TRADE SECRET

Study Title

H-24616: Bacterial Reverse Mutation Test

Authors

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Performing Laboratory

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Performing Laboratory Study Number
AA37AZ.501100.BTL

DuPont Project ID
DuPont-5234

Work Request Number
[redacted]

Service Code
[redacted]

Company Sanitized. Does not contain TSCA CBI

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Issued by Study Director: Valentine O. Wagner, III — 25 Jan 2001
Valentine O. Wagner III, M.S. — Date

Approved by Study Monitor: Maria Donner — 22 JAN 2001
Maria Donner, Ph.D. — Date
Senior Research Scientist

Company Sanitized. Does not contain TSCA CBI

TABLE OF CONTENTS

STUDY INFORMATION

Substance Tested: [redacted]

Synonyms/Codes: • H-24616
[redacted]

Haskell Number: 24616

Purity: [redacted]

Known Impurities: [redacted]

Physical Characteristics [redacted]

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Solubility: Aquatics: Dispersible in water
All Others: Dimethyl sulfoxide

Sponsor: E. I. du Pont de Nemours and Company
Wilmington, Delaware 19898
U.S.A.

Study Initiated/Completed: November, 16, 2000 / (see report cover page)

In-Life Initiated/Completed: November 28, 2000 / December 06, 2000

SUMMARY

The test substance, H-24616, [redacted] was tested in the bacterial reverse mutation test using *S. typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *E. coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. The test was performed using the plate incorporation method to evaluate the mutagenic potential of the test substance.

Water was selected as the solvent of choice based on solubility of the test substance, compatibility with the target cells, and stability of the test article in the solvent (per Sponsor supplied information). The test substance was soluble and but cloudy in water at approximately 10 mg/mL. The test substance was a workable suspension at 25 to 50 mg/mL.

In the mutagenicity assay, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and a 100 µL plating aliquot. The test article diluted to a clear solution at 1.0 and 3.3 mg/mL. The dose levels tested were 100, 333, 1000, 3333 and 5000 µg per plate. No positive responses were observed with any of the tester strains in the presence and absence of rat S9 activation.

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Test indicate that, under the conditions of this study, H-24616 did not induce a positive responses with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium*, and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of S9 metabolic activation.

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test substance, H-24616, was received by BioReliance on 06 November 2000 and was assigned the code number AA37AZ. The Sponsor characterized the test substance as an [redacted] that should be stored at ambient temperature. An expiration date was not provided. Upon receipt, the test substance was described as a[redacted] and stored at room temperature, protected from exposure to light.

The vehicle used to deliver H-24616 to the test system was water, (CAS# 7732-18-5), obtained from Life Technologies.

Positive controls plated concurrently with the mutagenicity test are listed below:

Strain	S9 Metabolic Activation	Positive Control	Concentration (µg/plate)
All *Salmonella* Strains	Rat	2-aminoanthracene (Sigma Chemical Co.)	1.0
WP2 *uvr*A			10
TA98	None	2-nitrofluorene (Aldrich Chemical Co., Inc.)	1.0
TA100, TA1535		sodium azide (Sigma Chemical Co.)	1.0
TA1537		9-aminoacridine (Sigma Chemical Co.)	75
WP2 *uvr*A		methyl methanesulfonate (Aldrich Chemical Co., Inc.)	1,000

To determine the sterility of the test substance, the highest test substance dose level used in the mutagenicity test was plated on selective agar with an aliquot volume equal to that used in the test.

MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975), and *Escherichia coli* tester strain WP2 *uvr*A. *Salmonella* tester strains were received on 11 August 1998 and 10 November 1998 directly from Dr. Bruce Ames, University of California, Berkeley. *E. coli* was received on 01 July 1987 from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to base-pair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a resting shaker/incubator at room temperature. The shaker/incubator was programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates, and the data is on file but not presented in this report. The study was conducted to comply with OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), adopted July 1997.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 was purchased from MolTox, batch prepared on 18 October 2000 and stored at -70°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared immediately before its use and contained 10% S9, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$

and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The Sham S9 mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use. To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar.

