FINAL REPORT

PURIFIED 8-2 ALCOHOL: *SALMONELLA-ESCHERICHIA COLI*/MAMMALIAN-MICROSOME REVERSE MUTATION ASSAY WITH A CONFIRMATORY ASSAY

COVANCE STUDY 22900-0-409OECD

STUDY SCHEDULE:

Study Initiation Date:	23 October 2001
Initial Dose Date:	29 October 2001
Study Termination Date:	10 December 2001
Final Report Date:	11 February 2002

STUDY DIRECTOR, SPONSOR, AND TESTING FACILITY

Study Director:	Leon F. Stankowski, Jr., PhD
Testing Facility:	Covance Laboratories, Inc. (Covance) 9200 Leesburg Pike Vienna, VA 22182
Sponsor:	Telomer Research Program Consortium administered by RAND Corp. 1700 Main Street Santa Monica, California 90407

This study was conducted in accordance with Covance Standard Operating Procedures; the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice, ENV/MC/CHEM(98)17 adopted November 26, 1997; and the Environmental Protection Agency (EPA-TSCA), Title 40 of the US Code of Federal Regulations Part 792, issued November 29, 1983 (effective December 29, 1983 [revision effective September 18, 1989]).

TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF APPENDICES

SUMMARY

Objective: The objective of this study was to evaluate the ability of Purified 8-2 Alcohol to induce reverse mutations at the histidine locus of selected *Salmonella typhimurium* strains, or the tryptophan locus of *Escherichia coli* strain WP2*uvr*A, in the presence and absence of an exogenous mammalian metabolic activation system (S9).

Study Design and Parameters: Purified 8-2 Alcohol was tested in the plate incorporation reverse mutation assay using *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and *E. coli* strain WP2*uvr*A, with and without a metabolic activation system (S9) containing induced hepatic microsomal enzymes from Aroclor™ 1254-treated rats. Initial and confirmatory mutagenicity assays were conducted. Each assay included vehicle and positive controls, and six dose levels of Purified 8-2 Alcohol, with and without metabolic activation. Doses of Purified 8-2 Alcohol evaluated, based on the results of a dose rangefinding assay and selected in conjunction with the Sponsor, were 33.3, 100, 333, 1000, 3330 and 5000 µg/plate with and without S9. Each test and control article dose was evaluated in triplicate plates in each strain. The dimethylsulfoxide (DMSO) vehicle control, as well as the test and positive control articles were administered in a volume of 50 µL.

Results: No toxicity was observed in any tester strain in the presence or absence of S9 up to the maximum dose tested of 5000 µg/plate. The mean number of revertants per plate did not meet the criteria for a positive response in any tester strain with or without S9. All criteria for a valid study, including appropriate vehicle and positive control responses (with the exception of positive control values for TA1537 without S9 in Experiment 22900-C1, see Protocol Deviation), were fulfilled.

Conclusion: Purified 8-2 Alcohol was not mutagenic in this test system.

1. OBJECTIVE

The objective of this study was to evaluate the test article for the ability to induce reverse mutations at the histidine locus in several strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537), and at the tryptophan locus in *Escherichia coli* tester strain WP2*uvr*A, in the presence or absence of an exogenous metabolic activation system (S9). The assay design was based on OECD Guideline 471, updated and adopted July 21, 1997.

2. MATERIALS

2.1 Tester Strains

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 (Ames et al., 1975) and the *Escherichia coli* tryptophan auxotroph WP2*uvr*A (Green and Muriel, 1976). The specific genotypes of the strains are shown below (Table 1).

Table 1. Tester Strain Genotypes

Tester Strain	*his/trp* Mutation	Additional Mutations		Plasmid
		Repair	LPS	
TA98	*his*D3052	*uvr*B	*rfa*	pKM101
TA100	*his*G46	*uvr*B	*rfa*	pKM101
TA1535	*his*G46	*uvr*B	*rfa*	–
TA1537	*his*C3076	*uvr*B	*rfa*	–
WP2*uvr*A	*trp*	*uvr*A	–	–

In addition to a mutation in the histidine or tryptophan operons, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. A mutation of the *uvr*A gene (*Escherichia coli*) or the *uvr*B gene (*Salmonella typhimurium*), results in a deficient DNA excision repair system which greatly enhances the sensitivity of

these strains to some mutagens. Since the *uvr*B deletion extends through the bio gene, the *Salmonella typhimurium* tester strains containing this deletion also require the vitamin biotin for growth.

