectedly cells exposed to this concentration did not grow well and could not serve as a positive control. In one replicate flask the cells died, in the other flask the toxicity allowed a selection of 0.6×10^6 cells which produced no mutants. The satisfactory EMS control consisted of CHO cells exposed to 600 ug/ml EMS on 2/11/82. The cells were carried over continuously and served as the second positive control which gave a mutation frequency of 1466×10^{-6} . It is not known why 360 ug/ml of EMS was more toxic than 600 ug/ml in this particular case.

The effect of Thiamin on the induction of HGPRT^r mutants in the presence of S9 mix is shown in Table 1. Seven concentrations were tested: 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, and 0.002. The background mutation frequencies were the following: for S9 mix - 1.3×10^{-6} and less than 1.1×10^{-6} for nonactivated benzo(a)pyrene - 16.7×10^{-6} and for thiamin - 34.1×10^{-6} . The last mutation frequency is relatively high and represents a mutation fluctuation. Only one experimental concentration, 0.10, showed a high mutation frequency, 42.9×10^{-6} , and since it was the highest concentration used, the experiment was repeated with three top concentrations. The mutation frequencies for benzo(a)pyrene (positive control) were 112×10^{-6} and 302×10^{-6} .

In experiment 02 (Table 3), 0.1, 0.05 and 0.005 were tested in the activated assay. The different background values in this experiment were 0.61×10^{-6} , 0.54×10^{-6} , 1.4×10^{-6} , 16×10^{-6} , 1.3×10^{-6} and less than 1.1×10^{-6} . None of the Thiamin concentrations induced mutations higher than the background. Benzo(a)pyrene gave mutation frequencies of 146×10^{-6} and 311×10^{-6} .

The toxicity of the administered concentrations (Table 3) verified the results presented in Table 1.

CONCLUSIONS

Under the conditions of the assay employed, the test sample, Thiamin 101, did not induce mutations at the HGPRT locus of Chinese hamster ovary (CHO) cells in culture. The tests were conducted in the presence and absence of an S9 metabolic activation system.

TABLE 1

The effect of Thermolin-101 on the survival of CHO cells with and without activation

Conc. µ	No. of cells Plated	Activated ¹		Non-Activated ²	
		No. of Colonies	Percent Survivors	No. of Colonies	Percent Survivors
0.1	2000	84/100	3	ND	ND
0.1	200	1/0	0.3	ND	ND
0.07	5000	ND	ND	200/232	9
0.07	200	ND	ND	5/4	4
0.06	1000	75	8	ND	ND
0.06	200	27/16	12	ND	ND
0.03	1000	117/125	23	220/232	44
0.03	200	45	24	60/30	44
0.01	1000	207/236	25	336/324	65
0.01	200	51/80	35	72/39	64
0.003	1000	860/304	67	456/452	89
0.003	200	154/184	91	107/116	109
0.001	1000	484/404	48	496/506	98
0.001	200	127/161	78	100/117	106
Media	200	159/212	100	106/89	100
DMSO	200	ND	ND	88/125	ND
S9 + Media	200	20/44	Not used	ND	ND
S9 + DMSO	200	20/25	Not used	ND	ND

^{*}The sample precipitated at this concentration

$$\text{Percent survivors} = 100 \times \frac{\text{PE of sample}}{\text{PE of control}}$$

$$\text{PE (Plating efficiency)} = \frac{\text{Av. No. of Cells}}{\text{No. of cells plated}}$$

Activation was accomplished by adding 1 ml of S9 mix (0.1 ml liver enzymes plus 0.9 ml cofactors) per 10 ml medium (one dish).

DMSO - dimethylsulfoxide 0.27%

ND - not done

¹The plating efficiency of cells exposed to S9 + Media and S9 + DMSO were unexpectedly low. This data was determined as non-reliable since cells exposed to Thermolin also contained the same concentrations of S9, DMSO, and media but had much higher plating efficiencies. Survival of cells exposed to media only was used as the 100% base.

²100% survival was average PE of Media and DMSO negative controls.