Solubility Test

A solubility test was conducted to select the vehicle using one or more of the following: water and dimethyl sulfoxide (DMSO). The test substance was tested to determine the vehicle, selected in order of preference, that permitted preparation of the highest soluble or workable stock concentration, up to 50 mg/mL for aqueous solvents and up to 500 mg/mL for organic solvents.

Mutagenicity Test

The mutagenicity test was used to evaluate the mutagenic potential of the test substance. Five dose levels of test substance (100, 333, 1000, 3333 and 5000 µg per plate) along with appropriate vehicle and positive controls were plated with tester strains TA98, TA100, TA1535, TA1537 and WP2 *uvr*A in the presence and absence of rat liver S9 activation. All dose levels of test substance, vehicle controls and positive controls were plated in triplicate.

Plating and Scoring Procedures

In the mutagenicity test, the test system was exposed to the test substance via the plate incorporation methodology as described by Ames *et* al. (1975) and updated by Maron and Ames (1983).

On the day of its use, minimal top agar, containing 0.8 % agar (W/V) and 0.5 % NaCl (W/V), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 50 µM each. Top agar not used with S9 or Sham mix was supplemented with 25 mL of water for each 100 mL of minimal top agar. For the preparation of media and reagents, all references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

Each plate was labeled with a code system that identified the test substance, test phase, dose level, tester strain, and activation, as described in detail in BioReliance's Standard Operating Procedures.

Test substance dilutions were prepared immediately before use. One-half (0.5) milliliter of S9 or sham mix, 100 µL of tester strain and 100 µL of vehicle or test substance were added to 2.0 mL of molten selective top agar at 45±2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test substance aliquot was replaced by a 50 µL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted (less than 10 days).

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plates using the codes shown below.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Severely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over ≥90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance precipitate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count (e.g., <3 particles on a plate with 30 revertants.)
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., >3 particles on a plate with 30 revertants.)

Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate exhibited toxicity. Plates with sufficient test substance precipitate to interfere with automated colony counting were counted manually.

Evaluation of Results

For each triplicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain with a minimum of two increasing concentrations of test substance. Data sets for strains TA1535 and TA1537 were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean vehicle control value. Data sets for strains TA98, TA100 and WP2 *uvr*A were judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean vehicle control value.

Criteria for a Valid Test

The following criteria must be met for the mutagenicity test to be considered valid. All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows (inclusive): TA98 (10 – 50); TA100 (80 – 240); TA1535 (5 – 45); TA1537 (3 – 21); and WP2 *uvr*A (10 – 60). To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to 0.3×10^9 cells/mL. The mean of each positive control must exhibit at least a three-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) A reduction in the background lawn.

Archives

Upon completion of the final report, all raw data and reports will be archived by BioReliance, Rockville, MD for a period of no less than 1 year from the study initiation date.

Deviations

No known deviations from the protocol or assay-method SOPs occurred during the conduct of this study.

RESULTS AND DISCUSSION

Solubility Test

Water was selected as the solvent of choice based on solubility of the test substance, compatibility with the target cells and stability of the test article in the solvent (per Sponsor supplied information). The test substance was soluble and but cloudy in water at approximately 10 mg/mL. The test substance was a workable suspension at 25 to 50 mg/mL.

Mutagenicity Test

The results of the mutagenicity test are presented in Tables 1 through 10 and summarized in Table 11. These data were generated in Experiment B1. In the mutagenicity assay, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and a 100 µL plating aliquot. The test article diluted to a clear solution at 1.0 and 3.3 mg/mL. The dose levels tested were 100, 333, 1000, 3333 and 5000 µg per plate. Neither precipitate nor toxicity was observed.

In Experiment B1, no positive responses were observed with any of the tester strains in the presence and absence of rat S9 activation.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Test indicate that, under the conditions of this study, **H-24616** did not induce a positive response with any of the tester strains in the presence and absence of Aroclor-induced rat liver S9.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp^+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), adopted July 1997.

Vogel, H.J. and D.M. Bonner (1956) Acetylornithinase of *E. coli*: Partial Purification and Some Properties, J. Biol. Chem., 218:97-106.

