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April 7, 1994

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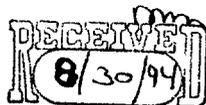
RE: Union Carbide Corporation's Submission of January 5, 1994
Concerning Thio PF6 Salt (CASRN 74227-35-3) and Bis PF6
Salt (CASRN 68156-13-8)

Dear Sir or Madam:

As a follow-up to the above-noted submission, Union Carbide Corporation ("Union Carbide") herewith submits the following reports:

- (1) "Bis Hexafluorophosphate Salt (Bis PF6 Salt): Mutagenic Potential in the Salmonella/Microsome (Ames) Assay", Bushy Run Research Center, BRRC Report 93U1321, March 22, 1994.
- (2) "Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential in the Salmonella/Microsome (Ames) Assay", Bushy Run Research Center, BRRC Report 93U1322, March 22, 1994.

In the attached reports the term "Confidential" may appear. This precautionary statement was for internal use at the time of issuance of the report, and is hereby waived for the purposes of the needs of the Agency in assessing health and safety information. The Agency is advised, however, that the publication rights to the contained information are the property of Union Carbide.



Very truly yours,

William C. Kuryla, Ph.D.
Associate Director
Product Safety

Attachments



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STUDY TITLE

Bis Hexafluorophosphate Salt (Bis PF6 Salt): Mutagenic Potential in
the Salmonella/Microsome (Ames) Assay

TEST SUBSTANCE

Bis Hexafluorophosphate Salt (Bis PF6 Salt)

DATA REQUIREMENT

Not Applicable

AUTHOR

J. S. Vergnes

STUDY COMPLETION DATE

March 22, 1994

PERFORMING LABORATORY

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93U1321

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**Bis Hexafluorophosphate Salt (Bis PF6 Salt): Mutagenic Potential in
the Salmonella/Microsome (Ames) Assay**

CONFIDENTIALITY STATEMENT

This report is Union Carbide Corporation Business Confidential and is not to be released outside of the Corporation without the written consent of the Sponsor.

**Bis Hexafluorophosphate Salt (Bis PF6 Salt): Mutagenic Potential in
the Salmonella/Microsome (Ames) Assay**

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

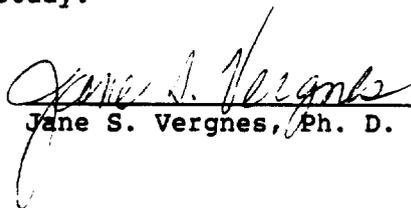
The portions of this study conducted by BRRC meet the requirements of the following Good Laboratory Practice Standards: Toxic Substances Control Act (TSCA), 40 CFR Part 792; and Organisation for Economic Co-operation and Development (OECD), C(81)30(Final), with exceptions. These exceptions are:

1. Analyses for stability and homogeneity of the test and control substances in the dosing solutions were not conducted.
2. The vehicle and positive control substances were not analyzed for chemical purity, stability, or uniformity.

The physical and chemical characterization of the test substance was not performed at BRRC and is considered the responsibility of the Sponsor.

These exceptions are not expected to compromise the integrity of the results and conclusion of the study.

Study Director:


Jane S. Vergnes, Ph. D.

3/22/94
Date

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**Bis Hexafluorophosphate Salt (Bis PF6 Salt): Mutagenic Potential in
the Salmonella/Microsome (Ames) Assay**

SUMMARY

Bis Hexafluorophosphate Salt (Bis PF6 Salt, CAS No. 74227-35-3) was tested for potential mutagenic activity using the Salmonella/microsome bacterial mutagenicity assay (Ames test). Doses were chosen on the basis of data obtained in a preliminary cytotoxicity study with strain TA100. Bis PF6 Salt was nontoxic to strain TA100 at doses of 3.0 mg/plate or less, both in the absence and in the presence of a rat liver S9 metabolic activation system. Sparse background lawn growth was observed at 10 mg/plate Bis PF6 Salt both in the absence and in the presence of S9 activation. The data suggest that the 5.0 mg/plate dose was also cytotoxic to strain TA100, since plate counts decreased by approximately 11-fold in the absence of S9 and 5-fold in the presence of S9 with respect to the 3.0 mg/plate dose.

Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538 were used for the mutagenicity assay. On the basis of the preliminary cytotoxicity study, 5 dose levels of Bis PF6 Salt, ranging from 0.10 to 5.0 mg/plate for the first mutagenicity test and from 0.03 to 3.0 mg/plate for the repeat mutagenicity test, were selected for treatments both in the absence and in the presence of metabolic activation and spaced at approximately half-log intervals. All 5 bacterial strains were treated in triplicate with the vehicle control substance (dimethylsulfoxide), an appropriate positive control substance, and 5 dose levels of Bis PF6 Salt using the plate incorporation method. Treated cultures were incubated at 37°C for 48 to 72 hr.

No mutagenic activity was observed in strains TA100 or TA1537, either in the absence or in the presence of S9. Increases in the mean number of colonies/plate of 2- to 3-fold were observed in strains TA1535 and TA1538 in the absence of S9. However, these increases were not reproducible in independent repetitions of the test. No mutagenic activity was observed in strains TA1535 or TA1538 in the presence of S9. Bis PF6 Salt was mutagenic to strain TA98, both in the absence and in the presence of S9. Increases in the mean number of colonies/plate ranged from approximately 3-fold at 0.03 mg/plate to 6- to 11-fold at 1.0 mg/plate in the absence of S9. Increases in the mean number of colonies/plate were approximately 2- to 3-fold at 0.10 mg/plate and 5- to 7-fold at 1.0 mg/plate in the presence of S9. All 5 bacterial strains exhibited mutagenic responses to the appropriate positive control substances. Vehicle controls were also tested with each strain, and the mean numbers of spontaneous revertants were considered acceptable. These results were observed in 2 independent tests.

To summarize, Bis PF6 Salt did not produce consistent, dose-related mutagenic effects in Salmonella strains TA100, TA1535, TA1537, or TA1538, either in the absence or in the presence of a rat liver S9 metabolic activation system. Bis PF6 Salt produced reproducible, dose-related increases in the number of colonies/plate of approximately 5-fold or greater in strain TA98 both in the absence and in the presence of S9 activation. Therefore, Bis PF6 Salt was considered mutagenic in strain TA98 under the conditions of this in vitro screening test.

OBJECTIVE

The objective of this study was to assess the mutagenic potential of Bis PF6 Salt in the Salmonella/microsome mutation assay.

BACKGROUND INFORMATION

The Salmonella/microsome mutation assay is a microbial screening test which detects the ability of chemicals to cause genetic alterations in the histidine gene of selected indicator strains of Salmonella. The indicator strains used for this test are all histidine-requiring mutants obtained from Dr. Bruce Ames, University of California, Berkeley, CA. Histidine-independent colonies can arise either spontaneously or by the mutagenic action of a chemical or physical agent. Base substitution mutagens cause a base change in the DNA molecule at the site of the original mutation or at a second site in the DNA which suppresses the original mutation. Frameshift mutagens cause the insertion or deletion of one or more base pairs in the DNA molecule. The frequency of induced or spontaneous reversion to histidine independence can be quantitated by plating the indicator strains on minimal agar and counting the number of revertant colonies.

DOSE SELECTION

Doses were selected on the basis of a preliminary cytotoxicity study, which is described in this report. The doses chosen for the first test were 0.10, 0.30, 1.0, 3.0, and 5.0 mg/plate, both in the absence and in the presence of S9. The doses chosen for the second test were 0.03, 0.10, 0.30, 1.0, and 3.0 mg/plate, both in the absence and in the presence of S9.

MATERIALS AND METHODS

The protocol detailing the design and conduct of this study is presented in Appendix 1.

Test Substance

One 2-ounce amber bottle (gross weight 84.56 g) of Bis Hexafluorophosphate Salt (Bis PF6 Salt), no Lot No. available, CAS No. 74227-35-3, was received on September 2, 1993, from Union Carbide Corporation, Bound Brook, NJ, and assigned BRRC Sample No. 56-352. The test substance was a white crystalline solid. The test substance was stored at room temperature. The purity of the test substance was not provided by the Sponsor. Test substance stability was not confirmed by BRRC.