0074

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TABLE 2

The effect of Thiamin-101 on the induction of HERT⁺ mutants in CHO cells in the absence of 50 Min

Sample	Conc.	No. of Plated Cells (x 10 ⁶)	Plating Efficiency (200 cells)	No. of Mutants	Mutation Frequency x 10 ⁶
Experiment 1					
Thiamin	0.07	1.2	Too toxic to select		
	0.07	1.2	Too toxic to select		
	0.05	1.2	Too toxic to select		
	0.05	1.2	110/125	3	4.1
	0.03	1.2	104/100	0	< 1.0
	0.03	1.2	111/114	3	4.4
	0.01	1.2	107/136	4	5.5
	0.01	1.2	89/92	4	7.4
	0.01	1.2	112/144	10	13.0
	0.000	1.2	134/97	23	33.2
	0.000	1.2	99/81	10	10.5
	0.000	1.2	105/145	10	13.3
	0.000	1.2	106/106	3	4.7
Media	100	1.2	121/97	4	6.1
DMSO	0.5	1.2	132/110	12	16.0
	0.5	1.2	124/127	9	12.0
EMS	360 ug/ml	1.2	90/107	11	10.6
		2.4	101/00	37	34.1
Experiment 2					
Thiamin	0.05	1.2	122/102	0	< 1.5
	0.05	1.0	140/142	4	5.7
	0.03	1.2	131/132	6	7.6
	0.03	1.2	140/135	10	11.0
	0.01	1.2	115/126	4	5.5
	0.01	1.2	106/170	3	2.0
	100	1.2	140/120	6	7.5
Media	100	1.2	127/110	2	2.7
	1	1.2	100/220	5	4.1
*EMS	360 ug/ml	1.2	162/150	3	3.1
		too toxic to select			
**EMS	600 ug/ml	0.6	0/0	0	---
		2.4	107/1.5	1953	1466

EMS - ethylmethanesulfonate; DM - dimethylsulfoxide

$$\text{Mutation frequency} = \frac{\text{No. of Mutants}}{\text{No. of Selected Cells} \times \text{PE}}$$

PE - Av. No. of colonies / 200

* In one replicate, EMS dose was high to select. In the other replicates, 10⁶ cells were used for selection.

** EMS dose was expanded to 100 ug/ml in one replicate. In the other replicates, 10⁶ cells were used for selection.

5

TABLE 3

The effect of Thiamin-101 on the induction of HGPRT⁺ mutants in CHO cells in the presence of S9 Mix

Sample	Conc. μ	No. of Plated Cells ($\times 10^7$)	Plating Efficiency	No. of Mutants	Mutation Frequency $\times 10^6$
Experiment 1					
Thiamin	0.2		Too toxic to select		—
	0.1	0.2*	102/191	8	42.9
	0.09	0.0*	126/113	7	14.6
	0.09	1.2	200/174	5	4.5
	0.08	1.2	135/116	12	15.9
	0.08	1.2	157/102	5	4.9
	0.06	1.2	89/118	6	9.7
	0.06	1.2	92/87	5	9.3
	0.04	1.2	115/126	21	29.1
	0.04	1.2	179/104	8	7.4
	0.02	1.2	110/103	1	1.6
	0.02	1.2	153/137	3	3.5
DMSO	1.0	1.2	139/124	1	1.3
	0.0	1.2	138/155	0	< 1.1
B(a)P + S9	13 μ g/ml	1.2	99/85	62	112
	13 μ g/ml	1.2	103/90	117	202
B(a)P - S9	13 μ g/ml	2.4	81/99	18	16.7
Media	100	2.4	88/117	42	34.1

*Due to toxicity of Thiamin at these concentrations, only limited number of cells were available for selection.

TABLE 3 Continued

Sample	Concn. ¹	No. of Plated Cells (x 10 ⁵)	Plating Efficiency	No. of Mutants	Mutation Frequency x 10 ⁶	Percentage Survivors
Experiment 2						
Thiourea	0.1	2.0	120/137	0	< 0.8	—
	0.50	1.2	142/128	0	< 1.3	—
	0.50	1.2	161/161	3	3.1	30%
	0.50	1.2	153/138	10	11.5	37%
	0.50	1.2	150/130	1	1.1	20%
B(a)P +SO	10 ug/ml	1.2	152/142	120	146	—
	10 ug/ml	1.2	164/147	200	311	—
B(a)P -SO	10 ug/ml	2.4	141/134	1	0.62	—
Media	100	2.4	155/155	1	0.54	8%
Media + SO	100	1.2	124/120	1	1.4	9%
	100	1.2	187/207	19	16	12%
DMSO +SO	1.0	1.2	122/127	1	1.3	—
	1.0	1.2	150/138	0	< 1.1	100%