Bacterial Mutation Assay

Table 1

Test Article Id : [redacted]
Study Number : AA37AZ.501100.BTL
Strain : TA98
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL
Experiment No : B1
Cells Seeded : 0.6×10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	10	1		
	02	21	1		
	03	18	1	16	6
100	01	12	1		
	02	14	1		
	03	13	1	13	1
333	01	21	1		
	02	12	1		
	03	15	1	16	5
1000	01	14	1		
	02	10	1		
	03	22	1	15	6
3333	01	8	1		
	02	12	1		
	03	14	1	11	3
5000	01	11	1		
	02	13	1		
	03	10	1	11	2
Positive Control 2-nitrofluorene 1.0 µg per plate					
	01	99	1		
	02	94	1		
	03	95	1	96	3

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Assay

Table 2

Test Article Id : {redacted}
Study Number : AA37AZ.501100.BTL
Strain : TA98
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 0.6 X 10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	23	1		
	02	15	1		
	03	20	1	19	4
100	01	27	1		
	02	15	1		
	03	14	1	19	7
333	01	14	1		
	02	11	1		
	03	16	1	14	3
1000	01	18	1		
	02	21	1		
	03	19	1	19	2
3333	01	16	1		
	02	21	1		
	03	17	1	18	3
5000	01	16	1		
	02	24	1		
	03	18	1	19	4
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	451	1		
	02	486	1		
	03	546	1	494	48

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Assay

Table 3

Test Article Id [redacted]
Study Number : AA37AZ.501100.BTL
Strain : TA100
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 0.9 X 10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	88	1		
	02	83	1		
	03	84	1	85	3
100	01	84	1		
	02	77	1		
	03	85	1	82	4
333	01	85	1		
	02	92	1		
	03	83	1	87	5
1000	01	79	1		
	02	71	1		
	03	86	1	79	8
3333	01	81	1		
	02	92	1		
	03	81	1	85	6
5000	01	116	1		
	02	90	1		
	03	100	1	102	13
Positive Control sodium azide 1.0 µg per plate					
	01	314	1		
	02	340	1		
	03	355	1	336	21

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Assay

Table 4

Test Article Id : [redacted]
Study Number : AA37AZ.501100.BTL
Strain : TA100
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 0.9 X 10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	98	1		
	02	82	1		
	03	89	1	90	8
100	01	78	1		
	02	91	1		
	03	79	1	83	7
333	01	83	1		
	02	78	1		
	03	83	1	81	3
1000	01	89	1		
	02	79	1		
	03	81	1	83	5
3333	01	98	1		
	02	110	1		
	03	85	1	98	13
5000	01	85	1		
	02	95	1		
	03	91	1	90	5
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	621	1		
	02	668	1		
	03	520	1	603	76

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Assay

Table 5

Test Article Id : [redacted]
Study Number : AA37AZ.501100.BTL
Strain : TA1535
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 2.7 X 10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	14	1		
	02	18	1		
	03	19	1	17	3
100	01	16	1		
	02	21	1		
	03	14	1	17	4
333	01	21	1		
	02	24	1		
	03	13	1	19	6
1000	01	20	1		
	02	13	1		
	03	15	1	16	4
3333	01	18	1		
	02	9	1		
	03	20	1	16	6
5000	01	15	1		
	02	13	1		
	03	16	1	15	2
Positive Control sodium azide 1.0 µg per plate					
	01	188	1		
	02	187	1		
	03	172	1	182	9

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Company Sanitized. Does not contain TSCA CBI

Bacterial Mutation Assay

Table 6

Test Article Id : [redacted]
Study Number : AA37AZ.501100.BTL
Strain : TA1535
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 2.7 X 10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	14	1		
	02	17	1		
	03	C		16	2
100	01	7	1		
	02	13	1		
	03	16	1	12	5
333	01	13	1		
	02	15	1		
	03	9	1	12	3
1000	01	17	1		
	02	21	1		
	03	16	1	18	3
3333	01	5	1		
	02	13	1		
	03	13	1	10	5
5000	01	17	1		
	02	18	1		
	03	21	1	19	2
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	120	1		
	02	90	1		
	03	105	1	105	15

