

77-94-0112  
INIT 07/14/94

FCI 0794 00120

# The Goodyear Tire & Rubber Company

Akron, Ohio 44316

July 19, 1982

04940000266

Mr Martin Greif, Executive Secretary  
TSCA Interagency Testing Committee  
Environmental Protection Agency (TS-792)  
401 M Street, S.W.  
Washington, D.C. 20460

Contains No CBI

RE: Comments of The Goodyear Tire & Rubber Company on "Chemicals to be Reviewed by the Toxic Substances Control Act Interagency Testing Committee". 47 FR 8244, February 25, 1982

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BUSINESS INFORMATION  
DOES NOT CONTAIN NAT  
SECURITY INFORMATION

Dear Mr Greif:

The Goodyear Tire & Rubber Company submits the following comments and information on two chemicals which have been selected for review by the Interagency Testing Committee.

CAS 95-32-9 2-(4-Morpholinylidithio) benzothiazole

Goodyear manufactures this product at one location by a continuous process in a closed system. As reported for the 1978 TSCA Inventory, the annual production quantity is Category 4: 1 @ 10 million pounds.

Goodyear manufactures the product primarily for captive use. Less than 10% of the volume is manufactured for the R T Vanderbilt Co for resale under the trade name MORFAX.

The material is produced as a powder and is packaged in 50 pound paper bags. It is treated with an anti-dusting agent. Less than 15 workers are employed in the packaging area.

The product is used in rubber compounding to give maximum processing safety and flat "curing" characteristics. It is formulated with both natural and synthetic rubber in the production of tires. It is structurally similar to certain other benzothiazole type accelerators which are used in the tire industry.

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Date 8-2  
Riley, DCO  
Office  
Letter from  
Goodyear  
8-25-82

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DPP/CBIC  
JUL 14 AM 9:28

The product has a low order of acute toxicity. The LD<sub>50</sub> in the rat is >16,000 mg/kg (R T Vanderbilt Safety Data Sheet).

Goodyear has evaluated the material for genetic activity in the Ames Test with and without metabolic activation. The results were negative. (January 25, 1980 report attached.)

In addition to the Ames Test, the product was tested by Litton Bionetics to determine if it could transform BALB/3T3 mouse cells in culture. The results of this assay were negative. (January 1981 Litton report attached.)

Since the material has a low order of acute toxicity and is negative in the above in vitro tests, no further testing is planned by Goodyear at this time.

Occupational exposure to the substance is limited since it is manufactured domestically by only one company. It is used principally in the production of tires at large manufacturing facilities where good industrial hygiene practice is followed. Since it reacts and binds to the polymer molecule during "curing" of a rubber tire, it becomes a part of the finished article. The material is not discharged into the environment.

Based on the limited occupational and non-occupational exposure to this material and the low order of toxicity, Goodyear does not believe that it should be recommended by the ITC for priority testing under TSCA.

CAS 2492-26-4 Sodium 2(3H)-benzothiazoethione

Goodyear manufactures this product at one location by a continuous process in a closed system. As reported for the 1978 TSCA Inventory, the annual production quantity is Category 4: 1 to 10 million pounds.

The material is produced as a 50% water solution, packaged in 50 gallon drums and resold by the R T Vanderbilt Co under the trade name NACAP. It is used primarily as a corrosion inhibitor in ethylene glycol antifreeze formulations.

NACAP is the sodium salt of mercaptobenzothiazole, a widely used rubber curing accelerator. Mercaptobenzothiazole is being tested by the National Toxicology Program for carcinogenicity.

Since NACAP is produced as a water solution in a continuous process, there is essentially no occupational exposure. Upon end use, some discharge to the environment occurs since vehicle antifreeze

solutions are eventually discarded. However, NACAP is used only in small amounts in the glycol formulations and disposal by consumers is widely dispersed.

The product has a low order of acute toxicity. The LD<sub>50</sub> in the rat is 3968 mg/kg (R T Vanderbilt Safety Data Sheet).

Goodyear has evaluated the material for genetic activity in the Ames Test with and without activation. The results were negative. (November 8, 1979 report attached.)

In addition to the Ames Test, the product was submitted to Litton Bionetics to determine if it could transform BALB/3T3 mouse cells in culture. The results of this assay were negative. (March 1982 Litton report attached.)

Because of these negative results, no further testing is planned by Goodyear at this time.