0.5 ml of SO mix (0.1 ml liver enzymes plus 0.9 ml cofactors) were added to 5 ml medium.

$$\text{Mutation frequency} = \frac{\text{No. of Mutants}}{\text{No. of Selected Cells} \times \text{PE}}$$

PE = $\frac{\text{No. of colonies}}{\text{No. of cells}}$

DMSO - 10 dimethylsulfoxide, B(a)P - Benzo(a)pyrene

¹ Toxicity of the three thiourea concentrations was reconfirmed with the mutagenicity experiment. 5×10^5 CIP cells were exposed to Thiourea. From 16 to 19 hours after the exposure, 200 cells (total count) were seeded for toxicity; the rest of the cells were replated for expression time and selection.

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QUALITY ASSURANCE REPORT

SPONSOR: Olin Corporation

TEST SUBSTANCE: Thiazolidin-101 (NSC 610904-1)

FINDINGS: The report on the effects of Thiazolidin on the HGPRT locus in Chinese hamster ovary cells was reviewed by Biosesay Systems Corporation Quality Assurance Unit personnel. All reported results were inspected and found to accurately reflect the original data. This study was performed in compliance with Good Laboratory Practice Regulations. However, dosage analysis was not performed on any of the dose formulations. Laboratory audits were not conducted for this study, however, sample formulation and administration, selection, and data observations were audited for other HGPRT assays conducted during the same time period.

DATE: 5-4-82

QUALITY ASSURANCE OFFICER:

Gregory A. Roscoe

REVISIONS TO THE PROPOSAL

REVISIONS TO THE PROPOSAL

REVISION: Olin Corporation

THE PROJECT NO. 10004

THEY: CARCINOGENICITY

REVISIONS TO THE PROPOSAL

1. Section III F:
Biosassy System Project number-10004
2. Section III G:
Proposed Study Schedule:
 - 1) Test substance received--12/14/81
 - 2) Study initiated--1/5/82
 - 3) Study completed--5/4/82
3. Section IV:
Request characterization. See technical data provided by Olin Corporation.
4. Section IV:
Positive control dimethylnitrosamine was replaced by benzo(a)pyrene. The characterization of benzo(a) pyrene is given in the final report.
5. Section V B:
F10 medium contains 10% newborn calf serum.
6. Section B:
Range-Finding with and without S9
Change "40⁵ hrs" to "4 to 5 hrs"

DISCLAIMER

The results and conclusions of this study are based upon tests conducted in the laboratories of Bioassay Systems Corporation. All tests were conducted in accordance with generally accepted analytical techniques and current procedures for biological testing. Bioassay Systems disclaims, however, any responsibility for the ultimate selection of said tests. No attempt has been made to either compare the results of these tests to the results obtained from other analytical techniques or to independently evaluate the efficacy of the laboratory procedures utilized. Consequently, no representations, either expressed or implied, are made or are intended to be made regarding the validity of these tests.

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LABORATORY
TESTING LABORATORIES, INC.

REPORT

TERATOLOGIC EVALUATION
OF
Therolin-101 *— ill*
IN
Dutch-Belged Rabbits

7-6-60

Submitted to **Olin Corporation**
New Haven, CT 06504

Michael Knickerbocker
Michael Knickerbocker
Associate Toxicologist

Date **January 28, 1960**

Laboratory No. **6038**

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ABSTRACT

To evaluate its teratogenic potential, Thermolin-101 was administered by oral intubation to pregnant Dutch-Belted rabbits at 0, 17, 50, 150 or 450 mg/kg body weight/day on days 6 through 18 of gestation. On day 29 of gestation, all animals were killed and subjected to uterine examination. The reproductive performance of each female was recorded, and the resultant fetuses were examined for skeletal and soft tissue abnormalities.