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate
C=Contaminated

Bacterial Mutation Assay

Table 7

Test Article Id : [redacted]
Study Number : AA37AZ.501100.BTL
Strain : TA1537
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL
Experiment No : B1
Cells Seeded : 1.6 X 10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	7	1		
	02	4	1		
	03	8	1	6	2
100	01	10	1		
	02	9	1		
	03	6	1	8	2
333	01	8	1		
	02	8	1		
	03	10	1	9	1
1000	01	6	1		
	02	9	1		
	03	5	1	7	2
3333	01	3	1		
	02	6	1		
	03	7	1	5	2
5000	01	7	1		
	02	6	1		
	03	8	1	7	1
Positive Control 9-aminoacridine 75 µg per plate					
	01	689	1		
	02	708	1		
	03	699	1	699	10

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Assay

Table 8

Test Article Id [redacted]
Study Number : AA37AZ.501100.BTL
Strain : TA1537
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 1.6 X 10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	6	1		
	02	8	1		
	03	6	1	7	1
100	01	6	1		
	02	5	1		
	03	3	1	5	2
333	01	10	1		
	02	8	1		
	03	8	1	9	1
1000	01	4	1		
	02	8	1		
	03	5	1	6	2
3333	01	9	1		
	02	9	1		
	03	6	1	8	2
5000	01	9	1		
	02	8	1		
	03	6	1	8	2
Positive Control 2-aminoanthracene 1.0 µg per plate					
	01	79	1		
	02	88	1		
	03	68	1	78	10

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Assay

Table 9

Test Article Id : [redacted]
Study Number : AA37AZ.501100.BTL
Strain : WP2 *uvrA*
Liver Microsomes : None
Vehicle : water
Plating Aliquot : 100 µL

Experiment No : B1
Cells Seeded : 3.2×10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	17	1		
	02	13	1		
	03	9	1	13	4
100	01	12	1		
	02	9	1		
	03	11	1	11	2
333	01	12	1		
	02	18	1		
	03	17	1	16	3
1000	01	14	1		
	02	14	1		
	03	9	1	12	3
3333	01	12	1		
	02	17	1		
	03	9	1	13	4
5000	01	17	1		
	02	18	1		
	03	18	1	18	1
Positive Control methyl methanesulfonate 1000 µg per plate					
	01	82	1		
	02	72	1		
	03	61	1	72	11

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Assay

Table 10

Test Article Id : [redacted]
Study Number : AA37AZ.501100.BTL
Strain : WP2 uvrA
Liver Microsomes : Rat liver S9
Vehicle : water
Plating Aliquot : 100 µL
Experiment No : B1
Cells Seeded : 3.2 X 10^8
Date Plated : 28 Nov 2000

Concentration µg per plate	Plate Number	Revertants per plate	Background Code	Average Revertants	Standard Deviation
Vehicle	01	18	1		
	02	13	1		
	03	12	1	14	3
100	01	14	1		
	02	13	1		
	03	13	1	13	1
333	01	10	1		
	02	11	1		
	03	15	1	12	3
1000	01	14	1		
	02	12	1		
	03	13	1	13	1
3333	01	10	1		
	02	13	1		
	03	10	1	11	2
5000	01	11	1		
	02	14	1		
	03	11	1	12	2
Positive Control 2-aminoanthracene 10 µg per plate					
	01	204	1		
	02	217	1		
	03	204	1	208	8

Background Lawn Code
1=Normal; 2=Slightly reduced; 3=Severely reduced
4=Extremely reduced; 5=Absent; 6=Obscured by precipitate
NP=Non-Interfering precipitate; IP=Interfering precipitate

Bacterial Mutation Assay
Summary of Results

Table 11

Test Article Id [redacted]
Study Number : AA37AZ.501100.BTL Experiment No : B1

Average Revertants Per Plate ± Standard Deviation

Liver Microsomes: None

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	16 ± 6	85 ± 3	17 ± 3	6 ± 2	13 ± 4
100	13 ± 1	82 ± 4	17 ± 4	8 ± 2	11 ± 2
333	16 ± 5	87 ± 5	19 ± 6	9 ± 1	16 ± 3
1000	15 ± 6	79 ± 8	16 ± 4	7 ± 2	12 ± 3
3333	11 ± 3	85 ± 6	16 ± 6	5 ± 2	13 ± 4
5000	11 ± 2	102 ± 13	15 ± 2	7 ± 1	18 ± 1
Positive	96 ± 3	336 ± 21	182 ± 9	699 ± 10	72 ± 11

