

TRADE SECRET

Study Title

H-24335: Bacterial Reverse Mutation Test in *Salmonella typhimurium* and *Escherichia coli*

Laboratory Project ID: DuPont-3827

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 23, 2000

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CERTIFICATION

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

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STUDY INFORMATION

Substance Tested: [REDACTED]

Synonyms/Codes: H-24335
[REDACTED]

Haskell Number: 24335

Composition [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: February 2, 2000 / (see report cover page)

In-Life Initiated/Completed: February 11, 2000 / February 22, 2000

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STUDY PERSONNEL

The following individuals participated in the conduct of this study:

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SUMMARY

H-24335 was evaluated in the bacterial reverse mutation test *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 test was performed using the plate incorporation method to evaluate the mutagenic potential of the test substance. Sterile water was chosen as the test substance solvent, diluent, and negative control.

Test substance concentrations of 5, 10, 50, 100, 500, 100, 2500, and 5000 $\mu\text{g}/\text{plate}$ were evaluated in comparison to negative (solvent) controls. Test substance-related toxicity, 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 prescribed negative control ranges.

Under the conditions of this study, no evidence of mutagenic activity was detected in any of the *Salmonella* strains or in the *Escherichia coli* strain in the presence or absence of the exogenous S9 metabolic activation system. Based on the findings, H-24335 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-24335, 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-24335, 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

Deionized water was the solvent for sodium azide (NAAZ). The solvent for 2AA, 2NF, ICR 191, DMBA, and ENNG was dimethyl sulfoxide (DMSO). The manufacturer, lot number, and purity of the solvent was recorded in the study records. Neither the amount and nature of the contaminants nor the use of the solvent was expected to affect the integrity or validity of the study. 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		LPS	R-factor	
	Reversion Target	DNA Repair*		(pKM101) Plasmid	pAQ1 Plasmid
<i>S. typhimurium</i> TA97a	<i>hisD6610</i> [#]	Δ <i>uvrB</i>	<i>rfa</i>	Present	Absent
<i>S. typhimurium</i> TA98	<i>hisD3052</i>	Δ <i>uvrB</i>	<i>rfa</i>	Present	Absent
<i>S. typhimurium</i> TA100	<i>hisG46</i>	Δ <i>uvrB</i>	<i>rfa</i>	Present	Absent
<i>S. typhimurium</i> TA102*	<i>hisG428</i>	(+)	<i>rfa</i>	Present	Present
<i>S. typhimurium</i> TA1535	<i>hisG46</i>	Δ <i>uvrB</i>	<i>rfa</i>	Absent	Absent
<i>S. typhimurium</i> TA1537	<i>hisC3076</i>	Δ <i>uvrB</i>	<i>rfa</i>	Absent	Absent
<i>E. coli</i> WP2 <i>uvrA</i> (pKM101)	<i>TrpE</i>	Δ <i>uvrA</i>	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.

Also *hisO1242*.

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.

The *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 test, 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. 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 test 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

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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 MgCl₂, 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 that start of the study. The stock concentration were 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 Test

This study consisted of a single trial that assessed the mutagenicity of the test substance. 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 1×10^8 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 4°C (± 3°C) prior to evaluation and counting of revertant colonies.

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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 pKM101 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 data collected at the testing facility are presented in the appendix of this report.

2. Tester Strain Titer

To ensure that appropriate numbers of bacteria were plated, all tester strain culture titers were approximately 1×10^9 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. Non-Toxic Concentration Levels

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, and data that met acceptability criteria are included in this report.

G. Classification Criteria

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

A test substance was classified as **NEGATIVE** (i.e., not mutagenic) if all positive classification criteria for all strains are not met.

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

RESULTS AND DISCUSSION

H-24335 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). The test was performed using the plate incorporation method 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, test substance concentrations of 5, 10, 50, 100, 500, 1000, 2500, and 5000 $\mu\text{g}/\text{plate}$ 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-24335 was concluded to be negative for the induction of mutagenicity in the bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli* in the presence or absence of an exogenous metabolic activation system.

