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PHILLIPS PETROLEUM COMPANY

1985 MAY -8 AM 10:43

BARTLESVILLE, OKLAHOMA 74004  
918 661-5956

*FYI-OTS-0585-0401 INITIAL  
SEQUENCE A*

Corporate Engineering  
JOHN J. MOON  
Manager, Environment and Consumer Protection

April 30, 1985

For Your Information  
t-Butyl Peroxybenzoate

TSCA Document Control Office  
Chemical Information Division  
Office of Toxic Substances (WH-557)  
Environmental Protection Agency  
401 M Street S.W.,  
Washington, D.C. 20460

RECEIVED  
5/10/85  
TOB/CSB

Dear Sir:

Phillips is by this communication voluntarily submitting "For Your Information", three laboratory reports on t-butyl peroxybenzoate [614-45-9] (TBPB). The study was performed under contract with us by the Hazleton Biotechnologies Corporation. The reports are as follows:

1. In Vitro Sister Chromatid Exchange in Chinese Hamster Ovary Cells (Attachment I)
2. Salmonella typhimurium/Mammalian Microsome Plate Incorporation Assay with Compound TBPB (Attachment II)
3. Mouse Lymphoma Forward Mutation Assay (Attachment III)

The reports are accompanied with two attachments:

1. A Summary of the Mutagenic Studies on TBPB (Attachment IV), and
2. A copy of the IARC Monograph, guidelines used for assessing the evidence for genetic activity. (Attachment V).

Following a review of this information, Phillips' TSCA Compliance Committee determined that although there was not sufficient evidence to indicate a substantial mutagenic risk to humans, it does merit attention and is therefore provided for your information.

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The results of our testing indicate the following:

Results of the in vitro Sister Chromatid Exchange assay in Chinese Hamster Ovary Cells showed dose-related, statistically significant increases at four dose levels without metabolic activation and at two dose levels with metabolic activation. None of the increases were 2-fold greater than the control values.

Negative results were obtained in the AMES Mutation assays.

Results obtained in the Mouse Lymphoma Forward Mutation Assay showed dose-related, greater than 2-fold increases in mutation frequency in duplicate cell cultures at the three highest dose levels without metabolic activation. A greater than 2-fold increase was seen only at the highest dose level with metabolic activation. These results have been summarized in Attachment IV.

The positive mutagenic results reported for two of the three test assays are considered to present new evidence of in vitro mutagenic activity with TBPB. However, we do not feel that these test results are sufficient to indicate a substantial mutagenic risk to humans. In this regard, we concur with the International Agency for Research on Cancer (IARC) Working group's scheme for assessing the evidence for genetic activity from short-term tests. Using this evaluation scheme, as listed in Attachment V, the test results would support a classification of limited evidence of genetic activity. It should be noted that the IARC definitions are considered operational and thus categorization is somewhat arbitrary. It is clear that short-term tests should not be used by themselves to conclude whether or not an agent is carcinogenic, nor as the sole criterion for setting priorities in carcinogenesis research and in selecting compounds for animal bioassays.

NTP selected TBPB for carcinogenesis testing. Testing was deferred as of 12/83. As of 4/84, NTP carcinogenicity testing on this product was still being deferred.

Physico-Chemical properties of TBPB were also considered in this hazard evaluation. TBPB is not shock-sensitive but does burn vigorously and is difficult to extinguish. Decomposition products generally are flammable vapors which can form explosive mixtures in air. The vapors may be hot enough to auto-ignite on contact with air if decomposition is rapid.

Physical hazards must be examined along with health hazards to decide which properties pose a greater potential or dominant danger to the employee. If the physical hazard is found to be dominant, the necessary careful and safe handling of the substance may preclude employee exposure.

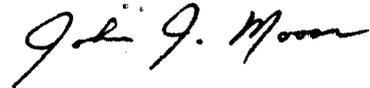
April 30, 1985

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For the purpose of such a risk assessment, all the information discussed above is being provided to the Organic Peroxide Producers Safety Division Committee of the Society of Plastics Industry.

If you have questions or need clarification on the information presented, please contact Mr. Russell Cook at (918) 661-4642.

Very truly yours,



RC:ccw/CE-404a  
Attachments (5)

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ATTACHMENT 1  
J. J. Moon  
April 30, 1985  
TBPB



**HAZLETON**

BIOTECHNOLOGIES CORPORATION

9200 LEESBURG TURNPIKE, VIENNA, VIRGINIA 22180, U S A

In Vitro Sister Chromatid Exchange in  
Chinese Hamster Ovary Cells

t-Butyl Peroxybenzoate

FINAL REPORT

Submitted to

Phillips Petroleum Company  
Bartlesville, Oklahoma

December 17, 1984



**HAZLETON**

BIOTECHNOLOGIES CORPORATION  
9200 LEBBURG TURNPIKE VIENNA VIRGINIA 22180 U.S.A.

SUBJECT: In vitro Sister Chromatid Exchange in Chinese Hamster Ovary  
Cells (CHO)  
Project No. 652-186

We, the undersigned, hereby declare that the work was performed under our supervision, according to the procedures herein described.

Study Director and Project Coordinator:

Deborah H. Pence 12/14/84  
DEBORAH H. PENCE, Ph.D. Date  
Diplomate, American Board of Toxicology  
Associate Director fo Toxicology/  
Scientific Coordination  
Life Sciences Division

Laboratory Supervision and Report Preparation:

Thomas A. Cortina 12/12/84  
THOMAS A. CORTINA, B.S. Date  
Research Associate  
Genetic and Immunotoxicology Department

RAW DATA STORAGE

At the completion of this study, the original copy of all raw data and the final report were sent to the Archives of Hazleton Laboratories America, Inc., Vienna, Virginia

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# HAZLETON

BIOTECHNOLOGIES CORPORATION

9200 LEESSBURG TURNPIKE, VIENNA, VIRGINIA 22180 U.S.A.

SPONSOR: Phillips Petroleum Company

SUBJECT: FINAL REPORT

In vitro Sister Chromatid Exchange in Chinese Hamster Ovary Cells

HLA Project Number: 652-186

Experiment Initiated: 7/12/84

Experiment Completed: 11/19/84

Test Material: t-Butyl Peroxybenzoate

Laboratory Number

699

LH Number

21,382A

Receipt Date

6/6/84

Control Articles:

Supplier

Lot Number

Purity

Positive Controls:

Ethylmethane sulfonate (EMS)

Eastman

AIA

Assumed 100%

Cyclophosphamide (CP)

Sigma

123F-0283

Assumed 100%

Solvent:

Dimethylsulfoxide (DMSO)

Fisher

730913

Assumed 100%

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652-186

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SUMMARY

This study was designed to evaluate the potential of t-Butyl Peroxybenzoate to induce sister chromatid exchanges (SCE) in Chinese Hamster Ovary Cells (CHO). CHO cells were exposed to six concentrations of the test material; 52, 18, 5.2, 1.8, 0.52, and 0.18 ug/ml for two hours in the presence of and in the absence of metabolic activation followed by a 24 hour expression period.

Results show that statistically significant increases in the frequency of SCE's were seen at 52, 18, 5.2, and 1.8 ug/ml without activation and at 18 and 5.2 ug/ml with activation.

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## OBJECTIVE

The purpose of this study was to evaluate t-Butyl Peroxybenzoate for the ability to induce SCE's in Chinese Hamster Ovary cells.

### A. Test Materials

#### 1. Storage

A colorless liquid designated t-Butyl Peroxybenzoate was submitted by the sponsor. Upon arrival in the laboratory, the sample was assigned Laboratory Number 699 and stored refrigerated until required for use. It should be noted that under the conditions of this in vitro assay, the test material was assumed stable.

#### 2. Preparation of Chemical Solutions

Preparation of test and control chemical was performed using precautions for handling toxic substances which conform to the safety standards of the Toxicologic Subcommittee for Carcinogen Standards, U.S. Department of Health and Human Services. All weighing was done by weight difference determinations in a chemical fume hood.

### B. Control Articles

#### 1. Positive Controls

Ethylmethane sulfonate (EMS) was used as the positive control without metabolic activation. Cyclophosphamide (CP) was used as the positive control with metabolic activation. Both compounds are assumed stable under the conditions of this assay.

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## 2. Solvent Controls

Both positive control compounds were dissolved in Ham's F-12 culture medium. The test material was dissolved in dimethylsulfoxide (DMSO).

### C. Cells

Chinese Hamster Ovary cells CHO, K-1, Number CCL61 were obtained from the American Type Culture Collection. CHO cells are a permanent cell line with an average modal number of 20, and an average cycling time of 10-14 hours.

CHO cells are maintained as a monolayer in Ham's F-12 medium with 10% fetal calf serum (FCS) and frozen stocks are held in a liquid nitrogen storage tank.

The cultures used for the mutation assay were thawed on September 18, 1984 and used at passage Px+6.

### D. Solubility

Test Compound t-Butyl Peroxybenzoate was soluble in DMSO at approximately 1000 mg/ml.

### E. Media and Reagents

All media and reagents used in this study were prepared and stored under the appropriate conditions and assigned a Batch Number and Expiration Date. Records are maintained in the Media and Reagent Log Books.

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F. Toxicity Testing

1. Design and Dosing

Six concentrations of t-Butyl Peroxybenzoate were prepared to contain the test dose in a 0.025 ml volume. The highest dose tested both with and without metabolic activation was 5,200 ug/ml with subsequent concentrations of 520, 52, 5.2, 0.52, and 0.052 ug/ml.