The maternal toxicity of Thermolin-101 at 450 mg/kg was indicated by increased mortality - all females died prior to term (day 29). At levels up to 150 mg/kg, there was no evidence of toxicity, and dam body weights and weight gains during gestation did not differ from the negative control. At 150 mg/kg, Thermolin-101 significantly increased the number of resorbed fetuses; however, the number of dams affected was no different from the sham control. Fetuses from dams treated with Thermolin-101 at levels up to 150 mg/kg/day showed no dose-related effects on skeletal or soft tissue development.



Table of Contents

<u>Contents</u>	<u>Page No.</u>
Abstract	1
Introduction	1
Procedures	2
Pilot Study	3
Animals and Husbandry	2
Treatment	2
Observations	4
Teratogenic Evaluation	5
Animals and Husbandry	5
Treatment	5
Observations	7
Statistical Evaluation	8
Results and Discussion	9
Conclusion	11

<u>Tables</u>	<u>Table No.</u>
Dam Body Weights During Gestation (Pilot Study)	1
Average Litter Reproduction Data of Rabbits (Pilot Study)	2
Dam Body Weights During Gestation	3
Average Litter Reproduction Data of Rabbits	4
Summary of Skeletal Findings in Fetuses	5
Summary of Soft Tissue Findings in Fetuses	6



Table of Contents (con't)

<u>Appendixes</u>	<u>Appendix No.</u>
Protocol	I
Individual Reproduction Data of Rabbits (Pilot Study)	II
Individual Reproduction Data of Rabbits	III
Quality Assurance Inspection Schedule	IV



Introduction

This report describes the results of a study designed to evaluate the teratogenic potential of Thermolin-101 following oral intubation in pregnant Dutch-Belted rabbits during organogenesis. The study was authorized and outlined by Dr. John E. Wedig, Senior Toxicologist, Corporate Health Affairs, Olin Corporation in a letter dated December 9, 1977. As requested by the sponsor, the testing included a pilot or dose-range finding study designed to establish dose levels suitable for teratogenic evaluation.

Procedures used in the studies were further defined in Food and Drug Research Laboratories, Inc., (FDRL) Protocols and approved by Dr. Wedig on June 19, 1979. The pilot study was initiated June 19, 1979, and terminated July 19, 1979. Based on the results of the pilot study and with the approval of the sponsor, dose levels for teratologic evaluation were established. The protocol was approved by Dr. Wedig on October 20, 1979. Minor modifications were made and approved by Dr. Wedig on November 5, 1979. Testing initiated October 29, 1979 and was completed December 6, 1979.

Supplied by the sponsor, the test material was received at the Waverly Research Center of FDRL on October 4, 1978. Contained in a one gallon metal pail the material was identified as Thermolin-101, Lot No. LCV145A-91578. Provided by F.S. Natoli



of Glin, the quality control analysis of the material was as follows:

SHIPPED T-101 MONDAY 9/18
GLIN NEW HAVEN CT MCLEAN 1/630 LBS. DRUM 054 033316
LOT LCV14SA91578
ASSAY 99
ACIDITY .04
CHP 0.12
WATER 0.03
VOLATILES WT % .06
SODIUM PPM 10
PYRIDINE PPM 120
CY-179 WT % .18
HAUGARD 1.51
COLOR- GARDNER -

Procedures

Pilot Study

Animals and Husbandry

To determine dosage levels for the complete teratologic evaluation of Thermolin-101, sexually mature (5 months of age and older) Dutch-Belted rabbits were purchased from Dutchland Laboratory Animals, Inc., Denver, PA. All females were virgin.

All animals were individually housed in wire-mesh bottom cages in temperature controlled (70 ± 30°F) quarters. Charles River Rabbit Chow and fresh tap water were available ad libitum throughout the study.

Treatment

On day 0 of gestation, each female was given an injection of 0.2 ml of human chorionic gonadotropin (200 IU) v.i.a



the marginal ear vein. The females were then mated with adult males; copulation was considered positive evidence of pregnancy.

The pregnant females were randomly distributed into five treatment groups using a random number assignment sheet. Four test groups and a vehicle control group each consisting of at least 3 pregnant rabbits were established. Five females considered to be pregnant were assigned to each group to allow for maternal deaths and false pregnancies.