Based on the low order of toxicity and limited exposure to this material and the fact that NTP is testing mercaptobenzothiazole, Goodyear does not believe that NACAP should be recommended by ITC for priority testing under TSCA.

THE GOODYEAR TIRE & RUBBER COMPANY

By W D Davis  
W D Davis, Manager  
Chemical Material Safety

WDD:bs

Attachments

# The Goodyear Tire & Rubber Company

FIBER & POLYMER PRODUCTS RESEARCH DIVISION  
130 JOHNS AVE.  
AKRON, OHIO 44316

**CONTAINS NO CBI**  
Per D. R. J. Date 5-28-77  
Name J Office DW 1012  
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SECURITY INFORMATION (E.O. 12065)~~

HEALTH, SAFETY AND ENVIRONMENTAL SECTION

January 25, 1980

Laboratory Report No 79-76  
Mutagenicity Evaluation of  
Morfax

**Material:**

<u>Sample Number</u>	<u>Identification</u>	<u>HS&amp;E Code</u>
	Morfax (96.1t)	7780-29-1

The test compound was evaluated for genetic activity in a microbial assay (Ames Test) with and without the addition of mammalian metabolic activation.

**Indicator Microorganisms**

Salmonella typhimurium, strains TA-1535, TA-98, TA-1537,  
(Ames Test) TA-100

**Activation System** (Ames et al. Mut Res 31, 347 (1975)).

Energy Resources Co, Inc, a 9000 XG supernatant fraction from adult male rat liver induced with Aroclor 1254.

**Positive Controls**

Nonactivation Assay: 2-nitrofluorene (2NF), Sodium Azide (NaAz),  
Quinacrine mustard (QM)

Activation Assay: 2-Aminoanthracene (2-AA)

Negative (solvent) control: DMSO

**Experimental Design:**

**A. Plate Test (HS&E Test Method 79-10)**

Approximately  $10^8$  cells from an overnight culture of each indicator strain were added to separate test tubes

containing the test chemical and 2.0 ml of molten agar supplemented with biotin and a trace of histidine. For nonactivation tests, contents of the appropriate tubes were poured over the surfaces of selective agar plates. In activation tests, just prior to pouring, an aliquot of reaction mixture (0.5 ml containing the 9000 XG liver homogenate) was added to each of the activation overlay tubes. The plates were incubated for 48 hours at 37°C and then scored for the number of colonies growing on each plate. The concentrations of all chemicals are given in the Results Section. Solvent controls and positive (using both directly active positive chemicals and those that require metabolic activation) were run with each assay. Each dose was run in triplicate.

B. Recording and Presenting Data

The number of colonies on each plate were counted and recorded directly on a printed form. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and the solvent controls are provided as reference points.

C. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for non-activation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls consist of the test compound solvent in the overlay agar together with the other essential components. The negative control plate for each strain gives a reference point to which the test data are compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

D. Test Criteria

To be considered positive (mutagenic), test results must satisfy two criteria; one for magnitude of response and one for dose dependency. For all five tester strains, the dose dependency criterion is satisfied if a dose-response effect is evident over three dose levels separated by at least one-half log units.

Since strains TA-1535, and TA-1537 exhibit relatively low spontaneous reversion frequencies (4-12), the minimum magnitude of response within the dose-dependent range must be at least twice the response of the solvent control.

Test Criteria (Cont'd)

Since strains TA-98 and TA-100 exhibit relatively high (23-65) spontaneous reversion rates, a test compound is judged positive if the maximum level of response within the dose-dependent range is at least twice the response of the solvent control.

Interpretation of Results and Conclusions:

A. Nonactivation Test Results (Table 1, Figure 1)

The test compound, Morfax, did not induce a significant increase in the number of revertant colonies per plate over those of the negative control plates. Toxicity was observed at 100-1000  $\mu$  g/plate.

B. Activation Test Results (Table 2, Figure 2)

In the presence of the S-9 metabolic activation system, the test compound, Morfax, did not induce a significant increase in the number of revertant colonies per plate over those of the negative control plates.

Conclusions:

When tested according to Health, Safety and Environmental Test Method 79-10, Morfax was not mutagenic to *Salmonella typhimurium*.