Sixteen 25 cm<sup>2</sup> tissue culture flasks were seeded with  $3.0 \times 10^5$  CHO cells in 5 ml Ham's F-12 medium. Twenty-four hours after culture initiation, the medium, S-9 mix, and test solutions were dispensed into the appropriate flasks as shown in Table I. Each flask was mixed, tightly capped, and incubated at  $37 \pm 0.5^\circ\text{C}$ . Following a four-hour exposure, each flask was rinsed twice with a balanced salt solution, 5 ml Ham's F-12 medium added to each flask, and the flasks incubated again at  $37 \pm 0.5^\circ\text{C}$ .

Approximately 24 hours after the flasks were rinsed, cell counts were performed on each flask using a Coulter Counter .

2. Results

Results for the toxicity test are presented in Table 2.

The test material reduced growth to 55% at 52 ug/ml without activation and to less than 1% at 52 ug/ml with activation. Based on these results, 52 ug/ml was chosen as the high dose without activation and 18 ug/ml the high dose with activation.

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G. SCE Assay

1. Design and Dosing

Six concentrations of t-Butyl Peroxybenzoate were prepared to contain the test dose in a 0.025 ml volume. The highest dose tested without metabolic activation was 52 ug/ml with the subsequent concentration of 18, 5.2, 1.8 and 0.52 ug/ml. The highest dose tested with metabolic activation was 18 ug/ml with subsequent concentrations of 5.2, 1.8, 0.52 and 0.18 ug/ml.

Thirty-two 25 cm<sup>2</sup> tissue culture flasks were seeded with  $3.0 \times 10^5$  CHO cells in 5 ml Ham's F-12 medium. Twenty-four hours after culture initiation, the medium, S-9 mix, and test solutions were dispensed into the appropriate flasks in duplicate as shown in Table 3. Each flask was mixed, tightly capped, and incubated at  $37 \pm 0.5^\circ\text{C}$ . Following a two-hour exposure, each flask was rinsed twice with a balanced salt solution, 5 ml Ham's F-12 medium containing 5-bromodeoxyuridine (BrdU) added to each flask, and the flasks incubated again at  $37 \pm 0.5^\circ\text{C}$ . Each flask was wrapped in aluminum foil to exclude light.

Approximately 24 hours after the flasks were rinsed, colcemid (0.2 ug/ml) was added to all flasks to arrest cells in metaphase.

2. Preparation of CHO Chromosomes

Two hours after the addition of colcemid, metaphases were collected by mitotic shake-off. Each flask was firmly tapped and the media was poured into an appropriately labelled 15 ml centrifuge tube. The cells were washed once in 0.075 M KCl, and washed twice in an acetic alcohol fixative (3:1, methanol: acetic acid).



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3. Preparation of Slides

Two slides were prepared for each treatment group, one from each duplicate flask. Slides were prepared by dropping the cells onto clean wet slides and air drying.

4. Slide Staining

Slides were stained for 10 minutes in Hoechst 33258 (5 ug/ml) in phosphate buffer (pH 6.8) and rinsed in glass distilled H<sub>2</sub>O. Coverslips were mounted with the same buffer and the slides exposed to a black light for approximately 1 hour.

Coverslips were removed and the slides stained in 2% Giemsa in buffer, rinsed twice, and air dried. Each slide was mounted with a glass coverslip using Coverbond®.

5. Slide Randomization

Flask numbers on each slide were covered with masking tape and each was assigned a temporary slide number. The coding was performed by an individual not involved in the scoring of the slides.

6. Slide Analysis

Fifty cells in the metaphase stage of mitosis were scored whenever possible at each dose level for the number of sister chromatid exchanges (SCE's). Results are presented as the number of SCE's per cell, and the number of SCE's per chromosome.

7. Statistical Analysis

All data were analyzed by analysis of variance (ANOVA) with individual group comparisons performed by a one-tailed students t- test. The data were analyzed at the 99% confidence interval ( $p < .01$ ).

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## RESULTS

The design for the SCE assay appears in Table 3. A summary of the SCE data appears in Table 4.

Results show that statistically significant increases in the number of SCE's per chromosome were seen at 52 ug/ml ( $p < 0.0001$ ), 18 ug/ml ( $p < 0.0001$ ), 5.2 ug/ml ( $p < 0.0001$ ) and 1.8 ( $p = 0.0023$ ) without activation and at 18 ug/ml ( $p < 0.0001$ ) and 5.2 ug/ml ( $p = 0.0033$ ) with metabolic activation.

Cyclophosphamide (CP) and Ethylmethane sulfonate (EMS) produced significant increases in the number of SCE's per chromosome ( $p < .0001$  CP,  $p < .0001$  EMS) both with and without metabolic activation, respectively, and both produced increases of greater than two-fold compared to the controls.

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### DISCUSSION AND CONCLUSIONS

The exposure of CHO cells to 52, 18, 5.2, 1.8, 0.52, and 0.18 ug/ml of t-Butyl Peroxybenzoate produced statistically significant increases in the frequency of sister chromatid exchanges (SCE) at 52, 18, 5.2, and 1.8 ug/ml without metabolic activation, and at 18 and 5.2 ug/ml with activation.

Therefore, under the conditions of this study, t-Butyl Peroxybenzoate is considered to be mutagenic in this test system.

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Table 1  
In vitro Sister Chromosome Exchange (CHO) Toxicity Test  
Experimental Design  
t-Butyl Peroxybenzoate

| Group and<br>Flask Number | Contents   |
|---------------------------|--|
| 1                         | 5 ml Ham's F-12  |
| 2                         | 5 ml Ham's F-12+.025 ml DMSO   |
| 3                         | 5 ml Ham's F-12+.025 ml t-Butyl Peroxybenzoate, 1039 mg/ml                                     |
| 4                         | 5 ml Ham's F-12+.025 ml t-Butyl Peroxybenzoate, 104 mg/ml                                      |
| 5                         | 5 ml Ham's F-12+.025 ml t-Butyl Peroxybenzoate, 10.4 mg/ml                                     |
| 6                         | 5 ml Ham's F-12+.025 ml t-Butyl Peroxybenzoate, 1.04 mg/ml                                     |
| 7                         | 5 ml Ham's F-12+.025 ml t-Butyl Peroxybenzoate, 0.104 mg/ml                                    |
| 8                         | 5 ml Ham's F-12+.025 ml t-Butyl Peroxybenzoate, 0.0104 mg/ml                                   |
| 9                         | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix   |
| 10                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+.025 ml DMSO                                    |
| 11                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+.025 ml<br>t-Butyl Peroxybenzoate, 1039 mg/ml   |
| 12                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+.025 ml<br>t-Butyl Peroxybenzoate, 104 mg/ml    |
| 13                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+.025 ml<br>t-Butyl Peroxybenzoate, 10.4 mg/ml   |
| 14                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+.025 ml<br>t-Butyl Peroxybenzoate, 1.04 mg/ml   |
| 15                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+.025 ml<br>t-Butyl Peroxybenzoate, 0.104 mg/ml  |
| 16                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+.025 ml<br>t-Butyl Peroxybenzoate, 0.0104 mg/ml |

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Table 2  
Preliminary Toxicity Test Results  
In vitro Sister Chromatid Exchange Assay with t-Butyl Peroxybenzoate

| <u>Treatment</u> | <u>S-9</u> | <u>Cell Counts/Flask</u><br><u>(x 10<sup>6</sup>)</u> | <u>Percentage<sup>1</sup></u><br><u>Growth</u> |
|------------------|------------|---|--|
| Media Control    | -          | 2.0   | -  |
| DMSO             | -          | 2.0   | -  |
| 5,200 ug/ml      | -          | 0.0046  | 0.23   |
| 520 ug/ml        | -          | 0.0032  | 0.16   |
| 52 ug/ml         | -          | 1.1   | 55   |
| 5.2 ug/ml        | -          | 1.5   | 75   |
| 0.52 ug/ml       | -          | 2.0   | 100  |
| 0.052 ug/ml      | -          | 2.2   | 110  |
| Media Control    | +          | 1.4   | -  |
| DMSO             | +          | 1.5   | -  |
| 5,200 ug/ml      | +          | 0.031   | 21   |
| 520 ug/ml        | +          | 0.0071  | 0.47   |
| 52 ug/ml         | +          | 0.0062  | 0.41   |
| 5.2 ug/ml        | +          | 1.1   | 73   |
| 0.52 ug/ml       | +          | 1.4   | 93   |
| 0.052 ug/ml      | +          | 1.5   | 100  |

<sup>1</sup> Percentage Growth =  $\frac{\text{Cell Counts Treated}}{\text{Cell Count Solvent Control}} \times 100$

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Table 3  
In vitro Sister Chromatid Exchange (CHO) Assay Experimental Design  
t-Butyl Peroxybenzoate

| Group and<br>Flask Number | Contents   |
|---------------------------|--|
| 1                         | 5 ml Ham's F-12  |
| 2                         | 5 ml Ham's F-12+0.025 ml EMS, 80 mg/ml   |
| 3                         | 5 ml Ham's F-12+0.025 ml DMSO  |
| 4                         | 5 ml Ham's F-12+0.025 ml t-Butyl Peroxybenzoate, 10.4 mg/ml                                    |
| 5                         | 5 ml Ham's F-12+0.025 ml t-Butyl Peroxybenzoate, 3.5 mg/ml                                     |
| 6                         | 5 ml Ham's F-12+0.025 ml t-Butyl Peroxybenzoate, 1.04 mg/ml                                    |
| 7                         | 5 ml Ham's F-12+0.025 ml t-Butyl Peroxybenzoate, 0.35 mg/ml                                    |
| 8                         | 5 ml Ham's F-12+0.025 ml t-Butyl Peroxybenzoate, 0.104 mg/ml                                   |
| 9                         | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix   |
| 10                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+0.025 ml CP,<br>0.28 mg/ml                      |
| 11                        | 4.5 ml Activation Dosing medium+0.5 ml S-9 Mix+0.025 ml DMSO                                   |
| 12                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+0.025 ml<br>t-Butyl Peroxybenzoate, 3.5 mg/ml   |
| 13                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+0.025 ml<br>t-Butyl Peroxybenzoate, 1.04 mg/ml  |
| 14                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+0.025 ml<br>t-Butyl Peroxybenzoate, 0.35 mg/ml  |
| 15                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+0.025 ml<br>t-Butyl Peroxybenzoate, 0.104 mg/ml |
| 16                        | 4.5 ml Activation Dosing Medium+0.5 ml S-9 Mix+0.025 ml<br>t-Butyl Peroxybenzoate, 0.035 mg/ml |

