TRADE SECRET

Study Title

H-24256: Bacterial Reverse Mutation Test in *Salmonella typhimurium* and *Escherichia coli*

Laboratory Project ID: DuPont-3906

TEST GUIDELINES: U.S. EPA Health Effects Test Guidelines
OPPTS 870.5100 (1998)

OECD Guidelines for Testing of Chemicals
Section 4: Health Effects, No. 471 (Adopted 1997)

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STUDY COMPLETED ON: May 8, 2000

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WORK REQUEST NUMBER: [redacted]

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DUPONT PHARMACEUTICALS COMPANY STUDY NO.: [redacted]

CERTIFICATION

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

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STUDY INFORMATION

9th Collective Nomenclature: [redacted]

Synonyms/Codes: [redacted]

- H-24256

Haskell Number: 24256

CAS Registry Number: [redacted]

Submitter's Notebook Number(s): [redacted]

Composition: [redacted]

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.

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

Study Initiated/Completed: 10-13-99 / (see report cover page)

In-Life Initiated/Completed: 10-14-99 / 10-20-99

STUDY PERSONNEL

The following individuals participated in the conduct of this study:

DuPont Pharmaceuticals Company

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Report Preparation: Brenda Tiffin

SUMMARY

H-24256 was evaluated in the bacterial reverse mutation assay using *Salmonella typhimurium* strains TA97a, TA98, TA100, TA1535, and *Escherichia coli* strain WP2 *uvrA* (pKM101) in the presence and absence of an exogenous metabolic activation system (Aroclor®-induced rat liver S9). A single trial assay was performed using the plate incorporation method to evaluate the mutagenic potential of the test substance. All tester strains exhibited appropriate phenotypic characteristics. The mean number of revertants seen in the negative controls for each strain was within the prescribed acceptable range.

Test substance concentrations of 5, 10, 50, 100, 500, 1000, 2500, and 5000 µg/plate were evaluated in comparison to the negative (solvent) control. The solvent diluent and negative control used in this study was sterile water. Test substance-related toxicity, as evidenced by the reduction of the microcolony background lawns and/or as a concentration-related reduction in the mean number of revertants per plate, was not observed. No precipitate was observed at any concentration with any strain. All tester strains exhibited appropriate phenotypic characteristics. The mean number of revertants seen in the negative controls for each strain was within the acceptable historical negative control ranges.

Under the conditions of this study, no evidence of mutagenic activity was detected in any of the *Salmonella* strains or the *Escherichia coli* strain. Based on the findings, H-24256 was concluded to be negative for the induction of mutagenicity in the bacterial reverse mutation test.

INTRODUCTION

This study evaluated the mutagenic potential of the test substance, H-24256, in the bacterial reverse mutation test using *Salmonella typhimurium* strains TA97a, TA98, TA100, and TA1535 and in *Escherichia coli* strain WP2 *uvrA* (pKM101). The bacterial reverse mutation test uses amino acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations, which involve substitution, addition or deletion of 1 or a few DNA base pairs.[1,2] The *Salmonella* tester strains are unable to synthesize histidine because of specific point mutations in genes coding for histidine biosynthesis. Additional mutations in the defective gene specific to the tester strain can result in individual bacteria regaining the ability to synthesize histidine. Tester strains TA97a and TA98 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) primarily by frameshift mutagens. Tester strains TA100 and TA1535 are reverted by mutagens that primarily cause base pair substitutions.
E. coli WP2 *uvrA* (pKM101) is unable to synthesize tryptophan due to an ochre mutation in the gene required for tryptophan biosynthesis. *E. coli* WP2 *uvrA* (pKM101) is primarily sensitive to mutagens that act at AT base pairs within the *trpE* gene, and may also revert to prototrophy from suppressor mutations at a locus in a tRNA gene.[2] By comparing the number of chemically induced revertants to the number of spontaneous revertants, the mutagenicity of the test substance can be assessed.

