



August 28, 1997

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Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
401 M St. S.W.
Washington, DC 20460-0001

Re: TSCA Section 8(e) Notification Followup
8EHQ-97-13981

Dear Sir:

ChemFirst Inc is submitting the enclosed report entitled "Mutagenicity Test with EPNB/Ethyl-4-nitrobenzoate in the Salmonella-Escherichia coli/Mammalian-microsome Reverse Mutation Assay" as a followup to the referenced 8(e) submission of preliminary data from this study.

Please let me know if you need additional information.

CAS No.
99-77-4

Sincerely,

Ellen R Stephens

Ellen R. Stephens, Ph.D. DABT
Manager, Toxicology
601-938-2219

enclosure

10-31-97
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MUTAGENICITY TEST WITH
EPNB/ETHYL-4-NITROBENZOATE
IN THE *SALMONELLA* - *ESCHERICHIA COLI*/MAMMALIAN-MICROSOME REVERSE
MUTATION ASSAY

FINAL REPORT

AUTHOR

PERFORMING LABORATORY

LABORATORY PROJECT ID

SUBMITTED TO

ChemFirst Inc.
Industrial Rd.
P.O. Box 7005
Pascagoula, MS 39568-7005

STUDY COMPLETION DATE

August 26, 1997

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SECTION I. SUMMARY

INTRODUCTION AND CONCLUSIONS

SUMMARY

A. Introduction

At the request of ChemFirst Inc., investigated EPNB/Ethyl-4-Nitrobenzoate for mutagenic activity in the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay. This assay evaluated the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains and at the tryptophan locus in an *Escherichia coli* tester strain both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver, (S9).

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strains TA100 and WP2uvrA and ten doses of test article ranging from 5,000 to 6.67 µg per plate, one plate per dose, both in the presence and absence of S9 mix.

The tester strains used in the mutagenicity assay were *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* tester strain WP2uvrA. The assay was conducted with six doses of test article in both the presence and absence of S9 mix along with concurrent vehicle and positive controls using three plates per dose. The doses tested were 5,000, 2,500, 1,000, 500, 250, and 100 µg per plate in both the presence and absence of S9 mix.

B. Conclusions

The results of the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay indicate that, under the conditions of this study, ChemFirst Inc.'s test article, EPNB/Ethyl-4-Nitrobenzoate, did cause positive increases in the number of revertants per plate with tester strains TA100 (2.3-fold) and TA1535 (53.8-fold) in the presence of S9 mix. No positive increases in the number of revertants per plate were observed with any of the remaining tester strain/activation condition combinations.

SECTION II. STUDY INFORMATION

STUDY INFORMATION

- A. Sponsor: **ChemFirst Inc.**

- B. Test Article: **EPNB/Ethyl-4-Nitrobenzoate**
EPNB #1447. #5
 - 1. Physical Description: **yellow solid**
 - 2. Date Received: **06/24/97**

- C. Type of Assay: *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay
 - 1. Protocol Number:
 - 2.

- D. Study Dates
 - 1. Study Initiation Date: **06/25/97**
 - 2. Experimental Start Date: **06/26/97**
 - 3. Experimental Termination Date: **07/07/97**

- E. Study Supervisory Personnel
 - Study Director:
 - Associate Scientist:
 - Laboratory Supervisor:

SECTION III. MATERIALS AND METHODS

MATERIALS AND METHODS

The experimental materials, methods and procedures are based on those described by Ames *et al* (1975) and Green and Muriel (1976).

MATERIALS

A. Tester Strains

1. *Salmonella typhimurium*

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, and TA1537 as described by Ames *et al* (1975). The specific genotypes of these strains are shown in Table I.

TABLE I. TESTER STRAIN GENOTYPES

| Histidine Mutation | | | Additional Mutations | | |
|--------------------|-----------------|-----------------|----------------------|-------------|----------|
| <i>hisG46</i> | <i>hisC3076</i> | <i>hisD3052</i> | LPS | Repair | R Factor |
| TA1535 | TA1537 | | <i>rfa</i> | <i>uvrB</i> | - |
| TA100 | | TA98 | <i>rfa</i> | <i>uvrB</i> | +R |

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall.

The second mutation, a deletion of the *uvrB* gene, results in a deficient DNA excision repair system which greatly enhances the sensitivity of these strains to some mutagens. Since the *uvrB* deletion extends through the *bio* gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA1535 is reverted by base

substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

2. *Escherichia coli*

The tester strain used was the tryptophan auxotroph WP2*uvrA* as described by Green and Muriel (1976).