The *Salmonella typhimurium* tester strains also contain the rfa wall mutation which 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.

Strains TA98 and TA100 also contain the pKM101 plasmid, 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. Tester strains TA100, TA1535 and WP2*uvr*A are reverted from auxotrophy to prototrophy by base substitution mutagens.

2.1.1 Source of Tester Strains

The *Salmonella typhimurium* tester strains were received from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley. The *Escherichia coli* tester strain, WP2*uvr*A, was received from The National Collection of Industrial Bacteria, Torrey Research Station, Scotland (United Kingdom).

2.1.2 Storage of Tester Strain

2.1.2.1 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 at ≤-70°C.

2.1.2.2 Master Plates

Master plates of the tester strains were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with either histidine and biotin (*S. typhimurium*) or tryptophan (*E. coli*), and for strains containing the pKM101 plasmid, ampicillin. Tester strain master plates were stored at 5 ± 3°C.

2.1.3 Preparation of Overnight Cultures

2.1.3.1 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 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began.

2.1.3.2 Harvest

To ensure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring of culture density. Cultures were harvested once a predetermined density was reached, which ensures that cultures have reached a density of at least 0.5 x 10^9 cells/mL and that the cultures have not overgrown. Overgrown of

stationary cultures may exhibit decreased sensitivity to some mutagens. Cultures were removed from incubation when the target density was reached and were placed at 5 ± 3°C until used in the assay.

2.1.4 Confirmation of Tester Strain Phenotypes

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

2.1.4.1 *rfa* Wall Mutation

For the *Salmonella* tester strains, 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.

2.1.4.2 pKM101 Plasmid R-factor

The presence of the pKM101 plasmid was confirmed for cultures of 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 growth in the zone immediately surrounding the disk.

2.1.4.3 Characteristic Number of Spontaneous Revertants

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

2.1.5 Tester Strain Media

2.1.5.1 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).

2.1.5.2 Minimal Bottom 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.

2.1.5.3 Top Agar for Selection of Revertants

Top (overlay) 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.

2.2 Test Article

Test Article:	Purified 8-2 Alcohol
Haskell Number:	24691
Physical Description:	White solid
Date Received:	8 August 2001

The test article, Purified 8-2 Alcohol, was supplied as a white solid. The test article was stored in a container at ambient temperature. The Sponsor was responsible for the determination and documentation of the analytical purity of the test article. Any unused test article and/or the original test article container were returned to the Sponsor.

2.3 Control Articles

2.3.1 Vehicle Controls

Dimethylsulfoxide (DMSO, CAS# 67-68-5, Acros Organics, Lot Nos. A014587401 and A012097501) was used as the vehicle. Vehicle controls were plated for all tester strains in the presence and absence of S9. The vehicle control was plated on selective agar using a 50 µL aliquot (equal to the maximum aliquot of test article plated), along with 100 µL of the appropriate tester strain and 500 µL of S9 mix (when necessary).

2.3.2 Positive Controls

The combinations of positive controls, activation condition, and tester strains plated concurrently with the assay are indicated below (Table 2, next page).

Table 2. Positive Controls

Tester Strain	S9 Mix	Positive Control	Dose (µg/plate)
TA98	+	benzo[a]pyrene	2.5
TA98	-	2-nitrofluorene	1.0
TA100	+	2-aminoanthracene	2.5
TA100	-	sodium azide	2.0
TA1535	+	2-aminoanthracene	2.5
TA1535	-	sodium azide	2.0
TA1537	+	2-aminoanthracene	2.5
TA1537	-	ICR-191	2.0
WP2*uvr*A	+	2-aminoanthracene	25.0
WP2*uvr*A	-	4-nitroquinoline-N-oxide	1.0

2.3.2.1 Source and Grade of Positive Control Articles

Benzo[a]pyrene (CAS# 50-32-8; purity ≥97%), 2-nitrofluorene (CAS# 607-57-8; purity ≥98%), sodium azide (CAS# 26628-22-8; purity ≥99%), ICR-191 (CAS# 1707-45-0; purity ≥90%), and 4-nitroquinoline-N-oxide (CAS# 56-57-5; purity ≥99%) were obtained from Sigma Chemical Co. All were prepared in DMSO, except for sodium azide, which was dissolved in deionized water.