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Table 4  
Summary of Sister Chromatid Exchange Data  
t-Butyl Peroxybenzoate

## Without Activation

| Treatment              | Dose Level (µg/ml) | Number of Cells Analyzed | Total Number of SCE's | Number of SCE's/Cell | P Value   | Fold Increase in SCE's/Cell | Number of Chromosomes Analyzed | Number of SCE's/Chromosome | P Value   | Fold Increase in SCE's/Chromosome |
|------------------------|--------------------|--------------------------|-----------------------|----------------------|-----------|-----------------------------|--------------------------------|----------------------------|-----------|-----------------------------------|
|                        |                    |                          |                       |                      |           |                             |                                |                            |           |                                   |
| Media Control          | -                  | 50                       | 496                   | 9.92                 | -         | -                           | 983                            | 0.50                       | -         | -                                 |
| DMSO                   | -                  | 50                       | 468                   | 9.36                 | -         | -                           | 988                            | 0.48                       | -         | -                                 |
| EMS                    | 400                | 50                       | 1,803                 | 36.06                | 0.0000(S) | 3.6                         | 1003                           | 1.80                       | 0.0000(S) | 3.6                               |
| t-Butyl Peroxybenzoate | 52                 | 50                       | 794                   | 15.88                | 0.0000(S) | 1.7                         | 994                            | 0.80                       | 0.0000(S) | 1.7                               |
|                        | 18                 | 50                       | 648                   | 12.96                | 0.0000(S) | 1.4                         | 993                            | 0.65                       | 0.0000(S) | 1.4                               |
|                        | 5.2                | 50                       | 641                   | 12.82                | 0.0000(S) | 1.4                         | 992                            | 0.65                       | 0.0000(S) | 1.4                               |
|                        | 1.8                | 50                       | 555                   | 11.10                | 0.0022(S) | 1.2                         | 981                            | 0.57                       | 0.0022(S) | 1.2                               |
|                        | 0.52               | 50                       | 482                   | 9.64                 | 0.31(NS)  | 1.0                         | 983                            | 0.49                       | 0.31(NS)  | 1.0                               |

## With Activation

| Treatment              | Dose Level (µg/ml) | Number of Cells Analyzed | Total Number of SCE's | Number of SCE's/Cell | P Value   | Fold Increase in SCE's/Cell | Number of Chromosomes Analyzed | Number of SCE's/Chromosome | P Value   | Fold Increase in SCE's/Chromosome |
|------------------------|--------------------|--------------------------|-----------------------|----------------------|-----------|-----------------------------|--------------------------------|----------------------------|-----------|-----------------------------------|
|                        |                    |                          |                       |                      |           |                             |                                |                            |           |                                   |
| Media Control          | -                  | 50                       | 456                   | 9.12                 | -         | -                           | 979                            | 0.47                       | -         | -                                 |
| DMSO                   | -                  | 50                       | 506                   | 10.12                | -         | -                           | 988                            | 0.51                       | -         | -                                 |
| CP                     | 1.4                | 50                       | 1,281                 | 25.62                | 0.0000(S) | 2.8                         | 980                            | 1.31                       | 0.0000(S) | 2.8                               |
| t-Butyl Peroxybenzoate | 18                 | 50                       | 783                   | 15.66                | 0.0000(S) | 1.5                         | 989                            | 0.79                       | 0.0000(S) | 1.5                               |
|                        | 5.2                | 50                       | 594                   | 11.88                | 0.0041(S) | 1.2                         | 985                            | 0.60                       | 0.0033(S) | 1.2                               |
|                        | 1.8                | 50                       | 515                   | 10.30                | 0.36(NS)  | 1.0                         | 985                            | 0.52                       | 0.34(NS)  | 1.0                               |
|                        | 0.52               | 50                       | 527                   | 10.54                | 0.24(NS)  | 1.0                         | 987                            | 0.54                       | 0.22(NS)  | 1.1                               |
|                        | 0.18               | 50                       | 532                   | 10.64                | 0.20(NS)  | 1.1                         | 985                            | 0.54                       | 0.19(NS)  | 1.1                               |

DMSO = Dimethylsulfoxide  
EMS = Ethylmethanesulfonate  
CP = Cyclophosphamide  
NS = Not significant  
S = Significant



**HAZLETON**

LABORATORIES AMERICA, INC.

9200 LEEBURG TURNPIKE, VIENNA, VIRGINIA 22180, U.S.A.

ATTACHMENT II  
J. J. Moon  
April 30, 1985  
TBPB

Salmonella typhimurium/Mammalian Microsome

Plate Incorporation Assay with

Compound t-BPB

FINAL REPORT

LH# 21,382A

Batch Not Listed

Submitted to:

PHILLIPS PETROLEUM  
Bartlesville, OK 74004

August 20, 1984

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# HAZLETON

LABORATORIES AMERICA, INC.

9200 LEESBURG TURNPIKE, VIENNA, VIRGINIA 22180, U.S.A.

SPONSOR: PHILLIPS PETROLEUM

SUBJECT: FINAL REPORT

Salmonella typhimurium/Mammalian Microsome Plate  
Incorporation Assay with Compound t-BPB

Laboratory Number: 699  
HLA Project Number: 652-184  
Compound: t-Butyl Peroxybenzoate (t-BPB)  
LH# Number: 21,382A  
Batch Number: Not Listed

Experiment Initiated: 07/03/84

Experiment Completed: 07/19/84

Receipt Date: 06/06/84

## PROJECT PERSONNEL

| <u>Title</u>                                      | <u>Name</u>        | <u>Signature</u>                  |
|---|--------------------|-----------------------------------|
| Study Director                                    | Deborah H. Pence   | <u>Deborah H. Pence</u>           |
| Scientific Investigator and<br>Report Preparation | Nancy E. McCarroll | <u>Nancy E. McCarroll 8-10-84</u> |

## RAW DATA STORAGE

At the completion of this study, the original copy of all raw data and the final report were sent to the Archives of Hazleton Laboratories America, Inc., Vienna, Virginia.



**HAZLETON**

LABORATORIES AMERICA, INC.

9200 LEESSBURG TURNPIKE, VIENNA, VIRGINIA 22180, U.S.A.

## OBJECTIVE

The purpose of this study was to examine an experimental compound for mutagenic activity in the Salmonella typhimurium/Mammalian Microsome mutagenicity test.

## EXPERIMENTAL PROCEDURES

### Materials and Supplies

#### A. Chemical

##### 1. Storage

A colorless to slightly yellow liquid designated t-Butyl Peroxybenzoate was furnished by the sponsor.

Upon arrival in the laboratory, the sample was assigned Laboratory Number 699 and stored at refrigerator temperature until required for use. It should be noted that under the conditions of this in vitro assay, the test material was assumed stable.

##### 2. Preparation of Chemical Solutions

Preparation of test and control chemical was performed using precautions for handling toxic substances which conform to the safety standards of the Toxicology Subcommittee for Carcinogen Standards, U.S. Department of Health, Education and Welfare. All weighing was done by weight difference determinations in a chemical fume hood.



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## B. Control Substances

The control substances listed below were used in this study and were similarly considered stable under the conditions of this in vitro assay.

| <u>Chemical</u>                    | <u>Manufacturer</u> | <u>Lot Number</u> | <u>Purity</u>   |
|------------------------------------|---------------------|-------------------|-----------------|
| Dimethylsulfoxide (DMSO)           | Fisher              | 730913            | Certified ACS   |
| Methylnitronitrosoguanidine (MNNG) | Aldrich             | 010247            | 97%             |
| 9-Aminoacridine (9-AA)             | Sigma               | 23F-0382          | Grade II        |
| 2-Nitrofluorene (2-NF)             | Aldrich             | 2610PE            | 98%             |
| 2-Aminoanthracene (2-AA)           | Sigma               | 11 of 0600        | Practical Grade |

## C. Reported Values

It should be noted that due to computer limitations, weights and volumes listed in the final report may be approximate representations when compared to raw data values. Actual weights and volumes are cited in the raw data.

## PROCEDURES

The experimental compound was investigated in accordance with the method of Ames et al (1).

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A. Solubility Studies

Based on the furnished specific gravity, it was assumed for the purposes of this study that one ml was equivalent to one gram. A 0.1 ml sample containing 100 mg was fully soluble when mixed with 0.9 ml of DMSO. This 100 mg/ml solution was, therefore, used as the starting dose for the toxicity test.

B. Toxicity Test

The stock solution prepared as described above was subsequently diluted in the same solvent to contain approximately 1000.0, 100.0, 10.00, 1.00, and .10 ug/0.1 ml.

The initial stock solution and the five ten-fold dilutions were used to establish the level at which approximately 50 percent of the bacterial cells would survive.