Positive Control Substances

Two 1 g bottles of 4-nitro-o-phenylenediamine (4 NPD), Lot No. 40-13A, CAS No. 99-56-9, were received on November 21, 1989, from Chem Service, Inc., and assigned BRRC Sample No. 52-729 A and B. It was a red crystalline solid and was stored at less than 0°C. One 25 g bottle of sodium azide (NaN₃), Lot No. 29F-0533, CAS No. 26628-22-8, was received on November 21, 1989, from Sigma Chemical Co., and assigned BRRC Sample No. 52-727. It was a white crystalline solid and was stored at less than 0°C. One 5 g bottle of 9-aminoacridine, (9-AA), Lot No. 96F-05641, CAS No. 90-45-9, was received on November 21, 1989,

from Sigma Chemical Co., and assigned BRRC Sample No. 52-728. It was a yellow crystalline solid and was stored at less than 0°C. One 1 g bottle of 2-aminoanthracene (2-AA), Lot No. 121H3475, CAS No. 613-13-8, was received on May 20, 1992, from Sigma Chemical Co., and assigned BRRC Sample No. 55-144. It was a crystalline solid and was stored at less than 0°C.

Vehicle Control Substance

A 1 liter bottle of dimethylsulfoxide (DMSO), Lot No. BD 469, CAS No. 67-68-5, was received on December 3, 1992, from Baxter Diagnostics, Inc., and assigned BRRC Sample No. 55-386. It was a nonviscous, liquid and was stored at room temperature.

Metabolic Activation System

Rat liver S9 homogenate, prepared from Aroclor 1254-induced rats, was purchased from Microbiological Associates. The complete S9 metabolic activation system contained the following: 8 mM MgCl₂, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9. Fresh S9 mix was prepared each day of testing and kept at 0-4°C.

Study Organization

Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538 were treated in triplicate with the vehicle control substance, an appropriate positive control substance, and 5 dose levels of Bis PF6 Salt both in the absence and in the presence of a rat liver S9 metabolic activation system using the plate incorporation method. Treated cultures were incubated at 37°C for 48-72 hr. Two independent repetitions of the complete assay were performed. The following table summarizes the testing schedule.

	<u>First Test</u>	<u>Second Test</u>
Test Initiated:	November 16, 1993	November 30, 1993
Test Completed:	November 18, 1993	December 2, 1993

Bacterial Strains and Culture Conditions

Test strains were stored as frozen permanent cultures at -80°C. Working cultures were prepared fresh for each test day by inoculating bacteria from frozen working cultures into nutrient broth. Cultures were incubated overnight (10 hr to 15 hr) at 37°C with gentle agitation. The working cultures were kept on ice throughout the test day.

A bacterial titer was determined by plating serial dilutions of the working cultures on nutrient agar plates and counting the resultant colonies approximately 24-48 hr after plating. The integrity of the genetic markers in each bacterial strain was verified each time frozen stock cultures were prepared.

Dosing Solution Preparation

Each dosing solution was prepared by dissolving the appropriate amount of Bis PF6 Salt in DMSO. Concentrations were not adjusted for percent of active ingredient of the test substance. Dosing solutions were prepared daily and stored at room temperature prior to use.

The activation-independent controls were 4-NPD for TA98 and TA1538, NaN_3 for TA100 and TA1535, and 9-AA for TA1537. The activation-dependent control was 2-AA for all strains. 4-NPD, 9-AA, and 2-AA were prepared by dissolving the control substances in dimethylsulfoxide. NaN_3 was prepared by dissolving it in deionized, distilled water. Dosing solutions were made by diluting concentrated stock solutions of the control substances. Dosing solutions were stored at less than 0°C for no longer than 3 months and concentrated stock solutions were stored at less than 0°C for no longer than 1 year.

Cytotoxicity Testing

Ten dose levels of Bis PF6 Salt ranging from 0.001 to 10.0 mg/plate were tested on strain TA100 with and without S9 metabolic activation to evaluate the cytotoxicity of the test substance. A 2 ml volume of complete top agar (6 g/l agar, 5 g/l NaCl, 0.05 mM L-histidine and 0.05 mM D-biotin) was added to the required number of sterile tubes. A 100 μl aliquot of strain TA100 was added to each tube followed by an aliquot of the appropriate dilution of Bis PF6 Salt. Then, either 0.5 ml of S9 mixture or 0.5 ml of phosphate buffered saline (PBS) was added. The mixture was vortexed briefly and poured onto a Vogel-Bonner Medium E agar plate (VBE plate). The top agar was allowed to harden and then the plates were incubated at 37°C for at least 48 hr. The plates were examined to determine the condition of their background lawns. Growth was recorded as either confluent, sparse, or absent. Confluence indicated nontoxicity, sparse growth indicated moderate toxicity, and no growth indicated extreme toxicity. Revertant colonies were counted either manually or on an Artek Model No. 880 colony counter.

Mutagenicity Testing

Based on the results of preliminary cytotoxicity studies, 5 dose levels of Bis PF6 Salt ranging from 0.10 to 5.0 mg/plate for the first test and from 0.03 to 3.0 mg/plate for the second test, were spaced approximately at half-log intervals. To a sterile tube containing 2 ml of complete top agar, a 100 μl aliquot of the appropriate bacterial culture was added followed by 50 μl of the appropriate control, 50 μl of vehicle, or 50 μl of test substance solution. Either 0.5 ml of S9 mix or 0.5 ml of PBS was added for tests with or without metabolic activation, respectively. The top agar mixture was vortexed briefly and then poured onto a VBE plate. Sterility testing was performed on the PBS, the S9 mix, the vehicle, and the highest concentration of test substance. The plates were transferred to a darkened 37°C incubator after hardening and incubated for 48-72 hr.

Observations and Measurements

Bacterial colonies were counted either manually or using an Artek Model No. 880 Colony Counter. The counter was calibrated for each test to check counting accuracy. The number of colonies/plate was counted and recorded. An

(Table 2) in the absence of S9. However, these increases were not reproducible in independent repetitions of the test. No mutagenic activity was observed in strains TA1535 or TA1538 in the presence of S9.

Bis PF6 Salt was mutagenic to strain TA98, both in the absence and in the presence of S9. Increases in the mean number of colonies/plate ranged from approximately 3-fold at 0.03 mg/plate to 6- to 11-fold at 1.0 mg/plate in the absence of S9. Increases in the mean number of colonies/plate were approximately 2- to 3-fold at 0.10 mg/plate and 5- to 7-fold at 1.0 mg/plate in the presence of S9.

Interpretation of the test data is problematic for 2 reasons. First, there is a discrepancy between the large magnitude of the increases in the numbers of revertant colonies in strain TA100 treated with Bis PF6 Salt during the cytotoxicity test, both in the absence and in the presence of S9, and the irreproducibility of this result in either repetition of the definitive test. Second, although treatment with Bis PF6 Salt consistently results in increases in the mean number of colonies/plate in strain TA98, both in the absence and in the presence of S9, the variability among the triplicate plates at each dose level is unusually large. However, the unusual characteristics of this data set are consistent with those of 2 related test substances (BRRC Reports 93U1322 and 93U1328). No other test substances tested at BRRC during the same time interval exhibited these unusual characteristics. For these reasons, Bis PF6 Salt was not considered to be mutagenic in strain TA100, but was considered mutagenic in strain TA98.

All 5 bacterial strains exhibited mutagenic responses to the appropriate positive control substances. Vehicle controls were also tested with each strain, and the mean numbers of spontaneous revertants were considered acceptable. All positive and negative controls were tested concurrently with the test substance. Concurrent sterility testing showed that the S9 mix, PBS, the test substance and the vehicle were sterile.

CONCLUSION

Bis PF6 Salt did not produce consistent, dose-related mutagenic effects in Salmonella strains TA100, TA1535, TA1537, or TA1538, either in the absence or in the presence of a rat liver S9 metabolic activation system. Bis PF6 Salt produced reproducible, dose-related increases in the number of colonies/plate of approximately 5-fold or greater in strain TA98 both in the absence and in the presence of S9 activation. These results were observed in 2 independent tests. Under the conditions of this Salmonella/microsome mutagenicity assay, Bis PF6 Salt was considered mutagenic in strain TA98.

examination was also made of the background lawn on each plate. If no background lawn was observed, the absence of the background lawn was noted and no plate count was recorded. If the background lawn was sparse, colonies were counted and the colony count was used to calculate a mean and standard deviation. A reduction in the number of spontaneous revertant colonies is also an indication of toxicity. A dose level was labeled "toxic" when the mean number of colonies/plate was less than one half the mean for the vehicle control.

Data Analyses

Various models of calculators, computers, and computer programs may have been used to analyze data for this study. Since various models round or truncate numbers differently, values in some tables may differ slightly from those in other tables or from independently calculated data. The integrity of the study and interpretation of the data were unaffected by these differences.