Tested by C. Bulman  
C Bulman

Reported & Compiled by E. N. Nowak  
E N Nowak  
Health, Safety & Environmental Section

D. A. Wampler  
D A Wampler

Approved by L. K. Hunt  
L K Hunt, Section Head  
Health, Safety and Environmental Section

ENN:dkp

Figure 1

MORFAX

NONACTIVATION

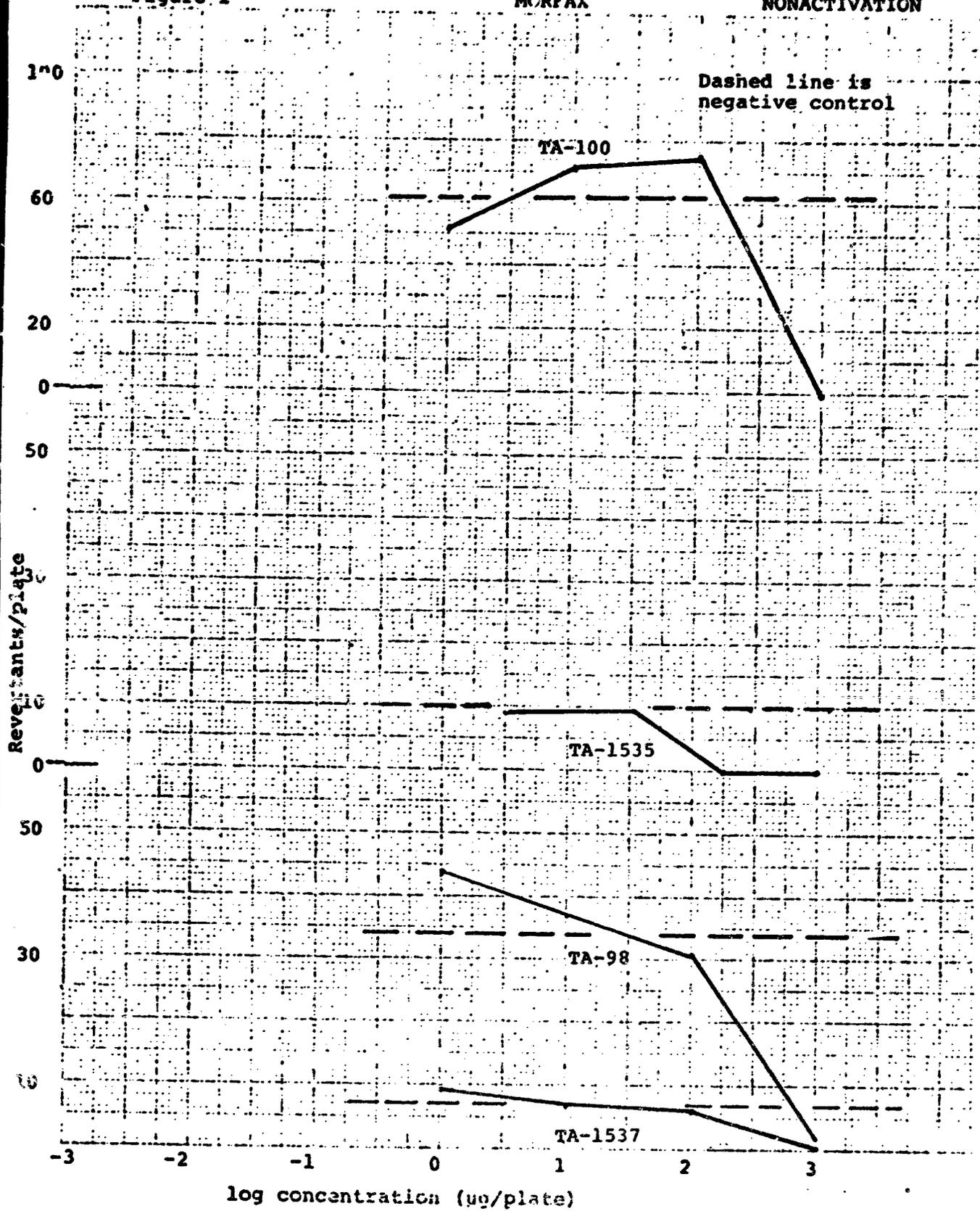
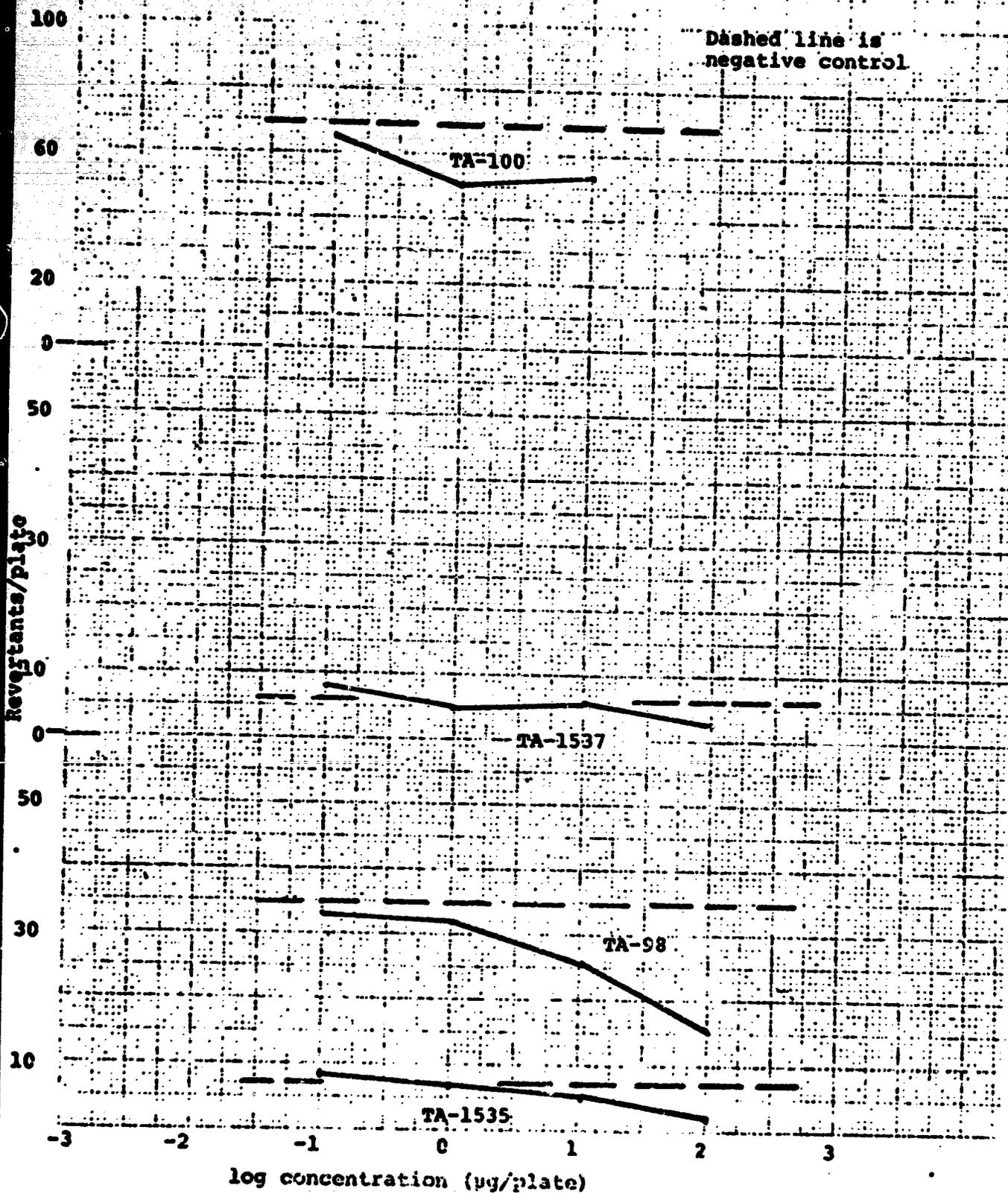


