

**CODING FORMS FOR SRC INDEXING**

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| <b>Submitting Organization</b> | BFGOODRICH CO  |                      |          |
| <b>Contractor</b>              |  |                      |          |
| <b>Document Title</b>          | INITIAL SUBMISSION: LETTER FROM BFGOODRICH CO TO USEPA REGARDING 2-METHYL-1,3-BUTADIENE WITH ATTACHMENTS, DATED 01/18/84 |                      |          |
| <b>Chemical Category</b>       | 2-METHYL-1,3-BUTADIENE   |                      |          |

CODING FORM FOR GLOBAL INDEXING

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| Chemical Name (300 per name)  | 25 | CAS No. (10)    | 24 |
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BF

*75-0774-001131*

*Will Perry  
#020384(4)*

The BFGoodrich Company  
500 South Main Street  
Akron, Ohio 44318



FYI-94-001131  
INIT 07/26/94

Address Reply To:  
Dept. 0020  
Bldg. 5-H



84940000210

January 18, 1984

Martin Greif, Executive Secretary  
Toxic Substances Control Act  
Interagency Testing Committee  
401 - M St., S.W.  
Washington, D.C. 20460

Dear Mr. Greif:

Thank you for your letter requesting information on the following chemicals:

|                | CAS #    |   |
|----------------|----------|---|
| <i>IR-403</i>  | 78-79-5  | 2-methyl-1,3-butadiene                  |
| <i>IR-414A</i> | 95-31-8  | N-tert-butyl-2-benzothiazolesulfenamide |
| <i>IR-414B</i> | 95-33-0  | N-cyclohexyl-2-benzothiazolesulfenamide |
| <i>IR-432</i>  | 120-78-5 | 2,2'dithiobisbenzothiazole              |
| <i>IR-438</i>  | 135-88-6 | N-phenyl-2-naphthylamine                |

*no production*

Two of these chemicals, CAS numbers 78-79-5 and 135-88-6, are no longer manufactured or used as raw materials by the BFGoodrich Company. For the other chemicals, CAS numbers 95-31-8, 95-33-0, and 120-78-5, we are enclosing a reprint of mutagenic evaluations conducted on two of these, material safety data sheets, and information on their production and usage. The number of employees listed are those who directly handle the product; exposures are daily.

Sincerely,

*Robert K. Hinderer*

Robert K. Hinderer, Ph.D.  
Sr. Toxicologist

v

*78-9*

14:3 13 93 700 46

RECEIVED

BFGoodrich Company - Production/Usage (1982)

|   | <u>CAS #95-31-8</u> | <u>CAS #95-33-0</u> | <u>CAS #120-78-5</u> |
|---|---------------------|---------------------|----------------------|
| Tire and Engineered Products<br>Groups* | 840,000 lbs.        | 20,000 lbs.         | 570,000 lbs.         |

Number of employees exposed: 179

|                  |                |      |              |
|------------------|----------------|------|--------------|
| Chemical Group** | 1,000,000 lbs. | None | 880,000 lbs. |
|------------------|----------------|------|--------------|

Number of employees exposed: 5

\*usage

\*\*production

# Goodrich

The BFGoodrich Company  
Chemical Group

6100 Oak Tree Boulevard  
Cleveland, Ohio 44131  
216-524-0200

September 23, 1980

Dear Customer:

In February of this year, we informed you of the results of some preliminary toxicity testing on our rubber accelerators OBTS and CURE-RITE®18. Those studies involved selected bacterial and animal cells. These tests were developed to indicate potential mutagenic and/or carcinogenic activity and are presently used for prioritization of whole animal studies.

The BFGoodrich Company has recently received results of the same tests on two other rubber accelerators, BBTS and MTBS. Although none of these accelerators have been active in all assays, all have produced a positive active response in one or more of these tests.

Whole animal studies are now being conducted on CURE-RITE®18 and OBTS. One series of screening tests, known as Dominant Lethal assays, has recently been completed. Results of these tests, in which rats were repeatedly fed either CURE-RITE®18 or OBTS, showed no evidence of a mutagenic effect.

As a next step, studies designed to evaluate chronic toxicity and carcinogenic potential of CURE-RITE®18 and OBTS are now underway, and we expect that the final results of these studies will be available in 1982 or 1983. An industry group is currently planning further studies of other accelerators.

Attached is the summary of the results of the short-term mutagenic assays. Until additional results are known, we continue to recommend that you minimize worker exposure to these chemicals, using standard industrial hygiene techniques routinely practiced when handling reactive substances. These techniques were reported in our previous letter. We would also suggest the attached information be transmitted to all appropriate personnel within your organization.

As new studies regarding these materials are completed, you will be informed. Should you have any questions, please feel free to contact E. N. Learner, Manager Product Stewardship, 216/447-7843 or A. M. Luxeder, Sr. Product Manager, 216/447-6324.

/kc

*Gene F. Krcmar*

Gene F. Krcmar  
Vice President & Genl. Manager  
Additives & Specialty  
Polymers Division

0005

PRELIMINARY MUTAGENIC ASSAYS

| BFGoodrich Name | Chemical Name  | Ames Test | Mouse Lymphoma | Cell Trans-formation (BALB/3T3) | E. coli WP2 uvr A- | E. coli POL A+ | CHO Chroma some Aberration | Dominant Lethal Assay (Rais) |
|-----------------|--|-----------|----------------|---------------------------------|--------------------|----------------|----------------------------|------------------------------|
| MBTS            | 2-benzothiazyl disulfide                               | -         | +              | -                               | -                  | -              | -                          | -                            |
| MBTS            | N-t-butyl-2-benzothiazole sulfenamide                  | -         | +A             | +                               | -                  | -              | B                          | -                            |
| OBTS*           | N-oxdiethylene-2-benzothiazole sulfenamide             | -         | +              | +                               | -                  | -              | -                          | -                            |
| Cure-Rite® 18*  | N-oxdiethylene thiocarbonyl-N-oxdiethylene Sulfenamide | -A        | +A             | +C                              | -                  | +              | +                          | -                            |

+ Is a mutagenic response in the given assay.

- Is the absence of a mutagenic response.

A Repeated and shown to be reproducible

B A significant increase seen at one dose, but others negative; called negative

C Positive in the first assay/negative when repeated.

\* Based on these data, The BFGoodrich Co. filed a notice of "Substantial Risk" under Sec. 8E of Toxic Substances Control Act (TSCA) on February 19, 1980.

A mutagenic response is an alteration or change in the genetic composition of a cell which is passed on to subsequent generations. This heritable change may be either deleterious, advantageous, or of no consequence.

9/9/80

PRECAUTIONARY LABEL

Product: Good-rite 3325 (BBTS)

Date: 12/1/81

Physical Form: Powder or pellet

Revision: Original

Container Type: PB 380 bags

Author: J. Tanzilli

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N-t-Butyl 1-2 Benzothiazole Sulfenamide (BBTS)

**CAUTION!**

HEATING ABOVE 302°F (150°C) WILL CAUSE RAPID DECOMPOSITION, POSSIBLY LIBERATING TOXIC VAPORS, INCLUDING t-BUTYLAMINE.

Avoid breathing dust. Wear proper NIOSH approved respirator when exposure to dust or decomposition products is possible. Use with adequate ventilation. Wear protective gloves and monogoggles. Wash thoroughly after handling.

**FIRST AID**

IN CASE OF EYE CONTACT: Flush eyes with plenty of water for at least five (5) minutes while holding eyelids open. See a physician.

IN CASE OF SKIN CONTACT: Remove contaminated clothing. Wash the affected area with plenty of soap and water. If irritation develops, see a physician.

**IN CASE OF**

FIRE: Use water, ABC dry powder or protein foam. CO<sub>2</sub> may be ineffective. Wear self-contained breathing apparatus.

SPILL OR LEAK: Vacuum or sweep into a closed, labeled container. Use care to avoid dust generation. Dispose of waste by incineration or landfill obeying all federal, state and local health and pollution regulations. Do not flush into public sewer or water system.

\*\*\*\*\*

IN CASE OF TRANSPORTATION EMERGENCY CALL CHEMTREC: (800) 424-9300

For further information, read material safety data sheet available from your employer or from The BFGoodrich Company, Chemical Group.

# Good-rite™

CHEMICALS

MATERIAL SAFETY DATA SHEET

GOOD-RITE® CBTS ACCELERATORS

ISSUED: MARCH, 1981

## SECTION I

Manufacturer's Name  
The BFGoodrich Company  
Chemical Group

Telephone Number  
(216) 447-6000

Address  
6100 Oak Tree Blvd.  
Cleveland, Ohio 44131

Chemical Family  
Benzothiazoles

Trademark  
Good-rite® 3321X2 Accelerator (pellets)  
Good-rite® 3321X4 Accelerator (powder)

Chemical Name and Synonyms  
N-Cyclohexyl-2-Benzothiazole  
Sulfenamide. CBTS

Formula  
C<sub>13</sub>H<sub>16</sub>N<sub>2</sub>S<sub>2</sub>

## SECTION II - HAZARDOUS INGREDIENTS

|                 | <u>Amount in<br/>Product</u> | <u>ACGIH<br/>TLV-TWA</u> | <u>OSHA<br/>PEL</u> |
|-----------------|------------------------------|--------------------------|---------------------|
| Cyclohexylamine | < 0.2%                       | None established.        |                     |

- Notes:
- TLV-TWA: Threshold Limit Value - Time Weighted Average for concentration of the chemical substance in the ambient workplace air. American Conference of Governmental Industrial Hygienists, 1980 Edition.
  - OSHA PEL: OSHA Permissible Exposure Limit, 8-hour TWA.
  - < means "less than"

B.F. Goodrich Chemical Company/6100 Oak Tree Blvd., Cleveland, Ohio 44131



The information contained herein is believed to be reliable, but no representations, guarantees, or warranties of any kind are made as to its accuracy, suitability for particular applications or the results to be obtained therefrom. Nothing contained herein is to be considered as permission, recommendation, nor as an inducement, to practice any patented invention without permission of the patent owner

SECTION III - PHYSICAL DATA (Typical data, not specifications)

Boiling Point  
Not Applicable (NA)

Specific Gravity (H<sub>2</sub>O=1)  
1.28 @ 25°C

Solubility in Water  
Insoluble

Percent Volatile by Weight  
0.5% Maximum

Melt Point  
198-216°F (92-102°C)

Appearance and Odor  
Light tan or buff powder or  
pellet. Very little odor.

pH  
NA

SECTION IV - FIRE AND EXPLOSION HAZARD DATA

Flash Point  
NA

Flammable Limits:  
Lower explosion limit: NA  
Upper explosion limit: NA

Extinguishing Media

Water. ABC dry powder. Protein type air foams. Carbon dioxide may be ineffective on larger fires due to a lack of cooling capacity which may result in reignition.

Special Fire Fighting Procedure

Positive pressure self-contained breathing apparatus should be used. Personnel not having suitable respiratory protection should leave the area to prevent significant exposure to toxic combustion gases from any source.