Beginning on day 6 of gestation and continuing daily through day 18 of gestation, the indicated materials were administered by oral intubation to the pregnant females as a solution of the appropriate concentration. The test material was prepared fresh daily using corn oil as the vehicle at concentrations necessary to be dosed on a 1 ml/kg basis. The amount of test material administered to each female was adjusted during gestation according to the most recent body weight. The dosage regimen was as follows:

<u>Group</u>	<u>Minimum Number of Pregnant Females</u>	<u>Treatment/Level</u>
K	3	Corn Oil: 1 ml/kg/day
L	3	Thermolin-101: 30 mg/kg/day
M	3	Thermolin-101: 100 mg/kg/day
N	3	Thermolin-101: 300 mg/kg/day
C	3	Thermolin-101: 1000 mg/kg/day

On day 29 of gestation, all females were killed with an overdose of sodium pentobarbital via intracardiac injection.

58



The uterine contents of each female were removed and the reproductive performance recorded. The urogenital tract of each female was examined for anatomical normality. All females that died or were sacrificed moribund during the course of the study were weighed; the weights were recorded; and all were subjected to a thorough uterine examination.

Observations

Body weights of females were recorded on days 0, 6, 9, 12, 15, 18 and 29 of gestation. All animals were observed daily for signs of toxicity and a record maintained.

At the time of sacrifice on day 29 (or if the animal died or was sacrificed moribund) the following observations were recorded: numbers of corpora lutea, implantation sites, resorption sites, live and dead fetuses, sex of fetuses and body weights of all fetuses.

All fetuses were examined immediately for external abnormalities. Then maintaining their identity as to both dam number and uterine position (ear mark fetuses) all live fetuses were placed in an incubator (-30°C) for 24 hours to determine neonatal survival. At 24 hours viability was recorded and surviving fetuses were killed by a 5-10 minute exposure to chloroform vapors.

All fetuses were examined by careful dissection and all anomalies recorded. Particular attention was paid

to any difference in size, shape and/or orientation of the major organs and blood vessels which might relate to treatment with the test material. Each fetus was then eviscerated, skinned, fixed in 70% isopropyl alcohol, and retained for possible future reference. Each fetus was processed, examined and stored in a manner retaining the identity of both dam number and uterine position.

Teratogenic Evaluation

The results of the pilot study were summarized. On the basis of the results and with the approval of the sponsor, levels suitable for teratogenic testing were chosen and the study initiated.

Animals and Husbandry

All animals used in this study were sexually mature (5 months of age or older) Dutch-Belted rabbits. All females were virgin. All rabbits were purchased from Dutchland Laboratory Animals, Inc., Denver, PA.

All animals were individually housed in wire-mesh bottom cages in temperature controlled quarters. Charles River Rabbit Chow and fresh tap water were available ad libitum throughout the study. Nesting materials were not provided to females because pregnancy was terminated prior to parturition.

Treatment

On day 0 of gestation, each female was given an



injection of 0.2 ml human chorionic gonadotropin (200 IU) via the marginal ear vein. The females were then mated with adult males as per Standard Operating Procedure. Matings took place in the male's cage and copulation was considered positive evidence of pregnancy.

The pregnant females were randomly distributed into six treatment groups using a random number assignment sheet. Four test groups, a vehicle (negative) control group, and a positive control group each consisting of at least 15 pregnant rabbits at term were established (unless excessive maternal mortality was evident at the higher doses). Twenty females considered to be pregnant were assigned to each group to allow for maternal deaths and false pregnancies. All pregnant females were individually identified by ear tags.

Beginning on day 6 of gestation and continuing daily through day 18 of gestation, the indicated materials were administered by oral intubation to the pregnant females as a solution of the appropriate concentration. The test material ~~was~~ prepared fresh daily using corn oil as the vehicle at concentrations necessary to be dosed on a 1 ml/kg basis. The positive control (6-aminonicotinamide) was administered by oral intubation as an aqueous solution at 2 ml/kg on day 9 of gestation only. The amount of test material administered to each female was adjusted during gestation according to the



most recent body weight. The dosage regimen was as follows:

Group	Minimum Number Examinant Females	Treatment/Level
A	15	Corn Oil: 1 ml/kg/day
B	15	0-aminocottinamide: 2.3 mg/kg
C	15	Thermalin-101: 17.0 mg/kg/day
D	15	Thermalin-101: 30.0 mg/kg/day
E	15	Thermalin-101: 130.0 mg/kg/day
F	15	Thermalin-101: 450.0 mg/kg/day