To each 2.5 ml of complete top agar, (0.6% agar, 0.05mM L-histidine-HCL, 0.05mM biotin), 0.1 ml of an overnight broth culture of S. typhimurium, TA100 and 0.1 ml of the appropriate dilution of the test compound or diluent were added. The contents of the tube were mixed and poured onto Vogel-Bonner Medium E (VBE) minimal agar plates. The plates were allowed to harden on a level surface, and inverted and placed in a dark  $37 \pm 0.5$  C incubator.

After two days of incubation, toxicity was observed at the 10,000 and 1,000 ug/plate levels. Slight compound precipitation was also noted at the highest test dose. Based on these findings, the starting dose selected for the mutational assay was 500 ug/plate.

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C. Media and Reagents

To ensure uniformity throughout the assay, all media and reagents used in this study were prepared in bulk volumes, stored under the appropriate conditions, and assigned a Batch Number and Expiration Date. Records are maintained in the Quality Control Log Book. The same batch of each ingredient was used for each phase of compound testing.

D. Mutational Test

Direct exposure of the auxotrophic S. typhimurium strains to the test compound was accomplished by the plate incorporation assay. Five concentrations of the test agent were evaluated in triplicate against the five bacterial strains with and without the metabolic activation system.

Approximately 0.2 ml of the test material weighing 200 mg were mixed with 3.8 ml of the solvent to contain approximately 50 mg/ml. This solution was subsequently diluted in the same solvent to 500, 166.7, 55.6, 18.5, and 6.2 ug/0.1 ml.



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To each 2.5 ml of complete top agar, 0.1 ml of an overnight broth culture of each tester strain, 0.1 ml of the appropriate dilution of the test agent or diluent and 0.5 ml of the S-9 mix for the activated tests were added. The contents of each tube were thoroughly mixed and poured onto the VBE minimal agar plates. Positive control chemicals were treated in a similar manner. Plates were gently rotated and tilted to assure uniform distribution of the top agar, allowed to harden on an even surface for approximately one hour, inverted, and placed in a dark  $37 \pm 0.5$  C incubator.

After two days, the colonies (revertants to histidine prototrophy) in both test and control plates were counted.

Due to high spontaneous reversion frequencies with strain TA100, this portion of the assay was repeated. Accordingly 0.2 ml of the test material were mixed with 3.8 ml of DMSO and diluted as described for the repeat test with strain TA100 both in the presence and absence of metabolic activation.



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## RESULTS AND DISCUSSION

Before an agent is reported to be either active or inactive in the Salmonella/microsomal assay, the following criteria must be met:

1. Demonstration of toxicity of the chemical for the bacterial strain(s), unless this is not possible due to a limited solubility of the test compound.
2. The spontaneous revertants for each strain must be within acceptable limits. The range of observed values are given below. The figures are from tests conducted in our laboratory compiled to the present.

3a. Without metabolic activation: TA1535: Range 10.0- 50.0  
Mean 21.7  
Standard Deviation (SD) 7.6

TA1537: Range 5.0- 19.0  
Mean 8.2  
SD 3.1

TA100: Range 100.0-200.0  
Mean 129.5  
SD 37.1

TA1538: Range 10.0- 30.0  
Mean 13.5  
SD 4.3

TA98: Range 15.0- 45.0  
Mean 28.5  
SD 17.3

b. With metabolic activation: TA1535: Range 4.0- 26.0  
Mean 15.3  
Standard Deviation (SD) 5.3

TA1537: Range 4.0- 20.0  
Mean 9.4  
SD 3.5

TA100: Range 100.0-200.0  
Mean 137.8  
SD 35.5

TA1538: Range 13.0- 39.0  
Mean 27.9  
SD 6.9

TA98: Range 15.0- 50.0  
Mean 39.9  
SD 16.5



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4. The solvent controls must have approximately the same number of colonies as spontaneous reversion controls.
5. Positive mutagens must give at least 2x the number of colonies as the controls for spontaneous reversion. Any test with a strain which does not meet these criteria must be repeated on a separate day.

All criteria noted above must be met before results with an unknown agent can be evaluated. A chemical that exhibits a positive dose response over three concentrations with the smallest of these increases equal to twice the solvent control is considered to be mutagenic in the S. typhimurium assay.

The aforementioned criteria were satisfied by the test conditions used with the experimental agent and the activity of the test material was evaluated accordingly.

S. typhimurium revertants to histidine prototrophy on test and control plates were counted with a New Brunswick scientific electronic colony counter.



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The results of the Salmonella assay are presented in Tables I and II.

As shown in Table I, the test compound without activation did not induce a significant increase in the number of revertant colonies over that shown in the solvent control plates for strains TA1535, TA1537, TA1538, TA100, TA98.

The inclusion of metabolic activation did not affect the activity of the test agent in this system as shown in Table II for strains TA1535, TA1537, TA1538, TA100, TA98.

Diagnostic controls which are included with Tables 1 and 2 confirm the sensitivity and responsiveness of the tester strains to detect genetic interaction with known mutagenic agents.



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### CONCLUSIONS

The aforementioned criteria were satisfied by the test conditions used with the test material. Under these conditions, the test material is not mutagenic in this test system.



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ABBREVIATIONS\*

|        |   |
|--------|---|
| 2-AA   | 2-Aminoanthracene   |
| 9-AA   | 9-Aminoacridine   |
| DMSO   | Dimethylsulfoxide**   |
| DGDH20 | Deionized Glass Distilled Water                                     |
| g      | Gram  |
| HIS+   | Histidine positive  |
| hr     | Hour  |
| M      | Molar   |
| mg     | Milligram   |
| min    | Minute  |
| ml     | Milliliter  |
| mm     | Millimeter  |
| mM     | Millimolar  |
| MNNG   | Methylnitronitrosoguanidine   |
| NaCl   | Sodium Chloride   |
| 2-NF   | 2-Nitrofluorene   |
| OD     | Optical density   |
| pH     | Acid-base scale; log of reciprocal of<br>hydrogen ion concentration |
| S-9    | Supernatant from 9000 x g centrifugation<br>of a liver homogenate   |
| SD     | Standard deviation  |
| u      | Micron  |
| ug     | Microgram   |
| ul     | Microliter  |
| 4-NPA  | 4-nitro-o-phenylenediamine  |
| A      | Heavy precipitation   |
| B      | Moderate Precipitation  |
| C      | Slight Precipitation  |
| D      | Repeat Test   |

\* It should be noted that all listed abbreviations may not appear in the text.

\*\* Solvent for positive control compounds.



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REFERENCES\*

1. Methods for Detecting Carcinogens and Mutagens with the Salmonella Mammalian-Microsome Mutagenicity Test. B. N. Ames, J. McCann and E. Yamasaki. *Mutation Research* 31 (1975) 347-364. (1981) 429-444.
2. Mutagenicity of N-nitrosamines in Salmonella. T. Yahagi, M. Nagao, Y. Seino, T. Matsushima, T. Sugimura and M. Okada. *Mutation Research* 48 (1977) 121-130.
3. Recommendations on data production and analysis using the Salmonella microsomal mutagenicity assay. F.J. de Serres, M.D. Shelby. *Mutation Research* 64 (1979) 159-165.

\* It should be noted that all listed references may not appear in text or tables.



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TABLE I

Results of SALMONELLA/MICROSOMAL Assay  
without metabolic activation on t-BPB

| COMPOUND                            | CONC<br>UNITS/<br>PLATE | REVERTANTS PER PLATE OF BACTERIAL TESTER STRAINS |                 |                  |                    |                  |
|-------------------------------------|-------------------------|--|-----------------|------------------|--------------------|------------------|
|                                     |                         | TA1535   | TA1537          | TA1538           | TA100 <sup>D</sup> | TA98             |
| BACTERIA ONLY<br>(NEGATIVE CONTROL) | 100.0000<br>ul          | 22.7<br>+ 3.1                                    | 6.7<br>+ 1.2    | 11.3<br>+ 7.5    | 102.0<br>+ 10.6    | 21.7<br>+ 2.5    |
| DMSO<br>(SOLVENT CONTROL)           | 100.0000<br>ul          | 18.3<br>+ 4.2                                    | 5.7<br>+ 2.3    | 10.0<br>+ 7.8    | 100.7<br>+ 16.2    | 20.7<br>+ 3.2    |
| MNNG<br>(POSITIVE CONTROL)          | 5.0000<br>ug            | 1862.7<br>+ 91.4                                 | NT              | NT               | 2025.3<br>+ 74.1   | NT               |
| 9-AA<br>(POSITIVE CONTROL)          | 75.0000<br>ug           | NT   | 924.7<br>+ 43.2 | NT               | NT                 | NT               |
| 2-NF<br>(POSITIVE CONTROL)          | 50.0000<br>ug           | NT   | NT              | 1758.3<br>+ 87.8 | NT                 | 2025.0<br>+ 57.9 |
| t-BPB                               | 500.0000<br>ug/plate    | 21.7<br>+ 4.7                                    | 8.3<br>+ 2.5    | 17.0<br>+ 1.0    | 104.7<br>+ 5.8     | 96.7<br>+ 21.5   |
|                                     | 166.7000<br>ug/plate    | 26.0<br>+ 5.3                                    | 11.7<br>+ 2.5   | 13.7<br>+ 1.5    | 100.3<br>+ 24.0    | 32.0<br>+ 9.0    |
|                                     | 55.6000<br>ug/plate     | 28.3<br>+ 1.5                                    | 9.0<br>+ 2.0    | 14.0<br>+ 3.5    | 102.7<br>+ 14.2    | 26.3<br>+ 3.2    |
|                                     | 18.5000<br>ug/plate     | 26.0<br>+ 3.6                                    | 5.7<br>+ .6     | 13.7<br>+ 4.2    | 95.7<br>+ 6.0      | 21.0<br>+ 7.5    |
|                                     | 6.2000<br>ug/plate      | 26.3<br>+ 4.0                                    | 6.3<br>+ 2.9    | 14.7<br>+ 3.8    | 94.3<br>+ 17.9     | 17.3<br>+ 3.1    |