Unusual Fire and Explosion Hazards

None known.

SECTION V - HEALTH HAZARD DATA

Threshold Limit Value  
None established.

Effects of Overexposure  
None Known.

Emergency and First Aid Procedure

IN CASE OF EYE CONTACT: Flush eyes with plenty of water for at least five (5) minutes while holding eyelids open. See a physician.

IN CASE OF SKIN CONTACT: Remove contaminated clothing. Wash the affected area with plenty of soap and water. If irritation develops, see a physician.

## SECTION VI - REACTIVITY DATA

### Stability

Decomposes rapidly above 302°F  
(150°C)

### Hazardous Polymerization

Will not occur

### Hazardous Decomposition Products

CO, CO<sub>2</sub>, oxides of nitrogen, sulfur dioxide and cyclohexylamine.

### Incompatibility (materials to avoid)

Avoid contact with strong oxidizing agents such as hydrogen peroxide, permanganates and perchlorates. Contact can result in intense heat, boiling, flame development or explosion depending on the amount and specific materials involved.

## SECTION VII - SPILL OR LEAK PROCEDURE

### Steps to be taken in case material is released or spilled

Vacuum or sweep into closed container using care to avoid dust generation. If conditions exist where dust is possible, wear a NIOSH approved respirator. Do not flush chemical into public sewer or water system.

### Waste Disposal Method

Landfill or incineration in accordance with federal, state and local regulations. These products are not defined or designated as hazardous by current provisions of the Federal Resource Conservation and Recovery Act (RCRA). 40CFR261.

## SECTION VIII - SPECIAL PROTECTION INFORMATION

### Ventilation

Provide efficient local exhaust at all operations capable of creating fumes, dust or vapors to prevent routine inhalation.

### Respiratory Protection

If conditions exist where dust is possible, wear NIOSH approved respirator.

### Protective Equipment

- Wear monogoggles.
- Wear protective gloves.

SECTION IX - SPECIAL PRECAUTIONS

- Avoid breathing dust.
- Open, handle and use under well-ventilated conditions.
- Wash hands thoroughly after handling. Always wash-up before eating, smoking or using toilet facilities.
- Store at temperatures below 110°F (43°C); avoid extremes of temperature and humidity.

SECTION X - TRANSPORTATION

For transportation purposes, these products are not defined or designated as a hazardous material by the U.S. Department of Transportation under Title 49 of the Code of Federal Regulations, 1980 Edition.

USER'S RESPONSIBILITY

A bulletin such as this cannot be expected to cover all possible individual situations. As the user has the responsibility to provide a safe workplace, all aspects of an individual operation should be examined to determine if, or where, precautions, in addition to those described herein, are required. Any health hazard and safety information contained herein should be passed on to your customers or employees, as the case may be. BFGoodrich must rely on the user to utilize the information we have supplied to develop work practice guidelines and employee instructional programs for the individual operation.

DISCLAIMER OF LIABILITY

As the conditions or methods of use are beyond our control, we do not assume any responsibility and expressly disclaim any liability for any use of this material. Information contained herein is believed to be true and accurate but all statements or suggestions are made without warranty, express or implied, regarding accuracy of the information, the hazards connected with the use of the material or the results to be obtained from the use thereof. Compliance with all applicable federal, state and local laws and regulations remains the responsibility of the user.

2915E/bh

PRECAUTIONARY LABEL

Product: Good-rite 3321\* (CBTS)

Date: 12/10/81

Physical Form: Powder or pellet

Revision: Original

Container Type: PB-370 bag or purchased

Author: J. D. Tanzilli

\*Applies to 3321X2 and 3321X4

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**N-Cyclohexyl-2-Benzothiazole Sulfenamide (CBTS)**

**CAUTION!**

**HEATING ABOVE 302°F (150°C) WILL CAUSE RAPID DECOMPOSITION**

Use with adequate ventilation. Avoid breathing dust. Wear proper NIOSH approved respirator when exposure to dust or decomposition products is possible. Wear protective gloves and monogoggles. Wash thoroughly after handling.

**FIRST AID**

**IN CASE OF EYE CONTACT:** Flush eyes with plenty of water for at least five (5) minutes while holding eyelids open. See a physician.

**IN CASE OF SKIN CONTACT:** Remove contaminated clothing. Wash the affected area with plenty of soap and water. If irritation develops, see a physician.

**IN CASE OF**

**FIRE:** Use water, ABC dry powder or protein foam. CO<sub>2</sub> may be ineffective. Wear self-contained breathing apparatus.

**SPILL OR LEAK:** Vacuum or sweep into a closed, labeled container. Use care to avoid dust generation. Dispose of waste by incineration or landfill obeying all federal, state and local health and pollution regulations. Do not flush into public sewer or water system.

\*\*\*\*\*

Store at a temperature below 110°F (43°C). Avoid extremes of temperatures and humidity.

**IN CASE OF TRANSPORTATION EMERGENCY CALL CHEMTREC: (800) 424-9300**

For further information, read material safety data sheet available from your employer or from The BFGoodrich Company, Chemical Group.

1052a/27

# Good-rite™

CHEMICALS

MATERIAL SAFETY DATA SHEET

GOOD-RITE® BBTS ACCELERATORS

ISSUED: MARCH, 1981

## SECTION I

Manufacturer's Name  
The BFGoodrich Company  
Chemical Group

Telephone Number  
(216) 447-6000

Address  
6100 Oak Tree Blvd.  
Cleveland, Ohio 44131

Trademark  
Good-rite® 3325X2 Accelerator (Pellet)  
Good-rite® 3325X3 Accelerator (Powder)

Chemical Family  
Benzothiazole

Formula  
C<sub>11</sub>H<sub>14</sub>N<sub>2</sub>S<sub>2</sub>

Chemical Name and Synonyms  
N-t-Butyl 1-2 Benzothiazole Sulfenamide.  
BBTS

## SECTION II - HAZARDOUS INGREDIENTS

|              | <u>Amount in</u><br><u>Product</u> | <u>ACGIH</u><br><u>TLV</u> | <u>OSHA</u><br><u>PEL</u> |
|--------------|------------------------------------|----------------------------|---------------------------|
| t-Butylamine | < 0.2%                             | 5ppm* (skin)               | 5 ppm* (skin)             |

- Notes: ● TLV: Threshold Limit Value for concentration of the chemical substance in the ambient workplace air. American Conference of Governmental Industrial Hygienists, 1980 Edition.
- OSHA PEL: OSHA Permissible Exposure Limit. 8-hour TWA. 29CFR 1910.1000.
- < means "less than"
- \* ceiling limit.

SECTION III - PHYSICAL DATA (Typical data, not specifications)

|   |   |
|---|---|
| <u>Boiling Point</u><br>Not applicable (NA)   | <u>Specific Gravity (H<sub>2</sub>O=1)</u><br>1.26-1.32 |
| <u>Solubility in Water</u><br>Insoluble   | <u>Percent Volatile by Weight</u><br>0.5% maximum       |
| <u>Melt Point</u><br>219-232°F (104-111°C)  |   |
| <u>Appearance and Odor</u><br>Light tan to buff powder or<br>pellet with faint aromatic odor. | <u>pH</u><br>NA   |

SECTION IV - FIRE AND EXPLOSION HAZARD DATA

|                                 |  |
|---------------------------------|--|
| <u>Flash Point</u><br>Not known | <u>Flammable Limits:</u><br>Lower explosion limit: 0.029 Oz./ft <sup>3</sup><br>Upper explosion limit: NA<br>Explosion severity vs.<br>Pittsburg Coal Dust (Valve = 1.0): 3.47 |
|---------------------------------|--|

Extinguishing Media

Water. ABC dry powder. Protein type air foams. Carbon dioxide may be ineffective on larger fires due to a lack of cooling capacity which may result in reignition.

Special Fire Fighting Procedure

Positive pressure self-contained breathing apparatus should be used. Personnel not having suitable respiratory protection should leave the area to prevent significant exposure to toxic combustion gases from any source.

Unusual Fire and Explosion Hazards

None known.

SECTION V - HEALTH HAZARD DATA

Threshold Limit Value  
None established

Effects of Overexposure  
See Appendix

Emergency and First Aid Procedure

IN CASE OF EYE CONTACT: Flush eyes with plenty of water for at least five (5) minutes while holding eyelids open. See a physician.

IN CASE OF SKIN CONTACT: Remove contaminated clothing. Wash the affected area with plenty of soap and water. If irritation develops, see a physician.

## SECTION VI - REACTIVITY DATA

### Stability

Decomposes rapidly above  
302°F. (150°C)

### Hazardous Polymerization

Will not occur

### Hazardous Decomposition Products

CO, CO<sub>2</sub>, oxides of nitrogen and sulfur, t-butylamine and small amounts of aliphatic and aromatic hydrocarbons.

### Incompatibility

Avoid contact with strong oxidizing agents such as hydrogen peroxide, permanganates and perchlorates. Contact can result in intense heat, boiling, flame development or explosion depending on the amount and specific materials involved.

## SECTION VII - SPILL OR LEAK PROCEDURE

### Steps to be taken in case material is released or spilled

Vacuum or sweep into closed container using care to avoid dust generation. If conditions exist where dust is possible, wear a NIOSH approved respirator. Do not flush chemical into public sewer or water system.

### Waste Disposal Method

Landfill or incineration in accordance with federal, state and local regulations. This product is not defined or designated as hazardous by current provisions of the Federal Resource Conservation and Recovery Act (RCRA). 40CFR261.

## SECTION VIII - SPECIAL PROTECTION INFORMATION

### Ventilation

Efficient exhaust ventilation should always be provided to draw fumes and vapors away from workers to prevent routine inhalation. Ventilation should be adequate to maintain the ambient workplace atmosphere below the TLVs listed in Section II.

### Respiratory Protection

Where dust is possible, wear NIOSH approved respirator.

### Protective Equipment

- Wear protective gloves.
- Wear monogoggles.

## SECTION IX - SPECIAL PRECAUTIONS

- Avoid breathing dust.
- Open, handle and use under well-ventilated conditions.
- Wash hands thoroughly after handling containers. Always wash-up before eating, smoking or using toilet facilities.

## SECTION X - TRANSPORTATION

For transportation purposes, these products are not defined or designated as a hazardous material by the U.S. Department of Transportation under Title 49 of the Code of Federal Regulations, 1980 Edition.

## USER'S RESPONSIBILITY

A bulletin such as this cannot be expected to cover all possible individual situations. As the user has the responsibility to provide a safe workplace, all aspects of an individual operation should be examined to determine if or where precautions, in addition to those described herein, are required. Any health hazard and safety information contained herein should be passed on to your customers or employees, as the case may be. BFGoodrich must rely on the user to utilize the information we have supplied to develop work practice guidelines and employee instructional programs for the individual operation.