MATERIALS AND METHODS

A. Study Protocol

The protocol consisted of the stand-alone Protocol and the Haskell General Testing Procedure [redacted] "Bacterial Reverse Mutation Test for Solids, Liquids, and Gases", effective 06/10/98). The study was designed to comply with:

- U.S. EPA, Office of Prevention, Pesticides and Toxic Substances (OPPTS) Guidelines (Subpart H, 40 CFR Part 870.5100 [1998]);
- Guidelines of the Organisation for Economic Cooperation and Development (OECD Guidelines for Testing of Chemicals, No. 471 [adopted 1997]);

The study design complied with the testing guidelines cited above with the following exception:

- One trial was conducted with the test substance as specified by the sponsor.

This exception did not affect the study validity as a second trial was not considered necessary by the sponsor to meet the objective of the study.

B. Test Materials

1. Test Substance

The test substance, H-24256, is a [redacted] that was stored at room temperature. Additional information regarding the test substance can be found on the study information page of this report. The test substance was assumed to be stable during the study and no evidence of instability was observed.

2. Negative and Positive Controls

Based on information supplied by the sponsor and on a solubility assessment at the testing facility, sterile water was chosen as the test substance solvent, diluent, and negative control. There were no impurities, known or reasonably anticipated, in the controls that might interfere with the validity of the study. Positive controls included the following:

Positive Controls	Chemical Abstracts Service (CAS) Registry Number
2-Nitrofluorene (2NF)	607-57-8
N-Ethyl-*N*-nitro-*N*-nitroguanidine (ENNG)	4245-77-6
Sodium azide (NAAZ)	26628-22-8
ICR 191 Acridine mutagen (ICR 191)	17070-45-0
9,10-Dimethyl-1,2-benzanthracene (DMBA)	57-97-6
2-Aminoanthracene (2AA)	613-13-8

The manufacturer lot number, and purity of the solvent referenced in the study records. Neither the amount and nature of the contaminants nor the use of solvent is expected to affect the integrity or validity of the study.

Deionized water was the solvent for sodium azide (NAAZ). The solvent for 2AA, 2NF, ICR 191, DMBA, and ENNG was dimethyl sulfoxide (DMSO). The positive controls were prepared in advance and maintained frozen at approximately -70°C. Previous experience with these negative and positive controls indicates that they are stable in this test system and no evidence of instability was observed during the study.

3. Tester Strain Source, Characterization, Storage, and Culture

S. typhimurium tester strains were obtained from Dr. Bruce Ames, Berkeley, CA, USA. *E. coli* WP2 *uvrA* (pKM101) was obtained from the National Collection of Industrial Bacteria,

Torrey Research Station, Scotland, UK. The characteristics of *S. typhimurium* tester strains TA97a, TA98, TA100, TA102, TA1535 and *E. coli* WP2 *uvrA* (pKM101) are as follows:

Strain	Gene Locus	Excision Repair	LPS	R-factor (pKM101)	pAQ1 Plasmid
S. typhimurium TA97a	*his*D6610$^{\phi}$	Δ*uvrB*	*rfa*	Present	Absent
S. typhimurium TA98	*his*D3052	Δ*uvrB*	*rfa*	Present	Absent
S. typhimurium TA100	*his*G46	Δ*uvrB*	*rfa*	Present	Absent
S. typhimurium TA102*	*his*G428	(+)	*rfa*	Present	Present
S. typhimurium TA1535	*his*G46	Δ*uvrB*	*rfa*	Absent	Absent
E. coli WP2 *uvrA* (pKM101)	*trpE*	Δ*uvrA*	NA	Present	Absent

Note: *uvrA* and *uvrB* are defective DNA repair genes.
LPS = lipopolysaccharide
RFA = deep rough mutation
NA = Not applicable
* Concurrent control strain used to distinguish differential sensitivity/resistance to ultraviolet light.
ϕ Also *his*O1242.
Δ symbol for "deletion"
(+) proficient in excision repair

The deletion (Δ) in *uvrB* (a gene that codes for a protein involved in DNA excision repair) increases the bacterial sensitivity to some mutagens.[3] The *uvrB* and *uvrA* traits are confirmed by demonstrating an increased bacterial sensitivity to ultraviolet light. Because the *uvrB* deletion also extends through a gene needed for biotin biosynthesis, the *S. typhimurium* tester strains require exogenous biotin to be added to culture media or plates for growth. The *rfa* mutation causes a partial loss in the integrity of the lipopolysaccharide (LPS) cell wall so that permeability to large molecules is increased.[4] The presence of the pKM101 or R-factor plasmid, conferring ampicillin resistance, also enhances an error-prone DNA repair system that is endogenous to these bacteria.[5] The pAQ1 plasmid confers tetracycline resistance to *S. typhimurium* TA102, that was used solely as a control for UV light sensitivity.