Figure 2

FORAM

WITH ACTIVATION

Dashed line is  
negative control



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Table 1

Sample	Dose µg	Activation	TA98	TA100	Revertants/plate TA1535	TA1537
Morfax	1000	-	2	0	0	0
	100	-	31	75	0	6
	10	-	37	71	9	7
	0.1	-	44	52	9	9
Negative Control	--	-	34	62	10	7
Positive Control	--	-	1080	994	832	1845
Morfax	10	-	34	79		
	1	-	31	83		
	0.1	-	36	80		
Negative Control	--	-	35	76		
Positive Control	--	-	835	806		

Table 2

Morfax	100	+	15	--	2	3
	10	+	26	53	5	6
	1	+	32	51	7	5
	0.1	+	33	66	8	8
Negative Control	--	+	35	69	7	6
Positive Control	--	+	1063	547	136	205

GENETICS ASSAY NO. 5418

LBI SAFETY NO. 6411

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WHICH IS UNCLASSIFIED  
EXCEPT WHERE SHOWN OTHERWISE  
IN THE SECURITY INFORMATION (E.O. 12065)~~

EVALUATION OF  
8048-59-5  
MORFAX  
IN THE  
IN VITRO TRANSFORMATION  
OF BALB/3T3 CELLS ASSAY  
FINAL REPORT

Per Name K. K. C.  
Date 5-21-77  
Office DC  
**CONTAINS NO CBI**

SUBMITTED TO:

GOODYEAR TIRE AND RUBBER CO.  
130 JOHNS AVENUE  
AKRON, OHIO 44316

SUBMITTED BY:

LITTON BIONETICS, INC.  
5516 NICHOLSON LANE  
KENSINGTON, MARYLAND 20795

LBI PROJECT NO. 20992

REPORT DATE: JANUARY, 1981



0013

## PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains items I-IX. Items I-IV provide sponsor and compound identification information, type of assay, and the assay design reference number. All assay design references indicate a standard procedure described in the Litton Bionetics, Inc. "Screening Program for the Identification of Potential Mutagens and Carcinogens." Item V provides the initiation and completion dates for the study, and Item VI provides identification of supervisory personnel. Item VII identifies the tables and/or figures containing the data used by the study director in interpreting the test results. The interpretation itself is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report, entitled Assay Design, describes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices. The evaluation criteria are included to acquaint the sponsor with the methods used to develop and analyze the test results.

All test and control results presented in this report are supported by fully documented raw data which are permanently maintained in the files of the Department of Genetics and Cell Biology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington Maryland, 20795.

Copies of raw data will be supplied to the sponsor upon request.



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0 0 1 4

- I. SPONSOR: Goodyear Tire and Rubber Co.
- II. MATERIAL (TEST COMPOUND): Genetics Assay Number: 5418
  - A. Identification: 8048-59-5
  - B. Date Received: October 24, 1980
  - C. Physical Description: White powder
- III. TYPE OF ASSAY: In Vitro Transformation of Balb/3T3 Cells Assay
- IV. ASSAY DESIGN NUMBER: 441
- V. STUDY DATES:
  - A. Initiation: November 14, 1980
  - B. Completion: January 14, 1981
- VI. SUPERVISORY PERSONNEL:
  - A. Study Director: John O. Rundell, Ph.D.
  - B. Laboratory Supervisor: M. Guntakatta
- VII. RESULTS:
- VIII. INTERPRETATION OF RESULTS:

The results of the assay are presented in Tables 1 and 2 on pages 4 and 5.

The test material, 8048-59-5, was insoluble in culture medium at a concentration of 1.0 mg/ml. Therefore, a 50 mg sample was dissolved in 0.4 ml DMSO and this entire stock was diluted with 49.6 ml culture medium to obtain a 1.0 mg/ml stock solution. A slight turbidity was observed at this concentration, but evidence of undissolved material was not observed at the lower concentrations obtained by serial dilution of the stock solution. Therefore, dilutions were performed with culture medium to obtain a series of 15 concentrations in 2-fold dilution steps for use in the preliminary cytotoxicity test. This test determines the effect of the test material on the ability of 3T3 cells to form colonies after 72 hours exposure and is used to select test concentrations for use in the transformation assay.

The results of the cytotoxicity test are shown in Table 1. The relative survivals ranged from 26.7% at 3.91  $\mu\text{g/ml}$  to 68.2% at 0.061  $\mu\text{g/ml}$ . No survivors were observed for test material



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## V. II. INTERPRETATION OF RESULTS: (continued)

concentrations of 7.81  $\mu\text{g/ml}$  and higher. The transformation assay is normally conducted using treatments that result in between 30%-50% and 100% survival and is considered to be most sensitive near 70% survival, since the observed frequency of transformed foci is not corrected for the number of cells surviving the treatments. Therefore, the wide concentration range of 0.01  $\mu\text{g/ml}$  to 2.0  $\mu\text{g/ml}$ , corresponding to a survival range of approximately 80% to 20% (estimated graphically), was selected for the assay.