## DISCLAIMER OF LIABILITY

As the conditions or methods of use are beyond our control, we do not assume any responsibility and expressly disclaim any liability for any use of this material. Information contained herein is believed to be true and accurate but all statements or suggestions are made without warranty, express or implied, regarding accuracy of the information, the hazards connected with the use of the material or the results to be obtained from the use thereof. Compliance with all applicable federal, state and local laws and regulations remains the responsibility of the user.

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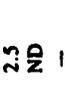
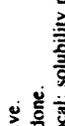
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## Erratum

Hinderer RK, Myhr B, Jagannath DR, Galloway SM, Mann SW, Riddle JC, Brusick DJ (1983): Mutagenic evaluations of four rubber accelerators in a battery of *in vitro* mutagenic assays. *Environ Mutagen* 5:193-215.

Table III from page 202 of the above article was printed with incorrect chemical structure diagrams for three rubber accelerators analyzed. The corrected table appears on the following page.

TABLE III. Comparison of the Effects of Several Rubber Accelerators in a Battery of In Vitro Mutagenic Screens

| Chemical name   | Structure   | S-9   | Salmonella <sup>a</sup> | E coli WP <sub>2</sub> uvrA <sup>-</sup> | E coli pol A + / A <sup>-</sup> b                                | Mouse lymphoma <sup>c</sup>                    | BALB/3T3 cell transformation <sup>c,f</sup> | CHO chromosome aberration <sup>d</sup> |
|-----------------|---|---|-------------------------|--|--|--|---|--|
| OTOS 1 and 2    |      | -S-9 <sup>h</sup><br>+NS9 <sup>h</sup><br>+IS9 <sup>m</sup> | -<br>-<br>-             | -<br>-<br>-                              | 2,500 <sup>e,j</sup><br>100 <sup>h,i</sup><br>100 <sup>h,i</sup> | -<br>ND <sup>o</sup><br>10.0-18.8 <sup>g</sup> | 0.10 <sup>f</sup><br>NT <sup>g</sup><br>ND  | 2.5<br>ND<br>-                         |
| OTOS (purified) | (Same as above)   | -S-9<br>+NS9<br>+IS9  | -<br>-<br>-             | -<br>-<br>-                              | 2,500 <sup>h</sup><br>100 <sup>h,i</sup><br>100 <sup>h,i</sup>   | 3.13<br>ND<br>6.25                             | 0.05<br>ND<br>ND                            | 5.0<br>ND<br>5.0                       |
| OBTS            |    | -S-9<br>+NS9<br>+IS9  | -<br>-<br>-             | -<br>-<br>-                              | 10 <sup>h</sup><br>ND<br>500 <sup>h</sup>                        | 10.0<br>ND<br>12.5                             | 0.20<br>ND<br>NT <sup>g</sup>               | -<br>ND<br>-                           |
| MBTS            |   | -S-9<br>+NS9<br>+IS9  | -<br>-<br>-             | -<br>-<br>-                              | EP<br>E<br>E   | -<br>ND<br>15.0                                | -<br>ND<br>ND                               | -<br>ND<br>-                           |
| BBTS            |  | -S-9<br>+NS9<br>+IS9  | -<br>-<br>-             | -<br>-<br>-                              | E<br>E<br>E  | -<br>ND<br>40.0                                | 35<br>ND<br>ND                              | -<br>ND<br>-                           |

<sup>a</sup>Strains TA-1535, TA-1537, TA-1538, TA-98, TA-100.

<sup>b</sup>Lowest effective dose: µg per plate.

<sup>c</sup>Lowest effective dose: µg/ml.

<sup>d</sup>Lowest dose tested at which aberrations increased (µg/ml).

<sup>e</sup>Two samples tested; only one OTOS 1 was active.

<sup>f</sup>Lowest dose tested.

<sup>g</sup>Plate assay.

<sup>h</sup>Suspension assay.

<sup>i</sup>Two samples tested.

<sup>j</sup>Only OTOS 2 tested.

<sup>k</sup>-S9 = nonactivation.

<sup>l</sup>+NS9 = noinduced activation.

<sup>m</sup>+IS9 = induced activation.

<sup>n</sup>- = negative.

<sup>o</sup>ND = not done.

<sup>p</sup>E = equivocal; solubility prevents obtaining toxic dose in the plate assay.

## Mutagenic Evaluations of Four Rubber Accelerators in a Battery of In Vitro Mutagenic Assays

R.K. Hinderer, B. Myhr, D.R. Jagannath, S.M. Galloway, S.W. Mann, J.C. Riddle, and D.J. Brusick

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The mutagenic/carcinogenic potential of four commercial accelerators were evaluated using a battery of in vitro assays. All of these compounds were mutagenic in one or more assays. Positive responses were noted in the *Escherichia coli* pol A<sup>+</sup>/pol A<sup>-</sup> DNA repair, mouse lymphoma L5178Y TK<sup>+</sup>/<sup>-</sup> forward mutation, BALB/3T3 cell transformation, and CHO cell chromosome aberration assays. In contrast to previous studies of accelerators, no mutagenic response was observed in the *E. coli* WP<sub>2</sub> uvrA<sup>-</sup> assay or in any of the *Salmonella typhimurium* strains tested. These studies have indicated that rubber accelerators should be regarded as potential human health hazards and that further in vitro and in vivo studies are needed to assess the potential genetic hazards of this large class of chemicals.

**Key words:** mutagenicity, in vitro, accelerators, rubber chemicals

### INTRODUCTION

Rubber accelerators are chemicals of a highly reactive nature. They are used to increase both the rate and "state" of curing, and function by attacking the sulfhydryl end groups in rubber. Since the sulfhydryl moiety also exists in proteins and many other essential cellular components, human exposure to rubber accelerators could result in deleterious biological reactions.

Several studies of mortality among rubber workers suggest that observed excesses in certain types of cancers might be occupationally related [Monson and Nakano, 1976; Andjelkovich et al, 1976, 1977; McMichael et al. 1976]. However,

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because of the myriad of raw materials and by-products present during rubber manufacture, it has been impossible to determine the underlying cause. Although rubber accelerators represent only a small fraction of the chemicals present during rubber manufacture, the potential biological reactivity of rubber accelerators makes them appropriate candidates for evaluation. Furthermore, studies by Hedenstedt et al [1979], show that certain thiuram and dithiocarbamate accelerators are mutagenic in various *Salmonella typhimurium* strains both with and without an S-9 activation system. These results suggest that other rubber accelerators also may be capable of producing mutagenic responses.

The present study of N-oxidiethylene thiocarbamyl-N-oxidiethylene sulfenamide, N-oxidiethylene-2-benzothiazole sulfenamide, 2-mercapto benzothiazole disulfide, and N-butyl-2-benzothiazole sulfenamide was conducted to evaluate their mutagenic potential and to assist in setting priority for in vivo studies of these chemicals. Sulfenamide and disulfide accelerators are produced by a number of manufacturers and were chosen because of their significant use in the rubber industry.

## MATERIALS AND METHODS

Samples of the accelerators used in this study were provided and analyzed by the BF Goodrich Company, Chemical Group. These materials were analyzed for general purity and levels of N-nitrosomorpholine and morpholine (Table I).

### Salmonella Plate Assays

*Salmonella typhimurium* strains TA-1535, TA-1537, TA-1538, TA-98 and TA-100 were obtained from Dr. Bruce Ames, University of California at Berkeley. All indicator strains were kept at 4°C on minimal medium plates supplemented with a trace of biotin and an excess of histidine [Ames, 1980]. In addition, the plates with the plasmid-carrying *Salmonella* strains (TA-98 and TA-100) were supplemented with 25 µg/ml of ampicillin to ensure stable maintenance of plasmid pKM101.

The bacterial strains were cultured at 37°C in Oxoid Media #2 (nutrient broth), and Vogel Bonner Medium E with 2% glucose was used as the selective medium [Vogel and Bonner, 1956]. The overlay agar was prepared according to the method of Ames et al [1975]. S-9 liver homogenates, which were prepared from Aroclor 1254-induced and noninduced adult Sprague-Dawley male rats as described by Ames et al [1975], were purchased from Bionetics Laboratory Products, Litton Bionetics, Inc. An S-9 mix was prepared by adding the following ingredients per milliliter of mix: 4 µmoles NADP (sodium salt), 5 µmoles D-glucose-6-phosphate, 8 µmoles MgCl<sub>2</sub>, 33 µmoles KCl, 100 µmoles sodium phosphate buffer (pH 7.4), and 100 µl of rat liver S-9 fraction.

All tests were based on the method of Ames et al [1975]. Test compounds were dissolved in dimethylsulfoxide (DMSO). Solvent and positive controls were used as listed in Table II. The highest dose was established as one which produced some toxicity.

For the nonactivation assay, the following materials were added (in the order presented) to a sterile 13 × 100 mm test tube placed in a 45°C water bath: 1) 2.00 ml of 0.6% agar containing 0.05 mM histidine and 0.05 mM biotin; 2) 0.05 ml of a solution of the test chemical to give an appropriate dose; 3) 0.1 ml of the overnight culture of indicator organism; and 4) 0.50 ml of 0.01 M phosphate buffer, pH 7.4.

TABLE I. Determinations of the Purity of Several Rubber Accelerators

| Chemical (CAS number)   | Acronym            | Purity<br>Wt %  | N-nitroso-<br>morpholine <sup>a</sup><br>ppm | Morpholine <sup>b</sup><br>ppm |
|---|--------------------|-----------------|--|--------------------------------|
| N-oxydiethylene thiocarbamyl-<br>N-oxydiethylene sulfenamide<br>(13752-51-7)              | OTOS 1             | ND <sup>c</sup> | ND   | ND                             |
|   | OTOS 2             | 95.6            | 0.440  | 500                            |
| N-oxydiethylene thiocarbamyl-N-<br>oxydiethylene sulfenamide <sup>d</sup><br>(13752-51-7) | OTOS<br>(purified) | 97.5            | 0.075  | —                              |
| N-oxydiethylene-2-benzo-<br>thiazole sulfenamide<br>(102-77-2)                            | OBTS               | 90-95           | 1.700  | 900                            |
| 2-mercapto benzothiazole<br>disulfide (120-78-5)  | MBTS               | 80              | ND   | 30                             |
| N-butyl-2-benzothiazole<br>sulfenamide (95-31-8)  | BBTS               | 92-95           | ND   | 40                             |

<sup>a</sup>Limit of detection—10 ppb.

<sup>b</sup>Values obtained from commercial samples other than ones used in this study.

<sup>c</sup>ND = not done.

<sup>d</sup>Purified sample: referred to in the text as OTOS (purified).

This mixture was swirled gently and then was poured into minimal agar plates. After the top agar had set, the plates were incubated at 37°C for approximately two days. The number of his<sup>+</sup> revertant colonies growing in the plates was counted and recorded.

The activation assay was run concurrently with the nonactivation assay. The only difference was the addition of 0.5 ml of S-9 to the tubes in place of the 0.5 ml of phosphate buffer which was added in nonactivation assays. All other details were similar to the procedure for nonactivation assays.