Salmonella tester strains were stored at approximately -70°C in ~8% (v/v) DMSO in Oxoid Nutrient Broth No. 2. The *E. coli* WP2 *uvrA* (pKM101) strain was stored at approximately -70°C in ~30% glycerol in Oxoid Nutrient Broth No. 2. Prior to the mutagenicity assays, overnight cultures were prepared by inoculating 20 mL of Oxoid Nutrient Broth No. 2 with 0.1 mL of a bacterial stock and incubating at approximately 37°C with shaking. appropriate tester strain phenotypes were confirmed on overnight cultures concurrently with the single trial.

4. Metabolic Activation System

Because the tester strains lack many of the enzymes required to convert some promutagens to a reactive state, the assay was performed in the presence and absence of an exogenous metabolic activation system similar to that described by Maron and Ames.[1] The exogenous metabolic activation system was a cofactor-supplemented post-mitochondrial fraction, (i.e., 9000 x g; homogenate of 1 g wet liver weight in 3 mL of an approximately 0.15 M KCl solution) prepared from the livers of young male Sprague Dawley rats treated with the enzyme-inducing agent

Aroclor® 1254 (500 mg/kg i.p.) as a single dose 5 days prior to sacrifice. The Aroclor®-induced rat liver S9 (purchased from MOLTOX™) was characterized for protein content and metabolic activity by the vendor. To confirm the sterility of the exogenous metabolic activation system, an aliquot was plated on nutrient agar capable of supporting the growth of viable bacteria. The amount of Aroclor®-1254 induced rat liver S9 in the exogenous metabolic activation system was 4.0 mg S9 protein (~10% [v/v]) / mL. The cofactor-supplement concentrations in the exogenous metabolic activation system were 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate (as a sodium salt), 4 mM $NADP^+$ (as a sodium salt), and 100 mM sodium phosphate buffer pH 7.4.

C. Test Substance, Concentration Selection, Stability and Verification

In accordance with testing guidelines, the highest concentration evaluated in this study was 5000 µg/plate. Solubility information was confirmed prior to study start. The stock concentration was calculated and adjusted for test substance displacement.

Solutions of the test substance were prepared immediately prior to treatment and were presumed to be stable under the conditions of the study. Treatment, control solutions, and the S9 mixture were not analyzed for concentration, uniformity, or stability. Top agar was not assayed for stability or concentration of the test or control substances, strain, or S9/PBS, since this assessment was not considered necessary to achieve the objectives of the study. Solutions of the test substance were assessed for sterility by plating a small amount of the highest test substance concentration onto the surface of agar plates capable of supporting bacterial growth.

D. Test Methods: Bacterial Mutagenicity Assay

This study consisted of a single trial that assessed the mutagenicity of the test substance mutagenicity. Three replicates were plated for each tester strain in the presence and absence of the exogenous metabolic activation system at each test substance concentration. Positive and negative controls were included for each strain and condition. Treatments with the exogenous metabolic activation system were conducted by adding 0.1 mL of negative or positive control or test substance solution, 0.5 mL of metabolic activation system, and 0.1 mL of an overnight culture containing approximately 1 x 10^8 bacteria to approximately 2 mL of top agar (0.6% [w/v] agar and NaCl) containing 0.05 mM L-histidine, D-biotin and L-tryptophan. These components were briefly mixed and poured onto a minimal glucose agar plate (25-30 mL, 0.4% [w/v] glucose with Davis salts, purchased from MOLTOX™). Treatments in the absence of the metabolic activation system were the same as those in the presence of the exogenous metabolic activation system with the exception that 0.5 mL of sterile buffer was used as a replacement for the volume of the exogenous metabolic activation system. After pouring onto the surface of minimal glucose agar plates, the top agar was allowed time to solidify, and the individually labeled plates were inverted and incubated at approximately 37°C for about 48 hours. Plates were refrigerated at approximately 4°C (± 3°C) prior to evaluation and counting of revertant colonies.