A test substance was considered a bacterial mutagen if it consistently produced a dose-related increase in the mean reversion frequency of at least one bacterial strain as compared to the vehicle control for that strain. At least one of those doses must have produced a mean reversion frequency at least twice that of the vehicle control. Alternatively, a test substance was considered a bacterial mutagen if there was a reproducible increase in the mean number of revertant colonies at a single dose level of at least 2-fold compared to the vehicle control. Increases in the mean reversion frequency that were not dose related, or could not be reproduced, were considered negative test results.

RETENTION OF RECORDS

All raw data, documentation, the protocol, and a copy of the final report generated as a result of this study will be retained in the BRRC Archives for at least 5 years.

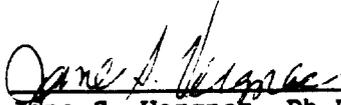
RESULTS AND DISCUSSION

The results of the preliminary cytotoxicity test are presented in Table 1. Bis PF6 Salt was nontoxic to strain TA100 at doses of 3.0 mg/plate or less, both in the absence and in the presence of a rat liver S9 metabolic activation system. Increases in the number of colonies/plate of approximately 13-fold in the absence of S9 and 8-fold in the presence of S9 were observed at 3.0 mg/plate Bis PF6 Salt. Sparse background lawn growth was observed at 10.0 mg/plate Bis PF6 Salt, both in the absence and in the presence of S9 activation. The data suggest that the 5.0 mg/plate dose was also cytotoxic to strain TA100, since the plate counts decreased by approximately 11-fold in the absence of S9 and 5-fold in the presence of S9 with respect to the plate counts at 3.0 mg/plate.

The results of the definitive mutagenicity tests are shown in Tables 2 and 4 (without activation) and in Tables 3 and 5 (with activation). No mutagenic activity was observed in strains TA100 or TA1537, either in the absence or in the presence of S9. Increases in the mean number of colonies/plate of 2- to 3-fold were observed in strains TA1535 (Table 4) and TA1538

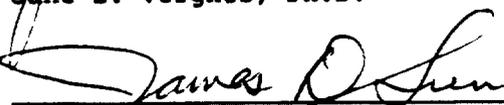
REVIEW AND APPROVAL

Study Director:


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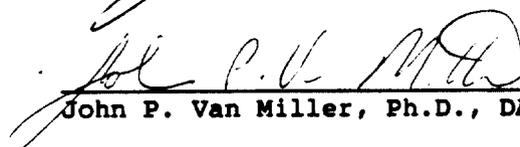
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REFERENCES

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

BRRC Report 93U1322 (In preparation). Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential in the Salmonella/Microsome (Ames) Assay. Bushy Run Research Center, Union Carbide Corporation, Export, PA. Prepared for Union Carbide Corporation.

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Kier, L. D., Brusick, D. J., Auletta, A. E., VonHalle, E. S., Brown, M. M., Simmon, V. F., Dunkel, V., McCann, J., Mortelmans, K., Prival, M., Rao, T. K., and Ray, V. (1986). The Salmonella typhimurium/Mammalian Microsomal Assay. A Report of the U. S. Environmental Protection Agency Gene-Tox Program. Mutation Research 168, 69-240.

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

TABLE 1
 BIS HEXAFLUOROPHOSPHATE SALT (BIS PF6 SALT): MUTAGENIC POTENTIAL IN
 THE SALMONELLA/MICROSOME (AMES) ASSAY

CYTOTOXICITY TESTING RESULTS IN STRAIN TA100

Test Substance (mg/plate)	Test without S9		Test with S9	
	Growth of Lawn*	Plate Count**	Growth of Lawn*	Plate Count**
0.001	C	76	C	103
0.003	C	92	C	137
0.01	C	101	C	100
0.03	C	92	C	109
0.10	C	92	C	109
0.30	C	98	C	117
1.0	C	165	C	131
3.0	C	1254	C	818
5.0	C	111	C	169
10.0	S	50	S	75
Vehicle - DMSO (55 mg/plate)	C	93	C	91

*Growth of the bacterial lawn was assessed with a dissecting microscope 48 hr after treatment.
 **One plate/test concentration.

Abbreviations: C-confluent growth; S-sparse growth; DMSO-dimethylsulfoxide.

TABLE 2
 BIS HEXAFLUOROPHOSPHATE SALT (BIS PF6 SALT): MUTAGENIC POTENTIAL IN
 THE SALMONELLA/NICROSOME (AMES) ASSAY

RESULTS OF THE SALMONELLA/REVERSE MUTATION ASSAY
 TEST 1 WITHOUT S9

Test Substance (mg/plate)	Plate Counts			Mean	Standard Deviation
	1	2	3		
STRAIN TA98					
DMSO (55)	14	20	19	18	3.2
4-NPD (0.01)	524	491	588	534	49.3
0.10	149	30	31	70	68.4
0.30	22	24	52	33	16.8
1.0	202	59	340	200	140.5
3.0	3 (S)	5 (S)	6 (S)	5 (T)	1.5
5.0	1 (S)	2 (S)	1 (S)	1 (T)	0.6
STRAIN TA100					
DMSO (55)	77	90	111	93	17.2
NaN ₃ (0.01)	1275	1224	1230	1243	27.9
0.10	100	97	88	95	6.2
0.30	125	97	84	102	21.0
1.0	98	126	105	110	14.6
3.0	92	102	100	98	5.3
5.0	45	79	88	71	22.7
STRAIN TA1535					
DMSO (55)	4	9	11	8	3.6
NaN ₃ (0.01)	1302	1268	1392	1321	64.1
0.10	10	11	13	11	1.5
0.30	12	5	9	9	3.5
1.0	9	12	12	11	1.7
3.0	1 (S)	1 (S)	3 (S)	2 (T)	1.2
5.0	0 (S)	0 (S)	0 (S)	0 (T)	0.0
STRAIN TA1537					
DMSO (55)	7	2	3	4	2.6
9-AA (0.06)	253	242	241	245	6.7
0.10	5	5	9	6	2.3
0.30	7	7	5	6	1.2
1.0	5 (S)	4 (S)	6	5	1.0
3.0	0 (S)	0 (S)	T	0 (T)	0.0
5.0	T	T	T	T	-
STRAIN TA1538					
DMSO (55)	6	6	4	5	1.2
4-NPD (0.01)	128	144	117	130	13.6
0.10	12	8	6	9	3.1
0.30	8	14	24	15	8.1
1.0	13	12	14	13	1.0
3.0	13	8	11	11	2.5
5.0	12	6	9	9	3.0

T: Toxic; absence of background lawn, or mean number of colonies < 1/2 solvent control value.
 S: Sparse growth of background lawn.
 4-NPD: 4-Nitro-o-phenylenediamine; NaN₃: Sodium azide; 9-AA: 9-Aminoacridine;
 DMSO: Dimethylsulfoxide.

TABLE 3
 BIS HEXAFLUOROPHOSPHATE SALT (BIS PF6 SALT): MUTAGENIC POTENTIAL IN
 THE SALMONELLA/MICROSOME (AMES) ASSAY

RESULTS OF THE SALMONELLA/REVERSE MUTATION ASSAY
 TEST 1 WITH S9

Test Substance (mg/plate)	Plate Counts			Mean	Standard Deviation
	1	2	3		
STRAIN TA98					
DMSO (55)	36	27	40	34	6.7
2-AA (2.5µg)	1576	1907	1504	1662	214.9
0.10	42	37	164	81	71.9
0.30	326	51	7	128	172.9
1.0	52	98	362	171	167.3
3.0	26	38	33	32	6.0
5.0	6 (S)	7 (S)	10 (S)	8 (T)	2.1
STRAIN TA100					
DMSO (55)	86	98	77	87	10.5
2-AA (2.5µg)	1527	1625	1877	1676	180.6
0.10	98	98	90	95	4.6
0.30	129	144	139	137	7.6
1.0	141	173	132	149	21.5
3.0	126	114	113	118	7.2
5.0	91	134	141	122	27.1
STRAIN TA1535					
DMSO (55)	8	14	19	14	5.5
2-AA (2.5µg)	183	185	179	182	3.1
0.10	7	15	12	11	4.0
0.30	13	12	20	15	4.4
1.0	16	11	9	12	3.6
3.0	7	5	5	6 (T)	1.2
5.0	3 (S)	5 (S)	3 (S)	4 (T)	1.2
STRAIN TA1537					
DMSO (55)	10	12	8	10	2.0
2-AA (2.5µg)	205	187	213	202	13.3
0.10	4	10	4	6	3.5
0.30	5	4	12	7	4.4
1.0	3	14	22	13	9.5
3.0	2	3	5	3 (T)	1.5
5.0	1 (S)	1 (S)	T	1 (T)	0.0
STRAIN TA1538					
DMSO (55)	21	15	18	18	3.0
2-AA (2.5µg)	1263	1311	1155	1243	79.9
0.10	24	14	17	18	5.1
0.30	17	14	25	19	5.7
1.0	16	20	18	18	2.0
3.0	22	20	12	18	5.3
5.0	19	25	21	22	3.1

T: Toxic; absence of background lawn, or mean number of colonies < 1/2 solvent control value.
 S: Sparse growth of background lawn.
 2-AA: 2-Aminoanthracene; DMSO: Dimethylsulfoxide.