Liver Microsomes: Rat liver S9

Dose (µg/plate)	TA98	TA100	TA1535	TA1537	WP2 uvrA
Vehicle	19 ± 4	90 ± 8	16 ± 2	7 ± 1	14 ± 3
100	19 ± 7	83 ± 7	12 ± 5	5 ± 2	13 ± 1
333	14 ± 3	81 ± 3	12 ± 3	9 ± 1	12 ± 3
1000	19 ± 2	83 ± 5	18 ± 3	6 ± 2	13 ± 1
3333	18 ± 3	98 ± 13	10 ± 5	8 ± 2	11 ± 2
5000	19 ± 4	90 ± 5	19 ± 2	8 ± 2	12 ± 2
Positive	494 ± 48	603 ± 76	105 ± 15	78 ± 10	208 ± 8

Vehicle = Vehicle Control
Positive = Positive Control
Plating aliquot: 100 µL

APPENDIX A

Historical Control Data

Historical Negative and Positive Control Values
1997 - 1999

revertants per plate

Strain	Control	Activation							
		None				Rat Liver			
		Mean	SD	Min	Max	Mean	SD	Min	Max
TA98	Neg	16	6	4	59	21	7	6	65
	Pos	397	216	21	1536	649	330	56	2454
TA100	Neg	128	29	53	288	144	30	62	258
	Pos	592	159	129	1920	785	300	106	2871
TA1535	Neg	11	5	1	45	12	5	1	42
	Pos	424	145	6	1024	98	79	8	1640
TA1537	Neg	6	3	0	30	7	3	1	29
	Pos	819	468	13	2786	102	128	2	1835
WP2 *uvr*A	Neg	16	5	3	48	17	6	2	115
	Pos	190	127	35	961	338	282	16	2632

SD=standard deviation; Min=minimum value; Max=maximum value; Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

APPENDIX B

Study Protocol

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

[redacted] Bacterial Reverse Mutation Test

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of Salmonella typhimurium and at the tryptophan locus of Escherichia coli WP2 *uvrA* in the presence and absence of S9 activation.

2.0 SPONSOR

2.1	Name:	E.I. du Pont de Nemours and Company
2.2	Address:	Stine Haskell Research Center DuPont Haskell Laboratory P.O. Box 50, 1090 Elkton Road Newark, DE 19714-0050
2.3	Representative:	Maria Donner, Ph.D. Phone: 302-366-5251 Fax: 302-235-7156 Email: maria.donner@usa.dupont.com
2.4	Sponsor Project No.:	DuPont-5234
2.5	WR#:	[redacted]
2.6	Haskell #:	24616
2.7	Service Code:	[redacted]

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Substance Name:	[redacted]	
3.2	Test Substance I.D.:	H-24616 (to be used in the report text)	
3.3	Controls:	Negative:	Test substance vehicle (Water)
		Positive:	9-aminoacridine
			2-aminoanthracene
			methyl methanesulfonate
			2-nitrofluorene
			sodium azide

or DMSO
Per call to Spencer Vow 16 Nov 2000

BIORELIANCE Formerly Microbiological Associates

Sponsor Project Number: DuPont - 5234
BioReliance Study Number: AA37AZ.501100.BTL

3.4 Determination of Strength, Purity, etc.

Unless alternate arrangements are made, the testing facility at BioReliance will not perform analysis of the dosing solutions. The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the substance, and the stability and strength of the substance in the solvent (or vehicle).