@ = POSITIVE CONTROL MEAN WAS LESS THAN 3.0 TIMES SOLVENT CONTROL MEAN

NT = NOT TESTED

TNTC = TOO NUMEROUS TO COUNT

T = TOXIC

VALUES REPRESENT MEAN AND STANDARD DEVIATION OF THE COUNTS OF 3 PLATES

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TABLE II

Results of SALMONELLA/MICROSOMAL Assay  
with metabolic activation on t-BPB

| COMPOUND                               | CONC<br>UNITS/<br>PLATE | REVERTANTS PER PLATE OF BACTERIAL TESTER STRAINS |                 |                   |                    |                  |
|--|-------------------------|--|-----------------|-------------------|--------------------|------------------|
|  |                         | TA1535   | TA1537          | TA1538            | TA100 <sup>D</sup> | TA98             |
| BACTERIA ONLY(a)<br>(NEGATIVE CONTROL) | 100.0000<br>u1          | 22.7<br>+ 3.1                                    | 6.7<br>+ 1.2    | 11.3<br>+ 7.5     | 102.0<br>+ 10.6    | 21.7<br>+ 2.5    |
| S-9 FRACTION<br>(NEGATIVE CONTROL)     | 100.0000<br>u1          | 11.3<br>+ 4.5                                    | 9.7<br>+ 3.2    | 31.0<br>+ 7.8     | 104.3<br>+ 6.4     | 42.0<br>+ 6.0    |
| DMSO<br>(SOLVENT CONTROL)              | 100.0000<br>u1          | 11.7<br>+ 3.5                                    | 8.0<br>+ .0     | 30.7<br>+ 9.5     | 88.7<br>+ 15.1     | 41.0<br>+ 6.0    |
| 2-AA<br>(POSITIVE CONTROL)             | 5.0000<br>ug            | 280.3<br>+ 67.0                                  | 126.7<br>+ 34.5 | 1287.7<br>+ 229.5 | 1676.7<br>+ 95.5   | 2000.7<br>+ 78.0 |
| t-BPB                                  | 500.0000<br>ug/plate    | 15.0<br>+ 2.0                                    | 12.0<br>+ 2.6   | T                 | 108.0<br>+ 17.6    | T                |
|  | 166.7000<br>ug/plate    | 12.3<br>+ 3.1                                    | 12.0<br>+ 5.3   | 39.0<br>+ 5.6     | 109.0<br>+ 14.5    | 68.7<br>+ 15.9   |
|  | 55.6000<br>ug/plate     | 12.7<br>+ 1.2                                    | 13.7<br>+ 1.2   | 35.3<br>+ 8.4     | 96.3<br>+ 4.2      | 53.3<br>+ 4.6    |
|  | 18.5000<br>ug/plate     | 10.0<br>+ 4.6                                    | 9.7<br>+ 1.5    | 32.7<br>+ 8.5     | 97.3<br>+ 5.5      | 44.0<br>+ 5.0    |
|  | 6.2000<br>ug/plate      | 17.3<br>+ 2.1                                    | 11.7<br>+ 3.1   | 45.3<br>+ 7.5     | 99.0<br>+ 14.0     | 42.0<br>+ 9.6    |

@ = POSITIVE CONTROL MEAN WAS LESS THAN 3.0 TIMES SOLVENT CONTROL MEAN  
 NT = NOT TESTED  
 TNTC = TOO NUMEROUS TO COUNT  
 T = TOXIC  
 VALUES REPRESENT MEAN AND STANDARD DEVIATION OF THE COUNTS OF 3 PLATES  
 (a) = NON ACTIVATED ORGANISM CONTROL INCLUDED FOR REFERENCE PURPOSES ONLY



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ATTACHMENT III

J. J. Moon

April 30, 1985

TBPB

**MOUSE LYMPHOMA FORWARD MUTATION ASSAY**

**t-Butyl Peroxybenzoate**

**FINAL REPORT**

**Submitted to:**

**Phillips Petroleum Company  
Bartlesville, Oklahoma**

**October 9, 1984**

12/6/87



# HAZLETON

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SPONSOR: Phillips Petroleum Company

SUBJECT: FINAL REPORT  
L5178Y Mouse Lymphoma Forward Mutation Assay with t-Butyl Peroxybenzoate

Lot Number: PW6406  
 Laboratory Number: 699  
 HLA Project Number: 652-185  
 LH Number: 21,382A  
 Assay Initiated: July 11, 1984 (toxicity) 072474  
 Assay Completed: August 13, 1984 (plate counts)  
 Receipt Date: May 5, 1984

### PROJECT PERSONNEL

| <u>Title</u>                              | <u>Name</u>         | <u>Signature</u>      | <u>Date</u> |
|---|---------------------|-----------------------|-------------|
| Study Director                            | Debbie Pence, Ph.D. | <i>Debbie Pence</i>   | 10/8/84     |
| Research Associate and Report Preparation | Russell C. Sernau   | <i>Russell Sernau</i> | 10/8/84     |

### RAW DATA STORAGE

At the completion of this study, the original copy of all raw data and the final report were sent to the Archives of Hazleton Laboratories America, Inc., Vienna, VA.

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**SUMMARY**

Mouse lymphoma cells were treated with eight dose levels of t-butyl peroxybenzoate ranging from 50 to 3 ug/ml. Duplicate doses at 22 ug/ml in the presence of metabolic activation and 15, 10 and 7. ug/ml in the absence of metabolic activation induced a mean mutation frequency greater than two-fold higher than the solvent control. Both positive and negative controls in this assay confirmed the sensitivity of the test system. Under the conditions of this assay, t-butyl peroxybenzoate was found to be mutagenic in the L5178Y Mouse Lymphoma Forward Mutation Assay.

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### OBJECTIVE

The purpose of this study was to evaluate t-butyl peroxybenzoate for the potential to induce forward mutations at the thymidine kinase (TK) locus in L5178Y mouse lymphoma cells when tested with and without metabolic activation.

### INTRODUCTION

The Mouse Lymphoma Assay is a short-term test for screening compounds for potential genetic activity. This test utilizes a mammalian cell line as a target to measure forward mutational events. This system has been shown to be sensitive and capable of detecting the activity of a wide range of chemical classes, some of which are not detected in the Ames Test. (Clive, D., et al., Mutation Research 59: 61-108, 1979).

The L5178Y mouse lymphoma cell line is presumed to be diploid in nature and three TK phenotypes have been recognized: TK+/, TK+/-, and TK-/- . The TK+/, and TK+/- cells are sensitive to trifluorothymidine (TFT) and resistant to a solution of thymidine, hypoxanthine, methotrexate and glycine (THMG). The TK-/- phenotype exhibits reverse sensitivity and resistance patterns. The heterozygous TK+/- phenotype is used as the target cell in this test system.

When TK+/- heterozygous phenotype cells are exposed to agents that can alter DNA, one of the possible consequences of this alteration is the induction of forward mutations which result in a change from TK+/- to the TK-/- phenotypes. This assay measures the induction of the TK-/- phenotype as its endpoint.

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## MATERIALS

### A. Test Materials

#### 1. Storage of Test Compound

A clear liquid designated t-butyl peroxybenzoate was submitted by the sponsor on May 5, 1984 and stored at approximately 4°C.

Upon receipt in the laboratory, the compound was assigned laboratory number 699.

Information on the method of synthesis, stability and composition of the test material reside with the sponsor. It should be noted that for the purpose of this study, the test material was assumed stable and 100% pure active ingredient.

All weighing was done by weight difference determinations in a chemical fume hood.

### B. Control Articles

#### 1. Positive Controls

Ethyl Methanesulfonate (EMS), a positive control not requiring activation, was dissolved in culture medium and used at a final treatment concentration of 300 ug/ml. 3-Methylcholanthrene (MCA), a positive control requiring metabolic activation, was dissolved in DMSO and used at a final treatment concentration of 4 ug/ml. Both positive controls were assumed stable and 100% active ingredient under the conditions of this assay.

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## 2. Solvent Control

The solvent control for the test compound was dimethylsulfoxide (DMSO).

| <u>Control Articles</u>      | <u>Supplier</u> | <u>Lot Number</u> |
|------------------------------|-----------------|-------------------|
| Ethyl Methanesulfonate (EMS) | Eastman Kodak   | A1A               |
| 3-Methylcholanthrene (MCA)   | Eastman Kodak   | A1A               |
| Dimethylsulfoxide (DMSO)     | Fisher          | 733138            |

## C. Cells

The in vitro system species is identified as L5178Y heterozygous TK+/- mouse lymphoma cells, subline 3.7.2 C which were received from Dr. Donald Clive, Research Triangle Park, North Carolina. Stocks of these cells were frozen and stored in liquid nitrogen until used. Prior to use in the assay, cells are routinely cleansed of spontaneous mutants by overnight treatment with methotrexate, thymidine, hypoxanthine and glycine. The culture used for the toxicity assay was thawed on 6/25/84 and cleansed on 7/5/84 and the culture used for the mutation assay was thawed on 7/10/84 and cleansed on 7/19/84.

## D. Solubility

The test material t-butyl peroxybenzoate was soluble in dimethylsulfoxide (DMSO) at 100 mg/ml (highest concentration used in the toxicity test).