The results of the transformation assay are shown in Table 2. The historical negative control for the subclone of 3T3 cells used in this assay consisted of 147 flasks containing a total of 21 transformed foci for an average of 0.14 foci/flask. In this assay, 2 foci were observed among 14 negative control flasks, giving an average frequency of 0.14 foci/flask. This spontaneous frequency was not significantly different from the historical value, using the Kastenbaum-Bowman Tables<sup>2</sup>. In fact, sets of 14 or 15 flasks with a total of 0 to 8 foci are included in the historical data base, so the effect of the test material was evaluated by comparison with the historical spontaneous transformation frequency.

As shown in Table 2, none of the applied concentrations of test material caused the induction of a significant number of transformed foci. In contrast, the MCA positive control treatment resulted in the appearance of an average of 3.13 foci/flask above the background frequency. This result was well within the expected range of 3 to 5 foci/flask, showing that the sensitivity of the assay was normal. Thus, concentrations of test material from 0.01  $\mu\text{g/ml}$  to 2.0  $\mu\text{g/ml}$  were not transforming to 3T3 cells.



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IX. CONCLUSIONS:

The test material, 8048-59-5, did not induce the appearance of a significant number of transformed foci over the concentration range of 0.01  $\mu\text{g}/\text{ml}$  to 2.0  $\mu\text{g}/\text{ml}$ . This concentration range corresponded to approximately 80% to 20% survival in the cytotoxicity test. Therefore, the test material is considered to be inactive in the Balb/3T3 In Vitro Transformation Assay.

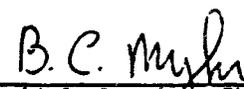
Submitted by:

Study Director

  
John O. Rundell, Ph.D.  
Assistant Section Chief  
Mammalian Genetics  
Department of  
Molecular Toxicology

1/26/81  
Date

Reviewed by:

for:   
David J. Brusich, Ph.D.  
Director  
Department of  
Molecular Toxicology

1/26/81  
Date



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TABLE 1

TOXICITY TEST IN BALB 3T3 CELLSCLIENT: Goodyear GENETICS ASSAY NO. 5418 DATE: November 14, 1980COMPOUND CODE: 8048-59-5SOLVENT: DMSO

<u>TEST COMPOUND DOSES TESTED</u>	<u>AVERAGE NUMBER OF COLONIES/PLATE</u>	<u>% SURVIVAL RELATIVE TO CONTROL</u>
1. 1000.0 µg/ml	0	0
2. 500.0 µg/ml	0	0
3. 250.0 µg/ml	0	0
4. 125.0 µg/ml	0	0
5. 62.50 µg/ml	0	0
6. 31.25 µg/ml	0	0
7. 15.63 µg/ml	0	0
8. 7.813 µg/ml	0	0
9. 3.906 µg/ml	19.3	26.7%
10. 1.953 µg/ml	34.0	47.0%
11. 0.9766 µg/ml	32.7	45.2%
12. 0.4883 µg/ml	40.3	55.8%
13. 0.2441 µg/ml	43.3	59.9%
14. 0.1221 µg/ml	46.7	64.5%
15. 0.0610 µg/ml	49.3	68.2%
16. 0 (control)	72.3	100.0%



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TABLE 2

SUMMARY OF DATA FROM TRANSFORMATION ASSAY

CLIENT: Goodyear GENETICS ASSAY NO. 5418 TEST DATE: December 9, 1980  
CLIENT'S COMPOUND CODE: 8048-59-5 SOLVENT: DMSO 3T3 CLONE: I<sub>13</sub>C<sub>14</sub>

TEST	DOSES TESTED	NUMBER OF FOCI PER FLASK SCORED															TOTAL NO. OF FOCI	NO. OF FOCI/FLASK	NO. OF FLASK CORRECTED FOR SPONTANEOUS
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
Negative Control (DMSO) 0.4% v/v	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0.14	-----
Positive Control (MCA) 5.0 µg/ml	4	2	5	4	2	3	4	2	2	2	3	4	5	4	3	49**	3.27	3.13	
<u>Test Material</u>																			
0.01 µg/ml	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0.07	-----	
0.10 µg/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00	-----	
0.50 µg/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00	-----	
1.00 µg/ml	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0.07	-----	
2.00 µg/ml	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0.07	-----	

MCA = 3-Methylcholanthrene  
C = Flask was contaminated and not scored

\*\* = Data significant from the negative control at p ≤ 0.01.

## ASSAY DESIGN (NO. 441)

### 1. OBJECTIVE

This assay evaluates the carcinogenic potential of test materials using mouse BALB/3T3 cells in culture. The objective of this semi-quantitative assay is to evaluate the test material for its ability to induce foci of transformed cells, recognized by dense, piled-up colonies on a monolayer of normal cells.