2.3.3 Sterility Controls

2.3.3.1 Test Article

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

2.3.3.2 S9 Mix

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

2.4 Liver Microsomal Enzyme Reaction Mixture (S9 Mix)

2.4.1 S9 Homogenate

S9 homogenate containing liver microsomal enzymes was purchased from Molecular Toxicology, Inc. [Lot Nos. 1302 (41.3 mg/mL protein) and 1296 (38.9 mg/mL protein). The homogenate was prepared from male Sprague-Dawley rats that had been injected (ip) with Aroclor™ 1254 (200 mg/mL in corn oil) at 500mg/kg (Ames *et al.*, 1975).

2.4.2 S9 Mix

The S9 mix was prepared immediately prior to use and contained the components indicated below (Table 3).

Table 3. S9 Mix Components

Component	Quantity
H_2O	0.70 mL
1M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 mL
0.242M Glucose-6-phosphate	0.02 mL
0.10M NADP	0.04 mL
0.825M KCl/0.2M $MgCl_2$	0.04 mL
S9 Homogenate	0.10 mL
	1.00 mL

3. EXPERIMENTAL DESIGN

3.1 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.

3.1.1 Design

The dose rangefinding study was performed using tester strains TA100 and WP2*uvr*A in the presence and absence of S9. Ten doses of test article, up to 5000 µg/plate, were tested for cytotoxicity (one plate per dose).

3.1.2 Rationale

The cytotoxicity of the test article observed in tester strain TA100 is generally representative of that observed on the other *Salmonella typhimurium* tester strains and because of the comparatively high number of spontaneous revertants per plate observed with this strain, gradations of cytotoxicity can be readily discerned from routine experimental variation. The *Escherichia coli* tester strain WP2*uvr*A 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 may vary greatly from that observed in the absence of S9. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the microsomal enzymes.

3.1.3 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.

3.1.4 Selection of the Maximum Dose for the Mutagenicity Assay

No cytotoxicity was observed in the dose rangefinding study and the highest dose level of test article used in the subsequent mutagenicity assay was that used in the dose rangefinding study (the reduction in background lawn observed in tester strain TA100 without S9 was not accompanied by a decrease in revertant frequency and likely was artifactual).

3.2 Mutagenicity Assay

3.2.1 Design

The assay was performed using tester strains TA98, TA100, TA1535, TA1537 and WP2*uvr*A in the presence and absence of S9. Doses of the test article were selected based on the results of the dose rangefinding study. The results of the initial mutagenicity assay were confirmed in an independent experiment.

3.2.2 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, revertant colonies were counted. All doses of the test article, the vehicle controls and the positive controls were plated in triplicate.

3.3 Plating Procedures

These procedures were used in 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 level. The S9 mix and dilutions of the test article were prepared immediately prior to their use.

When S9 was not required, 100 µL of tester strain and 50 µL of test or control article were added to 2.5 mL of molten selective top agar (maintained at 45 ± 2°C). When S9 was required, 500 µL of S9 mix, 100 µL of tester strain and 50 µL of test or control article were

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 solidified, the plates were inverted and incubated for 52 ± 4 hours at 37 ± 2°C.

3.4 Scoring the Plates

Plates which were not evaluated immediately following the incubation period were held at 5 ± 3°C until such time that colony counting and bacterial background lawn evaluation could take place.

3.4.1 Bacterial Background Lawn Evaluation

The condition of the bacterial background lawn was evaluated macroscopically and microscopically (using a dissecting microscope) for indications 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 described below (Appendix B).

3.4.2 Counting Revertant Colonies

Revertant colonies were counted by automated colony counter.

4. DATA

4.1 Data Presentation

For all replicate platings, the mean revertants per plate and the standard deviation were calculated (Appendix A).

4.2 Assay Acceptance Criteria

Before assay data were evaluated, the criteria for a valid assay had to be met. The following criteria were used to determine a valid assay:

4.2.1 Tester Strain Integrity

4.2.1.1 *rfa* Wall Mutation

All *Salmonella typhimurium* tester strain cultures exhibited sensitivity to crystal violet, demonstrating the presence of the *rfa* wall mutation.

4.2.1.2 pKM101 Plasmid

Tester strains TA98 and TA100 exhibited resistance to ampicillin, demonstrating the presence of the pKM101 plasmid.