On day 29 of gestation, all females were killed with an overdose of sodium pentobarbital via intracardiac injection. The uterine contents of each female were removed and the reproductive performance recorded. The urogenital tract of each female was examined for anatomical normality. All females that died or were sacrificed moribund during the course of the study were weighed; the weights were recorded; and all were subjected to a thorough uterine examination. Any female that showed signs of abortion or premature delivery was sacrificed on the day such evidence was observed and subjected to a thorough uterine examination. All fetuses obtained from these dams were saved in 10% formalin.

Observations

Body weights of females were recorded on days 0, 6, 9, 12, 15, 18 and 29 of gestation. All animals were observed daily for signs of toxicity and a record maintained.

At the time of sacrifice on day 29 (or if the animal died or was sacrificed moribund) the following observations

60



were recorded: uterine weight, numbers of corpora lutea, implantation sites, resorption sites, live and dead fetuses; sex of fetuses; and body weights of all fetuses.

All fetuses were examined immediately for gross external abnormalities. Then, maintaining their identity as to both dam number and uterine position, (ear mark fetuses), all live fetuses were placed in an incubator (-30°C) for 24 hours to determine neonatal survival. At 24 hours viability was recorded and surviving fetuses were killed by exposure to chloroform vapors.

All fetuses were examined by careful dissection and all anomalies recorded. Particular attention was paid to any differences in size, shape and/or orientation of the major organs and blood vessels which might relate to treatment with the test material. Each fetus was then eviscerated, skinned, fixed in 70% isopropyl alcohol, macerated in a 3% potassium hydroxide solution, stained with Alizarian - Red S dye, cleared in glycerine, and examined under low power magnification for skeletal variations and malformations. Each fetus was processed, examined and stored for future reference in a manner retaining the identity of both dam number and uterine position.

Statistical Evaluation

Incidences of occurrence were expressed as percent, and comparisons between the negative control and test groups were



made using either 95% confidence intervals for proportions or by computation of exact probabilities. Continuous data were analyzed using a one-way completely random classification analysis of variance for fixed effects. Differences were deemed significant when the probability of rejecting the null hypothesis when true was less than 0.05. The least significant difference test was then employed to determine which test group(s) differed from the negative control. For all comparisons, the litter rather than the fetus was considered the unit of observation.

Results and Discussion

To establish dosage levels for its teratologic evaluation, Thernalin-101 was administered by oral intubation to pregnant Dutch-Belted rabbits at 0, 30, 100, 300 or 1000 mg/kg/day on days 6 through 18 of gestation. All five females administered 1000 mg/kg died before day 29 of gestation while one female treated at 300 mg/kg died. Females treated at 300 mg/kg exhibited significantly lower body weights on days 15 and 18 of gestation. Dam body weight gains during the treatment period were significantly reduced at both 100 and 300 mg/kg (Table 1). Summarized in Table 2, the reproductive data of treated dams showed a significant reduction in live fetuses and an increase in resorption sites at 300 mg/kg/day. (The Individual Reproduction Data are presented in Appendix II.)

61



Based on the results of the pilot study, and in an effort to produce maternal toxicity, dose levels for the teratologic evaluation of Thermolin-101 were established. Thermolin-101 was administered by oral intubation to pregnant Dutch-Belted rabbits at 0, 17, 50, 150 or 450 mg/kg body weight/day on days 6 through 18 of gestation.

All twenty females treated with Thermolin-101 at 450 mg/kg/day either died or were sacrificed moribund prior to day 29 of gestation thus demonstrating the material's maternal toxicity. One female in the 150 mg/kg group was sacrificed after showing signs of abortion as was one control animal. No other noteworthy changes were seen in any test group as compared to the vehicle control.

Dam body weights during gestation are summarized in Table 3. While maternal toxicity and mortality were evident at 450 mg/kg, females treated at levels up to 150 mg/kg showed body weights and weight gains during gestation no different from the negative control.