In addition to a mutation in the tryptophan operon, the tester strain contains a *uvrA* DNA repair deficiency which enhances its sensitivity to some mutagenic compounds. This deficiency allows the strain to show enhanced mutability since the *uvrA* repair system would normally act to remove the damaged part of the DNA molecule and accurately repair it afterwards.

Tester strain WP2*uvrA* is reverted from tryptophan dependence (auxotrophy) to tryptophan independence (prototrophy) by base substitution mutagens.

3. Source of Tester Strains

a. *Salmonella typhimurium*

The tester strains in use were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

b. *Escherichia coli*

The tester strain, WP2*uvrA*, in use was received from the National Collection of Industrial Bacteria, Torrey Research Station, Scotland (United Kingdom).

4. Storage of the Tester Strains

a. Frozen Permanent Stocks

Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 ml/ml of culture) and freezing small aliquots (0.5-1.5 ml) at $\leq -70^{\circ}\text{C}$.

b. Master Plates

Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with 1) for *Salmonella typhimurium*, an excess of histidine, and biotin, and for tester strains TA98 and TA100,

ampicillin (25 µg/ml), to ensure the stable maintenance of the pKM101 plasmid; and 2) for *Escherichia coli*, an excess of tryptophan. Tester strain master plates were stored at $5 \pm 3^\circ\text{C}$.

5. Preparation of Overnight Cultures

a. Inoculation

Overnight cultures for use in all testing procedures were inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 ± 25 rpm; incubation, $37 \pm 2^\circ\text{C}$) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began.

b. Harvest

To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture turbidity. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance (%T) reading on a spectrophotometer. This target turbidity ensures that cultures have reached a density of at least 0.5×10^9 cells per ml and that the cultures have not overgrown. Overgrown (stationary) cultures may exhibit decreased sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached and were placed at $5 \pm 3^\circ\text{C}$.

6. Confirmation of Tester Strain Genotypes

Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

a. *Salmonella typhimurium*

1) *rfa* Wall Mutation

The presence of the *rfa* wall mutation was confirmed by demonstration of the sensitivity of the culture to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

2) pKM101 Plasmid R-factor

The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an

overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

3) Characteristic Number of Spontaneous Revertants

The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 µl aliquots of the culture along with the appropriate vehicle on selective media.

b. *Escherichia coli*

1) Characteristic Number of Spontaneous Revertants

The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 µl aliquots of the WP2*uvrA* culture along with the appropriate vehicle on selective media.

7. Tester Strain Media

a. Culturing Broth

The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

b. Agar Plates

Bottom agar (25 ml per 15 x 100 mm petri dish) was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956), supplemented with 1.5% (w/v) agar and 0.2% (w/v) glucose.

c. Overlay Agar for Selection of Revertants

Overlay (top) agar was prepared with 0.7% agar (w/v) and 0.5% NaCl (w/v) and was supplemented with 10 ml of 1) 0.5 mM histidine/biotin solution per 100 ml agar for selection of histidine revertants, or 2) 0.5 mM tryptophan solution per 100 ml of agar for selection of tryptophan revertants. When S9 mix was required, 2.0 ml of the supplemented top agar was used in the overlay. However, when S9 mix was not required, water was added to the supplemented top agar (0.5 ml of water per 2 ml of supplemented top agar) and the resulting 2.5 ml of diluted supplemented top agar was used for the overlay. This dilution

ensured that the final top agar and amino acid supplement concentrations remained the same both in the presence and absence of S9 mix.

B. Liver Microsomal Enzyme Reaction Mixture (S9 Mix)

1. S9 Homogenate

Liver microsomal enzymes (S9 homogenate) were purchased from Molecular Toxicology, Inc., Batch 0744 (35.0 mg of protein per ml) and Batch 0755 (35.03 mg of protein per ml). The homogenate was prepared from male Sprague-Dawley rats that had been injected (i.p.) with Aroclor™ 1254 (200 mg per ml in corn oil) at 500 mg/kg as described by Ames *et al*, 1975.

2. S9 Mix

The S9 mix was prepared immediately prior to its use in any experimental procedure. The S9 mix contained the components indicated in Table II.