Bacterial background lawns were evaluated for evidence of test substance toxicity and precipitation. Evidence of toxicity observed in the microcolony background lawns, was scored

relative to the concurrent negative control plates and recorded with the mean revertant count for the strain, condition, and concentration. Revertant colonies for a given tester strain and condition were counted by an automated colony counter.

E. Statistical Analysis

Data for each tester strain were evaluated independently. For each tester strain, the mean number of revertants and the standard deviation at each concentration in the presence of and absence of the exogenous metabolic activation system were calculated.

F. Acceptability Criteria

An individual trial must have included a negative and positive control and at least 5 concentration levels of the test substance for each tester strain and condition. A data point, concentration level or trial was excluded from analysis when acceptability criteria were not met. The acceptability criteria were as follows:

1. Tester Strain Integrity

All *S. typhimurium* tester strain cultures were required to exhibit L-histidine dependent growth. To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures were required to exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvrB* mutation, all *S. typhimurium* tester strain cultures were required to exhibit sensitivity to ultraviolet light in comparison to strain TA102, which is proficient in the repair of small amounts of ultraviolet light-induced DNA damage. *E. coli* tester strains were required to exhibit L-tryptophan dependent growth. To demonstrate the presence of the *uvrA* mutation, the *E. coli* tester strain was required to exhibit sensitivity to ultraviolet light. Tester strain cultures of *S. typhimurium* TA97a, TA98, TA100 and *E. coli* WP2 *uvrA* (pKM101) must have exhibited resistance to ampicillin to demonstrate the presence of the pKMI01 or R-factor plasmid.

All tester strain cultures were required to exhibit a characteristic number of spontaneous revertants per plate in the absence of the test substance. The acceptable mean revertants per plate of the negative controls in the presence or absence of the exogenous metabolic activation system were derived from the means of the historical negative control data and were within the following ranges: *S. typhimurium* strains TA97a (65-163); TA98 (8-40); TA100 (58-192); TA1535 (2-28), *E. coli* strain WP2 *uvrA* (pKM101) (90-227). Historical control data collected at the testing facility are presented in the appendix at the end of this report.

2. Tester Strain Culture Titer

To ensure that appropriate numbers of bacteria were plated, all tester strain culture titers were approximately 1 x 10^9 cells/mL.

3. Positive Control Values

Mean positive control values must have exhibited at least a three-fold increase over the respective mean of the concurrent negative control value for each tester strain and condition.

4. Revertant Toxicity

A minimum of 5 analyzable (of which 4 must be non-toxic) concentration levels were required to classify the test substance. A concentration level was considered toxic and analyzable if the test substance at that specific concentration caused a >50% reduction in the mean number of revertants per plate relative to the mean of the concurrent negative control and was not equal to 0.

5. Rejection of Plates, Concentration Levels, or Assays

A plate may have been rejected if contamination, test substance precipitation or conditions resulted on a treatment plate that prevented an accurate colony counting.

A concentration level (or a negative control) was rejected if there were less than 2 data points or if variability between replicate plates was judged to be excessive. Scientific judgement was used in determining the acceptability of the data.

An assay (for an individual strain) would have been rejected if the negative control was rejected, if the positive control was rejected, or if the tester strain failed to exhibit the appropriate phenotype.

All data from the study were retained, met acceptability criteria, and are included in this report.

G. Classification Guidelines

A test substance was classified as **POSITIVE** (i.e., mutagenic) if the mean number of revertants in any strain (except *Salmonella* strain TA1535) at any test substance concentration was at least two times greater than the mean of the concurrent strain specific concurrent negative control, and there was a concentration-related increase in the mean number of revertants per plate in that same strain. The mean number of revertants in *Salmonella* strain TA1535 must be at least three times greater than the mean number of revertants of its concurrent negative control.