4.2.1.3 Characteristic Number of Spontaneous Revertants

All vehicle control cultures exhibited their characteristic number of spontaneous revertants per plate, demonstrating the requirement for histidine (*Salmonella typhimurium*) or tryptophan (*Escherichia coli*). The acceptable ranges for the mean vehicle controls were as follows:

Strain	Number of Revertants
TA98	8 - 60
TA100	60 - 240
TA1535	4 - 45
TA1537	2 - 25
WP2*uvr*A	5 - 40

4.2.2 Tester Strain Culture Density

The cell densities of all tester strain cultures were greater than or equal to 0.5×10^9 bacteria/mL (with the exception of TA98 in Experiment 22900-B1, 0.4×10^9 bacteria/mL), or the optical densities of these cultures reached a target value demonstrated to produce cultures with at least 0.5×10^9 bacteria/mL, demonstrating that appropriate numbers of bacteria were plated.

4.2.3 Positive Control Values

4.2.3.1 Positive Control Values in the Absence of S9 Mix

The mean value of the positive control for each tester strain exhibited at least a 3-fold increase over the mean value of the vehicle control for that strain (with the exception of tester strain TA1537 in Experiment 22900-C1, see Protocol Deviation), demonstrating that the tester strains were capable of identifying a mutagen.

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

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

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

4.2.4 Cytotoxicity

A minimum of three non-toxic doses was used to evaluate assay data. Cytotoxicity can be detected as a decrease in the number of revertant colonies per plate and/or by a thinning or disappearance of the bacterial background lawn compared to the appropriate vehicle control.

4.3 Assay Evaluation Criteria

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

4.3.1 Tester Strains TA98, TA100 and WP2*uvr*A

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.

4.3.2 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.

5. 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 of Covance-Vienna 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 of Covance-Vienna for an additional period of time or sent to a storage facility designated by the Sponsor.

6. STUDY RESPONSIBILITIES

Function	Responsible Person(s)
Study Director	Leon F. Stankowski, Jr., PhD
Laboratory Supervisor	Magnus A. Evertson, BS

7. RESULTS

7.1 Test Article Handling

Purified 8-2 Alcohol formed a solution at 100 mg/mL in dimethylformamide after heating. In DMSO at 100 mg/mL, the test article formed a solution that foamed when heated and vortexed. DMSO was selected as the vehicle. At 100 mg/mL, which was the most concentrated stock prepared for the mutagenicity assay, the test article formed a transparent, colorless solution after heating to 45°C for 3 minutes; it remained freely soluble at all succeeding lower dilutions.

7.2 Dose Rangefinding Study (Appendix A, Tables 4 and 5)

A dose rangefinding assay was conducted on the test article using tester strains TA100 and WP2*uvr*A (one plate per dose; Experiment 22900-A1; Tables 4 and 5). Ten doses of test article, from 6.67 to 5000 µg/plate, were evaluated in the presence and absence of S9. Apparently normal growth was observed in both tester strains at all doses evaluated with and without S9 (the reduction in background lawn observed in tester strain TA100 without S9 was not accompanied by a decrease in revertant frequency and likely was artifactual). However, the test article was found to be incompletely soluble in the aqueous top agar at a dose of 5000 µg/plate with and without S9.

7.3 Mutagenicity Assay (Appendix A, Tables 6, 8 - Individual Data, Tables 7, 9- Summary Data)

Based upon the results of the dose rangefinding study, Purified 8-2 Alcohol was evaluated in the initial mutagenicity assay in all five tester strains at doses of 33.3, 100, 333, 1000, 3330 and 5000 µg/plate with and without S9 (Experiment 22900-B1, Tables 6 and 7). All doses of the test article, as well as the concurrent positive and vehicle controls, were evaluated using three plates per dose. Apparently normal growth again was observed in all tester strains at all doses of Purified 8-2 Alcohol evaluated with and without S9. In addition, the test article precipitated from solution at doses ≥1000 µg/plate with and without S9. Revertant frequencies for all doses of Purified 8-2 Alcohol, in all tester strains with and without S9, approximated or were less than those observed in the concurrent vehicle control cultures.

The test article was re-evaluated in an independent confirmatory experiment under identical conditions, and similar results were observed (Experiment 22900-C1; Tables 8 and 9). Normal growth was again observed in all tester strains at all doses evaluated with and without S9, and the test article again precipitated from solution at doses ≥1000 µg/plate with and without S9. Revertant frequencies for all doses of Purified 8-2 Alcohol, in all tester

strains with and without S9, again approximated or were less than control values. Except for tester train TA1537 without S9 in Experiment 22900-C1 (see Protocol Deviation), all positive and vehicle control values were within acceptable ranges in both assays. All criteria for a valid study were met.