TABLE II. S9 MIX COMPONENTS

| | |
|---|----------------|
| H ₂ O | 0.70 ml |
| 1M NaH ₂ PO ₄ /Na ₂ HPO ₄ , pH7.4 | 0.10 ml |
| 0.25M Glucose-6-phosphate | 0.02 ml |
| 0.10M NADP | 0.04 ml |
| 0.825M KCl/0.2M MgCl ₂ | 0.04 ml |
| S9 Homogenate | <u>0.10 ml</u> |
| | <u>1.00 ml</u> |

C. Controls

1. Vehicle Controls

Vehicle controls were plated for all tester strains both in the presence and absence of S9 mix. The vehicle control was plated, using a 50 µl aliquot of vehicle (equal to the maximum aliquot of test article dilution plated), along with a 100 µl aliquot of the appropriate tester strain and a 500 µl aliquot of S9 mix (when necessary), on selective agar.

2. Positive Controls

The combinations of positive controls, activation condition and tester strains plated concurrently with the assay are indicated in Table III.

TABLE III. POSITIVE CONTROLS

| Tester <u>Strain</u> | <u>S9 Mix</u> | <u>Positive Control</u> | Conc <u>per plate</u> |
|-------------------------|---------------|--------------------------|--------------------------|
| TA98 | + | 2-aminoanthracene | 2.5 µg |
| TA98 | - | 2-nitrofluorene | 1.0 µg |
| TA100 | + | 2-aminoanthracene | 2.5 µg |
| TA100 | - | sodium azide | 2.0 µg |
| TA1535 | + | 2-aminoanthracene | 2.5 µg |
| TA1535 | - | sodium azide | 2.0 µg |
| TA1537 | + | 2-aminoanthracene | 2.5 µg |
| TA1537 | - | ICR-191 | 2.0 µg |
| WP2 _{uvrA} | + | 2-aminoanthracene | 25.0 µg |
| WP2 _{uvrA} | - | 4-nitroquinoline-N-oxide | 1.0 µg |

a. Source and Grade of Positive Control Articles

2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., purity ≥ 97.5%; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., purity 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., purity >98%; ICR-191 (CAS #1707-45-0), Sigma Chemical Co., purity 98%; 4-nitroquinoline-N-oxide (CAS #56-57-5), Sigma Chemical Co., purity >99%.

3. Sterility Controls

a. Test Article

The most concentrated test article dilution was checked for sterility by plating a 50 µl aliquot (the same volume used in the assay) on selective agar.

b. S9 Mix

The S9 mix was checked for sterility by plating 0.5 ml on selective agar.

METHODS

A. Dose Rangefinding Study

The growth inhibitory effect (cytotoxicity) of the test article to the test system was determined in order to allow the selection of appropriate doses to be tested in the mutagenicity assay.

1. Design

The dose rangefinding study was performed using tester strains TA100 and WP2uvrA both in the presence and absence of S9 mix. Ten doses of test article were tested at one plate per dose. The test article was checked for cytotoxicity up to a maximum concentration of 5 mg per plate.

a. Rationale

The cytotoxicity of the test article observed on tester strain TA100 is generally representative of that observed on the other tester strains and because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. The *Escherichia coli* tester strain WP2uvrA does not possess the *rfa* wall mutation that the *Salmonella typhimurium* strains have and thus, a different range of cytotoxicity may be observed. Also, the cytotoxicity induced by a test article in the presence of S9 mix may vary greatly from that observed in the absence of S9 mix. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the S9 mix.

2. Evaluation of the Dose Rangefinding Study

Cytotoxicity is detectable as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn.

3. Selection of the Maximum Dose for the Mutagenicity Assay

a. Cytotoxicity Observed

Cytotoxicity was observed in the dose rangefinding study and the highest concentration of test article used in the subsequent mutagenicity assay was a dose which gave a reduction of revertants per plate and/or a thinning of the bacterial background lawn.

B. Mutagenicity Assay

1. Design

The assay was performed using tester strains TA98, TA100, TA1535, TA1537, and WP2*uvrA* both in the presence and absence of S9 mix. Six doses of test article were tested along with the appropriate vehicle and positive controls. The doses of test article were selected based on the results of the dose rangefinding study.

2. Frequency and Route of Administration

The tester strains were exposed to the test article via the plate incorporation methodology originally described by Ames *et al* (1975) and Maron and Ames (1983). This methodology has been shown to detect a wide range of classes of chemical mutagens. In the plate incorporation methodology, the test article, the tester strain and the S9 mix (where appropriate) were combined in molten agar which was overlaid onto a minimal agar plate. Following incubation at $37 \pm 2^\circ\text{C}$ for 48 ± 8 hr, revertant colonies were counted. All doses of the test article, the vehicle controls and the positive controls were plated in triplicate.

C. Plating Procedures

These procedures were used in both the dose rangefinding study and the mutagenicity assay.