3.5 Test Substance Retention Sample

The retention of a reserve sample of the substance will be the responsibility of the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
BioReliance

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Valentine O. Wagner III, M.S.
Phone: 301-610-2152
Fax: 301-738-2362
Email: swagner@bioreliance.com

5.0 PROPOSED STUDY DATES

5.1	Experimental Start Date:	28 Nov 2000
5.2	Experimental Termination Date:	19 Dec 2000
5.3	Draft Report Date:	04 Jan 2001
5.4	Final Report Date:	18 Jan 2001

6.0 TEST SYSTEM

The tester strains will include the S. typhimurium histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames et al. (1975) and the E. coli tester strain WP2 uvrA as described by Green and Muriel (1976).

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

Genotype of the Strains Used for Mutagen Testing						
Histidine Mutation			Tryptophan Mutation	Additional Mutations		
hisG46	hisC3076	HisD3052	TrpE	LPS	Repair	R-factor
TA1535	TA1537	-	-	Rfa	ΔuvrB	-
TA100	-	TA98	-	Rfa	ΔuvrB	+R
-	-	-	WP2 uvrA	-	ΔuvrA	-

Each S. typhimurium tester strain contains, in addition to a mutation in the histidine operon, additional mutations that enhance sensitivity to some mutagens. The rfa mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The deletion in the uvrB gene results in a deficient DNA excision-repair system. Tester strains TA98 and TA100 also contain the pKM101 plasmid (carrying the R-factor). It has been suggested that the plasmid increases sensitivity to mutagens by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA100 is reverted by both frameshift and base substitution mutagens and TA1535 is reverted only by mutagens that cause base substitutions.

The E. coli tester strain has an AT base pair at the critical mutation site within the *trp*E gene (Wilcox *et al.*, 1990). Tester strain WP2 uvrA has a deletion in the uvrA gene resulting in a deficient DNA excision-repair system. Tryptophan revertants can arise due to a base change at the originally mutated site or by a base change elsewhere in the chromosome causing the original mutation to be suppressed. Thus, the specificity of the reversion mechanism is sensitive to base-pair substitution mutations (Green and Muriel, 1976).

The S. typhimurium tester strains were received directly from Dr. Bruce Ames, University of California, Berkeley. The E. coli tester strain was received from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The test substance will be tested at a minimum of five dose levels along with appropriate negative and positive controls with tester strains TA98, TA100, TA1535, TA1537 and WP2 uvrA with and without S9 activation. All dose levels of test substance, negative controls and positive controls will be plated in triplicate.

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

7.1 Solubility Determination

The Sponsor has indicated that the test substance is soluble in water. A solubility test will be conducted per SOP. or DMSO

7.2 Dose Levels Recall to Sponsor. Vow 16 Nov 2000

The Sponsor will provide information for selection of the dose levels. This will be based on a previous study the Sponsor has conducted.

7.3 Frequency and Route of Administration

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the test. Verification of a clear positive response is not required. Negative results will not be retested when justification can be provided. Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method). This guidance is based on the OECD Guideline 471 (adopted July 1997) and ICH Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals (1997).

7.4 Controls

7.4.1 Positive Controls

All combinations of positive controls and tester strains plated concurrently with the test are listed below:

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
Salmonella Strains	Rat	2-aminoanthracene	1.0
WP2 uvrA			10
TA98	None	2-nitrofluorene	1.0
TA100, TA1535		Sodium azide	1.0
TA1537		9-aminoacridine	75
WP2 uvrA		methyl methanesulfonate	1,000

7.4.2 Negative Controls

Appropriate negative controls will be plated for each tester strain with and without S9 activation. The negative control will be the vehicle alone,

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

unless there is no historical basis for use of the selected vehicle. In the latter case, both untreated and vehicle controls will be used.

7.4.3 Sterility Controls

The most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

7.5 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S9 will be used as the metabolic activation system. The S9 homogenate will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S9 will be batch prepared and stored frozen at approximately -70°C until used. Each batch of S9 homogenate will be tested for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenzanthracene to forms mutagenic to *S. typhimurium* TA100.

Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 10% S9 homogenate, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$ and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. This mixture is referred to as S9 mix. Sham mix will be 100 mM phosphate buffer at pH 7.4.