On day 29 of gestation, all females were killed and subjected to a thorough uterine examination. The reproductive performance of each female was recorded and is summarized in Table 4. Treatment with Thermolin-101 at 150 mg/kg/day significantly increased the percentage of implantation sites that were resorbed; however, the percentage of dams affected did not differ from the vehicle control. No other effects on



pregnancy, implantation or gestation were ascribed to Thermolin-101 at the levels tested. (The Individual Reproduction Data are presented in Appendix III.)

Fetuses from treated dams were evaluated for skeletal anomalies, and the results are summarized in Table 5. The abnormalities noted in fetuses whose dams received Thermolin-101 at 17, 50 or 150 mg/kg/day were generally variations rather than malformations. There were no dose-related differences in type or frequency of findings as compared to the negative control. Thus, no skeletal effects were ascribed to treatment with Thermolin-101 in this study.

Similarly, gross and soft tissue examinations of fetuses revealed no significant differences between the negative control and any test group (c.f. Table 6).

Utilized as the positive control, treatment with 2.5 mg/kg of 6-aminonicotinamide (6-AN) on day 9 of gestation resulted in many noteworthy findings. As seen in Table 4, 6-AN significantly reduced fetal weights and decreased neonatal survival. In addition, the number of live fetuses per dam was reduced while the percentage of resorption sites and resorbed litters were increased. Fetal examinations revealed many skeletal anomalies as well as soft tissue abnormalities (Tables 5 and 6).

Conclusion

To evaluate its teratogenic potential, Thermolin-101 was



administered by oral intubation to pregnant Dutch-Belted rabbits at 0, 17, 50, 150 or 450 mg/kg body weight/day on days 6 through 18 of gestation. On day 29 of gestation, all animals were killed and subjected to uterine examination. The reproductive performance of each female was recorded, and the resultant fetuses were examined for skeletal soft tissue abnormalities.

The maternal toxicity of Thermolin-101 at 450 mg/kg was indicated by increased mortality. All females in the treatment group died prior to term (day 29). Females administered Thermolin-101 at levels up to 150 mg/kg showed no toxic signs attributable to treatment. Dam body weights and weight gains during gestation did not differ from the negative control. The percentage of implantation sites resulting in resorption was significantly increased in dams administered 150 mg/kg/day Thermolin-101; however, the number of dams affected was no different from the negative control. Fetuses from dams treated with Thermolin-101 at levels up to 150 mg/kg/day showed no dose-related differences in type or frequency of skeletal or soft tissue findings as compared to the negative control.



Table 1
 Dam Body Weights During Gestation¹
 (Pilot Study)

Treatment ²	-Days-										weight gain	
	0	6	9	12	15	18	21	29	0-29	0-18		
	(kg)											
Control Corn Oil (1.1 mL/kg)	2.36	2.40	2.41	2.42	2.45	2.49	2.69	2.69	0.33	0.09		
	2.27 (5)	2.25 (5)	2.24 (5)	2.23 (5)	2.23 (5)	2.20 (5)	2.24 (5)	2.00 (5)	2.05 (5)			
Thromalin-101 (20 mg/kg)	2.50	2.55	2.53	2.54	2.59	2.59	2.79	2.79	0.29	0.05		
	2.14 (4)	2.10 (4)	2.12 (4)	2.11 (4)	2.13 (4)	2.14 (4)	2.14 (4)	2.01 (4)	2.05 (4)			
Thromalin-101 (100 mg/kg)	2.21	2.24	2.26	2.26	2.32	2.31	2.45	2.45	0.13	-0.03		
	2.00 (3)	2.04 (3)	2.12 (3)	2.03 (3)	2.03 (3)	2.03 (3)	2.00 (3)	2.16 (3)	2.05 (3)			
Thromalin-101 (500 mg/kg)	2.17	2.26	2.21	2.21	2.10*	2.20*	2.47	2.47	0.20	-0.05		
	2.24 (3)	2.17 (3)	2.19 (3)	2.09 (3)	2.10 (3)	2.13 (3)	2.06 (3)	2.24 (3)	2.09 (3)			
Thromalin-101 (1000 mg/kg)	All animals died (during gestation).											

¹ All values are means ± S.D. for the number of pregnancies in ().
² All materials administered by groups daily days 0-18 of gestation using corn oil as the vehicle.
 * Significantly different from control (p<0.05).

63

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