Each plate was labeled with a code which identified the test article, test phase, tester strain, activation condition and dose. The S9 mix and dilutions of the test article were prepared immediately prior to their use.

When S9 mix was not required, 100 μl of tester strain and 50 μl of vehicle or test article dose was added to 2.5 ml of molten selective top agar (maintained at $45 \pm 2^\circ\text{C}$). When S9 mix was required, 500 μl of S9 mix, 100 μl of tester strain and 50 μl of vehicle or test article dose was added to 2.0 ml of molten selective top agar. After the required components had been added, the mixture was vortexed and overlaid onto the surface of 25 ml of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for 48 ± 8 hr at $37 \pm 2^\circ\text{C}$. Positive control articles were plated using a 50 μl plating aliquot.

D. Scoring the Plates

Plates which were not evaluated immediately following the incubation period were held at $5 \pm 3^\circ\text{C}$ until such time that colony counting and bacterial background lawn evaluation could take place.

1. Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn was evaluated for evidence of cytotoxicity and test article precipitate. Evidence of cytotoxicity was scored relative to the vehicle control plate and was recorded along with the revertant counts for all plates at that dose on the data tables using the code system presented at the end of the Materials and Methods Section.

2. Counting Revertant Colonies

The number of revertant colonies per plate for the vehicle controls and all plates containing test article were counted manually. The number of revertant colonies per plate for the positive controls were counted by automated colony counter.

E. Analysis of Data

For all replicate platings, the mean revertants per plate and the standard deviation were calculated. The results of these calculations are presented in tabular form in the Data Tables Section of this report.

EVALUATION OF TEST RESULTS

Before assay data were evaluated, the criteria for a valid assay had to be met.

A. Criteria For A Valid Assay

The following criteria were used to determine a valid assay:

1. Tester Strain Integrity : *Salmonella typhimurium*

a. *rfa* Wall Mutation

To demonstrate the presence of the *rfa* wall mutation, tester strain cultures exhibited sensitivity to crystal violet.

b. pKM101 Plasmid

To demonstrate the presence of the R-factor plasmid, pKM101, cultures of tester strains TA98 and TA100 exhibited resistance to ampicillin.

c. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for histidine, the tester strain cultures exhibited a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable ranges for the mean vehicle controls were as follows:

| | |
|--------|----------|
| TA98 | 8 - 60 |
| TA100 | 60 - 240 |
| TA1535 | 4 - 45 |
| TA1537 | 2 - 25 |

2. Tester Strain Integrity : *Escherichia coli*

a. Characteristic Number of Spontaneous Revertants

To demonstrate the requirement for tryptophan, the tester strain culture exhibited a characteristic number of spontaneous revertants per plate when plated along with the vehicle under selective conditions. The acceptable range for the WP2uvrA mean vehicle controls was 5 to 40 revertants per plate.

3. Tester Strain Culture Density

To demonstrate that appropriate numbers of bacteria are plated, the density of tester strain cultures were greater than or equal to 0.5×10^9 bacteria per ml and/or had reached a target level of turbidity demonstrated to produce cultures with a density greater than or equal to 0.5×10^9 bacteria per ml.

4. Positive Control Values

a. Positive Control Values in the Absence of S9 Mix

To demonstrate that the tester strains were capable of identifying a mutagen, the mean value of a positive control for a respective tester strain exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain.

b. Positive Control Values in the Presence of S9 Mix
(S9 Mix Integrity)

To demonstrate that the S9 mix was capable of metabolizing a promutagen to its mutagenic form(s), the mean value of the positive control for a respective tester strain in the presence of the S9 mix exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain.

An acceptable positive control in the presence of S9 mix for a specific strain was evaluated as having demonstrated both the integrity of the S9 mix and the ability of the tester strain to detect a mutagen.

5. Cytotoxicity

A minimum of three non-toxic doses were required to evaluate assay data.

B. Criteria For A Positive Response

Once the criteria for a valid assay had been met, responses observed in the assay were evaluated as follows:

1. Tester Strains TA98, TA100, and WP2_{uvrA}

For a test article to be considered positive, it had to produce at least a 2-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

2. Tester Strains TA1535 and TA1537

For a test article to be considered positive, it had to produce at least a 3-fold increase in the mean revertants per plate of at least one of these tester strains over the mean revertants per plate of the appropriate vehicle control. This increase in the mean number of revertants per plate had to be accompanied by a dose response to increasing concentrations of the test article.