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.
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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.
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5. McCann, J., N. E. Springarn, J. Kabori, 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

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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 <i>uvrA</i> (pKM101)	-	+	R	S	R	S
TA102 ^e	N/A ^f	N/A	N/A	R	N/A	N/A

^aTested for histidine (*S. typhimurium*)/tryptophan (*E. coli*) requirement by the ability to grow in the absence and presence of histidine/tryptophan; + = Growth; - = No growth.

^bTested for *rfa* deletion by demonstrating sensitivity to crystal violet; S = Sensitive; R = Resistant.

^cTested for *uvrA* and *uvrB* deletion by demonstrating sensitivity to UV light; S = Sensitive; R = Resistant.

^dTested for presence of pKM101 plasmid by demonstrating resistance to ampicillin and sensitivity to tetracycline; R = Resistant; S = Sensitive.

^eTA102 used as a control for UV Light sensitivity only.

^fN/A = Not applicable

Table 2
Mutagenic Activity in *Salmonella typhimurium* TA97a

Concentration (μ g/plate)	Revertants			Mean (S.D.)	Observations
	Plate 1	Plate 2	Plate 3		
A. WITHOUT METABOLIC ACTIVATION					
0	100	115	148	121 (25)	T0,P0
5	114	145	150	136 (20)	T0,P0
10	125	125	137	129 (7)	T0,P0
50	123	137	130	130 (7)	T0,P0
100	134	152	134	140 (10)	T0,P0
500	133	134	119	129 (8)	T0,P0
1000	138	142	141	140 (2)	T0,P0
2500	122	123	128	124 (3)	T0,P0
5000	94	89	97	93 (4)	T0,P0
ICR 191					
2 μ g/plate	2034	2319	1858	2070 (233)	N
B. WITH METABOLIC ACTIVATION (Aroclor [®] -induced rat liver S9)					
0	142	145	156	148 (7)	T0,P0
5	142	159	165	155 (12)	T0,P0
10	151	162	149	154 (7)	T0,P0
50	134	143	138	138 (5)	T0,P0
100	152	174	161	162 (11)	T0,P0
500	170	174	176	173 (3)	T0,P0
1000	153	140	136	143 (9)	T0,P0
2500	108	125	144	126 (18)	T0,P0
5000	116	147	130	131 (16)	T0,P0
DMBA					
20 μ g/plate	880	855	1222	986 (205)	N

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Table 3

Mutagenic Activity in *Salmonella typhimurium* TA98

Concentration ($\mu\text{g}/\text{plate}$)	Revertants			Mean (S.D.)	Observations
	Plate 1	Plate 2	Plate 3		
A. WITHOUT METABOLIC ACTIVATION					
0	28	14	14	19 (8)	T0,P0
5	27	17	24	23 (5)	T0,P0
10	15	18	17	17 (2)	T0,P0
50	23	32	13	23 (10)	T0,P0
100	20	13	23	19 (5)	T0,P0
500	18	17	23	19 (3)	T0,P0
1000	24	12	16	17 (6)	T0,P0
2500	14	9	18	14 (5)	T0,P0
5000	14	13	10	12 (2)	T0,P0
2NF					
25 $\mu\text{g}/\text{plate}$	1985	1509	1482	1659 (283)	N
B. WITH METABOLIC ACTIVATION (Aroclor [®] -induced rat liver S9)					
0	20	20	36	25 (9)	T0,P0
5	30	24	23	26 (4)	T0,P0
10	24	19	20	21 (3)	T0,P0
50	14	21	26	20 (6)	T0,P0
100	20	25	25	23 (3)	T0,P0
500	19	22	28	23 (5)	T0,P0
1000	20	20	26	22 (3)	T0,P0
2500	20	23	12	18 (6)	T0,P0
5000	18	21	26	22 (4)	T0,P0
2AA					
2 $\mu\text{g}/\text{plate}$	1544	1518	1594	1552 (39)	N