### 2. RATIONALE

BALB/3T3 mouse cells will multiply in culture until a monolayer is achieved and will then cease further division. These cells, if injected into immunosuppressed, syngeneic host animals, will not produce neoplastic tumors. However, cells treated *in vitro* with chemical carcinogens will give rise to foci of cellular growth super-imposed on the cell monolayer. If these foci are picked from the cultures, grown to larger numbers and injected into animals, a malignant tumor will in most cases be obtained. Thus, the appearance of piled-up colonies in treated cell cultures at a higher frequency than in control cultures is highly correlated with malignant transformation.

### 3. MATERIALS

#### A. Indicator Cells

Clone I13 of BALB/3T3 mouse cells was obtained from Dr. Takeo Kakunaga. Further subclones, selected for low spontaneous frequencies of foci formation, are used for assays. Stocks are maintained in liquid nitrogen and laboratory cultures are checked periodically to ensure the absence of mycoplasma contamination. Cultures are grown and passaged weekly in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum.

#### B. Control Compounds

##### 1. Negative Controls

A negative control consisting of assays performed on untreated cells is performed. If the test compound is not soluble in growth medium, an organic solvent (normally DMSO) is used; the final concentration of solvent in the growth medium will be 1% or less. Cells exposed to solvent in the medium are assayed as the solvent negative control to determine any effects on survival or transformation caused by the solvent alone. Fifteen flasks of the



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### 3. MATERIALS (continued)

#### B. Control Compounds

##### 1. Negative Controls

appropriate type of negative control are prepared for each assay.

##### 2. Positive Control

3-methylcholanthrene (MCA) is a known carcinogen and is used as a positive control for the transformation of 3T3 cells. Fifteen flasks are treated with 5 µg MCA per milliliter for each assay.

#### C. Sample Forms

Solid materials are dissolved in growth medium, if possible, or in DMSO, unless another solvent is requested. Liquids are tested by direct addition to the test system at predetermined concentrations or following dilution in a suitable solvent.

### 4. EXPERIMENTAL DESIGN

#### A. Dosage Selection

The solubility of the test chemical in growth medium, DMSO or other solvent is first determined. Fifteen dose levels of the test compound are then chosen, starting with a maximum applied dose of 1 mg/ml for solid compounds or 1 µl/ml for liquid samples and decreasing in twofold-dilution steps. Each dose is applied to three culture dishes seeded 24 hours earlier with 200 cells per dish. After an exposure period of three days, the cells are washed and incubated in growth medium for an additional four days. The surviving colonies are stained and counted by an automatic colony counter. A relative survival for each dose is obtained by comparing the number of colonies surviving treatment to the colony counts in negative control dishes. The highest dose chosen for subsequent transformation assays should cause no more than a 50% reduction in colony forming ability and is best located near 30% reduction. Four lower doses (including one or two doses with low or no apparent toxicity) are also selected for the transformation assay.

#### B. Transformation Assay

The procedure used at LBI is based on that reported by Kakunaga (1973)<sup>1</sup>. Twenty-four hours prior to treatment, a series of 25-cm<sup>2</sup> flasks is seeded with 10<sup>6</sup> cells/flask



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#### 4. EXPERIMENTAL DESIGN (continued)

##### B. Transformation Assay

and incubated. Fifteen flasks are then treated for each of the following conditions: Five preselected doses of test chemical; positive control; and solvent negative control, if applicable. The flasks are incubated for a three-day exposure period; the cells are then washed and incubation is continued for four weeks with refeeding twice a week. The assay is terminated by fixing the cell monolayers with methanol and staining with Giemsa. The stained flasks are examined by eye and by microscope to determine the number of foci of transformed cells.

##### 5. SCORING OF TRANSFORMED FOCI

At the end of the four-week incubation period, cultures of normal cells yield a uniformly stained monolayer of round, closely-packed cells. Transformed cells form a dense mass (focus or colony) that stains deeply (usually blue) and is superimposed on the surrounding monolayer of normal cells. The foci are variable in size.

Scored foci have several variations in morphological features. Most foci consist of a dense piling up of cells and exhibit a random, criss-cross orientation of fibroblastic cells at the periphery of the focus. Other scored foci are composed of more rounded cells with little criss-crossing at the periphery but with necrosis at the center caused by the dense piling up of a large number of cells. A third variation is a focus without the necrotic center and large number of cells but which exhibit the criss-cross pattern of overlapping cells throughout most of the colony.