RECORDS TO BE MAINTAINED

All raw data, documentation, records, the protocol, and the final report generated as a result of this study will be archived in the storage facilities for at least one year following submission of the final report to the Sponsor. After the one year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities for an additional period of time or sent to a storage facility designated by the Sponsor.

REFERENCES

- Ames, B.N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test. *Mutation Research* 31:347-364 (1975).
- Brusick, D.J., V.F. Simmon, H.S. Rosenkranz, V.A. Ray, and R.S. Stafford. An evaluation of the *Escherichia coli* WP2 and WP2uvrA reverse mutation assay. *Mutation Research* 76:169-190 (1980).
- Green, M.H.L. and W.J. Muriel. Mutagen testing using *trp*⁺ reversion in *Escherichia coli*. *Mutation Research* 38:3-32 (1976).
- Maron, D.M., and B. Ames. Revised Methods for the *Salmonella* Mutagenicity Test. *Mutation Research* 113:173-215 (1983).
- Vogel, H.J., and D.M. Bonner. Acetylornithinase of *E. coli*: Partial purification and some properties. *J. Biol. Chem.* 218:97-106 (1956).

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

| CODE | DEFINITION | <u>CHARACTERISTICS OF BACKGROUND LAWN</u> |
|------|----------------------------|---|
| 1 | Normal | A healthy microcolony lawn. |
| 2 | Slightly Reduced | A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate. |
| 3 | Moderately Reduced | A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate. |
| 4 | Extremely Reduced | An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate. |
| 5 | Absent | A complete lack of any microcolony lawn. |
| 6 | Obscured by Precipitate | The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate. |

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

| | | |
|----|-------------------------|--|
| sp | Slight Precipitate | Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate. |
| mp | Moderate Precipitate | The amount of macroscopic precipitate on the plate would interfere with automated counting, thus requiring the plate to be hand counted. |
| hp | Heavy Precipitate | The large amount of macroscopic precipitate on the plate makes the required hand counting difficult. |

Example: 4mp would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION IV. RESULTS AND CONCLUSIONS

RESULTS

A. Test Article Handling

The test article, **EPNB/Ethyl-4-Nitrobenzoate**, was stored at room temperature. Dimethylsulfoxide (DMSO, CAS# 67-68-5, Sigma Chemical Co., Lot 116H0703, 99+%) was used as the vehicle. At 100 mg per ml, which was the most concentrated stock dilution prepared, the test article formed a clear, pale yellow solution. The test article remained a solution in all succeeding dilutions prepared for the mutagenicity assay.

B. Dose Rangefinding Study

Doses to be tested in the mutagenicity assay were selected based on the results of the dose rangefinding study conducted on the test article using tester strains TA100 and WP2uvrA in both the presence and absence of S9 mix (one plate per dose). Ten doses of test article, from 5,000 to 6.67 µg per plate, were tested and the results are presented in Tables 1 and 2. These data were generated in Experiment 18699-A1. Cytotoxicity was observed with tester strain TA100 at 3,330 µg per plate and above in both the presence and absence of S9 mix as evidenced by the reduced number of revertants per plate and/or the thinning of the bacterial background lawn. With tester strain WP2uvrA, cytotoxicity was observed at 5,000 µg per plate in the absence of S9 mix as evidenced by the reduced number of revertants per plate and/or the thinning of the bacterial background lawn. No cytotoxicity was observed with tester strain WP2uvrA in the presence of S9 mix as evidenced by a normal background lawn and no decrease in the number of revertants per plate.

C. Mutagenicity Assay

The mutagenicity assay results for **EPNB/Ethyl-4-Nitrobenzoate** are presented in Tables 3 and 4. These data were generated in Experiment 18699-B1. The data are presented as mean revertants per plate ± standard deviation for each treatment and control group (Table 4) and as individual plate counts (Table 3).

The results of the dose rangefinding study were used to select six doses to be tested in the mutagenicity assay. The doses tested were 5,000, 2,500, 1,000, 500, 250, and 100 µg per plate in both the presence and absence of S9 mix.

In Experiment 18699-B1 (Tables 3 and 4), all data were acceptable and positive increases in the number of revertants per plate were observed with tester strains TA100 (2.3-fold) and TA1535 (53.8-fold) in the presence of S9 mix. No positive increases in the number of revertants per plate were observed with any of the remaining tester strain/activation condition combinations.

All criteria for a valid study were met.