Statistical methods were not used to evaluate the data. Criteria which were used to determine whether a chemical was mutagenic were: 1) an increase in revertants in strains TA-1535, TA-1537, and TA-1538 of three times the solvent control; 2) an increase in revertants in strains TA-98 and TA-100 of twice the solvent control; 3) reproducibility; and 4) a dose-related response, and a consistent pattern of response between strains derived from the same parental strain (TA-1535 vs TA-100, both derived from strain G-46; TA-1538 vs TA-98, both derived from strain D3052).

#### Escherichia coli Plate Assay

The E coli strain WP<sub>2</sub> urvA<sup>-</sup> was obtained from Dr. M.H.L. Green, MRC Cell Mutation Unit, University of Sussex, England. The indicator strain was kept at 4°C on standard methods agar plates or minimal medium plates supplemented with an excess of tryptophan. Laboratory cultures were grown at 37°C in Oxoid #2 (nutrient broth). Vogel Bonner Medium E [Vogel and Bonner, 1956] with 2% glucose was used as the selective medium. The overlay agar was prepared according to the method of Green and Muriel [1976].

The S-9 activation system was prepared as described for the Salmonella plate assay.



The procedures were based on a modification of the methods described by Ames et al [1975]. Test compounds were dissolved in DMSO. Solvent and positive controls were used as listed in Table II. Doses were established as described for the Salmonella plate assay.

In the nonactivation assay, the following materials were added (in the following order) to a sterile 13 × 100 mm test tube placed in a 45°C water bath: 1) 2.00 ml of 0.6% agar containing 0.05 mM tryptophan and 0.1 M NaCl; 2) 0.05 ml of a solution of the test chemical to give an appropriate dose; 3) 0.1 ml - 0.2 ml of indicator organism/s, and 4) 0.50 ml of 0.01 M phosphate buffer, pH 7.4. All other procedures for the nonactivation assay and those for the activation assay were the same as described for the Salmonella plate assay.

A chemical was considered mutagenic if there was a dose-related response over a minimum of three test concentrations and if it was reproducible.

#### DNA Repair Plate Test

E coli strain W3110 (pol A<sup>+</sup>), which is repair competent, and strain p3078 (pol A<sup>-</sup>), which is repair deficient, were obtained from Dr. H. Rosenkranz, Columbia University, New York. The indicator strains were kept at 4°C on standard methods agar plates or HA + T medium [Slater et al, 1971]. For each experiment, the bacterial strains were cultured overnight at 37°C in HA + T medium without agar. A thymine-supplemented HA agar was used as the selective medium [Slater et al, 1971].

The procedure used was based on the method of Slater et al [1971]. Test compounds were dissolved in DMSO. Solvent and positive controls were used as described in Table II. The S-9 activation system was prepared as described for the Salmonella plate assay.

The following materials were added (in order) to a sterile 13 × 100 mm test tube placed in a 45°C water bath: 1) 2.0 ml of HA + T overlay agar and 2) 0.1 ml - 0.2 ml of indicator organism/s. This mixture was swirled gently and then poured onto HA + T agar plates. After the top agar had set, wells of uniform diameter were made in the center of each agar plate. Equal volumes of at least four doses of the test chemical and a single dose of the control chemical were added to the wells of the appropriate plates. For the nonactivation assays, 0.05 ml of phosphate buffer, pH 7.4, was added to each well; for the activation assays, 0.05 ml of the S-9 mix was added. The plates were incubated at 37°C for approximately 24 hours. The zones of inhibition were measured and recorded in millimeters.

Any chemical which caused a differential of 4 mm or greater between the competent and noncompetent strains was considered to have produced a DNA-modifying effect. If no zones were induced, the results were judged a no-test. Additional criteria such as reproducibility also were considered.

#### DNA Repair Suspension Assay

The origin and the maintenance of the E coli strains W3110 (pol A<sup>+</sup>) and p 3078 (pol A<sup>-</sup>) were the same as described for the DNA repair plate test. The procedure for the suspension assay was based on the method of Rosenkranz and Leifer [1980]. Test compounds were dissolved in DMSO. Solvent and positive controls were used as described in Table II. The S-9 activation system was prepared as described for the Salmonella plate assay.

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In the nonactivation assay, the following materials were added (in the following order) to a sterile 13 × 100 mm test tube placed in a 45°C water bath: 1) 0.05 ml of a solution of the test chemical; 2) 0.1 - 2.0 ml of indicator organism; and 3) 0.5 ml of phosphate buffer, pH 7.4. This mixture was incubated at 37°C on a rotary shaker for two hours. After incubation, 2.0 ml HA + T medium containing 0.75% agar was added to the tubes and gently mixed. The mixture was then poured into HA + T agar plates. After the top agar had set, the plates were incubated at 37°C for approximately two days. The number of surviving colonies growing in the plates was counted and recorded.

The activation assay was run concurrently with the nonactivation assay. Both assays were conducted identically, except that 0.5 ml of the S-9 mix [Ames et al, 1975] was added in place of the phosphate buffer, and the incubation period was reduced to one hour.

Any chemical which reduced the survival index (Table V) to 0.85 or less was considered to have produced a DNA-modifying effect. Additional criteria such as reproducibility were considered.

#### Mouse Lymphoma Assay

The mouse lymphoma cell line, L5178Y TK<sup>+</sup> (Clone 3.7.2C), used in this assay was derived from the Fischer L5178Y line by Dr. Donald Clive, Burroughs Wellcome, Research Triangle Park, North Carolina. Stocks were maintained in liquid nitrogen, and laboratory cultures were periodically checked for the absence of mycoplasma contamination by culturing methods. To reduce the negative control frequency (background frequency) of TK<sup>-</sup> mutants to as low level as possible, cell stock cultures were routinely exposed to medium containing THMG (3 µg/ml thymidine, 5 µg/ml hypoxanthine, 0.1 µg/ml methotrexate, and 7.5 µg/ml glycine). The cultures were then returned to THG medium for one day and then to normal growth medium for two or more days before use in mutagenesis experiments.

The cells were maintained in Fischer's mouse leukemia medium supplemented with 2 mM L-glutamine, 110 µg/ml sodium pyruvate, 96 units/ml penicillin, 96 µg/ml streptomycin, 0.05% Pluronic F68 and horse serum (10% by volume). Cloning medium consisted of the preceding growth medium (minus Pluronic F68) with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. The selection medium was the cloning medium containing 100 µg/ml of 5-bromodeoxyuridine (BrdUrd).

The test compounds were dissolved in DMSO (or acetone for OBTS) just prior to use, and the DMSO (or acetone) solutions were diluted 1:100 into the cell cultures to initiate the treatments. Negative, solvent, and positive controls were used. Solvent controls were prepared by adding 0.1 ml DMSO per 10-ml culture. The positive control for the nonactivation portion of the assay was 0.5 µl/ml ethylmethane sulfonate (EMS). This treatment was initiated by adding 0.5 ml of freshly prepared aqueous stock (10 µl/ml) to a 9.5-ml cell culture. Dimethylnitrosamine (DMN) at 0.3 µl/ml was the positive control for the activation assays. A 0.3-ml aliquot of a freshly prepared aqueous stock (10 µl/ml) was added to a 9.7-ml cell culture containing the S-9 activation system.

The assay procedure was very similar to the method described by Clive and Spector [1975]. Each treated culture consisted of 3 × 10<sup>6</sup> cells suspended in 10-ml final volumes in 15-ml centrifuge tubes. The nonactivation and activation assays were

conducted in the same manner, except the cell cultures in the activation assay contained the 50  $\mu$ l/ml of S-9 supernatant and the cofactors, 2.7 mg/ml NADP and 4.5 mg/ml isocitric acid. The S-9 was prepared from the livers of Aroclor 1254-induced Fischer 344 male rats [Ames et al, 1975]. The cell cultures were exposed to the test material or control chemical for four hours, were washed, and were allowed an expression time of two days in growth medium. Cell counts were determined by hemacytometer and the cell cultures were diluted to  $3 \times 10^5$  cells/ml each day if the density had increased beyond  $4 \times 10^5$  cells/ml.

At the end of the expression period,  $3 \times 10^6$  cells from each treated culture were seeded in selection medium at  $1 \times 10^6$  cells per 100-mm dish. To determine the efficiency of cloning, a portion of each cell suspension was serially diluted and 300 cells were seeded in cloning medium (nonselective) at 100 cells per 100-mm dish. BrdUrd-resistant colonies (TK<sup>-</sup> mutants) and viable colonies (nonselective medium) were counted after 10 days of incubation at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. A Biotran II automatic counter was used at settings allowing the scoring of colonies with diameters of approximately 0.3 mm and larger. The ratio of resistant colonies to viable colonies yielded the mutant frequency in units of 10<sup>-4</sup>.

The average of the solvent and untreated negative control mutant frequencies was used as the background or spontaneous mutant frequency for each trial. A treated culture was considered to have a significantly elevated mutant frequency if the frequency exceeded  $10 \times 10^{-6}$  plus 1.5 times the background frequency. Additional criteria, such as the presence of a dose-related or toxicity-related response and repeatability between trials were used to help assess the presence of any mutagenic activity.

#### Cell Transformation Assay

Clone 113 of BALB/3T3 mouse cells was obtained from Dr. Takeo Kakunaga, National Cancer Institute, Bethesda, Maryland. Further subclones, selected for low spontaneous frequencies of foci formation, were used for these assays. Stocks were maintained in liquid nitrogen, and laboratory cultures were checked periodically to ensure the absence of mycoplasma contamination. Cultures were grown and passaged weekly in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum.

Each test chemical was dissolved in a small quantity of DMSO (or culture media alone for OTOS 2) and subsequently diluted with EMEM such that the final concentration of DMSO was less than 0.5%. Lower concentrations were achieved by serial dilutions with EMEM. The dose range was based on the results of a clonal toxicity assay in which a series of cultures at 200 cells/dish were exposed in triplicate to a wide range of test chemical concentrations for 3 days. Five doses were selected to cover a range of toxicities from little or no reduction in colony-forming ability to a 50% reduction in the colony number. A negative control consisting of cells exposed to 0.5% DMSO (or culture media alone for OTOS 2) in EMEM and a positive control treatment of 5  $\mu$ g/ml 3-methylcholanthrene (3-MCA) were also employed.

The transformation assay was based on the method of Kakunaga [1973]. Twenty-four hours prior to treatment, a series of 25-cm<sup>2</sup> flasks was seeded with 10<sup>4</sup> cells/flask and incubated. Fifteen flasks were then treated for each test dose, the positive control, and the negative control. The flasks were incubated for a three-day exposure period at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were then

washed, and the incubation was continued for four weeks with refeeding twice a week. The assay was terminated by fixing the cell monolayers with methanol and staining Giemsa. The stained flasks were examined by eye and by microscope to determine the number of foci of transformed cells. The foci consisted of piled up and randomly oriented cells, sometime with necrotic centers, on a surrounding monolayer of normal cells.