TABLE 4
 BIS HEXAFLUOROPHOSPHATE SALT (BIS PF6 SALT): MUTAGENIC POTENTIAL IN
 THE SALMONELLA/MICROSOME (AMES) ASSAY

RESULTS OF THE SALMONELLA/REVERSE MUTATION ASSAY
 TEST 2 WITHOUT S9

Test Substance (mg/plate)	Plate Counts			Mean	Standard Deviation
	1	2	3		
STRAIN TA98					
DMSO (55)	12	6	16	11	5.0
4-NPD (0.01)	483	451	342	425	73.9
0.03	58	17	17	31	23.7
0.10	21	38	153	71	71.8
0.30	254	24	30	103	131.1
1.0	58	73	61	64	7.9
3.0	1 (S)	11 (S)	4 (S)	5 (T)	5.1
STRAIN TA100					
DMSO (55)	60	67	84	70	12.3
NaN ₃ (0.01)	899	858	1017	925	82.5
0.03	55	69	76	67	10.7
0.10	80	64	84	76	10.6
0.30	62	55	65	61	5.1
1.0	126	104	99	110	14.4
3.0	60	122	120	101	35.2
STRAIN TA1535					
DMSO (55)	2	6	5	4	2.1
NaN ₃ (0.01)	984	863	913	920	60.8
0.03	7	7	6	7	0.6
0.10	6	7	7	7	0.6
0.30	11	8	11	10	1.7
1.0	6	4	4	5	1.2
3.0	0 (S)	1 (S)	0 (S)	0 (T)	0.6
STRAIN TA1537					
DMSO (55)	5	3	3	4	1.2
9-AA (0.06)	75	130	81	95	30.2
0.03	9	4	8	7	2.6
0.10	6	7	4	6	1.5
0.30	4	3	6	4	1.5
1.0	1 (S)	2 (S)	3	2	1.0
3.0	T	T	T	T	-
STRAIN TA1538					
DMSO (55)	11	9	8	9	1.5
4-NPD (0.01)	111	96	101	103	7.6
0.03	10	13	6	10	3.5
0.10	8	8	10	9	1.2
0.30	14	8	17	13	4.6
1.0	5	17	10	11	6.0
3.0	5	8	11	8	3.0

T: Toxic; absence of background lawn, or mean number of colonies < 1/2 solvent control value.
 S: Sparse growth of background lawn.
 4-NPD: 4-Nitro-o-phenylenediamine; NaN₃: Sodium azide; 9-AA: 9-Aminoacridine;
 DMSO: Dimethylsulfoxide.

TABLE 5
 BIS HEXAFLUOROPHOSPHATE SALT (BIS PF6 SALT): MUTAGENIC POTENTIAL IN
 THE SALMONELLA/MICROSOME (AMES) ASSAY

RESULTS OF THE SALMONELLA/REVERSE MUTATION ASSAY
 TEST 2 WITH S9

Test Substance (mg/plate)	Plate Counts			Mean	Standard Deviation
	1	2	3		
STRAIN TA98					
DMSO (55)	25	33	24	27	4.9
2-AA (2.5µg)	1663	1700	1713	1692	25.9
0.03	17	31	53	34	18.1
0.10	140	33	40	71	59.9
0.30	40	60	417	172	212.1
1.0	79	384	65	176	180.3
3.0	T	T	T	T	-
STRAIN TA100					
DMSO (55)	78	88	85	84	5.1
2-AA (2.5µg)	1456	1292	1288	1345	95.9
0.03	78	109	88	92	15.8
0.10	89	109	87	95	12.2
0.30	80	157	90	109	41.9
1.0	110	129	110	116	11.0
3.0	108	110	128	115	11.0
STRAIN TA1535					
DMSO (55)	9	12	4	8	4.0
2-AA (2.5µg)	105	116	126	116	10.5
0.03	4	7	9	7	2.5
0.10	8	12	6	9	3.1
0.30	8	5	10	8	2.5
1.0	4	11	8	8	3.5
3.0	7 (S)	5 (S)	9 (S)	7	2.0
STRAIN TA1537					
DMSO (55)	7	7	9	8	1.2
2-AA (2.5µg)	182	168	192	181	12.1
0.03	7	6	6	6	0.6
0.10	21	8	7	12	7.8
0.30	10	4	14	9	5.0
1.0	12	9	9	10	1.7
3.0	2 (S)	4 (S)	T	3 (T)	1.2
STRAIN TA1538					
DMSO (55)	9	16	16	14	4.0
2-AA (2.5µg)	1061	950	1000	1004	55.6
0.03	11	10	12	11	1.0
0.10	18	17	18	18	0.6
0.30	19	18	15	17	2.1
1.0	22	14	17	18	4.0
3.0	23	19	26	23	3.5

T: Toxic; absence of background lawn, or mean number of colonies < 1/2 solvent control value.
 S: Sparse growth of background lawn.
 2-AA: 2-Aminoanthracene; DMSO: Dimethylsulfoxide.

Bis Hexafluorophosphate Salt (Bis PF6 Salt): Mutagenic Potential in the Salmonella/Microsome (Ames) Assay
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QUALITY ASSURANCE UNIT INSPECTION SUMMARY

<u>Inspection Date(s)</u>	<u>Inspection Type</u>	<u>Date QAU Report Issued To</u>	
		<u>Study Director</u>	<u>Management</u>
09-20-93	PROTOCOL	09-20-93	09-20-93
12-02-93	EVENT-CELL COUNTING	12-02-93	12-02-93
01-21-94	RAW DATA, REPORT	01-24-94	03-22-94
03-22-94	ARCHIVES	03-22-94	03-22-94


 Linda J. Calisti, Manager

3/22/94
 Date

Good Laboratory Practices/Quality Assurance

**Bis Hexafluorophosphate Salt (Bis PF6 Salt): Mutagenic Potential in
the Salmonella/Microsome (Ames) Assay**

Protocol

(9 Pages)



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PROTOCOL

TITLE: Bis Hexafluorophosphate Salt (Bis PF₆ Salt): Mutagenic Potential in the Salmonella/Microsome (Ames) Assay

BRRC PROJECT ID: 93U1321

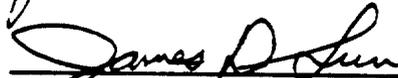
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Union Carbide Corporation
39 Old Ridgebury Road
Danbury, CT 06817-0001

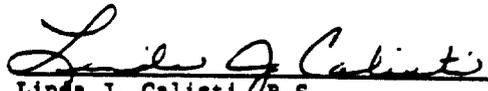
TESTING FACILITY: Bushy Run Research Center (BRRC)
Union Carbide Corporation
6702 Mellon Road
Export, PA 15632-8902

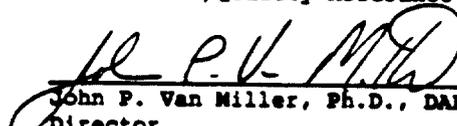
Reviewed and Approved by:

Bushy Run Research Center:

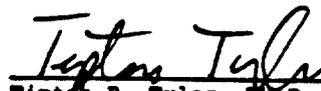
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Richard C. Wise Date
Manager of Product Safety

Union Carbide Chemicals and Plastics Company Inc.
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OBJECTIVE

The objective of this study is to assess the mutagenic potential of Bis Hexafluorophosphate Salt (Bis PF6 Salt) in the Salmonella/microsome mutation assay.