A test substance was classified as **NEGATIVE** (i.e., not mutagenic) if in all strains, except for TA1535, there were no test substance concentrations with a mean number of revertants that was at least two times greater than the mean number of revertants of the concurrent negative control, and there was no concentration-related increase in the mean number of revertants per plate in that same strain. For *S. typhimurium* strain TA1535, there must be no test substance concentration with a mean number of revertants that is at least three times greater than the mean number of revertants of the concurrent negative control, and no concentration-related increase in the mean number of revertants per plate.

Results not meeting criteria for positive or negative classification were evaluated using scientific judgment and experience and may have been reported as **EQUIVOCAL.**

RESULTS AND DISCUSSION

H-24256 was evaluated in the bacterial reverse mutation test using *Salmonella typhimurium* strains TA97a, TA98, TA100, TA1535, and *Escherichia coli* strain WP2 *uvrA* (pKM101) in the presence and absence of an exogenous metabolic activation system (Aroclor®-induced rat liver S9). The test was performed using the plate incorporation method in order to evaluate the mutagenic potential of the test substance. Sterile water was chosen as the test substance solvent, diluent, and negative control.

In this study, concentrations of 5, 10, 50, 100, 500, 1000, 2500, and 5000 μg/plate were tested in comparison to negative (solvent) controls. Mean positive control values, measured as revertants per plate, exhibited greater than a three-fold increase over the means of the respective negative control values for each tester strain (Tables 2-6). All tester strains exhibited appropriate phenotypic characteristics (Table 1). The mean number of revertants observed in the negative control for each strain was within the prescribed acceptable range.

No test substance-related precipitate or evidence of toxicity was observed in any of the *Salmonella typhimurium* strains or in the *Escherichia coli* strain (Tables 2-6). In tester strains TA97a, TA98, TA100, or *E. coli* WP2 *uvrA*(pKM101), there were no test substance concentrations with a mean number of revertants that were two times greater than the mean of the concurrent vehicle control (Tables 2, 3, 4, and 6). In tester strain TA1535, there were no test substance concentrations with a mean number of revertants three times greater than the mean of the concurrent vehicle control (Table 5). There was no concentration-related increase in the mean revertants per plate in any strain.

CONCLUSIONS

Under the conditions of this study, no evidence of mutagenic activity was detected. Based on the findings, H-24256 was concluded to be negative for the induction of mutagenicity in the bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli.*

RECORDS AND SAMPLE STORAGE

Laboratory-specific or site-specific data, such as personnel files and equipment records will be retained by the facility where the work was done.

At the request of the sponsor, raw data and the final report will be retained at DuPont Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware.

REFERENCES

1. Maron, D. M. and B. N. Ames (1983). Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* **113**, 173-215.

2. Green, M.H.L. and W. J. Muriel (1976). Mutagen testing using TRP^+ reversion in *Escherichia* coli. *Mutat. Res.* **38**, 3-32.

3. Claxton, L.D., Allen, J., Auletta, A., Mortelmans, K., Nestmann, E., and E. Zeiger (1987). Guide for the *Salmonella typhimurium* / mammalian microsome tests for bacterial mutagenicity. *Mutat. Res.* **189**, 83-91.

4. Ames, B. N., F. D. Lee, and W. E. Durston (1973). An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proc. Natl. Acad. Sci. USA* **70**, 782-786.

5. McCann, J., N. E. Springarn, J. Kobori, and B. N. Ames (1975). Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. *Proc. Natl. Acad. Sci. USA* **72**, 979-983.

TABLES

ABBREVIATIONS FOR TABLES

Evidence for test substance toxicity to the bacteria was documented by recording the appearance of the plates and background lawn using the following key:

T0 **Normal**, background microcolony lawn appeared normal.
T1 **Slightly reduced**, background microcolony lawn was noticeably thinner.
T2 **Moderately reduced**, background lawn was markedly thinner resulting in an increase in the size of microcolonies compared to the vehicle control plate(s).
T3 **Severely reduced**, background lawn was distinguished by an extreme thinning resulting in an increase in the size of the microcolonies compared to the vehicle control plate(s). Microcolonies were seen readily by the unaided eye and were greatly enlarged relative to controls.
T4 **Absent**, plate(s) were distinguished by a complete lack of any microcolony lawn over a majority of the area of the plate(s).