Statistical tables provided by Kastenbaum and Bowman [1970] were used to determine statistical significance.

#### Chromosome Aberration Assay

Chinese Hamster Ovary (CHO) cells were obtained from the American Type Culture Collection, Repository No. CCL61, Rockville, Maryland. CHO cells were grown in McCoy's 5a medium supplemented with 10% fetal calf serum, L-glutamine, and penicillin-streptomycin. Cultures were set up approximately 24 hours prior to treatment by seeding  $8 \times 10^5$  cells per 75-cm<sup>2</sup> plastic flask in 10 ml of fresh medium.

The test chemicals were dissolved in DMSO or acetone (OBTS). Untreated and solvent (final concentration 1%) control cultures were used, and the positive controls were triethylenemelamine (0.5, 0.75, or 1.0  $\mu\text{g}/\text{ml}$ ) without activation or cyclophosphamide (62.25, 130.5, or 261  $\mu\text{g}/\text{ml}$ ) with S-9 activation. The dose range was selected on the basis of survival of L5178Y cells 24 hours after treatment in the mouse lymphoma forward mutation assay. While cell lines differ in responses to cytotoxic chemicals, an estimate of toxicity for CHO cells can be obtained from L5178Y cells, bearing in mind that the exposure period (2 hours) in the CHO aberration test is shorter than in the forward mutation assay (4 hours). The highest doses for cytogenetics assays were selected to produce little or no toxicity.

In the nonactivation assay, approximately  $2-3 \times 10^6$  cells were treated with the test chemical for two hours at 37°C in growth medium. The exposure period was terminated by washing the cells twice with saline containing 10% FBS, after which fresh medium was added. Incubation was continued for 15½-17 hours. Colcemid was added for the last two hours of incubation ( $2 \times 10^{-7}$  M final concentration), and metaphase cells were collected by mitotic shake-off [Terasima and Tolmach, 1951]. The cells were treated with 0.075 M KCl hypotonic solution, then washed three times in fixative (methanol:acetic acid, 3:1, v/v), dropped into slides, and air-dried. The slides were then stained with 10% Giemsa at pH 6.8. Fifty or 100 cells were scored at each dose level.

The activation assay differed from the nonactivation assay in that S-9 reaction mixture (2.4 mg NADP, 4.5 mg isocitric acid and 15  $\mu\text{l}$  S-9 fraction per ml) was added to the growth medium, together with the test chemical, for two hours. After exposure, the cultures were treated as described above.

Statistical evaluations were conducted using the Student *t*-test [Bancroft, 1957].

## RESULTS

### Salmonella and E coli WP<sub>2</sub> uvrA<sup>-</sup> Plate Assays

Numerous dose levels were evaluated for each of the rubber accelerators. These dose levels ranged from 0.5 to 5,000  $\mu\text{g}$  per plate. Evidence of toxicity was observed at the high dose levels. None of the dose levels of any of the rubber accelerators

increased the number of revertants by a factor of three or more times the control in any of the *S typhimurium* or *E coli* strains either in the presence or absence of S-9 rat liver homogenate (Table III).

#### ***E coli* pol A<sup>+</sup>/pol A<sup>-</sup> Plate and Suspension Assays**

The dose levels for each rubber accelerator ranged from 0.5 to 5,000  $\mu\text{g}$  (plate) and 0.5 to 2,500  $\mu\text{g}$  (suspension). All accelerators were evaluated in the plate assay; however, only OBTS was tested in the suspension assay. In the plate assay, only OTOS 2 and OTOS (purified) evaluations were considered reliable (Table III). Unequivocal determinations of the mutagenic potentials of OBTS, MBTS, and BBTS were precluded since they failed to produce any zone of inhibition in either the normal repair or repair-deficient strains. Both OTOS 2 and OTOS (purified) produced zones of inhibition to similar extents in the repair-deficient strain under both activation and nonactivation conditions (Table IV). However, under activation conditions toxicity at high doses was seen in the repair-competent strains only for OTOS 2 and not for OTOS (purified), thereby precluding any meaningful quantitative comparison. In the absence of activation, DNA damage appeared quantitatively similar for both chemicals. At the lowest effective dose in all tests for both chemicals, zones of inhibition occurred only in the repair-deficient strain and ranged from 11 to 16 mm.

Since OBTS, MBTS, and BBTS failed to produce any evidence of killing in the *E coli* pol A<sup>+</sup>/pol A<sup>-</sup> plate assay, OBTS was tested in the suspension assay. Evidence of the preferential killing of the DNA polymerase-deficient strain was obtained (Table V). A dose-related decrease in the survival index was observed starting at 10 and 500  $\mu\text{g}$  in the nonactivation and activation systems, respectively.

#### **Mouse Lymphoma L5178Y Assay**

The mouse lymphoma assays were conducted with and without an Aroclor 1254-induced rat liver homogenate. Although multiple trials were conducted, only the results of representative trials are listed in Table VIa and b.

In these assays, there was a distinct contrast in response with the nonactivation versus the activation conditions. In the former case, only OTOS (purified) and OBTS were mutagenic, with positive responses occurring only at doses which were very toxic. However, in the presence of induced rat liver homogenate, all chemicals elicited mutagenic responses. This increase in the number of positive responses also paralleled a general decrease in the toxicity of these accelerators to the cells.

#### **Cell Transformation**

Each accelerator was incubated with cultured BALB/3T3 cells. No activation system was added. Each flask was scored for the number of foci present. All of the rubber accelerators except MBTS exhibited an increase in the number of foci. As it is shown in Table III, the lowest effective doses for these differed by orders of magnitude.

#### **Chromosomal Aberrations**

Five accelerators were evaluated in the CHO cell chromosome aberration assay, both with and without an in vitro metabolic activation system (Aroclor 1254-induced rat liver S-9 homogenate). At each dose, a total of 50-100 cells were evaluated to determine the number and types of aberrations. The results are shown in Table VIII.

TABLE III. Comparison of the Effects of Several Rubber Accelerators in a Battery of In Vitro Mutagenic Screens

| Chemical name   | Structure       | S-9   | Salmonella <sup>a</sup> | E. coli WP <sub>2</sub> uvrA <sup>-</sup> | E. coli pol A + / A <sup>-b</sup>                          | Mouse lymphoma <sup>c</sup>                    | BALB/3T3 cell transformation <sup>d</sup> | CHO chromosome aberration <sup>d</sup> |
|-----------------|-----------------|---|-------------------------|---|--|--|---|--|
| OTOS 1 and 2    |                 | -S-9 <sup>e</sup><br>+NS9 <sup>f</sup><br>+IS9 <sup>m</sup> | -<br>-<br>-             | - <sup>n</sup><br>-<br>-                  | 2,500 <sup>g</sup><br>100 <sup>h</sup><br>100 <sup>h</sup> | -<br>ND <sup>i</sup><br>10.0-18.8 <sup>i</sup> | 0.10 <sup>g</sup><br>ND<br>ND             | 2.5<br>ND<br>-                         |
| OTOS (purified) | (Same as above) | -S-9<br>+NS9<br>+IS9  | -<br>-<br>-             | -<br>-<br>-                               | 2,500 <sup>g</sup><br>100 <sup>h</sup><br>100 <sup>h</sup> | 3.13<br>ND<br>6.25                             | 0.05<br>ND<br>ND                          | 5.0<br>ND<br>5.0                       |
| OBTS            |                 | -S-9<br>+NS9<br>+IS9  | -<br>-<br>-             | -<br>-<br>-                               | 10 <sup>h</sup><br>ND<br>500 <sup>h</sup>                  | 10.0<br>ND<br>12.5                             | 0.20<br>ND<br>ND                          | -<br>ND<br>-                           |
| MBTS            |                 | -S-9<br>+NS9<br>+IS9  | -<br>-<br>-             | -<br>-<br>-                               | E <sup>p</sup><br>E<br>E                                   | -<br>ND<br>15.0                                | -<br>ND<br>ND                             | -<br>ND<br>-                           |
| BBTS            |                 | -S-9<br>+NS9<br>+IS9  | -<br>-<br>-             | -<br>-<br>-                               | E<br>E<br>E  | -<br>ND<br>40.0                                | 35.0<br>ND<br>ND                          | -<br>ND<br>-                           |

<sup>a</sup>Strains TA-1535, TA-1537, TA-1538, TA-98, TA-100.<sup>b</sup>Lowest effective dose: µg per plate.<sup>c</sup>Lowest effective dose: µg/ml.<sup>d</sup>Lowest dose tested at which aberrations increased (µg/ml).<sup>e</sup>Two samples tested; only one OTOS 1 was active.<sup>f</sup>Lowest dose tested.<sup>g</sup>Plate assay.<sup>h</sup>Suspension assay.<sup>i</sup>Two samples tested.<sup>k</sup>-S9 = nonactivation.<sup>l</sup>+NS9 = noninduced activation.<sup>m</sup>+IS9 = induced activation.<sup>n</sup>- = negative.<sup>o</sup>ND = not done.<sup>p</sup>E = equivocal; solubility prevents obtaining toxic dose in the plate assay.

TABLE IV. Determination of the Potential of Certain Rubber Accelerators to Cause DNA Damage in the *Escherichia coli* pol A+/pol A- Assay

| Dose                          | OTOS 2  |                 |         |         |         |         |         |         |         |         |
|-------------------------------|---------|-----------------|---------|---------|---------|---------|---------|---------|---------|---------|
|                               | pol A+  |                 | pol A-  |         | pol A+  |         | pol A-  |         | pol A-  |         |
|                               | Trial 1 | Trial 2         | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| Solvent control <sup>a</sup>  | 0       | 0               | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       |
| Positive control <sup>a</sup> | 39      | 45              | 56      | 56      | 39      | 45      | 56      | 56      | 56      | 56      |
| 100 µg                        | 0       | ND <sup>b</sup> | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       |
| 500 µg                        | 0       | 0               | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       |
| 1,000 µg                      | 0       | 0               | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       |
| 2,500 µg                      | 0       | 0               | 11      | 12      | 0       | 0       | 0       | 13      | 13      | 12      |
| 5,000 µg                      | 0       | 0               | 13      | 15      | 0       | 0       | 0       | 15      | 15      | 14      |
| Solvent control <sup>a</sup>  | 0       | 0               | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       |
| Positive control <sup>a</sup> | 13      | 13              | 23      | 23      | 13      | 13      | 13      | 23      | 23      | 23      |
| 100 µg                        | 0       | ND              | 16      | ND      | 0       | 0       | ND      | 13      | 13      | ND      |
| 500 µg                        | 0       | 0               | 21      | 21      | 0       | 0       | 0       | 18      | 18      | ND      |
| 1,000 µg                      | 0       | 0               | 21      | 21      | 0       | 0       | 0       | 16      | 16      | 17      |
| 2,500 µg                      | 0       | 0               | 20      | 21      | 0       | 0       | 0       | 14      | 14      | 16      |
| 5,000 µg                      | 13      | 14              | 22      | 22      | 0       | 0       | 0       | 17      | 17      | 16      |
| Solvent control <sup>a</sup>  | 0       | 0               | 0       | 0       | 0       | 0       | 0       | 0       | 0       | 0       |
| Positive control <sup>a</sup> | 13      | 13              | 20      | 21      | 13      | 13      | 13      | 20      | 20      | 21      |
| 100 µg                        | 0       | ND              | 15      | ND      | 0       | 0       | ND      | 15      | 15      | ND      |
| 500 µg                        | 0       | 0               | 15      | 16      | 0       | 0       | 0       | 20      | 20      | 20      |
| 1,000 µg                      | 0       | 0               | 17      | 21      | 0       | 0       | 0       | 21      | 21      | 19      |
| 2,500 µg                      | 13      | 14              | 21      | 22      | 0       | 0       | 0       | 16      | 16      | 18      |
| 5,000 µg                      | 14      | 14              | 21      | 21      | 0       | 0       | 0       | 17      | 17      | 20      |

<sup>a</sup>See Table II.