Design and Basis for the Study

Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538 will be used for the mutagenicity assay. Five dose levels of the test substance, spaced at approximately half-log intervals, will be selected for treatments both in the absence and in the presence of a rat liver S9 metabolic activation system. All 5 strains will be treated in triplicate with the vehicle control substance, an appropriate positive control substance, and 5 dose levels of the test substance using the plate incorporation method. Treated cultures will be incubated at approximately 37°C for 48-72 hours. Two independent assays will be performed.

The portions of this study conducted at BRRC will be in compliance with the following guidelines and standards:

U.S. Environmental Protection Agency (EPA), Toxic Substances Control Act (TSCA) Good Laboratory Practice (GLP) Standards, 40 CFR Part 792.

Organisation for Economic Co-operation and Development (OECD) (1981).
OECD Principles of Good Laboratory Practice, C(81)30(Final).

PERSONNEL

All personnel who participate in the conduct of the study will be documented in the raw data.

PROJECT DATES

Proposed Starting Dates of Test Substance Administration

Test 1	October 5, 1993
Test 2	October 12, 1993

Proposed Completion Dates

Test 1	October 7, 1993
Test 2	October 14, 1993

<u>Proposed Date for Submission of Draft Final Report</u>	November 12, 1993
---	-------------------

METHODSTest Substance

Product Name Bis Hexafluorophosphate Salt

Chemical Name (Thiodi-4,1-phenylene)bis[diphenylsulfonium] bishexafluorophosphate

CAS Registry Number 74227-35-3

Source Union Carbide Corporation, Bound Brook, NJ

Sponsor Identification Number Not available

BRRC Sample Number 56-352

Description White crystals according to the Material Safety Data Sheet (MSDS).

Purity Purity information will be provided by the Sponsor and documented in the raw data and final report.

Stability The test substance is considered to be stable at room temperature for the duration of the study.

Storage Conditions The test substance will be stored under well-ventilated conditions at ambient temperature in an appropriate storage area.

Quantity A two ounce amber bottle (gross weight 84.56 g) of the test substance was received for the proposed study [ies]. After the assigned study [ies] has been completed, all unused test substance will be returned to the Sponsor.

Reserve Sample A reserve sample will not be retained at BRRC.

Safety An MSDS supplied by the Sponsor will be reviewed by all relevant personnel before their participation in the study. This review will be documented. Normal precautions for untested substances will be used. These procedures include the use of disposable Tyvek® or plastic coats or smocks and disposable gloves. Eye protection will include the use of safety glasses at all times. All manipulations of the test substance will be performed in an approved safety hood. Access to the laboratory will be restricted to essential personnel while testing is in progress.

Bacterial Cultures

Strains Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538

Supplier Dr. Bruce N. Ames, University of California, Berkeley, CA

Rationale Salmonella strains TA98, TA100, TA1535, and TA1537 are the strains of choice for this assay. Strain TA1538 has been selected because it occasionally detects mutagens that strain TA98 does not.

Culture Conditions The test strains are stored as frozen broth cultures at -80°C. Daily working cultures are prepared fresh for each test day by inoculating bacteria from frozen working cultures into nutrient broth. Cultures are incubated overnight (10-15 hours) at 37°C with gentle agitation. The daily working cultures are kept on ice throughout the test day.

A bacterial titer will be determined by plating serial dilutions of the daily working cultures on nutrient agar plates and counting the resultant colonies approximately 24-48 hours after plating. The integrity of the genetic markers in each bacterial strain will be verified each time frozen permanent stock cultures or frozen working cultures are prepared.

Administration of Test Substance

Metabolic Activation S9 liver homogenate, prepared from Aroclor 1254-induced rats, is purchased from Microbiological Associates. The complete S9 metabolic activation system contains the following: 8 mM MgCl₂, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9. Fresh S9 mix is prepared each day of testing and kept at 0-4°C.

Vehicle Control Substances The preferred vehicle control substances are water (CAS No. 7732-18-5), dimethyl sulfoxide (CAS No. 67-68-5), ethanol (CAS No. 64-17-5), methanol (CAS No. 67-56-1), and acetone (CAS No. 67-64-1). Other substances may be used as long as they are compatible with the test substance, noncytotoxic, and nonmutagenic in this test system. The chosen vehicle control substance will be documented in the raw data.

**Positive Control
Substances**

Both activation-dependent and activation-independent positive controls will be used. The activation-independent controls are 4-nitro-o-phenylenediamine (CAS No. 99-56-9) for TA98 and TA1538, sodium azide (CAS No. 26628-22-8) for TA100 and TA1535, and 9-aminoacridine (CAS No. 90-45-9) for TA1537. The activation-dependent control is 2-aminoanthracene (CAS No. 613-13-8) for all strains. The appropriate concentrations are determined from prior dose-response experiments with these substances. Analyses for stability, homogeneity, and concentration of the positive control substances in dosing solutions will not be performed.

**Preparation of
Dosing Solutions**

Dosing solutions will be prepared by mixing appropriate amounts of the test substance (g) (uncorrected for percent active ingredient) and vehicle. The concentration of the test substance in dosing solutions will be verified gravimetrically. Mixing procedures and storage conditions will be specified in the raw data and final report. Dosing solutions will be prepared on the day they are used and will be discarded by the end of that day. Analyses for stability, homogeneity, and concentration of the test substance in dosing solutions will not be performed.

**Preliminary
Toxicity
Testing**

Five to 10 dose levels of the test substance will be tested on strain TA100 with and without S9 metabolic activation to evaluate its cytotoxicity. Generally, the maximum dose tested in the cytotoxicity assay will be 10 mg/plate. However, if a solution, suspension, or emulsion cannot be prepared at the maximum dose level, then a lower dose level will be chosen. A 2 ml volume of complete top agar (6 g/l agar, 5 g/l NaCl, 0.05 mM L-histidine and 0.05 mM D-biotin) will be added to the required number of sterile tubes. A 100 µl aliquot of strain TA100 will be added to each tube followed by an aliquot of the vehicle control substance or the appropriate dilution of the test substance. Then, either 0.5 ml of S9 mixture or 0.5 ml of phosphate buffered saline (PBS) will be added for testing with or without metabolic activation. The mixture will be vortexed briefly and poured onto a Vogel-Bonner Medium E (VBE) agar plate. The top agar will be allowed to harden and then the plates will be incubated at 37°C for at least 48 hours. The plates will be examined to determine the condition of their background lawns. Lawns will be recorded as either confluent, sparse, or absent. Confluence indicates no toxicity, sparse growth indicates moderate toxicity, and no growth indicates extreme toxicity. Revertant colonies will be counted

either manually or with an Artek Model No. 880 colony counter. A significant ($\geq 50\%$) reduction in the number of revertant colonies/plate will be considered a toxic effect.

Mutagenicity Testing

A minimum of 5 dose levels, spaced approximately at half-log intervals, will be tested. The highest dose level will be selected to produce either a significant ($\geq 50\%$) reduction in the number of revertant colonies as compared to the vehicle control or a significant inhibition of background lawn growth (sparse or absent lawns). The highest dose level for noncytotoxic test substances will be 10 mg/plate unless the test substance cannot be kept in solution, suspension or emulsion at that dose level. At least 2 dose levels should not be cytotoxic. To a sterile tube containing 2 ml of complete top agar, a 100 μ l aliquot of the appropriate bacterial culture will be added followed by 50 μ l of the appropriate control, 50 μ l of vehicle, or 50 μ l of test substance solution. Either 0.5 ml of S9 mix or 0.5 ml of PBS will be added for tests with or without metabolic activation. The top agar mixture will be vortexed briefly and then poured onto a VBE plate. Sterility testing will be performed on the PBS, the S9 mix, all vehicles, and the highest concentration of test substance. The plates will be transferred to a darkened 37°C incubator after hardening and incubated for 48-72 hours.

Experimental Evaluations

Data Collection

The number of bacterial colonies/plate will be counted either manually or using an Artek Model No. 880 Colony Counter and recorded. The counter will be calibrated prior to use to verify counting accuracy.

The background lawn on each plate will also be evaluated. If the background lawn on a plate is sparse, an "S" will be entered beside the colony count for that plate. If the background lawn on a plate is absent, there will be no colony count.

The mean number of revertant colonies and the standard deviation of that mean will be calculated for each dose level and bacterial strain. Sparse plates will be used to calculate the mean number of revertant colonies. Whether sparse dose levels are used in the evaluation of mutagenicity will be decided by the Study Director on a case-by-case basis. When the mean number of revertant colonies/plate is less than 50% that of the vehicle control, the dose level is labeled toxic and a "T" is entered beside the mean on the report table.