## E. Toxicity Test

An initial toxicity test was performed with and without metabolic activation using dose levels ranging from 1000 to 3 ug/ml. Due to less than 90% cell survival at all but the lowest dose, a second toxicity test was required in order to establish a dose range for the

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forward mutation assay. The dose levels for the second toxicity test ranged from 100 to 0.3 ug/ml. The toxicity test was performed by adding 0.1 ml of the solvent, medium control, 100x positive control, or 100x test agent to the appropriate 50 ml centrifuge tubes containing  $6.0 \times 10^6$  TK+/- cells in 6 ml of cell culture medium. An additional 4 ml of S-9 mix were added to the tubes for metabolic activation; four ml of F10P were added to the nonactivated tubes. The cells were exposed to the chemical for four hours, washed twice, resuspended in 20 ml F10P, and incubated overnight.

On the day following treatment, all cultures were set back to  $0.3 \times 10^6$  cells per ml when necessary and incubated until the following day. On the second post treatment day the cell density was again determined for each culture and relative growth was determined.

The dose range for the forward mutation assay was based on the second toxicity test. The high dose level (50 ug/ml) was chosen to demonstrate a high level of toxicity. Seven subsequent dilutions were made to yield dose levels of approximately 34, 22, 15, 10, 7, 5 and 3 ug/ml.

#### F. Forward Mutation Assay

The eight 100x concentrations of the test compound were prepared to contain the test dose in 0.1 ml volumes. The highest final concentration used for the mutation assay with and without activation was 50 ug/ml. Duplicate cultures for each test compound concentration and triplicate cultures of positive, solvent and medium controls were dosed as follows:

Six million precleaved cells in six ml of cell culture medium were added to 50 ml centrifuge tubes. An additional four ml of F<sub>0</sub> were added to the tubes not requiring activation, and four ml of the S-9

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mix were added to those tubes being tested with metabolic activation. Next, 0.1 ml of either the 100x test chemical dilution, 100x positive control, solvent or media control was added to the appropriate tubes. Each tube was mixed, gassed with a mixture of 5% CO<sub>2</sub> in air, sealed and incubated at 37.5° ± .5°C on a revolving roller drum for four hours. Following this incubation, the tubes were centrifuged and the treatment solutions decanted. The cells were washed twice with F10P and resuspended in 20 ml F10P after the second wash. The tube cultures were then gassed and reincubated as described above for a two-day expression time. Growth of the cells was monitored at one day postexposure and the cultures adjusted to  $.3 \times 10^6$  cells/ml, if necessary. At the end of the expression period, a sample from each of the cultures to be cloned was centrifuged, and the cells resuspended at  $1.0 \times 10^6$  viable cells/ml in F10P.

Five concentrations of the test compound with and without metabolic activation were used for cloning.

Approximately  $1.0 \times 10^6$  cells were plated in each of three selective medium plates containing trifluorothymidine (TFT), and approximately 200 cells were cloned in each of three nonselective plates for each test and control tube. After incubation, the mutant colonies (TK-/-) were counted on the selective TFT-containing plates; similarly, colonies in the non-selective medium plates were counted.

Due to contamination in the positive control (MCA) plates, it was necessary to repeat activated portion of this study. The data pertaining to activation that follow are the result of this retest.

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## RESULTS AND DISCUSSION

The five highest dose levels with a relative percent suspension growth of at least 10% were subjected to TFT selection. As shown in Table 1, the doses selected with metabolic activation ranged from 22 to 5 ug/ml and without metabolic activation from 15 to 3 mg/ml. The mutant colony counts and cloning efficiencies are expressed in Table 2. Table 3 presents the percentage total survival, mutation frequencies and fold increases obtained from the cloned test doses and controls.

For evaluation of the mouse lymphoma assay, the following criteria have been established by our laboratory:

1. A test chemical will be considered positive if a dose-related response is obtained in which the mutation frequencies at least two test concentrations are at least two-fold higher than the mutation frequency of the solvent control.
2. Mutation frequencies of the test compound will be calculated for dose levels with 10% or greater cell survival.

As shown in Table 3 the test material, t-butyl peroxybenzoate, induced a greater than two-fold increase in the mean mutation frequency with duplicate doses of the high dose level (22 ug/ml) with metabolic activation. A greater than two-fold increase was produced in a dose-related response at the three high doses (15, 10 and 7 ug/ml) in the absence of metabolic activation.

The spontaneous mutation frequencies and the levels of activity of the positive controls in this assay confirmed the sensitivity of the test system species.

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**CONCLUSION**

Under the conditions of this assay, t-butyl peroxybenzoate is considered to be mutagenic.

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## ABBREVIATIONS

|                  |   |
|------------------|---|
| CON              | Contaminated  |
| H <sub>2</sub> O | Deionized glass distilled water                                     |
| DMSO             | Dimethylsulfoxide   |
| EMS              | Ethyl Methanesulfonate  |
| FOP              | Fischer's Medium with Antibiotics, Pluronic F68 and Sodium Pyruvate |
| F10P             | FOP with 10% Horse Serum  |
| MCA              | 3-Methylcholanthrene  |
| MF               | Mutation Frequency  |
| N/R              | No Result   |
| NS               | Nonselective  |
| S                | Selective   |
| T                | Toxic dose  |
| T/E              | Technical Error   |
| TFT              | Trifluorothymidine  |
| N/A              | Not Applicable  |

All abbreviations may not appear in the text or Tables 1, 2 and 3.

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Key to Tables 1, 2 and 3

$$\text{Total Suspension Growth} = \text{Day 1 Counts} \times \frac{\text{Day 2 Counts}}{3 \times 10^5}$$

$$\text{Relative Suspension Growth (\%)} = \frac{\text{Total Suspension Growth (Treated)}}{\text{Total Suspension Growth (Solvent)*}} \times 100$$

$$\text{Percent Cloning Efficiency} = \frac{\text{Mean No. NS colonies in Treated Dishes}}{\text{Mean No. NS Colonies in Solvent}} \times 100$$

Percentage Total Survival =

$$\text{Relative Suspension Growth} \times \frac{\text{Mean No. Colonies NS Treated}}{\text{Mean No. Colonies NS Controls}}$$

$$\text{Mutation Frequency} = \frac{\text{Mean No. Colonies S Plates}}{\text{Mean No. Colonies NS Plates (5 x 10}^3\text{)}}$$

$$\text{Fold Increase} = \frac{\text{Treated Mutation Frequency}}{\text{Solvent Control Mutation Frequency}}$$

<sup>1</sup>When Day 1 cell counts are equal to or less than  $.3 \times 10^6$ , then the total suspension growth will be the Day 2 cell counts.

\*Control tubes are calculated individually; mean value of each control is then calculated from these individual values.

Dose levels reported in Tables 1-3 have been rounded to the nearest whole number.

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REFERENCES

Clive, D., K. O. Johnson, J. F. S. Spector, A. G. Batson, and M. M. M. Brown (1979) Validation and Characterization of the L5178Y/TK+/- Mouse Lymphoma Mutagen Assay System. *Mutation Res.* 59: 61-108.

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Table 1  
Suspension Growth Results of Mouse Lymphoma Assay  
t-Butyl Peroxybenzoate

| Treatment              | S-9 | Cell Counts<br>(x 10 <sup>6</sup> per/ml) |       | Total<br>Suspension<br>Growth | Relative<br>Suspension<br>Growth (%) |
|------------------------|-----|---|-------|-------------------------------|--------------------------------------|
|                        |     | Day 1                                     | Day 2 |                               |                                      |
| Solvent Control (DMSO) | -   | 1.30                                      | 1.22  | 5.29                          | } 5.22 100.0                         |
| Solvent Control (DMSO) | -   | 1.23                                      | 1.26  | 5.17                          |                                      |
| Solvent Control (DMSO) | -   | 1.24                                      | 1.26  | 5.21                          |                                      |
| 50 ug/ml               | -   | .02                                       | N/R   | N/R                           | N/R                                  |
| 50 ug/ml               | -   | .02                                       | N/R   | N/R                           | N/R                                  |
| 34 ug/ml               | -   | .05                                       | N/R   | N/R                           | N/R                                  |
| 34 ug/ml               | -   | .06                                       | N/R   | N/R                           | N/R                                  |
| 22 ug/ml               | -   | .28                                       | N/R   | N/R                           | N/R                                  |
| 22 ug/ml               | -   | .25                                       | N/R   | N/R                           | N/R                                  |
| 15 ug/ml               | -   | .63                                       | .93   | 1.95                          | 37.4                                 |
| 15 ug/ml               | -   | .63                                       | .95   | 2.00                          | 38.3                                 |
| 10 ug/ml               | -   | .95                                       | 1.07  | 3.39                          | 64.9                                 |
| 10 ug/ml               | -   | .90                                       | 1.02  | 3.06                          | 58.6                                 |
| 7 ug/ml                | -   | 1.08                                      | 1.03  | 3.71                          | 70.9                                 |
| 7 ug/ml                | -   | 1.09                                      | 1.15  | 4.17                          | 79.9                                 |
| 5 ug/ml                | -   | 1.08                                      | 1.50  | 5.40                          | 103.4                                |
| 5 ug/ml                | -   | 1.09                                      | 1.44  | 5.23                          | 100.2                                |
| 3 ug/ml                | -   | 1.07                                      | 1.36  | 4.85                          | 92.9                                 |
| 3 ug/ml                | -   | 1.22                                      | 1.38  | 5.61                          | 107.5                                |
|                        |     |   |       |                               |                                      |
| Solvent Control (DMSO) | +   | .66                                       | 1.03  | 2.27                          | } 2.24 100.0                         |
| Solvent Control (DMSO) | +   | .62                                       | 1.10  | 2.27                          |                                      |
| Solvent Control (DMSO) | +   | .60                                       | 1.09  | 2.18                          |                                      |
| 50 ug/ml               | +   | .03                                       | N/R   | N/R                           | N/R                                  |
| 50 ug/ml               | +   | .03                                       | N/R   | N/R                           | N/R                                  |
| 34 ug/ml               | +   | .06                                       | N/R   | N/R                           | N/R                                  |
| 34 ug/ml               | +   | .03                                       | N/R   | N/R                           | N/R                                  |
| 22 ug/ml               | +   | .39                                       | .69   | .90                           | 40.2                                 |
| 22 ug/ml               | +   | .37                                       | .86   | 1.06                          | 47.3                                 |
| 15 ug/ml               | +   | .34                                       | .78   | .88                           | 39.3                                 |
| 15 ug/ml               | +   | .34                                       | .75   | .85                           | 37.9                                 |
| 10 ug/ml               | +   | .56                                       | 1.09  | 2.03                          | 90.6                                 |
| 10 ug/ml               | +   | .53                                       | .96   | 1.70                          | 75.9                                 |
| 7 ug/ml                | +   | .60                                       | 1.00  | 2.00                          | 89.3                                 |
| 7 ug/ml                | +   | .61                                       | 1.08  | 2.20                          | 98.2                                 |
| 5 ug/ml                | +   | .55                                       | 1.18  | 2.16                          | 96.4                                 |
| 5 ug/ml                | +   | .62                                       | 1.14  | 2.36                          | 105.4                                |
| 3 ug/ml                | +   | .58                                       | 1.06  | 2.05                          | 91.5                                 |
| 3 ug/ml                | +   | .65                                       | 1.00  | 2.17                          | 96.9                                 |