<sup>b</sup>ND = not done.

TABLE V. Results of the *Escherichia coli* DNA Polymerase Suspension Assay With OBTS

| Dose (amount/tube)            | Indicator organism  |                       |                     |                       |                             |
|-------------------------------|---------------------|-----------------------|---------------------|-----------------------|-----------------------------|
|                               | pol A+              |                       | pol A-              |                       | Survival index <sup>a</sup> |
|                               | Survivors/<br>plate | Percentage<br>control | Survivors/<br>plate | Percentage<br>control |                             |
|                               | Nonactivation       |                       |                     |                       |                             |
| Solvent control <sup>b</sup>  | 521                 | 100.00                | 515                 | 100.00                | 1.00                        |
| Positive control <sup>c</sup> | 201                 | 38.58                 | 101                 | 19.61                 | 0.51                        |
| 0.5 µg                        | 526                 | 100.96                | 496                 | 96.31                 | 0.95                        |
| 1.0 µg                        | 505                 | 96.93                 | 489                 | 94.95                 | 0.96                        |
| 5.0 µg                        | 498                 | 95.59                 | 465                 | 90.29                 | 0.94                        |
| 10.0 µg                       | 465                 | 89.25                 | 360                 | 69.90                 | 0.78                        |
| 100.0 µg                      | 361                 | 69.29                 | 201                 | 39.03                 | 0.56                        |
| 500.0 µg                      | 216                 | 41.46                 | 98                  | 19.03                 | 0.46                        |
| 1000.0 µg                     | 191                 | 36.66                 | 47                  | 9.13                  | 0.26                        |
| 2500.0 µg                     | 109                 | 20.92                 | 30                  | 5.83                  | 0.28                        |
|                               | Activation          |                       |                     |                       |                             |
| Solvent control <sup>d</sup>  | 678                 | 100.00                | 625                 | 100.00                | 1.00                        |
| Positive control <sup>d</sup> | 176                 | 25.96                 | 65                  | 10.40                 | 0.40                        |
| 0.5 µg                        | 675                 | 99.56                 | 612                 | 97.92                 | 0.98                        |
| 1.0 µg                        | 677                 | 99.85                 | 628                 | 100.48                | 1.01                        |
| 5.0 µg                        | 669                 | 98.67                 | 610                 | 97.60                 | 0.99                        |
| 10.0 µg                       | 671                 | 98.97                 | 608                 | 97.28                 | 0.98                        |
| 100.0 µg                      | 678                 | 100.00                | 601                 | 96.16                 | 0.96                        |
| 500.0 µg                      | 656                 | 96.76                 | 516                 | 82.56                 | 0.85                        |
| 1000.0 µg                     | 631                 | 93.07                 | 289                 | 46.24                 | 0.49                        |
| 2500.0 µg                     | 602                 | 88.79                 | 165                 | 26.40                 | 0.29                        |

<sup>a</sup>Percentage survivors pol A- divided by percentage survivors pol A+.

<sup>b</sup>50 µl dimethylsulfoxide.

<sup>c</sup>10 µl methylmethane sulfonate.

<sup>d</sup>10 µl dimethylnitrosamine.

The frequency of cells that contained aberrations in the negative and solvent controls (exposed to 1% DMSO or acetone) was 0-5%. This frequency was within the normal background range for the CHO cell line. The positive control compound triethylenemelamine (0.5, 0.75, or 1.0 µg/ml) induced a large amount of chromosome damage, including the chromatid interchanges typical of chemical clastogen effects. The high frequency of aberrations induced by the cyclophosphamide positive control demonstrated the effective metabolic activation of this compound by the S-9 mix.

Significant increases in aberrations were induced by OTOS 2 and by purified OTOS. Purified OTOS was effective both with and without the activation system, but the commercial sample, OTOS 2, induced aberrations only in the absence of metabolic activation. Although the aberrations were predominantly simple chromatid and chromosome breaks, complex aberrations such as quadriradials, triradials, and ring chromosomes were also noted. Without activation, OTOS 2 caused a higher frequency of aberrations than did purified OTOS at the same concentration (5 µg/ml). There was also a higher frequency of complex aberrations with OTOS.

TABLE VIa. Evaluation of the Mutagenic Potential of Several Rubber Accelerators in the Mouse Lymphoma L5178Y Assay Under Nonactivation Conditions

| Dose<br>( $\mu\text{g/ml}$ )  | Mutation frequency <sup>a</sup> (percentage relative growth <sup>b</sup> ) |                           |                           |                           |                           |                           |
|-------------------------------|--|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
|                               | OTOS 1 <sup>c</sup>  | OTOS 2 <sup>c</sup>       | OTOS<br>(purified)        | OBTS                      | MBTS                      | BBTS                      |
| Positive control <sup>d</sup> | 698.0 (6.5) <sup>e</sup>   | 462.2 (23.1) <sup>f</sup> | 765.7 (11.1) <sup>f</sup> | 538.6 (8.5) <sup>f</sup>  | 1107.7 (6.9) <sup>f</sup> | 602.4 (10.7) <sup>f</sup> |
| 0                             | 33.4 <sup>g</sup> (100.0)  | 16.1 <sup>h</sup> (100.0) | 14.4 <sup>g</sup> (100.0) | 31.5 <sup>g</sup> (100.0) | 19.0 <sup>g</sup> (100.0) | 13.0 <sup>g</sup> (100.0) |
| 0.313                         | 50.6 (80.9)  | 18.6 (25.6)               | ND                        | ND                        | ND                        | ND                        |
| 0.625                         | 38.3 (43.0)  | 13.1 (15.0)               | ND                        | ND                        | ND                        | ND                        |
| 0.780                         | ND <sup>h</sup>  | ND                        | ND                        | ND                        | ND                        | ND                        |
| 0.938                         | 49.4 (58.8)  | ND                        | ND                        | ND                        | 30.1 (162.6)              | ND                        |
| 1.250                         | 63.2 (12.3)  | 29.5 (11.9)               | 24.6 (29.7)               | 44.0 (14.1)               | ND                        | ND                        |
| 1.560                         | ND   | ND                        | ND                        | ND                        | ND                        | ND                        |
| 1.880                         | 33.7 (7.9)   | ND                        | ND                        | ND                        | 35.5 (86.2)               | ND                        |
| 2.500                         | ND   | 31.5 (11.6)               | ND                        | 34.2 (14.1)               | ND                        | ND                        |
| 3.130                         | ND   | ND                        | ND                        | ND                        | ND                        | ND                        |
| 3.750                         | ND   | ND                        | ND                        | ND                        | 21.0 (131.8)              | 12.7 (81.9)               |
| 5.000                         | ND   | 26.6 (3.8)                | 62.0 (7.7) <sup>f</sup>   | 39.0 (12.3)               | ND                        | 18.1 (62.1)               |
| 6.25 <sup>e</sup>             | ND   | ND                        | ND                        | ND                        | ND                        | ND                        |
| 7.500                         | ND   | ND                        | ND                        | ND                        | ND                        | 12.5 (55.8)               |
| 7.800                         | ND   | ND                        | ND                        | ND                        | 25.4 (70.4)               | ND                        |
| 10.000                        | ND   | ND                        | ND                        | 57.4 (7.8) <sup>f</sup>   | ND                        | 21.8 (13.9)               |
| 15.000                        | ND   | ND                        | 54.2 (7.7) <sup>f</sup>   | 88.3 (3.2) <sup>f</sup>   | 31.4 (9.4)                | 26.5 (4.8)                |
| 20.000                        | ND   | ND                        | 35.4 (11.2) <sup>f</sup>  | ND                        | ND                        | ND                        |

<sup>a</sup>( $\times 10^{-6}$ ).

<sup>b</sup>Relative suspension growth  $\times$  relative cloning efficiency/100.

<sup>c</sup>Commercial samples.

<sup>d</sup>Average mutant frequency of duplicate solvent controls and an unactivated negative control.

<sup>e</sup>Positive response.

<sup>f</sup>0.5  $\mu\text{l/ml}$  ethyl methanesulfonate.

<sup>g</sup>ND = not done.

TABLE V/D. Evaluation of the Mutagenic Potential of Several Rubber Accelerators in the Mouse Lymphoma L5178Y Assay Under Activation Conditions

| Dose<br>( $\mu\text{g/ml}$ ) | Mutation frequency <sup>a</sup> (percentage relative growth) <sup>b</sup> |                           |                           |                           |                           |                           |  |
|------------------------------|---|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|--|
|                              | OTOS 1 <sup>c</sup>   | OTOS 2 <sup>c</sup>       | OTOS<br>(purified)        | OBTS                      | MBTS                      | BBTS                      |  |
| Positive control             | 526.5 (4.1) <sup>f</sup>  | 259.7 (48.2) <sup>f</sup> | 297.5 (11.2) <sup>f</sup> | 229.9 (34.1) <sup>f</sup> | 297.4 (11.2) <sup>f</sup> | 491.3 (10.3) <sup>f</sup> |  |
| 0 <sup>a</sup>               | 13.0 (100.0)  | 51.4 (100.0)              | 36.9 (100.0)              | 31.4 (100.0)              | 36.9 (100.0)              | 39.9 (100.0)              |  |
| 0.625                        | ND <sup>b</sup>   | ND                        | ND                        | ND                        | ND                        | ND                        |  |
| 0.780                        | ND  | ND                        | ND                        | ND                        | ND                        | ND                        |  |
| 1.250                        | ND  | ND                        | ND                        | ND                        | ND                        | ND                        |  |
| 1.560                        | ND  | ND                        | 45.7 (64.9)               | ND                        | ND                        | ND                        |  |
| 1.880                        | ND  | ND                        | ND                        | ND                        | 36.8 (130.2)              | ND                        |  |
| 2.500                        | ND  | ND                        | ND                        | ND                        | ND                        | ND                        |  |
| 3.120                        | ND  | ND                        | 48.3 (50.1)               | ND                        | ND                        | ND                        |  |
| 3.750                        | ND  | ND                        | ND                        | ND                        | 58.5 (70.7)               | ND                        |  |
| 5.000                        | ND  | ND                        | ND                        | ND                        | ND                        | ND                        |  |
| 6.250                        | ND  | ND                        | 73.5 (58.6) <sup>f</sup>  | ND                        | ND                        | ND                        |  |
| 7.800                        | ND  | ND                        | ND                        | ND                        | 56.5 (97.1)               | ND                        |  |
| 9.400                        | ND  | ND                        | ND                        | ND                        | ND                        | ND                        |  |
| 10.000                       | ND  | ND                        | ND                        | ND                        | ND                        | ND                        |  |
| 12.500                       | 16.8 (43.2)   | ND                        | 140.2 (24...)             | ND                        | ND                        | ND                        |  |