Data Interpretation

The mean spontaneous reversion frequencies for the vehicle controls should be within this laboratory's historical ranges. The positive controls should demonstrate that the test system is responsive to known mutagens. A test substance will be considered a bacterial mutagen if it produces a dose-related increase in the mean reversion frequency of at least one bacterial strain as compared to the vehicle control for that strain. Furthermore, at least one of those doses must produce a mean reversion frequency at least twice that of the vehicle control.

Alternatively, a test substance will be considered a bacterial mutagen if there is a reproducible increase in the mean number of revertant colonies at a single dose level of at least 2-fold compared to the vehicle control. A test substance will not be considered a bacterial mutagen if it does not meet these criteria. Increases which are not dose related, or which cannot be reproduced, will be considered negative test results.

ALTERATION OF PROTOCOL

Alterations to this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, such change will be honored. However, it then becomes the responsibility of the Sponsor to follow such verbal change with a written verification. BRRC reserves the right to revise the protocol or deviate therefrom solely at the discretion of the Study Director if prior approval of the Sponsor cannot be obtained and the integrity of the study is considered in jeopardy. In this event, the Sponsor will be notified of the alteration as soon as possible, and documentation of the change will be the responsibility of the Study Director.

RETENTION OF RECORDS

All raw data, documentation, the protocol and any amendments, and a copy of the final report generated as a result of this study will be retained in the BRRC Archives for at least 5 years.

Following the retention period specified above, the Sponsor will be contacted and given the option of taking receipt, destroying, or arranging for other storage of the data and materials. All data and materials mentioned above will remain the sole property of the Sponsor and can be removed from BRRC at the Sponsor's discretion.

REPORTS**Draft Final Report**

An unaudited draft of the final report will be submitted to the Sponsor approximately 1 month after the completion of the testing. This report will be a comprehensive report which will include all information necessary to

provide a complete and accurate description and evaluation of the test procedures and results. It will include: a summary and appropriate text discussions of the experimental design, materials and methods, and results. In addition, it will contain appendices with other pertinent information.

Final Report

The draft final report will be reviewed by the Sponsor, and comments on the report will be provided to BRRRC within 4 weeks from the date of submission of the draft version. BRRRC will consider these comments in preparing the final report. Assuming the Sponsor's comments are received at the specified time and no major revisions are required, BRRRC will submit a final report within 8 weeks of issuance of the draft report.

The final report will be audited by the Quality Assurance Unit and contain a signed quality assurance statement. It will conform to the formatting specifications of EPA PR notice 86-5.

GOOD LABORATORY PRACTICE COMPLIANCE

BRRRC, through the administration of a quality assurance program by the Good Laboratory Practice Committee and Quality Assurance Unit, assures compliance of all phases of studies conducted at BRRRC with existing regulations and generally accepted good laboratory practices.

The study will be subjected to periodic inspections and the final report will be reviewed by the BRRRC Quality Assurance Unit.

REFERENCES

- Ames, B. N., McCann, J., and Yamasaki, F. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Research* 31, 347-364.
- Kier, L. D., Brusick, D. J., Auletta, A. E., VonHalle, E. S., Brown, M. M., Simmon, V. F., Dunkel, V., McCann, J., Mortelmans, K., Prival, M., Rao, T. K., and Ray, V. (1986). The Salmonella typhimurium/Mammalian microsomal assay. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutation Research* 168, 69-240.
- Maron, D. M. and Ames, B. N. (1983). Revised Methods for the Salmonella mutagenicity test. *Mutation Research* 113, 173-215.



BUSHY RUN RESEARCH CENTER

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STUDY TITLE

Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential
in the Salmonella/Microsome (Ames) Assay

TEST SUBSTANCE

Thio Hexafluorophosphate Salt (Thio PF6 Salt)

DATA REQUIREMENT

Not Applicable

AUTHOR

J. S. Vergnes

STUDY COMPLETION DATE

March 22, 1994

PERFORMING LABORATORY

Bushy Run Research Center (BRRC)
Union Carbide Corporation (UCC)
6702 Mellon Road
Export, PA 15632-8902

LABORATORY PROJECT ID

93U1322

SPONSOR

Solvents and Coatings Materials Division
Union Carbide Corporation
39 Old Ridgebury Road
Danbury, CT 06817-0001

**Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential
in the Salmonella/Microsome (Ames) Assay**

CONFIDENTIALITY STATEMENT

This report is Union Carbide Corporation Business Confidential and is not to be released outside of the Corporation without the written consent of the Sponsor.

**Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential
in the Salmonella/Microsome (Ames) Assay**

COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

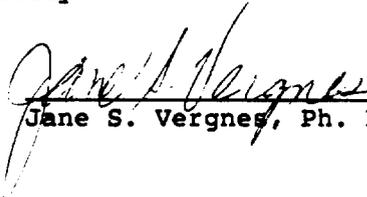
The portions of this study conducted by BRRC meet the requirements of the following Good Laboratory Practice Standards: Toxic Substances Control Act (TSCA), 40 CFR Part 792; and Organisation for Economic Co-operation and Development (OECD), C(81)30(Final) with exceptions. These exceptions are:

1. Analyses for stability and homogeneity of the test and control substances in the dosing solutions were not conducted.
2. The vehicle and positive control substances were not analyzed for chemical purity, stability, or uniformity.

The physical and chemical characterization of the test substance was not performed at BRRC and is considered the responsibility of the Sponsor.

These exceptions are not expected to compromise the integrity of the results and conclusion of the study.

Study Director:


Jane S. Vergnes, Ph. D.

3/22/94
Date

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Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential
in the Salmonella/Microsome (Ames) Assay

SUMMARY

Thio Hexafluorophosphate Salt (Thio PF6 Salt, CAS No. 68156-13-8) was tested for potential mutagenic activity using the Salmonella/microsome bacterial mutagenicity assay (Ames test). Doses were chosen on the basis of data obtained in a preliminary cytotoxicity study with strain TA100. Thio PF6 Salt was nontoxic to strain TA100 at doses of 0.30 mg/plate or less in the absence of a rat liver S9 metabolic activation system and at doses of 1.0 mg/plate or less in the presence of S9 activation. Sparse background lawn growth was observed at 1.0 mg/plate Thio PF6 Salt in the absence of S9 activation and at 3.0 mg/plate in the presence of S9 activation. Complete absence of background lawn growth was observed at Thio PF6 Salt doses of 5.0 mg/plate or greater, both in the absence and in the presence of metabolic activation.

Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538 were used for the mutagenicity assay. On the basis of the preliminary cytotoxicity study, 5 dose levels of Thio PF6 Salt, ranging from 0.10 to 5.0 mg/plate for the first mutagenicity test and from 0.01 to 1.0 mg/plate for the repeat mutagenicity test, were selected for treatments both in the absence and in the presence of metabolic activation and spaced at approximately half-log intervals. All 5 bacterial strains were treated in triplicate with the vehicle control substance (dimethylsulfoxide), an appropriate positive control substance, and 5 dose levels of Thio PF6 Salt using the plate incorporation method. Treated cultures were incubated at 37°C for 48 to 72 hours.

No mutagenic activity was observed in strains TA100, TA1535, TA1537, or TA1538, either in the absence or in the presence of S9 activation. These results were observed in 2 independent experiments. Thio PF6 Salt was mutagenic to strain TA98, both in the absence and in the presence of S9. Increases in the mean number of colonies/plate ranged from approximately 5-fold at 0.01 mg/plate to 8- to 11-fold at 0.1 mg/plate in the absence of S9. Increases in the mean number of colonies/plate were approximately 7-fold at 0.01 mg/plate and 11- to 15-fold at 0.10 mg/plate in the presence of S9. All 5 bacterial strains exhibited mutagenic responses to the appropriate positive control substances. Vehicle controls were also tested with each strain, and the mean numbers of spontaneous revertants were considered acceptable.

To summarize, Thio PF6 Salt did not produce consistent, dose-related mutagenic effects in Salmonella strains TA100, TA1535, TA1537, or TA1538, either in the absence or in the presence of a rat liver S9 metabolic activation system. Thio PF6 Salt produced reproducible, dose-related increases in the number of colonies/plate of approximately 5-fold or greater in strain TA98 both in the absence and in the presence of S9 activation. Therefore, Thio PF6 Salt was considered mutagenic in strain TA98 under the conditions of this in vitro screening test.

OBJECTIVE

The objective of this study was to assess the mutagenic potential of Thio PF6 Salt in the Salmonella/microsome mutation assay.