8. CONCLUSIONS

The results of the *Salmonella* - *Escherichia coli*/Mammalian- Microsome Reverse Mutation Assay indicate that under the conditions of this study, Purified 8-2 Alcohol was not mutagenic in any of the tester strains in the presence or absence of an exogenous metabolic activation system containing induced hepatic microsomal enzymes from Aroclor™ 1254-treated rats.

9. PROTOCOL DEVIATIONS

In Experiment 22900-C1, the test article-treated cultures for tester strain TA1537 without S9 were scored even though the concurrent positive control values were below acceptable limits (all plates had no revertants). However, the vehicle control values for tester strain TA1537 without S9 were within acceptable ranges. In addition, the positive control values for tester strain TA1537 with S9 also were within acceptable limits, indicating that the tester strain and S9 were functioning properly. Thus, the observed low values were likely due to a technical error. This deviation is not considered to have had an adverse impact upon the integrity of the study or the conclusions derived from it.

10. LIST OF REFERENCES

Ames et al., 1975.
Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test. *Mutation Research* 1975; 31:347-64.

Green and Muriel, 1976.
Green MHL, Muriel WJ. Mutagen testing using *trp*$^+$ reversion in *Escherichia coli*. *Mutation Research* 1976; 38:3-32.

Maron and Ames, 1983.
Maron DM, Ames B. Revised methods for the *Salmonella* Mutagenicity Test. *Mutation Research* 1983; 113:173-215.

Vogel and Bonner, 1956.
Vogel HJ, Bonner DM. Acetylornithinase of *E. coli*: Partial purification and some properties. *J Biol Chem* 1956; 218:97-106.

Appendix A. Experimental Data Tables

Table 4 Dose Rangefinding Study – TA100

Test Article ID:	Purified 8-2 Alcohol	Experiment ID:	22900-A1
Date Plated:	29-Oct-01	Date Counted:	01-Nov-01
Vehicle:	DMSO	Plating Aliquot:	50 µL

TA100 Revertants Per Plate

µg/Plate	With S9		Without S9	
	Revertants Per Plate	Background Lawn Evaluation[a]	Revertants Per Plate	Background Lawn Evaluation[a]
0.00 (Vehicle) (50 µL)	113	N	82	N
Test Article 6.67	107	N	86	N
10.0	113	N	107	N
33.3	136	N	101	N
66.7	97	N	94	N
100	139	N	87	N
333	97	N	67	N
667	93	N	98	N
1000	106	N	89	N
3330	108	N	107	N
5000	105	NP	103	RP

[a] Background Lawn Evaluation Codes:
N = normal R = reduced A = absent
P = precipitate O = obscured by precipitate

Table 5 Dose Rangefinding Study – WP2*uvr*A

Test Article ID:	Purified 8-2 Alcohol	Experiment ID:	22900-A1
Date Plated:	29-Oct-01	Date Counted:	01-Nov-01
Vehicle:	DMSO	Plating Aliquot:	50 µL

		WP2*uvr*A Revertants Per Plate			
		With S9		Without S9	
µg/Plate		Revertants Per Plate	Background Lawn Evaluation[a]	Revertants Per Plate	Background Lawn Evaluation[a]
0.00 (Vehicle) (50 µL)		23	N	12	N
Test Article	6.67	21	N	12	N
	10.0	15	N	11	N
	33.3	13	N	15	N
	66.7	17	N	11	N
	100	12	N	18	N
	333	14	N	12	N
	667	18	N	11	N
	1000	23	N	12	N
	3330	12	N	14	N
	5000	17	NP	13	NP

[a] Background Lawn Evaluation Codes:
N = normal R = reduced A = absent
P = precipitate O = obscured by precipitate

Table 6 Initial Mutagenicity Assay Results - Individual Plate Counts

Test Article ID:	Purified 8-2 Alcohol	Experiment ID:	22900-B1
Date Plated:	13-Nov-01	Date Counted:	26-Nov-01
Vehicle:	DMSO	Plating Aliquot:	50 µL