|         |                            |                           |                          |                           |                          |                           |
|---------|----------------------------|---------------------------|--------------------------|---------------------------|--------------------------|---------------------------|
| 15,000  | ND                         | ND                        | ND                       | 78.4 (34.7) <sup>f</sup>  | 93.8 (58.9) <sup>f</sup> | ND                        |
| 18,800  | 42.9 (23.6) <sup>e,g</sup> | ND                        | ND                       | ND                        | ND                       | ND                        |
| 20,000  | ND                         | 69.4 (78.1)               | ND                       | 92.2 (27.9) <sup>f</sup>  | ND                       | ND                        |
| 25,000  | 51.1 (15.3) <sup>e,g</sup> | 83.7 (31.6)               | 275.5 (5.2) <sup>f</sup> | ND                        | ND                       | 64.2 (35.6)               |
| 30,000  | ND                         | 190.3 (21.4) <sup>f</sup> | ND                       | 84.2 (29.9) <sup>f</sup>  | 85.4 (38.8) <sup>f</sup> | ND                        |
| 35,000  | ND                         | 375.9 (5.7) <sup>f</sup>  | ND                       | ND                        | ND                       | ND                        |
| 37,500  | NC <sup>d</sup>            | ND                        | ND                       | ND                        | ND                       | ND                        |
| 40,000  | ND                         | ND                        | ND                       | 191.3 (13.1) <sup>f</sup> | ND                       | 73.0 (28.4) <sup>f</sup>  |
| 50,000  | 57.0 (4.2) <sup>f</sup>    | ND                        | ND                       | 266.3 (2.6) <sup>f</sup>  | ND                       | 105.0 (18.1) <sup>f</sup> |
| 60,000  | ND                         | ND                        | ND                       | ND                        | ND                       | 96.9 (20.7) <sup>f</sup>  |
| 80,000  | ND                         | ND                        | ND                       | ND                        | ND                       | 134.8 (12.9) <sup>f</sup> |
| 100,000 | ND                         | ND                        | ND                       | ND                        | ND                       | 121.0 (15.1) <sup>f</sup> |
|         |                            |                           |                          |                           |                          | ND                        |
|         |                            |                           |                          |                           |                          | ND                        |

<sup>a</sup> × 10<sup>-6</sup>

<sup>b</sup>(Relative suspension growth × relative cloning efficiency)/100.

<sup>c</sup>Commercial samples.

<sup>d</sup>Average mutant frequency of duplicate solvent controls and an untreated negative control.

<sup>e</sup>One plate contaminated: value based on remaining two plates.

<sup>f</sup>0.3 μl/ml dimethylnitrosamine.

<sup>g</sup>Positive response.

<sup>h</sup>ND = not done.

<sup>i</sup>NC = not counted; two or more plates contaminated.

No significant increases in chromosome damage were noted in the tests for OBTS and MBTS (Table VIII). A slight, but significant, increase at one dose of BBTS with activation was observed. However, the aberration frequency was not significantly higher than historical control levels. No complex aberrations were found. Since a dose-related response was not noted, BBTS was considered to be negative in this assay.

## DISCUSSION

A battery of *in vitro* mutagenic assays was used for the initial evaluation of the mutagenic/carcinogenic potential of a number of rubber accelerators. Although Hed-enstedt et al [1979] reported on mutagenic evaluations of certain thiuram and dithiocarbamates, no *in vitro* mutagenic evaluations of other accelerators have been reported.

All of the sulfenamide and disulfide accelerators induced genotoxic activity in one or more of the assays. However, all of the chemicals failed to produce any evidence of mutagenicity in either the *Salmonella* or *E coli* WP<sub>2</sub> uvrA<sup>-</sup> strains.

The lack of activity of these accelerators in these bacterial strains, while not surprising, is worthy of note. These results are particularly interesting in light of the mutagenic activity of thiuram and dithiocarbamates in *Salmonella* test systems [Hed-enstedt et al, 1979]. While all these accelerators share some structural similarities, the absence of a secondary amine group in those studied here may provide an explanation for their lack of activity in the *Salmonella* assay. The fact that small structural changes can apparently produce quantitative differences in responses is illustrated by comparing tetramethylthiuram disulfide with tetraethylthiuram disulfide. However, the observations of genotoxic activities in other assays suggest that the lack of response in the bacterial strains also could be attributed to physical factors such as solubility and/or cell wall permeability.

When the accelerators were evaluated in the *E coli* pol A<sup>+</sup>/pol A<sup>-</sup> plate assay, all chemicals except OTOS 2 and OTOS (purified) failed to show any evidence of killing (ie, no zone of inhibition) (Table V). This suggested that low solubility might have been responsible. For this reason we evaluated OBTS in the *E coli* pol A<sup>+</sup>/pol A<sup>-</sup> suspension assay, which involved a two-hour preincubation at 37°C with the indicator organisms (with or without the S-9 preparation) in solution. In the suspension assay OBTS was mutagenic both with and without activation (Table V), thus supporting the contention that low solubility was the reason for the lack of activity in the plate assay. Positive responses were found in all of the other assays which involved mammalian cells and utilized a nutrient medium incubation step which was physically similar to the preincubation step in the *E coli* pol A<sup>+</sup>/pol A<sup>-</sup> suspension assay. Further studies with modification in the bacterial reverse mutation assays will be required to determine whether this lack of mutagenic responses was an artifact of the experimental methodology or a true indication of the absence of mutagenic activity.

In the mouse lymphoma assay the assessment of mutagenic activity by each test chemical was facilitated by plotting the increase in mutagenic frequency as a function of the relative growth (Fig. 1). This comparison was necessary because the toxicity was not always directly related to the applied concentrations within and between trials. However, for all test chemicals the mutation response showed upward trends as the degree of interaction with the cells increased (decreased relative growth). Furthermore, there was an increase in the actual numbers of mutants for each dose-response curve shown in Figure 1.



the much higher solubility for the other materials). Such observations suggest that uncharacterized (and possibly soluble) impurities could play an important role in the resultant mutagenic response.

The purified OTOS sample was tested to address the effect of impurities on the compound's mutagenic response, particularly because one of the impurities, N-nitrosomorpholine, is a known animal carcinogen [Newberne and Shank, 1973; Shank and Newberne, 1976]. Although N-nitrosomorpholine was reduced to very low levels (Table I), no corresponding decrease in the general mutagenic response with this sample was observed. Still, in the mouse lymphoma assay, the mutagenic activities of the commercial and purified samples were found to be quite different in the nonactivation system. Without S-9, purified OTOS curiously exhibited a positive response, while both commercial samples were negative. These results suggested that purifications removed some component(s) toxic to mouse lymphoma cells, thereby permitting exposure to higher, mutagenic amounts of OTOS; or that the removed hypothetical components were able to inhibit mutagenesis specifically in the mouse lymphoma cells. Considering the low survivals and relatively weak responses noted, assay variability cannot be ruled out as a possible explanation.

The relative mutagenic potency of each accelerator in the activation system was estimated by dividing the induced mutation frequency (numbers of mutants/ $10^6$  cells minus the background frequency) at the lowest effective dose by that dose. The following relationship was observed: CTOS (purified) (5.9) > OTOS 2 (4.6) > MBTS (3.8) > OBTS (3.1) > BBTS (0.8). However, the lowest effective dose levels were not found to differ greatly (Table III).

In the transformation assay, all of the chemicals except MBTS and OTOS 2 were active (Table VII). The relative transformation response (total number of foci at the lowest effective dose/lowest effective dose) for these compounds was OTOS (purified) (220) > OTOS 1 (40) > OBTS (25) > BBTS (0.1). It should be noted that direct quantitative comparisons of activity in this assay are difficult because of its somewhat limited detection capability, its inability to assay very toxic doses, the absence of any correction for toxicity, and the inability to detect shifts in toxicity between trials and the preliminary clonal toxicity assay. Furthermore, since these chemicals were evaluated over a period of time, comparison of qualitative differences must be tempered because of possible subtle shifts in the ability of the cells to metabolize test chemicals.

Of the accelerators tested in the chromosomal aberration assay only OTOS 2 and OTOS (purified) produced clear evidence of a clastogenic effect. Both chemicals were positive without activation, but only the purified OTOS was positive with activation. Without activation the percentage of cells with aberrations was higher with the commercial sample than with the purified material, and a significant increase was achieved at one quarter of the dose required for the purified OTOS. Although some genotoxic contaminant(s) may have played a role in these responses, it is important to note that OTOS remained clastogenic after removing about one half of the contaminants (by weight).