Formation of a precipitate by the test material was documented using the following key:

P0 **No precipitate,** no precipitate observed.
P1 **Microscopic precipitate**, precipitate present that did not interfere with background lawn evaluation or automated colony counting.
P2 **Non-interfering precipitate,** precipitate present that was visible to the naked eye that did not interfere with automated colony counting.
P3 **Interfering precipitate,** precipitate present that required plate to be counted by hand.
P4 **Heavy interfering precipitate**, precipitate present that prevented accurate colony counting and obscured the background lawn requiring plate rejection (R).

Additional abbreviations may include the following:

N Absence of any noteworthy observation
R Plate rejected

Positive controls were abbreviated as follows:

ICR 191	Acridine mutagen
DMBA	9,10-Dimethyl-1,2-benzanthracene
2NF	2-Nitrofluorene
2AA	2-Aminoanthracene
NAAZ	Sodium azide
ENNG	*N*-Ethyl-*N*-nitro-*N*-nitroguanidine

TABLE 1
STRAIN PHENOTYPE CONFIRMATION

Strain	L-Histidine/L-Tryptophan Dependent Growth[a]		Additional Growth Characteristics			
	In Absence	In Presence	Crystal Violet[b]	UV Light[c]	Ampicillin[d]	Tetracycline[d]
TA97a	-	+	S	S	R	S
TA98	-	+	S	S	R	S
TA100	-	+	S	S	R	S
TA1535	-	+	S	S	S	S
WP2 *uvr*A (pKM101)	-	+	R	S	R	S
TA102[e]	N/A[f]	N/A	N/A	R	N/A	N/A

[a]Tested for histidine (*S. typhimurium*)/tryptophan (*E. coli*) requirement by the ability to grow in the absence and presence of histidine/tryptophan; + = Growth; - = No growth.
[b]Tested for *rfa* deletion by demonstrating sensitivity to crystal violet; S = Sensitive; R = Resistant.
[c]Tested for *uvrA* and *uvrB* deletion by demonstrating sensitivity to UV light; S = Sensitive; R = Resistant.
[d]Tested for presence of pKM101 plasmid by demonstrating resistance to ampicillin and sensitivity to tetracycline; R = Resistant; S = Sensitive.
[e]TA102 used as a control for UV Light sensitivity only.
[f]N/A = Not applicable

TABLE 2

Mutagenic Activity in *Salmonella typhimurium* TA97a

Concentration (μg/plate)	Revertants Plate 1	Plate 2	Plate 3	Mean (S.D.)	Observations
A. Without Metabolic Activation					
0	144	152	157	151 (7)	T0,P0
5	129	140	134	134 (6)	T0,P0
10	148	139	145	144 (5)	T0,P0
50	165	143	141	150 (13)	T0,P0
100	138	155	144	146 (9)	T0,P0
500	130	140	155	142 (13)	T0,P0
1000	127	153	151	144 (14)	T0,P0
2500	132	134	142	136 (5)	T0,P0
5000	137	140	157	145 (11)	T0,P0
ICR 191					
2 μg/plate	1384	1659	1962	1668 (289)	N
B. With Metabolic Activation (Aroclor®-induced rat liver S9)					
0	157	156	163	159 (4)	T0,P0
5	148	152	161	154 (7)	T0,P0
10	139	157	153	150 (9)	T0,P0
50	153	151	167	157 (9)	T0,P0
100	149	173	181	168 (17)	T0,P0
500	146	139	149	145 (5)	T0,P0
1000	146	172	157	158 (3)	T0,P0
2500	146	179	139	155 (21)	T0,P0
5000	154	160	152	155 (4)	T0,P0
DMBA					
20 μg/plate	2197	1659	2196	2017 (310)	N