Dose/Plate	Revertants Per Plate															Back-ground Lawn[a]
	TA98			TA100			TA1535			TA1537			WP2*uvr*A			
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
Microsomes: Rat Liver																
Vehicle Control	33	30	27	115	125	112	14	8	18	7	7	10	27	14	5	N
Test Article 33.3 µg	32	24	30	112	91	103	16	17	16	5	10	14	10	16	18	N
100 µg	25	20	28	108	102	117	11	9	10	10	9	5	19	19	19	N
333 µg	34	35	18	88	114	108	12	9	14	10	10	9	11	10	21	N
1000 µg	34	33	27	119	115	116	18	9	17	6	12	9	8	26	15	NP
3330 µg	23	45	28	115	99	104	16	14	19	10	14	9	23	19	19	NP
5000 µg	28	45	30	111	120	116	14	16	8	11	9	12	20	20	21	NP
Positive Control[b]	574	512	542	984	1048	1090	141	165	138	181	148	178	233	241	293	N
Microsomes: None																
Vehicle Control	12	25	9	116	86	90	10	12	17	6	5	3	16	14	17	N
Test Article 33.3 µg	18	14	11	87	89	70	17	14	6	3	5	9	17	12	18	N
100 µg	12	17	15	82	86	70	9	7	17	7	9	2	15	17	17	N
333 µg	21	18	8	88	97	86	14	11	11	10	8	2	10	7	12	N
1000 µg	20	17	16	97	94	90	11	7	10	8	6	10	11	9	12	NP
3330 µg	23	11	29	79	112	84	17	16	10	3	7	12	10	21	20	NP
5000 µg	18	10	19	94	93	97	11	15	16	6	9	3	18	26	19	NP
Positive Control[c]	208	192	216	1021	1028	948	664	656	579	1064	867	898	158	128	89	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]

TA98	benzo[a]pyrene	2.5 µg/plate
TA100	2-aminoanthracene	2.5 µg/plate
TA1535	2-aminoanthracene	2.5 µg/plate
TA1537	2-aminoanthracene	2.5 µg/plate
WP2*uvr*A	2-aminoanthracene	25.0 µg/plate

[c]

TA98	2-nitrofluorene	1.0 µg/plate
TA100	sodium azide	2.0 µg/plate
TA1535	sodium azide	2.0 µg/plate
TA1537	ICR-191	2.0 µg/plate
WP2*uvr*A	4-nitroquinolone-N-oxide	1.0 µg/plate

Table 7 Initial Mutagenicity Assay Results - Summary

Test Article ID:	Purified 8-2 Alcohol	Experiment ID:	22900-B1
Date Plated:	13-Nov-01	Date Counted:	26-Nov-01
Vehicle:	DMSO	Plating Aliquot:	50 µL

		Mean Revertants Per Plate with Standard Deviation										Back-ground Lawn[a]
Dose/Plate		TA98		TA100		TA1535		TA1537		WP2*uvr*A		
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Microsomes: Rat Liver												
Vehicle Control		30	3	117	7	13	5	8	2	15	11	N
Test Article	33.3 µg	29	4	102	11	16	1	10	5	15	4	N
	100 µg	24	4	109	8	10	1	8	3	19	0	N
	333 µg	29	10	103	14	12	3	10	1	14	6	N
	1000 µg	31	4	117	2	15	5	9	3	16	9	NP
	3330 µg	32	12	106	8	16	3	11	3	20	2	NP
	5000 µg	34	9	116	5	13	4	11	2	20	1	NP
Positive Control[b]		543	31	1041	53	148	15	169	18	256	33	N
Microsomes: None												
Vehicle Control		15	9	97	16	13	4	5	2	16	2	N
Test Article	33.3 µg	14	4	82	10	12	6	6	3	16	3	N
	100 µg	15	3	79	8	11	5	6	4	16	1	N
	333 µg	16	7	90	6	12	2	7	4	10	3	N
	1000 µg	18	2	94	4	9	2	8	2	11	2	NP
	3330 µg	21	9	92	18	14	4	7	5	17	6	NP
	5000 µg	16	5	95	2	14	3	6	3	21	4	NP
Positive Control[c]		205	12	999	44	633	47	943	106	125	35	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]

TA98	benzo[a]pyrene	2.5 µg/plate
TA100	2-aminoanthracene	2.5 µg/plate
TA1535	2-aminoanthracene	2.5 µg/plate
TA1537	2-aminoanthracene	2.5 µg/plate
WP2*uvr*A	2-aminoanthracene	25.0 µg/plate

[c]

TA98	2-nitrofluorene	1.0 µg/plate
TA100	sodium azide	2.0 µg/plate
TA1535	sodium azide	2.0 µg/plate
TA1537	ICR-191	2.0 µg/plate
WP2*uvr*A	4-nitroquinolone-N-oxide	1.0 µg/plate

Table 8 Confirmatory Mutagenicity Assay Results - Individual Plate Counts

Test Article ID:	Purified 8-2 Alcohol	Experiment ID:	22900-B1
Date Plated:	29-Nov-01	Date Counted:	07-Dec-01, 10-Dec-01
Vehicle:	DMSO	Plating Aliquot:	50 µL