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Table 1 (continued)  
 Suspension Growth Results of Mouse Lymphoma Assay  
 t-Butyl Peroxybenzoate

| <u>Treatment</u>             | <u>S-9</u> | <u>Cell Counts</u><br>(x 10 <sup>6</sup> per/ml) |              | <u>Total</u><br><u>Suspension</u><br><u>Growth</u> | <u>Relative</u><br><u>Suspension</u><br><u>Growth (%)</u> |
|------------------------------|------------|--|--------------|--|---|
|                              |            | <u>Day 1</u>                                     | <u>Day 2</u> |  |   |
| <b>Positive Controls</b>     |            |  |              |  |   |
| EMS (300 ug/ml) <sup>1</sup> | -          | 1.09   | 1.28         | 4.65   | 81.0  |
| EMS (300 ug/ml)              | -          | 1.08   | 1.29         | 4.64   | 80.8  |
| EMS (300 ug/ml)              | -          | 1.07   | 1.27         | 4.53   | 78.9  |
| MCA (4 ug/ml) <sup>2</sup>   | +          | .31  | .95          | .98  | 43.8  |
| MCA (4 ug/ml)                | +          | .33  | .81          | .89  | 39.7  |
| MCA (4 ug/ml)                | +          | .29  | .79          | .79  | 35.3  |
| <b>Medium Control</b>        |            |  |              |  |   |
| Cell Culture Medium          | -          | 1.33   | 1.19         | 5.28   | } 5.74<br>N/A   |
| Cell Culture Medium          | -          | 1.12   | 1.37         | 5.11   |   |
| Cell Culture Medium          | -          | 1.35   | 1.52         | 6.84   |   |
| Cell Culture Medium          | +          | .77  | .90          | 2.31   | N/A   |
| Cell Culture Medium          | +          | .65  | 1.10         | 2.38   | N/A   |
| Cell Culture Medium          | +          | .63  | 1.09         | 2.29   | N/A   |

<sup>1</sup> Compared with cell culture medium - S9

<sup>2</sup> Compared with DMSO + S9

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Table 2  
Results of Mouse Lymphoma Assay  
t-Butyl Peroxybenzoate

| Treatment              | S-9 | Number Colonies<br>Selective Plates |     |     |      | Number Colonies<br>Nonselective<br>Plates |     |     |      | %<br>Cloning<br>Efficiency |     |
|------------------------|-----|-------------------------------------|-----|-----|------|---|-----|-----|------|----------------------------|-----|
|                        |     | (1)                                 | (2) | (3) | Mean | (1)                                       | (2) | (3) | Mean |                            |     |
| Solvent Control (DMSO) | -   | 24                                  | 34  | 19  | 26   | 120                                       | 100 | 117 | 112  | 126.5                      | 100 |
| Solvent Control (DMSO) | -   | 24                                  | 33  | 14  | 24   | CON                                       | CON | CON | N/R  |                            |     |
| Solvent Control (DMSO) | -   | 9                                   | 7   | 16  | 11   | 161                                       | 133 | 129 | 141  |                            |     |
| 50 ug/ml               | -   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 50 ug/ml               | -   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 34 ug/ml               | -   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 34 ug/ml               | -   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 22 ug/ml               | -   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 22 ug/ml               | -   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 15 ug/ml               | -   | 71                                  | 55  | 42  | 56   | 45  | 67  | 53  | 55   | 43                         |     |
| 15 ug/ml               | -   | 31                                  | 41  | 55  | 42   | 29  | 69  | 45  | 48   | 38                         |     |
| 10 ug/ml               | -   | 40                                  | 44  | 23  | 36   | 64  | 95  | 79  | 79   | 62                         |     |
| 10 ug/ml               | -   | 36                                  | 48  | 40  | 41   | 70  | 38  | 44  | 51   | 40                         |     |
| 7 ug/ml                | -   | 39                                  | 48  | 42  | 43   | 66  | 56  | 74  | 65   | 51                         |     |
| 7 ug/ml                | -   | 46                                  | 48  | 48  | 47   | 71  | 84  | 99  | 85   | 67                         |     |
| 5 ug/ml                | -   | 25                                  | 24  | 33  | 27   | 65  | 64  | 78  | 69   | 54                         |     |
| 5 ug/ml                | -   | 25                                  | 28  | 20  | 24   | 131                                       | 124 | 125 | 127  | 100                        |     |
| 3 ug/ml                | -   | 26                                  | 16  | 20  | 21   | 113                                       | 106 | 131 | 117  | 92                         |     |
| 3 ug/ml                | -   | 25                                  | 9   | 15  | 16   | 125                                       | 58  | 129 | 104  | 82                         |     |
| Solvent Control (DMSO) | +   | 19                                  | 15  | 20  | 18   | 124                                       | 174 | 129 | 142  | 149.0                      | 100 |
| Solvent Control (DMSO) | +   | 21                                  | 42  | 19  | 27   | 137                                       | 168 | 159 | 155  |                            |     |
| Solvent Control (DMSO) | +   | 20                                  | 57  | 22  | 33   | 139                                       | 175 | 137 | 150  |                            |     |
| 50 ug/ml               | +   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 50 ug/ml               | +   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 34 ug/ml               | +   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 34 ug/ml               | +   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 22 ug/ml               | +   | 103                                 | 100 | 114 | 106  | 110                                       | 97  | 95  | 101  | 68                         |     |
| 22 ug/ml               | +   | 72                                  | 71  | 70  | 71   | 99  | 94  | 97  | 97   | 65                         |     |
| 15 ug/ml               | +   | 20                                  | 14  | 19  | 18   | 83  | 92  | 105 | 93   | 62                         |     |
| 15 ug/ml               | +   | 21                                  | 7   | 18  | 15   | 83  | 91  | 92  | 89   | 60                         |     |
| 10 ug/ml               | +   | 21                                  | 37  | 35  | 31   | 155                                       | 155 | 143 | 151  | 101                        |     |
| 10 ug/ml               | +   | 33                                  | 28  | 17  | 26   | 153                                       | 157 | 141 | 150  | 101                        |     |
| 7 ug/ml                | +   | 8                                   | 17  | 9   | 11   | 156                                       | 128 | 140 | 141  | 95                         |     |
| 7 ug/ml                | +   | 5                                   | 19  | 15  | 13   | 152                                       | 183 | 191 | 175  | 117                        |     |
| 5 ug/ml                | +   | 8                                   | 6   | 5   | 6    | 175                                       | 198 | 169 | 181  | 121                        |     |
| 5 ug/ml                | +   | 14                                  | 14  | 28  | 19   | 172                                       | 182 | 176 | 177  | 119                        |     |
| 3 ug/ml                | +   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |
| 3 ug/ml                | +   | N/R                                 | N/R | N/R | N/R  | N/R                                       | N/R | N/R | N/R  | N/R                        | N/R |

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Table 2 (continued)  
 Results of Mouse Lymphoma Assay  
 t-Butyl Peroxybenzoate

| Treatment                    | S-9 | Number Colonies<br>Selective Plates |     |     |      | Number Colonies<br>Nonselective<br>Plates |     |     |      | %<br>Cloning<br>Efficiency |
|------------------------------|-----|-------------------------------------|-----|-----|------|---|-----|-----|------|----------------------------|
|                              |     | (1)                                 | (2) | (3) | Mean | (1)                                       | (2) | (3) | Mean |                            |
| <b>Positive Controls</b>     |     |                                     |     |     |      |   |     |     |      |                            |
| EMS (300 ug/ml) <sup>1</sup> | -   | 172                                 | 204 | 182 | 186  | 72  | 63  | 102 | 79   | 66                         |
| EMS (300 ug/ml)              | -   | 151                                 | 134 | 170 | 152  | 143                                       | 93  | 72  | 103  | 87                         |
| EMS (300 ug/ml)              | -   | 192                                 | 205 | 204 | 200  | 138                                       | 143 | 88  | 123  | 103                        |
| MCA (4 ug/ml) <sup>2</sup>   | +   | 142                                 | 290 | 275 | 236  | 95  | 90  | 62  | 82   | 55                         |
| MCA (4 ug/ml)                | +   | 202                                 | 226 | 191 | 206  | 83  | 80  | 91  | 85   | 57                         |
| MCA (4 ug/ml)                | +   | 268                                 | 143 | 187 | 199  | 67  | 62  | 97  | 75   | 50                         |
| <b>Medium Control</b>        |     |                                     |     |     |      |   |     |     |      |                            |
| Cell Culture Medium          | -   | 3                                   | 9   | 14  | 9    | 153                                       | 149 | 101 | 134  | } 119<br>N/A               |
| Cell Culture Medium          | -   | 26                                  | 9   | 9   | 15   | 115                                       | 135 | 105 | 118  |                            |
| Cell Culture Medium          | -   | 18                                  | 18  | 19  | 18   | 124                                       | 108 | 84  | 105  |                            |
| Cell Culture Medium          | +   | 37                                  | 17  | 37  | 30   | 137                                       | 161 | 176 | 158  |                            |
| Cell Culture Medium          | +   | 14                                  | 18  | 5   | 12   | 128                                       | 141 | 155 | 141  |                            |
| Cell Culture Medium          | +   | 29                                  | 30  | 16  | 25   | 176                                       | 190 | 143 | 170  | N/A                        |