TABLE 3

Mutagenic Activity in *Salmonella typhimurium* TA98

Concentration (μg/plate)	Revertants Plate 1	Plate 2	Plate 3	Mean (S.D.)	Observations
A. Without Metabolic Activation					
0	24	33	27	28 (5)	T0,P0
5	23	29	28	27 (3)	T0,P0
10	25	25	26	25 (1)	T0,P0
50	25	31	30	29 (3)	T0,P0
100	24	28	29	27 (3)	T0,P0
500	15	26	20	20 (6)	T0,P0
1000	30	21	16	22 (7)	T0,P0
2500	27	24	25	25 (2)	T0,P0
5000	25	15	17	19 (5)	T0,P0
2NF 25 μg/plate	1262	1450	1544	1419 (144)	N
B. With Metabolic Activation (Aroclor®-induced rat liver S9)					
0	27	30	25	27 (3)	T0,P0
5	25	24	25	25 (1)	T0,P0
10	29	31	32	31 (2)	T0,P0
50	27	26	35	29 (5)	T0,P0
100	26	39	29	31 (7)	T0,P0
500	29	22	29	27 (4)	T0,P0
1000	31	27	32	30 (3)	T0,P0
2500	30	27	29	29 (2)	T0,P0
5000	34	37	25	32 (6)	T0,P0
2AA 2 μg/plate	849	866	747	821 (64)	N

TABLE 4

Mutagenic Activity in *Salmonella typhimurium* TA100

Concentration (μg/plate)	Revertants Plate 1	Plate 2	Plate 3	Mean (S.D.)	Observations
A. Without Metabolic Activation					
0	146	158	164	156 (9)	T0,P0
5	146	165	132	148 (17)	T0,P0
10	157	157	163	159 (3)	T0,P0
50	136	140	150	142 (7)	T0,P0
100	155	156	151	154 (3)	T0,P0
500	157	153	137	149 (11)	T0,P0
1000	131	154	130	138 (14)	T0,P0
2500	127	145	135	136 (9)	T0,P0
5000	169	159	150	159 (10)	T0,P0
NAAZ					
2 μg/plate	877	790	790	819 (50)	N
B. With Metabolic Activation (Aroclor®-induced rat liver S9)					
0	171	166	154	164 (9)	T0,P0
5	167	167	153	162 (8)	T0,P0
10	176	162	162	167 (8)	T0,P0
50	163	153	169	162 (8)	T0,P0
100	169	160	178	169 (9)	T0,P0
500	172	163	182	172 (10)	T0,P0
1000	167	172	192	177 (13)	T0,P0
2500	157	184	157	166 (16)	T0,P0
5000	156	131	172	153 (21)	T0,P0
2AA					
2 μg/plate	745	900	901	849 (90)	N

TABLE 5

Mutagenic Activity In *Salmonella typhimurium* TA1535

Concentration (μg/plate)	Revertants Plate 1	Plate 2	Plate 3	Mean (S.D.)	Observations
A. Without Metabolic Activation					
0	21	21	21	21 (0)	T0,P0
5	22	26	24	24 (2)	T0,P0
10	19	21	20	20 (1)	T0,P0
50	22	16	17	18 (3)	T0,P0
100	22	17	21	20 (3)	T0,P0
500	16	25	12	18 (7)	T0,P0
1000	23	28	14	22 (7)	T0,P0
2500	20	12	24	19 (6)	T0,P0
5000	24	24	17	22 (4)	T0,P0
NAAZ					
2 μg/plate	699	760	783	747 (43)	N
B. With Metabolic Activation (Aroclor®-induced rat liver S9)					
0	14	18	12	15 (3)	T0,P0
5	13	14	15	14 (1)	T0,P0
10	21	12	13	15 (5)	T0,P0
50	15	26	14	18 (7)	T0,P0
100	17	14	10	14 (4)	T0,P0
500	13	17	20	17 (4)	T0,P0
1000	19	14	13	15 (3)	T0,P0
2500	16	15	13	15 (2)	T0,P0
5000	18	11	14	14 (4)	T0,P0
2AA					
2 μg/plate	229	218	235	227 (9)	N