Dose/Plate		Revertants Per Plate															Back-ground Lawn[a]
		TA98			TA100			TA1535			TA1537			WP2*uvr*A			
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
Microsomes: Rat Liver																	
Vehicle Control		29	27	26	97	98	122	16	17	16	11	8	11	15	23	15	N
Test Article 33.3	µg	24	34	26	114	98	132	9	21	11	8	14	7	18	8	20	N
100	µg	39	34	38	126	115	122	14	15	15	10	5	14	15	19	18	N
333	µg	19	40	25	111	111	112	14	17	12	10	10	9	17	9	24	N
1000	µg	18	26	28	106	113	99	8	5	15	7	8	9	17	12	20	NP
3330	µg	40	32	42	85	97	103	11	11	15	12	12	7	26	25	29	NP
5000	µg	49	32	39	93	97	97	15	13	10	11	11	16	23	19	21	NP
Positive Control[b]		398	321	180	1131	1228	1189	138	192	125	156	180	141	468	389	461	N
Microsomes: None																	
Vehicle Control		24	17	12	95	96	85	6	8	5	11	3	5	21	20	16	N
Test Article 33.3	µg	20	21	26	110	116	85	7	8	10	6	7	8	12	12	21	N
100	µg	24	14	18	72	97	89	10	12	10	8	7	2	16	17	21	N
333	µg	14	24	18	96	93	95	9	16	8	3	7	5	11	29	20	N
1000	µg	18	13	18	105	91	103	8	13	14	6	4	3	17	17	16	NP
3330	µg	21	18	20	92	81	84	10	10	8	4	2	3	20	14	17	NP
5000	µg	23	30	24	90	84	111	13	3	13	6	8	4	16	18	20	NP
Positive Control[c]		287	263	220	1100	1089	1216	821	881	832	0	0	0	298	321	328	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]

TA98	benzo[a]pyrene	2.5 µg/plate
TA100	2-aminoanthracene	2.5 µg/plate
TA1535	2-aminoanthracene	2.5 µg/plate
TA1537	2-aminoanthracene	2.5 µg/plate
WP2*uvr*A	2-aminoanthracene	25.0 µg/plate

[c]

TA98	2-nitrofluorene	1.0 µg/plate
TA100	sodium azide	2.0 µg/plate
TA1535	sodium azide	2.0 µg/plate
TA1537	ICR-191	2.0 µg/plate
WP2*uvr*A	4-nitroquinolone-N-oxide	1.0 µg/plate

Table 9 Confirmatory Mutagenicity Assay Results - Summary

Test Article ID:	Purified 8-2 Alcohol	Experiment ID:	22900-B1
Date Plated:	29-Nov-01	Date Counted:	07-Dec-01, 10-Dec-01
Vehicle:	DMSO	Plating Aliquot:	50 µL

	Dose/Plate		Mean Revertants Per Plate with Standard Deviation										Back-ground Lawn[a]
			TA98		TA100		TA1535		TA1537		WP2*uvr*A		
			Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Microsomes: Rat Liver													
Vehicle Control			27	2	106	14	16	1	10	2	18	5	N
Test Article	33.3	µg	28	5	115	17	14	6	10	4	15	6	N
	100	µg	37	3	121	6	15	1	10	5	17	2	N
	333	µg	28	11	111	1	14	3	10	1	17	8	N
	1000	µg	24	5	106	7	9	5	8	1	16	4	NP
	3330	µg	38	5	95	9	12	2	10	3	27	2	NP
	5000	µg	40	9	96	2	13	3	13	3	21	2	NP
Positive Control[b]			300	111	1183	49	152	36	159	20	439	44	N
Microsomes: None													
Vehicle Control			18	6	92	6	6	2	6	4	19	3	N
Test Article	33.3	µg	22	3	104	16	8	2	7	1	15	5	N
	100	µg	19	5	86	13	11	1	6	3	18	3	N
	333	µg	19	5	95	2	11	4	5	2	20	9	N
	1000	µg	16	3	100	8	12	3	4	2	17	1	NP
	3330	µg	20	2	86	6	9	1	3	1	17	3	NP
	5000	µg	26	4	95	14	10	6	6	2	18	2	NP
Positive Control[c]			257	34	1135	70	845	32	0	0	316	16	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b]