<sup>1</sup> Compared with cell culture medium - S9  
<sup>2</sup> Compared with DMSO + S9

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Table 3 (continued)  
 Summary of Mutation Frequencies from the  
 Mouse Lymphoma Assay of t-Butyl Peroxybenzoate

| <u>Treatment</u>             | <u>S-9</u> | <u>Percentage<br/>Total<br/>Survival</u> | <u>Mutation<br/>Frequency<br/>(x 10<sup>-5</sup>)</u> | <u>Fold<br/>Increase</u> |
|------------------------------|------------|--|---|--------------------------|
| <b>Positive Controls</b>     |            |  |   |                          |
| EMS (300 ug/ml) <sup>1</sup> | -          | 53.5                                     | 47.1  | 19.6                     |
| EMS (300 ug/ml)              | -          | 70.3                                     | 29.5  | 12.3                     |
| EMS (300 ug/ml)              | -          | 81.3                                     | 32.5  | 13.5                     |
| MCA (4 ug/ml) <sup>2</sup>   | +          | 24.1                                     | 57.6  | 16.5                     |
| MCA (4 ug/ml)                | +          | 22.6                                     | 48.5  | 13.9                     |
| MCA (4 ug/ml)                | +          | 17.7                                     | 53.1  | 15.2                     |
| <b>Medium Controls</b>       |            |  |   |                          |
| Cell Culture Medium          | -          | N/A                                      | 1.3   | N/A                      |
| Cell Culture Medium          | -          | N/A                                      | 2.5   | N/A                      |
| Cell Culture Medium          | -          | N/A                                      | 3.4   | N/A                      |
| Cell Culture Medium          | +          | N/A                                      | 3.8   | N/A                      |
| Cell Culture Medium          | +          | N/A                                      | 1.7   | N/A                      |
| Cell Culture Medium          | +          | N/A                                      | 2.9   | N/A                      |

\* Solvent control values are the mean of two tubes.

1 Compared with cell culture medium - S9

2 Compared with DMSO + S9

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Table 3  
 Summary of Mutation Frequencies from the  
 Mouse Lymphoma Assay of t-Butyl Peroxybenzoate

| <u>Treatment</u>       | <u>S-9</u> | <u>Percentage Total Survival</u> | <u>Mutation Frequency (x 10<sup>-5</sup>)</u> | <u>Fold Increase</u> |     |
|------------------------|------------|----------------------------------|---|----------------------|-----|
| Solvent Control (DMSO) | -          | -                                | 4.6   | } 3.1                | 1.0 |
| Solvent Control (DMSO) | -          | 100*                             | N/R   |                      |     |
| Solvent Control (DMSO) | -          | -                                | 1.6   |                      |     |
| 15 ug/ml               | -          | 16.1                             | 20.4  | } 6.6                | }   |
| 15 ug/ml               | -          | 14.6                             | 17.5  |                      |     |
| 10 ug/ml               | -          | 40.2                             | 9.1   | } 2.9                | }   |
| 10 ug/ml               | -          | 23.4                             | 16.1  |                      |     |
| 7 ug/ml                | -          | 36.2                             | 13.2  | } 4.3                | }   |
| 7 ug/ml                | -          | 53.5                             | 11.1  |                      |     |
| 5 ug/ml                | -          | 55.8                             | 7.8   | } 2.5                | }   |
| 5 ug/ml                | -          | 100.2                            | 3.8   |                      |     |
| 3 ug/ml                | -          | 85.5                             | 3.6   | 1.2                  | }   |
| 3 ug/ml                | -          | 88.2                             | 3.1   | 1.0                  |     |
| Solvent Control (DMSO) | +          | N/A                              | 2.5   | } 3.5                | 1.0 |
| Solvent Control (DMSO) | +          | N/A                              | 3.5   |                      |     |
| Solvent Control (DMSO) | +          | N/A                              | 4.4   |                      |     |
| 22 ug/ml               | +          | 27.3                             | 21.0  | } 6.0                | }   |
| 22 ug/ml               | +          | 30.7                             | 14.6  |                      |     |
| 15 ug/ml               | +          | 24.4                             | 3.9   | 1.1                  | }   |
| 15 ug/ml               | +          | 22.7                             | 3.4   | 1.0                  |     |
| 10 ug/ml               | +          | 91.5                             | 4.1   | 1.2                  | }   |
| 10 ug/ml               | +          | 76.7                             | 3.5   | 1.0                  |     |
| 7 ug/ml                | +          | 84.8                             | 1.6   | .5                   | }   |
| 7 ug/ml                | +          | 114.9                            | 1.5   | .4                   |     |
| 5 ug/ml                | +          | 116.6                            | .7  | .2                   | }   |
| 5 ug/ml                | +          | 125.4                            | 2.1   | .6                   |     |

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LABORATORIES AMERICA, INC.

SPONSOR: Phillips Petroleum Company

DATE: January 9, 1985

MATERIAL: t-Butyl Peroxybenzoate

SUBJECT: FINAL REPORT  
Mouse Lymphoma Forward Mutation Assay  
Project No. 652-185

Page 7 is being submitted as a correction page for incorporation into the subject report, dated October 9, 1984.

Study Director:

*Deborah H. Pence*

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**CORRECTION PAGE**

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### RESULTS AND DISCUSSION

The five highest dose levels with a relative percent suspension growth of at least 10% were subjected to TFT selection. As shown in Table 1, the doses selected with metabolic activation ranged from 22 to 5 ug/ml and without metabolic activation from 15 to 3 ug/ml. The mutant colony counts and cloning efficiencies are expressed in Table 2. Table 3 presents the percentage total survival, mutation frequencies and fold increases obtained from the cloned test doses and controls.

For evaluation of the mouse lymphoma assay, the following criteria have been established by our laboratory:

1. A test chemical will be considered positive if a dose-related response is obtained in which the mutation frequencies of at least two test concentrations are at least two-fold higher than the mutation frequency of the solvent control.
2. Mutation frequencies of the test compound will be calculated for dose levels with 10% or greater cell survival.

As shown in Table 3 the test material, t-butyl peroxybenzoate, induced a greater than two-fold increase in the mean mutation frequency with duplicate doses of the high dose level (22 ug/ml) with metabolic activation. A greater than two-fold increase was produced in a dose-related response at the three high doses (15, 10 and 7 ug/ml) in the absence of metabolic activation.

The spontaneous mutation frequencies and the levels of activity of the positive controls in this assay confirmed the sensitivity of the test system species.

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SUMMARY OF MUTAGENIC STUDIES ON t-BUTYL PEROXYBENZOATE

PRODUCT NAME

TEST DESCRIPTION

TEST RESULTS

METABOLIC ACTIVATION  
WITHOUT                      WITH

t-BUTYL PEROXYBENZOATE

IN VITRO SISTER CHROMATID  
EXCHANGE

POSITIVE

POSITIVE

SUMMARY:

Statistically significant, less than 2-fold, increases in the number of SCE's, were seen at 52, 18, 5.2, and 1.8 µg/ml without metabolic activation. Statistically significant, less than 2-fold, increases were seen at 18, 5.2 µg/ml with metabolic activation.

AMES MUTATION ASSAY

NEGATIVE

NEGATIVE

SUMMARY:

Lucidol MSDS gives results for the AMES test as slightly positive. Published mutation data is not available on this product.

MOUSE LYMPHOMA FORWARD  
MUTATION ASSAY

POSITIVE

NEGATIVE

SUMMARY:

Greater than 2-fold increases were seen at 15, 10, and 7 µg/ml (in duplicate) without metabolic activation and a greater than 2-fold increase at 22 µg/ml (in duplicate) was seen with metabolic activation.

IARC MONOGRAPH PROGRAMME ON THE EVALUATION OF THE CARCINOGENIC RISK  
OF CHEMICALS TO HUMANS

Overall assessment of data from short-term tests

An overall assessment of the evidence for genetic activity is then made on the basis of the entries in the table, and the evidence is judged to fall into one of four categories, defined as follows:

- (i) Sufficient evidence is provided by at least three positive entries, one of which must involve mammalian cells in vitro or in vivo and which must include at least two of three end-points - DNA damage, mutation and chromosomal effects.
- (ii) Limited evidence is provided by at least two positive entries.
- (iii) Inadequate evidence is available when there is only one positive entry or when there are too few data to permit an evaluation of an absence of genetic activity or when there are unexplained, inconsistent findings in different test systems.
- (iv) No evidence applies when there are only negative entries; these must include entries for at least two end-points and two levels of biological complexity, one of which must involve mammalian cells in vitro or in vivo.

It is emphasized that the above definitions are operational, and that the assignment of a chemical into one of these categories is thus arbitrary.