TABLE 6

Mutagenic Activity In *Escherichia coli* WP2 u*vrA* (pKM101)

Concentration (μg/plate)	Revertants Plate 1	Plate 2	Plate 3	Mean (S.D.)	Observations
A. Without Metabolic Activation					
0	183	187	190	187 (4)	T0,P0
5	182	210	211	201 (16)	T0,P0
10	196	214	196	202 (10)	T0,P0
50	199	189	195	194 (5)	T0,P0
100	202	193	208	201 (8)	T0,P0
500	206	195	208	203 (7)	T0,P0
1000	180	202	174	185 (15)	T0,P0
2500	144	172	173	163 (16)	T0,P0
5000	148	187	169	168 (20)	T0,P0
ENNG					
2 μg/plate	1456	1350	1402	1403 (53)	N
B. With Metabolic Activation (Aroclor®-induced rat liver S9)					
0	138	140	174	151 (20)	T0,P0
5	164	178	174	172 (7)	T0,P0
10	153	163	167	161 (7)	T0,P0
50	157	176	155	163 (12)	T0,P0
100	156	166	158	160 (5)	T0,P0
500	188	169	168	175 (11)	T0,P0
1000	169	168	202	180 (19)	T0,P0
2500	171	163	174	169 (6)	T0,P0
5000	166	173	192	177 (13)	T0,P0
2AA					
25 μg/plate	1513	1383	1371	1422 (79)	N

APPENDIX -
HISTORICAL CONTROL DATA

Historical Control Data[a]

Tester Strain Control [Positive Control[b]]	Exogenous Metabolic Activation System	Mean (S.D.)	Range Minimum - Maximum
S. typhimurium TA100			
Negative	Absent	122 (29)	54 - 218
Negative	Present	128 (29)	65 - 253
Positive [NAAZ-2]	Absent	872 (274)	339 - 2604
Positive [2AA-1]	Present	1187 (476)	94 - 2682
S. typhimurium TA 1535			
Negative	Absent	15 (6)	4 - 46
Negative	Present	14 (6)	4 - 39
Positive [NAAZ-2]	Absent	618 (199)	127 - 1270
Positive [2AA-2]	Present	362 (136)	44 - 1323
S. typhimurium TA97a			
Negative	Absent	103 (18)	59 - 164
Negative	Present	124 (26)	67 - 196
Positive [ICR 191-2]	Absent	1822 (662)	476 - 3359
Positive [DMBA-20]	Present	1534 (559)	563 - 2773
Positive [2AA-1]	Present	960 (348)	308 - 2281
S. typhimurium TA98			
Negative	Absent	22 (7)	7 - 47
Negative	Present	26 (7)	11 - 53
Positive [2NF-25]	Absent	1403 (372)	567 - 2774
Positive [2AA-2]	Present	1552 (598)	250 - 3114
E. coli WP2 *uvrA* (pKM101)			
Negative	Absent	148 (29)	82 - 221
Negative	Present	167 (29)	93 - 255
Positive [MMS-1000]	Absent	1656 (449)	208 - 2453
Positive [ENNG-2]	Absent	1627 (317)	1049 - 2253
Positive [2AA-25]	Present	1577 (450)	485 - 2484
Positive [2AA-250]	Present	1682 (372)	929 - 2230

a Historical data for tester strains used in the reported study. Data are based on studies reported during the period 1996 to 1998. Data include all control solvents or diluents, metabolic activation systems based on Aroclor®-induced rat liver S9, and all forms of study modification (e.g., plate incorporation, pre-incubation/gas, waste water).

b Abbreviations for positive controls: NAAZ (sodium azide); 2AA (2-aminoanthracene); 2NF (2-nitrofluorene); MMS (methyl methanesulfonate); ICR 191 (ICR 191 Acridine mutagen); DMBA (9,10-dimethyl-1,2-benzanthracene), ENNG (*N*-ethyl-*N*-nitro-*N*-nitrosoguanidine). The number following abbreviation is the microgram (μg) amount per plate or vial used for the positive control.