Some foci are not scored. These include small foci of transformed morphology that are found in close proximity to larger foci; these foci are regarded as being formed from cells which migrated from the larger colony. Other unscored foci are small areas where some piling up of rounded cells has occurred but the random orientation of fibroblastic cells is not observed. Microscopic examination is employed for scored foci and in the final judgement of transformed character for any marginal foci.

##### 6. CONFIRMATION OF TUMORIGENICITY OF TRANSFORMED CLONES

Most transformed clones will produce malignant tumors when collected from an unstained transformation plate and injected into syngeneic host animals. Although not routinely performed, this confirmation step can be conducted at additional cost.



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### ASSAY ACCEPTANCE CRITERIA

The assay will be considered acceptable for evaluation of the test results if the following criteria are met:

- 1) The negative control flasks consist of a contiguous monolayer of cells which may or may not contain transformed foci. The lack of contiguous sheet of cells indicates growth conditions too poor to allow the reliable detection of weak transforming agents
- 2) The negative control transformation frequency does not exceed an average of about 2 foci/flask. Attempts are made to isolate and maintain cell stocks (subclones of BALB/3T3 I<sub>13</sub>) with a very low spontaneous frequency of transformation.
- 3) The positive control yields an average number of foci/flask that is significantly different from the negative control at the 99% confidence level.<sup>2</sup>
- 4) A minimum of 8 flasks per test condition are available for analysis. At least 4 dose levels of test substance are assayed.
- 5) The dose range of test substance assayed falls within the 50-100% survival range as determined by the preliminary toxicity test, which measures relative cloning efficiencies.



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### EVALUATION CRITERIA

In many cases, no transformed foci will be observed in the set of flasks comprising the negative control. This does not necessarily mean that any foci found in the treated flasks constitutes a positive response in this assay. In order to determine what minimum number of foci will allow a conclusion that the frequency of transformed foci has been elevated over the negative control, a historical negative control data base is used. This data base consists of the ten most recent assays in which 100 to 150 negative control flasks have been scored. The total number of flasks and transformed foci in this set will be provided in each report.

The statistical tables provided by Kastenbaum and Bowman<sup>2</sup> are used to determine whether the results at each dose level are significantly different from the historical control at the 95% or 99% confidence levels. This test compares variables distributed according to Poissonian expectations by summing the probabilities in the tails of two binomial distributions. The 95% confidence level must be met to consider the test substance active at a particular dose level.

If the negative control is found by the same test to be significantly different from the historical control ( $p < 0.05$ ), the assay will be evaluated independently. Comparisons between the current negative control and tested dose levels will be analyzed by the Kastenbaum-Bowman tables.

The number of induced foci usually does not increase proportionately with the applied dose in this assay. In fact, above a minimum dose level where the number of foci is elevated, further increases in dose may result in little or no increase in the number of foci. The number of foci can be reduced at the highest dose assayed if the toxicity is too high. A response at only one dose level (other than the highest tested dose) that just meets the 95% confidence level will normally not be considered sufficient evidence for activity in this assay. All other degrees of response will usually provide evidence for classifying a test substance as active, although the study director exercises scientific judgement and may obtain expert opinion in the evaluation of each test substance.



7. REFERENCES

1. Kakunaga, T.: A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/3T3. *Int. J. Cancer*, 12:463-473, 1973.
2. Kastenbaum, M.A. and Bowman, K.O.: Tables for determining the statistical significance of mutation frequencies. *Mutation Res.*, 9:527-549, 1970.



**BIONETICS**

Q.A. Inspection Statement  
(reference 2) CFR 56.35(b)(7))

PROJECT 20992

LBI Assay No. 5418.

TYPE OF STUDY In Vitro Transformation of BALB/3T3 Cells Assay

This final study report was reviewed by the LBI Quality Assurance Unit on 1/21/91. A report of findings was submitted to the Study Director and to Management on 1/21/91.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately once per month to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Michael S. Elshil  
Auditor, Quality Assurance Unit



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**CERTIFICATE OF AUTHENTICITY**

**THIS IS TO CERTIFY** that the microimages appearing on this microfiche are accurate and complete reproductions of the records of U.S. Environmental Protection Agency documents as delivered in the regular course of business for microfilming.

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