CONCLUSIONS

The results of the *Salmonella - Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay indicate that, under the conditions of this study, ChemFirst Inc.'s test article, **EPNB/Ethyl-4-Nitrobenzoate**, did cause positive increases in the number of revertants per plate with tester strains TA100 (2.3-fold) and TA1535 (53.8-fold) in the presence of S9 mix. No positive increases in the number of revertants per plate were observed with any of the remaining tester strain/activation condition combinations.

SECTION V. DATA TABLES

TABLE 2

DOSE RANGE-FINDING STUDY

TEST ARTICLE ID: EPNB/Ethyl-4-Nitrobenzoate

EXPERIMENT ID:

DATE PLATED: 26-Jun-97

VEHICLE: DMSO

DATE COUNTED: 28-Jun-97, 30-Jun-97

| µg/PLATE | WP2uvrA REVERTANTS PER PLATE | | | |
|---------------------------|------------------------------|-----------------------------------|----------------------------|-----------------------------------|
| | WITH S9 | | WITHOUT S9 | |
| | REVERTANTS PER PLATE | BACKGROUND LAWN EVALUATION* | REVERTANTS PER PLATE | BACKGROUND LAWN EVALUATION* |
| 0.00 (Vehicle) (50 µl) | 8 | 1 | 14 | 1 |
| Test Article 6.67 | 12 | 1 | 17 | 1 |
| 10.0 | 12 | 1 | 11 | 1 |
| 33.3 | 11 | 1 | 17 | 1 |
| 66.7 | 17 | 1 | 14 | 1 |
| 100 | 5 | 1 | 11 | 1 |
| 333 | 11 | 1 | 10 | 1 |
| 667 | 17 | 1 | 8 | 1 |
| 1000 | 11 | 1 | 6 | 1 |
| 3330 | 8 | 1 | 5 | 1 |
| 5000 | 11 | 1 | 0 | 3 |

* Background Lawn Evaluation Codes:

1 = normal

2 = slightly reduced

3 = moderately reduced

4 = extremely reduced

5 = absent

6 = obscured by precipitate

sp = slight precipitate

mp = moderate precipitate
(requires hand count)hp = heavy precipitate
(requires hand count)

TABLE 1

DOSE RANGE-FINDING STUDY

TEST ARTICLE ID: EPNB/Ethyl-4-Nitrobenzoate

EXPERIMENT ID:

DATE PLATED: 26-Jun-97

VEHICLE: DMSO

DATE COUNTED: 28-Jun-97

| µg/PLATE | TA100 REVERTANTS PER PLATE | | | |
|---------------------------|----------------------------|-----------------------------------|----------------------------|-----------------------------------|
| | WITH S9 | | WITHOUT S9 | |
| | REVERTANTS PER PLATE | BACKGROUND LAWN EVALUATION* | REVERTANTS PER PLATE | BACKGROUND LAWN EVALUATION* |
| 0.00 (Vehicle) (50 µl) | 73 | 1 | 70 | 1 |
| Test Article 6.67 | 108 | 1 | 91 | 1 |
| 10.0 | 87 | 1 | 100 | 1 |
| 33.3 | 103 | 1 | 94 | 1 |
| 66.7 | 108 | 1 | 86 | 1 |
| 100 | 96 | 1 | 84 | 1 |
| 333 | 150 | 1 | 91 | 1 |
| 667 | 214 | 1 | 100 | 1 |
| 1000 | 332 | 1 | 71 | 1 |
| 3330 | 0 | 1 | 0 | 5 |
| 5000 | 0 | 2 | 0 | 5 |

* Background Lawn Evaluation Codes:

| | | |
|-------------------------|--|---|
| 1 = normal | 2 = slightly reduced | 3 = moderately reduced |
| 4 = extremely reduced | 5 = absent | 6 = obscured by precipitate |
| sp = slight precipitate | mp = moderate precipitate (requires hand count) | hp = heavy precipitate (requires hand count) |

TABLE 3
MUTAGENICITY ASSAY RESULTS
INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: EPNB/Ethyl-4-Nitrobenzoate

EXPERIMENT ID:

DATE PLATED: 01-Jul-97

VEHICLE: DMSO

DATE COUNTED: 03-Jul-97

PLATING ALIQUOT: 50 µl

| | DOSE/PLATE | REVERTANTS PER PLATE | | | | | | | | | | | | BACKGROUND LAWN* | | | |
|-----------------------|------------|----------------------|------|-----|-------|------------------|------|--------|-----|-----|--------|-----|-----|------------------|-----|-----|----------------|
| | | TA98 | | | TA100 | | | TA1535 | | | TA1537 | | | WP2uvrA | | | |
| | | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | |
| MICROSOMES: Rat Liver | | | | | | | | | | | | | | | | | |
| VEHICLE CONTROL | | 21 | 13 | 12 | 81 | 88 | 70 | 5 | 10 | 8 | 7 | 9 | 6 | 11 | 17 | 13 | 1 |
| TEST ARTICLE | 100 µg | 16 | 19 | 17 | 107 | 90 | 101 | 23 | 27 | 23 | 5 | 8 | 3 | 14 | 12 | 8 | 1 |
| | 250 µg | 12 | 10 | 14 | 108 | 95 | 110 | 23 | 26 | 26 | 3 | 4 | 10 | 10 | 11 | 9 | 1 |
| | 500 µg | 20 | 24 | 20 | 162 | 153 | 150 | 64 | 63 | 90 | 4 | 4 | 4 | 6 | 11 | 8 | 1 |
| | 1000 µg | 20 | 17 | 14 | 169 | 182 | 202 | 156 | 148 | 174 | 1 | 2 | 3 | 9 | 12 | 10 | 1 |
| | 2500 µg | 15 | 18 | 12 | 0 | 0 | 0 | 429 | 396 | 465 | 3 | 1 | 1 | 8 | 8 | 9 | 1 |
| | 5000 µg | 18 | 22 | 16 | 0 | 0 | 0 | 416 | 427 | 397 | 0 | 0 | 0 | 12 | 14 | 13 | 1 |
| POSITIVE CONTROL ** | | 964 | 1041 | 992 | 1050 | 1073 | 1085 | 121 | 128 | 115 | 188 | 206 | 151 | 309 | 259 | 200 | 1 |
| MICROSOMES: None | | | | | | | | | | | | | | | | | |
| VEHICLE CONTROL | | 10 | 23 | 6 | 60 | 62 | 74 | 7 | 6 | 8 | 6 | 8 | 6 | 11 | 12 | 10 | 1 |
| TEST ARTICLE | 100 µg | 9 | 13 | 12 | 50 | 73 | 69 | 5 | 3 | 8 | 2 | 10 | 5 | 11 | 12 | 10 | 1 |
| | 250 µg | 12 | 11 | 8 | 63 | 78 | 69 | 4 | 9 | 7 | 8 | 5 | 3 | 7 | 21 | 6 | 1 |
| | 500 µg | 15 | 14 | 12 | 64 | 61 _{mp} | 57 | 9 | 3 | 4 | 8 | 3 | 4 | 13 | 12 | 20 | 1 |
| | 1000 µg | 11 | 12 | 8 | 48 | 75 | 62 | 6 | 8 | 5 | 5 | 6 | 12 | 5 | 14 | 6 | 1 |
| | 2500 µg | 5 | 2 | 4 | 10 | 7 | 10 | 3 | 6 | 1 | 3 | 2 | 1 | 7 | 5 | 1 | 2 _e |
| | 5000 µg | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 5 _e |
| POSITIVE CONTROL *** | | 110 | 108 | 99 | 356 | 348 | 358 | 375 | 411 | 396 | 326 | 376 | 370 | 191 | 150 | 161 | 1 |

| | | | | | |
|---------|-------------------|---------------|----------|--------------------------|--------------|
| ** TA98 | 2-aminoanthracene | 2.5 µg/plate | *** TA98 | 2-nitrofluorene | 1.0 µg/plate |
| TA100 | 2-aminoanthracene | 2.5 µg/plate | TA100 | sodium azide | 2.0 µg/plate |
| TA1535 | 2-aminoanthracene | 2.5 µg/plate | TA1535 | sodium azide | 2.0 µg/plate |
| TA1537 | 2-aminoanthracene | 2.5 µg/plate | TA1537 | ICR-191 | 2.0 µg/plate |
| WP2uvrA | 2-aminoanthracene | 25.0 µg/plate | WP2uvrA | 4-nitroquinoline-N-oxide | 1.0 µg/plate |

* Background Lawn Evaluation Codes:

| | | |
|-------------------------|--|---|
| 1 = normal | 2 = slightly reduced | 3 = moderately reduced |
| 4 = extremely reduced | 5 = absent | 6 = obscured by precipitate |
| sp = slight precipitate | mp = moderate precipitate (requires hand count) | hp = heavy precipitate (requires hand count) |

• The bacterial background lawn for tester strain WP2uvrA was evaluated as normal (1) at this dose.

• The bacterial background lawn for tester strain WP2uvrA was evaluated as slightly reduced (2) at this dose.