TA98	benzo[a]pyrene	2.5 µg/plate
TA100	2-aminoanthracene	2.5 µg/plate
TA1535	2-aminoanthracene	2.5 µg/plate
TA1537	2-aminoanthracene	2.5 µg/plate
WP2*uvr*A	2-aminoanthracene	25.0 µg/plate

[c]

TA98	2-nitrofluorene	1.0 µg/plate
TA100	sodium azide	2.0 µg/plate
TA1535	sodium azide	2.0 µg/plate
TA1537	ICR-191	2.0 µg/plate
WP2*uvr*A	4-nitroquinolone-N-oxide	1.0 µg/plate

Table 10 Historical Control Data

Plate Incorporation Method - Report Period 01E

Vehicle Controls with S9 Mix					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	28.2	105.7	13.3	10.4	20.0
Standard Deviation	7.2	14.3	4.4	4.6	6.5
Maximum	61	143	30	26	41
Minimum	14	61	5	2	9
Count	290	294	209	185	203
Vehicle Controls without S9 Mix					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	16.4	86.8	12.6	8.6	19.0
Standard Deviation	5.4	13.8	4.5	4.3	5.8
Maximum	36	128	28	26	38
Minimum	3	46	3	1	5
Count	289	282	207	183	194
Positive Controls with S9 Mix* **					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	325.8	837.3	132.1	128.1	568.4
Standard Deviation	72.3	285.9	26.1	28.2	173.3
Maximum	614	1985	203	221	1055
Minimum	146	317	77	46	192
Count	278	281	204	181	198
Positive Controls without S9 Mix**					
Strain	TA98	TA100	TA1535	TA1537	WP2*uvr*A
Mean Revertants per Plate	255.6	990.5	708.4	980.9	227.0
Standard Deviation	59.8	166.9	119.1	269.5	77.3
Maximum	464	1572	1037	2135	481
Minimum	130	386	333	522	10
Count	276	273	202	178	190

** TA98	benzo[a]pyrene	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

Appendix B. Definitions of Bacterial Background Lawn Evaluation Codes

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	CHARACTERISTICS OF BACKGROUND LAWN
N	Normal	A healthy microcolony lawn.
R	Reduced	A distinct thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
A	Absent	A complete lack of any microcolony lawn.
O	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate, macroscopic test article precipitate, or plate coloration.
E	Enhanced	A distinct thickening of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.

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.

P	Precipitate	Macroscopic precipitate observed on the plate.

Appendix C. Quality Assurance and Compliance Statements

QUALITY ASSURANCE STATEMENT

Purified 8-2 Alcohol: *Salmonella-Escherichia Coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay

The report has been reviewed by the Quality Assurance Unit of Covance Laboratories Inc., in accordance with the Good Laboratory Practice regulations as set forth in the Environmental Protection Agency (EPA - TSCA), Title 40 of the U.S. Code of Federal Regulations Part 792; and the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice ENV/MC/CHEM (98)17; and any applicable amendments. The following inspections were conducted and the findings reported to the Study Director and study director management. Written status reports of inspections and findings are issued to Covance management according to standard operating procedures.

Inspection Dates	Phase	Dates Reported to Study Director and Study Director Management	Auditor
24-Oct-2001	Protocol Review	24-Oct-2001	P. Cáceres
13-Nov-2001	S9 Mix Preparation	14-Nov-2001	J. Howard
07-Jan-2002	Draft Report Review	07-Jan-2002	C. Smith
08-Feb-2002	Final Report Review	08-Feb-2002	C. Smith

Representative, Quality Assurance Unit

11 Feb 02
Date

STUDY COMPLIANCE AND CERTIFICATION

Except that the test and control article dosing solutions were not analyzed for stability, homogeneity or accuracy of preparation, this study was conducted in compliance with the Good Laboratory Practice regulations as set forth by the Environmental Protection Agency (EPA - TSCA), Title 40 of the U.S. Code of Federal Regulations Part 792; and the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice ENV/MC/CHEM (98)17; and any applicable amendments. There were no other deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria.

Study Director:

Leon F. Stankowski, Jr., PhD
Bacterial Mutagenesis
Genetic and Molecular Toxicology
Covance-Vienna

2/11/02
Study Completion Date

Testing Facility Management:

Brian C. Myhr, PhD
Bacterial Mutagenesis
Genetic and Molecular Toxicology

2/11/02
Date

Report Reviewed and Accepted for E.I. du Pont de Nemours and Co. by:

Gerald Kennedy, PhD
Sponsor Study Monitor

Date

Maria Donner, PhD
Sponsor Technical Project Monitor

Date