BACKGROUND INFORMATION

The Salmonella/microsome mutation assay is a microbial screening test which detects the ability of chemicals to cause genetic alterations in the histidine gene of selected indicator strains of Salmonella. The indicator strains used for this test are all histidine-requiring mutants obtained from Dr. Bruce Ames, University of California, Berkeley, CA. Histidine-independent colonies can arise either spontaneously or by the mutagenic action of a chemical or physical agent. Base substitution mutagens cause a base change in the DNA molecule at the site of the original mutation or at a second site in the DNA which suppresses the original mutation. Frameshift mutagens cause the insertion or deletion of one or more base pairs in the DNA molecule. The frequency of induced or spontaneous reversion to histidine independence can be quantitated by plating the indicator strains on minimal agar and counting the number of revertant colonies.

DOSE SELECTION

Doses were selected on the basis of a preliminary cytotoxicity study, which is described in this report. The doses chosen for the first test were 0.10, 0.30, 1.0, 3.0, and 5.0 mg/plate, both in the absence and in the presence of S9. The doses chosen for the second test were 0.01, 0.03, 0.10, 0.30, and 1.0 mg/plate, both in the absence and in the presence of S9.

MATERIALS AND METHODS

The protocol detailing the design and conduct of this study is presented in Appendix 1.

Test Substance

One 2-ounce amber bottle (gross weight 84.81 g) of Thio Hexafluorophosphate Salt (Thio PF6 Salt), Lot No. 18687-35, CAS No. 68156-13-8, was received on September 2, 1993, from Union Carbide Corporation, Bound Brook, NJ, and assigned BRRC Sample No. 56-353. The test substance was a white, crystalline solid. The test substance was stored at room temperature. The purity of the test substance was not provided by the Sponsor. Test substance stability was not confirmed by BRRC.

Positive Control Substances

Two 1 g bottles of 4-nitro-o-phenylenediamine (4 NPD), Lot No. 40-13A, CAS No. 99-56-9, were received on November 21, 1989, from Chem Service, Inc., and assigned BRRC Sample No. 52-729 A and B. It was a red crystalline solid and was stored at less than 0°C. One 25 g bottle of sodium azide (NaN₃), Lot No. 29F-0533, CAS No. 26628-22-8, was received on November 21, 1989, from Sigma Chemical Co., and assigned BRRC Sample No. 52-727. It was a white crystalline solid and was stored at less than 0°C. One 5 g bottle of 9-aminoacridine, (9-AA), Lot No. 96F-05641, CAS No. 90-45-9, was received on November 21, 1989,

examination was also made of the background lawn on each plate. If no background lawn was observed, the absence of the background lawn was noted and no plate count was recorded. If the background lawn was sparse, colonies were counted and the colony count was used to calculate a mean and standard deviation. A reduction in the number of spontaneous revertant colonies is also an indication of toxicity. A dose level was labeled "toxic" when the mean number of colonies/plate was less than one half the mean for the vehicle control.

Data Analyses

Various models of calculators, computers, and computer programs may have been used to analyze data for this study. Since various models round or truncate numbers differently, values in some tables may differ slightly from those in other tables or from independently calculated data. The integrity of the study and interpretation of the data were unaffected by these differences.

A test substance was considered a bacterial mutagen if it consistently produced a dose-related increase in the mean reversion frequency of at least one bacterial strain as compared to the vehicle control for that strain. At least one of those doses must have produced a mean reversion frequency at least twice that of the vehicle control. Alternatively, a test substance was considered a bacterial mutagen if there was a reproducible increase in the mean number of revertant colonies at a single dose level of at least 2-fold compared to the vehicle control. Increases in the mean reversion frequency that were not dose related, or could not be reproduced, were considered negative test results.

RETENTION OF RECORDS

All raw data, documentation, the protocol, and a copy of the final report generated as a result of this study will be retained in the BRRC Archives for at least 5 years.

RESULTS AND DISCUSSION

The results of the preliminary cytotoxicity test in the absence and in the presence of an S9 metabolic activation system are shown in Table 1. Thio PF6 Salt was nontoxic to strain TA100 at doses of 0.30 mg/plate or less in the absence of an S9 rat liver metabolic activation system. In the presence of a rat liver S9 metabolic activation system, Thio PF6 Salt was nontoxic to strain TA100 at doses of 1.0 mg/plate or less. Sparse background lawn growth was observed at 1.0 mg/plate Thio PF6 Salt in the absence of S9 activation and at 3.0 mg/plate in the presence of S9 activation. Complete absence of background lawn growth was observed at Thio PF6 Salt doses of 5.0 mg/plate or greater, both in the absence and in the presence of metabolic activation.

The results of the definitive mutagenicity tests are shown in Tables 2 and 4 (without activation) and in Tables 3 and 5 (with activation). No mutagenic activity was observed in strains TA100, TA1535, TA1537, or TA1538, either in the absence or in the presence of S9 activation. These results were observed in 2 independent experiments.

Thio PF6 Salt was mutagenic to strain TA98, both in the absence and in the presence of S9. Increases in the mean number of colonies/plate ranged from approximately 5-fold at 0.01 mg/plate to 8- to 11-fold at 0.1 mg/plate in the absence of S9. Increases in the mean number of colonies/plate were approximately 7-fold at 0.01 mg/plate and 11- to 15-fold at 0.10 mg/plate in the presence of S9.

Interpretation of the test data is problematic for 2 reasons. First, there is a discrepancy between the approximately 2 to 5-fold increases in the numbers of revertant colonies in strain TA100 treated with Thio PF6 Salt during the cytotoxicity test, both in the absence and in the presence of S9, and the irreproducibility of this result in either repetition of the definitive test. Second, although treatment with Thio PF6 Salt consistently results in increases in the mean number of colonies/plate in strain TA98, both in the absence and in the presence of S9, the variability among the triplicate plates at each dose level is unusually large. However, the unusual characteristics of this data set are consistent with those of 2 related test substances (BRRC Reports 93U1321 and 93U1328). No other test substances tested at BRRC during the same time interval exhibited these unusual characteristics. For these reasons, Thio PF6 Salt was not considered to be mutagenic in strain TA100, but was considered mutagenic in strain TA98.

All 5 bacterial strains exhibited mutagenic responses to the appropriate positive control substances. Vehicle controls were also tested with each strain, and the mean numbers of spontaneous revertants were considered acceptable. All positive and negative controls were tested concurrently with the test substance. Concurrent sterility testing showed that the S9 mix, PBS, the test substance and the vehicle were sterile.

CONCLUSION

Thio PF6 Salt did not produce consistent, dose-related mutagenic effects in Salmonella strains TA100, TA1535, TA1537, or TA1538, either in the absence or in the presence of a rat liver S9 metabolic activation system. Thio PF6 Salt produced reproducible, dose-related increases in the number of colonies/plate of approximately 5-fold or greater in strain TA98 both in the absence and in the presence of S9 activation. These results were observed in 2 independent tests. Under the conditions of this Salmonella/microsome mutagenicity assay, Thio PF6 Salt was considered mutagenic in strain TA98.

from Sigma Chemical Co., and assigned BRRC Sample No. 52-728. It was a yellow crystalline solid and was stored at less than 0°C. One 1 g bottle of 2-aminoanthracene (2-AA), Lot No. 121H3475, CAS No. 613-13-8, was received on May 20, 1992, from Sigma Chemical Co., and assigned BRRC Sample No. 55-144. It was a crystalline solid and was stored at less than 0°C.

Vehicle Control Substance

A 1 liter bottle of dimethylsulfoxide (DMSO), Lot No. BD 469, CAS No. 67-68-5, was received on December 3, 1992, from Baxter Diagnostics, Inc., and assigned BRRC Sample No. 55-386. It was a nonviscous liquid and was stored at room temperature.

Metabolic Activation System

Rat liver S9 homogenate, prepared from Aroclor 1254-induced rats, was purchased from Microbiological Associates. The complete S9 metabolic activation system contained the following: 8 mM MgCl₂, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9. Fresh S9 mix was prepared each day of testing and kept at 0-4°C.

Study Organization

Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538 were treated in triplicate with the vehicle control substance, an appropriate positive control substance, and 5 dose levels of Thio PF6 Salt both in the absence and in the presence of a rat liver S9 metabolic activation system using the plate incorporation method. Treated cultures were incubated at 37°C for 48-72 hours. Two independent repetitions of the complete assay were performed. The following table summarizes the testing schedule.