TABLE 4
MUTAGENICITY ASSAY RESULTS
SUMMARY

TEST ARTICLE ID: EPNB/Ethyl-4-Nitrobenzoate

EXPERIMENT ID:

DATE PLATED: 01-Jul-97

VEHICLE: DMSO

DATE COUNTED: 03-Jul-97

PLATING ALIQUOT: 50 µl

MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATION

| | DOSE/PLATE | TA98 | | TA100 | | TA1535 | | TA1537 | | WP2uvrA | | BACKGROUND LAWN* |
|-----------------------|------------|------|------|-------|-----------------|--------|------|--------|------|---------|------|------------------|
| | | MEAN | S.D. | MEAN | S.D. | MEAN | S.D. | MEAN | S.D. | MEAN | S.D. | |
| MICROSOMES: Rat Liver | | | | | | | | | | | | |
| VEHICLE CONTROL | | 15 | 5 | 80 | 9 | 8 | 3 | 7 | 2 | 14 | 3 | 1 |
| TEST ARTICLE | 100 µg | 17 | 2 | 99 | 9 | 24 | 2 | 5 | 3 | 11 | 3 | 1 |
| | 250 µg | 12 | 2 | 104 | 8 | 25 | 2 | 6 | 4 | 10 | 1 | 1 |
| | 500 µg | 21 | 2 | 155 | 6 | 72 | 15 | 4 | 0 | 8 | 3 | 1 |
| | 1000 µg | 17 | 3 | 184 | 17 | 159 | 13 | 2 | 1 | 10 | 2 | 1 |
| | 2500 µg | 15 | 3 | 0 | 0 | 430 | 35 | 2 | 1 | 8 | 1 | 1 |
| | 5000 µg | 19 | 3 | 0 | 0 | 413 | 15 | 0 | 0 | 13 | 1 | 1 |
| POSITIVE CONTROL ** | | 999 | 39 | 1069 | 18 | 121 | 7 | 182 | 28 | 256 | 55 | 1 |
| MICROSOMES: None | | | | | | | | | | | | |
| VEHICLE CONTROL | | 13 | 9 | 65 | 8 | 7 | 1 | 7 | 1 | 11 | 1 | 1 |
| TEST ARTICLE | 100 µg | 11 | 2 | 64 | 12 | 5 | 3 | 6 | 4 | 11 | 1 | 1 |
| | 250 µg | 10 | 2 | 70 | 8 | 7 | 3 | 5 | 3 | 11 | 8 | 1 |
| | 500 µg | 14 | 2 | 61 | 4 ^{mp} | 5 | 3 | 5 | 3 | 15 | 4 | 1 |
| | 1000 µg | 10 | 2 | 62 | 14 | 6 | 2 | 8 | 4 | 8 | 5 | 1 |
| | 2500 µg | 4 | 2 | 9 | 2 | 3 | 3 | 2 | 1 | 4 | 3 | 2 ^{sp} |
| | 5000 µg | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 5 ^{sp} |
| POSITIVE CONTROL *** | | 106 | 6 | 354 | 5 | 394 | 18 | 357 | 27 | 167 | 21 | 1 |

| | | | | | |
|---------|-------------------|---------------|----------|--------------------------|--------------|
| ** TA98 | 2-aminoanthracene | 2.5 µg/plate | *** TA98 | 2-nitrofluorene | 1.0 µg/plate |
| TA100 | 2-aminoanthracene | 2.5 µg/plate | TA100 | sodium azide | 2.0 µg/plate |
| TA1535 | 2-aminoanthracene | 2.5 µg/plate | TA1535 | sodium azide | 2.0 µg/plate |
| TA1537 | 2-aminoanthracene | 2.5 µg/plate | TA1537 | ICR-191 | 2.0 µg/plate |
| WP2uvrA | 2-aminoanthracene | 25.0 µg/plate | WP2uvrA | 4-nitroquinoline-N-oxide | 1.0 µg/plate |

* Background Lawn Evaluation Codes:

| | | |
|-------------------------|--|---|
| 1 = normal | 2 = slightly reduced | 3 = moderately reduced |
| 4 = extremely reduced | 5 = absent | 6 = obscured by precipitate |
| sp = slight precipitate | mp = moderate precipitate (requires hand count) | hp = heavy precipitate (requires hand count) |

• The bacterial background lawn for tester strain WP2uvrA was evaluated as normal (1) at this dose.

• The bacterial background lawn for tester strain WP2uvrA was evaluated as slightly reduced (2) at this dose.