The absence of a clastogenic effect in the CHO assay with OBTS, BBTS, or MBTS is quite interesting particularly in light of their apparent chemical "similarities" to OTOS. While it is possible that this might be the result of some specific structural difference, it also is possible that the dose levels may not have been high enough (little to no toxicity) to detect a weak clastogen. This, however, seems less

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TABLE VII. Evaluation of the Transformation Potential of Several Rubber Accelerators in the BALB/3T3 Cell Transformation Assay Under Nonactivation Conditions

| Dose                          | Total number of foci (number of foci/flask) |                       |                        |                        |                        |                        |
|-------------------------------|---|-----------------------|------------------------|------------------------|------------------------|------------------------|
|                               | OTOS 1 <sup>a</sup>                         | OTOS 2 <sup>a</sup>   | OTOS (purified)        | OBTS                   | MBTS                   | BBTS                   |
| Solvent control <sup>b</sup>  | 2 (0.13)                                    | 0 (0)                 | 3 (0.21)               | 2 (0.13)               | 1 (0.07)               | 1 (0.07)               |
| Positive control <sup>c</sup> | 38 <sup>d</sup> (3.80)                      | 69 <sup>d</sup> (4.6) | 63 <sup>d</sup> (4.2)  | 68 <sup>d</sup> (4.53) | 74 <sup>d</sup> (4.93) | 76 <sup>d</sup> (5.07) |
| 0.00625 µg/ml                 | 1 (0.11)                                    | ND <sup>e</sup>       | ND                     | ND                     | ND                     | ND                     |
| 0.01000 µg/ml                 | ND  | ND                    | 0 (0)                  | ND                     | ND                     | ND                     |
| 0.01250 µg/ml                 | 1 (0.13)                                    | ND                    | ND                     | ND                     | ND                     | ND                     |
| 0.02500 µg/ml                 | 0 (0.00)                                    | ND                    | 5 (0.36)               | ND                     | ND                     | ND                     |
| 0.05000 µg/ml                 | 2 (0.25)                                    | 0 (0)                 | 14 <sup>d</sup> (0.93) | 4 (0.27)               | 0 (0)                  | ND                     |
| 0.10000 µg/ml                 | 6 <sup>e</sup> (0.60)                       | 1 (0.07)              | 15 <sup>d</sup> (1.0)  | ND                     | ND                     | ND                     |
| 0.17500 µg/ml                 | ND  | 1 (0.07)              | ND                     | ND                     | ND                     | ND                     |
| 0.20000 µg/ml                 | ND  | ND                    | 15 <sup>d</sup> (1.0)  | 7 <sup>d</sup> (0.47)  | ND                     | ND                     |
| 0.25000 µg/ml                 | ND  | 2 (0.13)              | ND                     | ND                     | ND                     | ND                     |
| 0.35000 µg/ml                 | ND  | 2 (0.20)              | ND                     | ND                     | ND                     | ND                     |
| 0.50000 µg/ml                 | ND  | ND                    | ND                     | ND                     | 1 (0.07)               | 0 (0)                  |
| 2.00000 µg/ml                 | ND  | ND                    | ND                     | 7 <sup>d</sup> (0.47)  | ND                     | ND                     |
| 5.00000 µg/ml                 | ND  | ND                    | ND                     | ND                     | 1 (0.07)               | 1 (0.07)               |
| 10.00000 µg/ml                | ND  | ND                    | ND                     | ND                     | 0 (0)                  | 0 (0)                  |
| 15.00000 µg/ml                | ND  | ND                    | ND                     | 8 <sup>d</sup> (0.53)  | ND                     | ND                     |
| 20.00000 µg/ml                | ND  | ND                    | ND                     | ND                     | 1 (0.07)               | 1 (0.07)               |
| 35.00000 µg/ml                | ND  | ND                    | ND                     | 15 <sup>d</sup> (1.0)  | ND                     | 6 <sup>d</sup> (0.40)  |

<sup>a</sup>Commercial samples.<sup>b</sup>Growth medium contains 0.5% dimethylsulfoxide.<sup>c</sup>5 µg/ml 3-methylcholanthrene.<sup>d</sup>Significant at  $P \leq 0.01$ .<sup>e</sup>ND = not done.

TABLE VIII. Determination of the Mutagenic Potential of Several Rubber Accelerators in the CHO Chromosome Aberration Assay

| Dose                          | Frequency of aberrations/cell (percentage of cells with aberrations) <sup>a</sup> |                              |                            |                              |                              |                       |
|-------------------------------|---|------------------------------|----------------------------|------------------------------|------------------------------|-----------------------|
|                               | OTOS 2  | OTOS (purified)              | OBTS                       | MBTS                         | BBTS                         |                       |
| Untreated control             | > 0.06 (5) <sup>c,f,g</sup>   | 0 (0)                        | 0 (0)                      | 0 (0)                        | 0 (0)                        | 0.02 (2) <sup>c</sup> |
| Solvent control <sup>b</sup>  | 0.02 (1) <sup>f</sup>   | 0.04 (4) <sup>c,f</sup>      | 0 (0)                      | 0.14 (2) <sup>c</sup>        | 0.02 (2) <sup>c</sup>        | 0.02 (2) <sup>c</sup> |
| Positive control <sup>c</sup> | > 0.50 (26) <sup>c,f,g</sup>  | > 2.26 (70) <sup>c,f,g</sup> | 0.34 (26) <sup>c,f,g</sup> | > 1.04 (46) <sup>c,f,g</sup> | > 0.64 (32) <sup>c,f,g</sup> | >                     |
| 0.313 µg/ml                   | 0.01 (1) <sup>f</sup>   | ND                           | ND                         | 0 (0)                        | ND                           | ND                    |
| 0.625 µl/ml                   | 0.01 (1) <sup>f</sup>   | ND                           | 0(0)                       | 0.04 (4) <sup>c,f</sup>      | 0.04 (2) <sup>c</sup>        | 0.04 (2) <sup>c</sup> |
| 1.250 µg/ml                   | 0.06 (6) <sup>c,f,g</sup>   | ND                           | 0.02 (2) <sup>f</sup>      | 0.04 (4) <sup>c,f</sup>      | 0 (0)                        | 0 (0)                 |
| 2.500 µg/ml                   | > 0.55 (31) <sup>c,f,g</sup>  | ND                           | 0 (0)                      | 0 (0)                        | 0 (0)                        | 0 (0)                 |
| 5.000 µg/ml                   | > 0.81 (34) <sup>c,f,g</sup>  | 0.22 (18) <sup>c,f</sup>     | 0 (0)                      | 0.02 (2) <sup>c</sup>        | 0 (0)                        | 0 (0)                 |
| 7.500 µg/ml                   | ND <sup>h</sup>   | ND                           | 0 (0)                      | ND                           | 0 (0)                        | 0 (0)                 |
| 10.000 µg/ml                  | ND  | 0.28 (18) <sup>c,f</sup>     | 0 (0)                      | 0 (0)                        | 0 (0)                        | 0.02 (2) <sup>f</sup> |
| 15.000 µg/ml                  | ND  | 0.34 (26) <sup>c,f</sup>     | ND                         | ND                           | ND                           | ND                    |
| 20.000 µg/ml                  | ND  | 0.18 (12) <sup>c,f</sup>     | ND                         | ND                           | ND                           | ND                    |
| Evaluation                    | +   | +                            | -                          | -                            | -                            | -                     |

Nonactivation

|                               | Activation                |                            |                            |                           |                             |
|-------------------------------|---------------------------|----------------------------|----------------------------|---------------------------|-----------------------------|
| Untreated control             | 0 (0)                     | 0.02 (2)                   | 0 (0)                      | 0.02 (2) <sup>f</sup>     | 0 (0)                       |
| Solvent control <sup>b</sup>  | 0 (0)                     | 0.02 (2)                   | 0 (0)                      | 0 (0)                     | 0 (0)                       |
| Positive control <sup>d</sup> | 0.22 (16) <sup>c,f*</sup> | > 1.60 (66) <sup>*</sup>   | 0.12 (12) <sup>c,f,*</sup> | 0.26 (20) <sup>c,f*</sup> | 0.30 (14) <sup>c,f,g*</sup> |
| 0.313 µg/ml                   | 0 (0)                     | ND                         | ND                         | 0.04 (4) <sup>c,f</sup>   | ND                          |
| 0.625 µg/ml                   | 0.06 (4) <sup>f</sup>     | ND                         | 0 (0)                      | 0.02 (2) <sup>f</sup>     | 0.02 (2) <sup>f</sup>       |
| 1.250 µg/ml                   | 0 (0)                     | ND                         | 0 (0)                      | 0 (0)                     | 0 (0)                       |
| 2.500 µg/ml                   | > 0.04 (4) <sup>f,g</sup> | 0 (0)                      | 0 (0)                      | 0 (0)                     | > 0.12 (8) <sup>c,g*</sup>  |
| 5.000 µg/ml                   | 0.02 (2) <sup>f</sup>     | 0.10 (8) <sup>c,*</sup>    | 0 (0)                      | 0 (0)                     | 0 (0)                       |
| 7.500 µg/ml                   | ND                        | ND                         | 0 (0)                      | ND                        | 0.10 (2) <sup>f</sup>       |
| 10.000 µg/ml                  | ND                        | ND                         | 0.02 (2) <sup>f</sup>      | 0 (0)                     | 0.02 (2) <sup>f</sup>       |
| 15.000 µg/ml                  | ND                        | 0.32 (28) <sup>c,f,*</sup> | ND                         | ND                        | ND                          |
| 20.000 µg/ml                  | ND                        | 0.42 (34) <sup>c,f,*</sup> | ND                         | ND                        | ND                          |
| Evaluation                    | -                         | +                          | -                          | -                         | -                           |

<sup>a</sup>Number of cells evaluated was 50 per culture, except for OTOS (without activation) where 100 cells per culture were examined.

<sup>b</sup>Dimethylsulfoxide: 1% as final concentration in growth medium for OTOS, BBTS, OTOS (purified), and MBTS, 1% acetone for OBTS.

<sup>c</sup>Triethylenemelamine: 0.5 µg/ml for OTOS (purified), MBTS, and BBTS; 0.75 µg/ml for OBTS; 1.0 µg/ml for OTOS.

<sup>d</sup>Cyclophosphamide: 261 µg/ml for OTOS; 62.25 µg/ml for BBTS, MBTS, and BBTS; 130.5 µg/ml for OTOS (purified).

<sup>e</sup>Simple aberrations: chromatid break, acentric fragment, chromosome break, chromatide deletions and fragments.

<sup>f</sup>Complex aberrations: triradial, quadriradial, dicentric chromosome, ring chromosome, complex rearrangement, translocation.

<sup>g</sup>Other aberrations: cell or cells with greater than ten aberrations, pulverized chromosomes, endoreduplication.

<sup>h</sup>ND = not done.

<sup>\*</sup>Significantly increased, P < 0.01.

> Denotes the presence of one or more heavily damaged cells (> 10 aberrations in those cells) and/or pulverized chromatin.

- = negative.

+ = positive.

likely since the L5178Y cell rangefinding and test trial data was used to select doses which exhibited significant toxicity. The only possible exception is with BBTS under activation conditions. Although there is no reason to believe that the top dose is not adequate, it cannot be clearly argued that dose selection erred on the high side for BBTS.

The results of this battery of in vitro assays clearly show that all of the tested chemicals can cause genetic damage to mammalian cells. While little information is available on the extrapolation of such data to predict the risk of germinal mutations in man, prudence suggests that such a consequence should be considered. Positive responses in these in vitro tests for genotoxicity correlate reasonably well with carcinogenic activity. Because of the results obtained in this study and in the study by Hedenstedt et al [1979] rubber accelerators should be regarded as possible human health hazards, and further in vitro and in vivo studies should be conducted to more clearly evaluate the mutagenic/carcinogenic potential of this large class of chemicals.

While many factors such as health effects, exposure potential, and the number of individuals exposed must be considered in setting priorities for further in vivo testing, a battery of in vitro mutagenic screens can play an important role in this process. However, it is not possible simply to say that a chemical which is mutagenic in five out of six assays is of greater concern than one which is mutagenic in three or only two assays. One must consider the events that the assays measure and not just the number of positives. Chemicals which exhibit two or more different types of mutagenic events should be of greater concern simply because this suggests that there are a greater number of ways that could lead to a deleterious event. As we learn more about the relationship of these in vitro events to those in vivo, the degree of differences and their very nature will become more important in determining what future studies are appropriate.

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