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Table 4

Mutagenic Activity in *Salmonella typhimurium* TA100

Concentration (μ g/plate)	Revertants			Mean (S.D.)	Observations
	Plate 1	Plate 2	Plate 3		
A. WITHOUT METABOLIC ACTIVATION					
0	160	140	145	148 (10)	T0,P0
5	146	162	185	164 (20)	T0,P0
10	143	136	154	144 (9)	T0,P0
50	155	132	139	142 (12)	T0,P0
100	126	136	118	127 (9)	T0,P0
500	151	156	151	153 (3)	T0,P0
1000	103	105	116	108 (7)	T0,P0
2500	63	68	64	65 (3)	T1,P0
5000	52	59	61	57 (5)	T2,P0
NAAZ					
2 μ g/plate	665	759	720	715 (47)	N
B. WITH METABOLIC ACTIVATION (Aroclor [®] -induced rat liver S9)					
0	161	178	178	172 (10)	T0,P0
5	151	144	174	156 (16)	T0,P0
10	159	149	155	154 (5)	T0,P0
50	153	138	158	150 (10)	T0,P0
100	124	152	159	145 (19)	T0,P0
500	176	169	176	174 (4)	T0,P0
1000	162	162	164	163 (1)	T0,P0
2500	91	121	127	113 (19)	T0,P0
5000	117	95	107	106 (11)	T0,P0
2AA					
2 μ g/plate	1846	2213	1697	1919 (266)	N

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Table 5
Mutagenic Activity in *Salmonella typhimurium* TA1535

Concentration ($\mu\text{g}/\text{plate}$)	Revertants			Mean (S.D.)	Observations
	Plate 1	Plate 2	Plate 3		
A. WITHOUT METABOLIC ACTIVATION					
0	13	12	16	14 (2)	T0,P0
5	9	14	13	12 (3)	T0,P0
10	10	10	11	10 (1)	T0,P0
50	11	17	18	15 (4)	T0,P0
100	16	14	13	14 (2)	T0,P0
500	13	16	10	13 (3)	T0,P0
1000	8	14	20	14 (6)	T0,P0
2500	8	6	10	8 (2)	T0,P0
5000	14	8	8	10 (3)	T0,P0
NAAZ					
2 $\mu\text{g}/\text{plate}$	931	1020	796	916 (113)	N
B. WITH METABOLIC ACTIVATION (Aroclor [®] -induced rat liver S9)					
0	16	18	13	16 (3)	T0,P0
5	14	23	20	19 (5)	T0,P0
10	14	7	9	10 (4)	T0,P0
50	15	17	14	15 (2)	T0,P0
100	11	15	16	14 (3)	T0,P0
500	10	8	15	11 (4)	T0,P0
1000	9	14	10	11 (3)	T0,P0
2500	15	14	8	12 (4)	T0,P0
5000	13	16	13	14 (2)	T0,P0
2AA					
2 $\mu\text{g}/\text{plate}$	350	323	334	336 (14)	N

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Table 6

Mutagenic Activity in *Escherichia coli* WP2 *uvrA* (pKM101)

Concentration (μ g/plate)	Revertants			Mean (S.D.)	Observations
	Plate 1	Plate 2	Plate 3		
A. WITHOUT METABOLIC ACTIVATION					
0	107	120	89	105 (16)	T0,P0
5	117	132	97	115 (18)	T0,P0
10	129	130	97	119 (19)	T0,P0
50	115	118	96	110 (12)	T0,P0
100	111	116	110	112 (3)	T0,P0
500	120	129	115	121 (7)	T0,P0
1000	103	120	95	106 (13)	T0,P0
2500	121	139	107	122 (16)	T0,P0
5000	106	91	89	95 (9)	T0,P0
ENNG					
2 μ g/plate	1081	1280	1191	1184 (100)	N
B. WITH METABOLIC ACTIVATION (Aroclor [®] -induced rat liver S9)					
0	161	160	166	162 (3)	T0,P0
5	164	174	176	171 (6)	T0,P0
10	152	176	168	165 (12)	T0,P0
50	173	177	147	166 (16)	T0,P0
100	200	169	199	189 (18)	T0,P0
500	207	213	150	190 (35)	T0,P0
1000	184	157	186	176 (16)	T0,P0
2500	179	175	164	173 (8)	T0,P0
5000	143	148	149	147 (3)	T0,P0
2AA					
25 μ g/plate	1583	1224	1313	1373 (187)	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
<i>S. typhimurium</i> 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
<i>S. typhimurium</i> 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
<i>S. typhimurium</i> 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
<i>S. typhimurium</i> 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
<i>E. coli</i> WP2 <i>uvrA</i> (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 (μ g) amount per plate or vial used for the positive control.

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