7.6 Preparation of Tester Strain

Overnight cultures will be inoculated from the appropriate master plate or from the appropriate frozen stock. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. At the end of the working day, each inoculated flask will be placed in a resting shaker/incubator at room temperature. The shaker/incubator will be programmed to begin shaking at approximately 125 rpm at 37±2°C approximately 12 hours before the anticipated time of harvest.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

7.7 Test System Identification

Each plate will be labeled with a code system that identifies the test substance, test phase, dose level, tester strain and activation type as described in BioReliance's Standard Operating Procedures.

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

7.8 Test Substance Preparation

Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under yellow light.

7.9 Treatment of Test System

One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary to achieve the target concentration or eliminate toxic vehicle effects, aliquots of other than 50 µL of test substance/vehicle/positive control will be plated. The mixture will be vortex mixed and overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay has solidified, the plates will be inverted and incubated for approximately 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

7.10 Scoring

The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the negative control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification.

7.11 Tester Strain Verification

On the day of use in the mutagenicity test, all tester strain cultures will be checked for the appropriate genetic markers cited in §6.0.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the mutagenicity test to be considered valid:

8.1 Tester Strain Integrity

To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

8.2 Spontaneous Revertant Background Frequency

Based on historical control data, all tester strain cultures must exhibit characteristic number of spontaneous revertants per plate in the negative controls (vehicle). The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60.

8.3 Tester Strain Titers

To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than 0.3×10^9 cells per milliliter.

8.4 Positive Control Values

Each mean positive control value must exhibit at least a three fold increase over the respective mean negative control value (vehicle) for each tester strain.

8.5 Toxicity

A minimum of three non-toxic dose levels will be required to evaluate test data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean negative control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the test will be repeated with an appropriate change in dose levels.

9.0 EVALUATION OF TEST RESULTS

For a test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

9.1 Strains TA1535 and TA1537

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than three times the mean negative control value (vehicle).

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

9.2 Strains TA98, TA100 and WP2 *uvr*A

Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than two times the mean negative control value (vehicle).

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. The report will include:

- Test Substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test substance, if known.
- Solvent/Vehicle: justification for choice of vehicle; solubility and stability of test substance in solvent/vehicle, if known.
- Strains: strains used; number of cells/mL per culture; strain characteristics.
- Test conditions: amount of test substance per plate with rationale for dose selection and number of plates per concentration; media used; type and composition of metabolic activation system, including acceptability criteria; treatment procedures.
- Results: signs of toxicity; signs of precipitation; individual plate counts; the mean number of revertant colonies per plate and standard deviation; dose-response relationship, where possible; statistical analysis, if any; concurrent negative and positive control data means and standard deviations; historical negative and positive control data with ranges, means and standard deviation.
- Discussion of results.
- Conclusion.

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be archived by BioReliance, Rockville, MD for a period of no less than 1 year from the study initiation date.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 471 for Testing of Chemicals (Genetic Toxicology: Bacterial Reverse Mutation Assay), adopted July 1997.

This study will be performed using the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies as a guideline; however, the study will not meet GLP requirements.

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

Unless arrangements are made to the contrary, unused dosing solutions will be disposed of following administration to the test system and all residual test substance will be disposed of following finalization of the report.

13.0 REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp$^+$ reversion in *Escherichia coli*. Mutation Research 38:3-32.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. S2A document recommended for adoption at step 4 of the ICH process on July 19, 1995. Federal Register 61:18198-18202, April 24, 1996.

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals. S2B document recommended for adoption at step 4 of the ICH process on July 16, 1997. Federal Register 62:16026-16030, November 21, 1997.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

OECD Guideline 471 for Testing of Chemicals (Genetic Toxicology: Bacterial Reverse Mutation Assay), adopted July 1997.

Wilcox, P., Naidoo, A., Wedd, D.J. and Gatehouse, D.G. (1990). Comparison of *Salmonella typhimurium* TA102 with *Escherichia coli* WP2 tester strains. Mutagenesis 5:285-291.

Sponsor Project Number: DuPont - 5234

BioReliance Study Number: AA37AZ.501100.BTL

14.0 APPROVAL

Maria Donner — Sponsor Representative — 07 Nov 2000 — Date

Maria Donner — (Print or Type Name)

Valentine O. Wagner, III — BioReliance Study Director — 16 Nov 2000 — Date