	<u>First Test</u>	<u>Second Test</u>
Test Initiated:	November 17, 1993	December 1, 1993
Test Completed:	November 19, 1993	December 3, 1993

Bacterial Strains and Culture Conditions

Test strains were stored as frozen permanent cultures at -80°C. Working cultures were prepared fresh for each test day by inoculating bacteria from frozen working cultures into nutrient broth. Cultures were incubated overnight (10 to 15 hours) at 37°C with gentle agitation. The working cultures were kept on ice throughout the test day.

A bacterial titer was determined by plating serial dilutions of the working cultures on nutrient agar plates and counting the resultant colonies approximately 24-48 hours after plating. The integrity of the genetic markers in each bacterial strain was verified each time frozen stock cultures were prepared.

Dosing Solution Preparation

Each dosing solution was prepared by dissolving the appropriate amount of Thio PF6 Salt in DMSO. Concentrations were not adjusted for percent of active ingredient of the test substance. Dosing solutions were prepared daily and stored at room temperature prior to use.

The activation-independent controls were 4-NPD for TA98 and TA1538, NaN₃ for TA100 and TA1535, and 9-AA for TA1537. The activation-dependent control was 2-AA for all strains. 4-NPD, 9-AA, and 2-AA were prepared by dissolving the control substances in dimethylsulfoxide. NaN₃ was prepared by dissolving it in deionized, distilled water. Dosing solutions were made by diluting concentrated stock solutions of the control substances. Dosing solutions were stored at less than 0°C for no longer than 3 months and concentrated stock solutions were stored at less than 0°C for no longer than 1 year.

Cytotoxicity Testing

Ten dose levels of Thio PF6 Salt ranging from 0.001 to 10.0 mg/plate were tested on strain TA100 with and without S9 metabolic activation to evaluate the cytotoxicity of the test substance. A 2 ml volume of complete top agar (6 g/l agar, 5 g/l NaCl, 0.05 mM L-histidine and 0.05 mM D-biotin) was added to the required number of sterile tubes. A 100 µl aliquot of strain TA100 was added to each tube followed by an aliquot of the appropriate dilution of Thio PF6 Salt. Then, either 0.5 ml of S9 mixture or 0.5 ml of phosphate buffered saline (PBS) was added. The mixture was vortexed briefly and poured onto a Vogel-Bonner Medium E agar plate (VBE plate). The top agar was allowed to harden and then the plates were incubated at 37°C for at least 48 hours. The plates were examined to determine the condition of their background lawns. Growth was recorded as either confluent, sparse, or absent. Confluence indicated nontoxicity, sparse growth indicated moderate toxicity, and no growth indicated extreme toxicity. Revertant colonies were counted either manually or using an Artek Model No. 880 colony counter.

Mutagenicity Testing

Based on the results of preliminary cytotoxicity studies, 5 dose levels of Thio PF6 Salt ranging from 0.10 to 5.0 mg/plate for the first test and from 0.01 to 1.0 mg/plate for the second test, were spaced approximately at half-log intervals. To a sterile tube containing 2 ml of complete top agar, a 100 µl aliquot of the appropriate bacterial culture was added followed by 50 µl of the appropriate control, 50 µl of vehicle, or 50 µl of test substance solution. Either 0.5 ml of S9 mix or 0.5 ml of PBS was added for tests with or without metabolic activation, respectively. The top agar mixture was vortexed briefly and then poured onto a VBE plate. Sterility testing was performed on the PBS, the S9 mix, the vehicle, and the highest concentration of test substance. The plates were transferred to a darkened 37°C incubator after hardening and incubated for 48-72 hours.

Observations and Measurements

Bacterial colonies were counted either manually or using an Artek Model No. 880 Colony Counter. The counter was calibrated for each test to check counting accuracy. The number of colonies/plate was counted and recorded. An

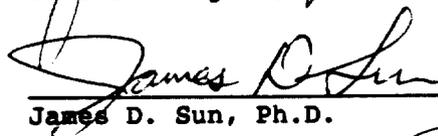
REVIEW AND APPROVAL

Study Director:


 Jane S. Vergnes, Ph.D.

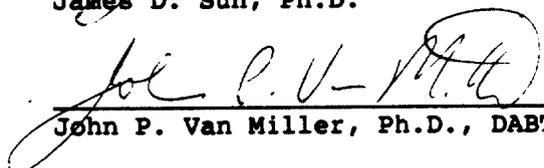
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 Date

Associate Director:


 James D. Sun, Ph.D.

 3-22-94
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Director:


 John P. Van Miller, Ph.D., DABT

 3-22-94
 Date
KEY PERSONNEL

Study Director:

J. S. Vergnes

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TABLE 1
 THIO HEXAFLUOROPHOSPHATE SALT (THIO PF6 SALT): MUTAGENIC POTENTIAL
 IN THE SALMONELLA/MICROSOME (AMES) ASSAY

CYTOTOXICITY TESTING RESULTS IN STRAIN TA100

Test Substance (mg/plate)	Test without S9		Test with S9	
	Growth of Lawn*	Plate Count**	Growth of Lawn*	Plate Count**
0.001	C	62	C	98
0.003	C	92	C	97
0.01	C	76	C	110
0.03	C	92	C	110
0.10	C	89	C	115
0.30	C	176	C	199
1.0	S	80	C	114
3.0	S	471	S	90
5.0	A	-	A	-
10.0	A	-	A	-
Vehicle - DMSO (55 mg/plate)	C	93	C	91

*Growth of the bacterial lawn was assessed with a dissecting microscope 48 hours after treatment.
 **One plate/test concentration.

Abbreviations: C-confluent growth; S-sparse growth; A-absence of background lawn;
 DMSO-dimethylsulfoxide.

TABLE 2
 THIO HEXAFLUOROPHOSPHATE SALT (THIO PF6 SALT): MUTAGENIC POTENTIAL
 IN THE SALMONELLA/MICROSOME (AMES) ASSAY

RESULTS OF THE SALMONELLA/REVERSE MUTATION ASSAY
 TEST 1 WITHOUT S9

Test Substance (mg/plate)	Plate Counts			Mean	Standard Deviation
	1	2	3		
STRAIN TA98					
DMSO (55)	17	10	17	15	4.0
4-NPD (0.01)	431	575	632	546	103.6
0.10	375	65	62	167	179.9
0.30	56	19	22	32	20.6
1.0	T	T	T	T	-
3.0	T	T	T	T	-
5.0	T	T	T	T	-
STRAIN TA100					
DMSO (55)	129	161	133	141	17.4
NaN ₃ (0.01)	1169	1177	979	1108	112.1
0.10	203	189	202	198	7.8
0.30	157	131	162	150	16.6
1.0	98 (S)	81 (S)	189	123	58.1
3.0	T	T	T	T	-
5.0	T	T	T	T	-
STRAIN TA1535					
DMSO (55)	4	5	10	6	3.2
NaN ₃ (0.01)	C	1518	1348	1433	104.1
0.10	11	6	10	9	2.6
0.30	6	0	4	3	3.1
1.0	T	T	T	T	-
3.0	T	T	T	T	-
5.0	T	T	T	T	-
STRAIN TA1537					
DMSO (55)	4	4	10	6	3.5
9-AA (0.06)	253	133	275	220	76.4
0.10	10	4	14	9	5.0
0.30	6 (S)	1 (S)	1 (S)	3	2.9
1.0	T	T	T	T	-
3.0	T	T	T	T	-
5.0	T	T	T	T	-
STRAIN TA1538					
DMSO (55)	13	17	10	13	3.5
4-NPD (0.01)	117	111	134	121	11.9
0.10	10	9	9	9	0.6
0.30	9	11	5	8	3.1
1.0	8	9	5	7	2.1
3.0	T	T	T	T	-
5.0	T	T	T	T	-

T: Toxic; absence of background lawn, or mean number of colonies < 1/2 solvent control value.
 S: Sparse growth of background lawn; C: Contaminated; DMSO: Dimethylsulfoxide.
 4-NPD: 4-Nitro-o-phenylenediamine; NaN₃: Sodium Azide; 9-AA: 9-Aminoacridine.

TABLE 3
 THIO HEXAFLUOROPHOSPHATE SALT (THIO PF6 SALT): MUTAGENIC POTENTIAL
 IN THE SALMONELLA/MICROSOME (AMES) ASSAY

RESULTS OF THE SALMONELLA/REVERSE MUTATION ASSAY
 TEST 1 WITH S9

Test Substance (mg/plate)	Plate Counts			Mean	Standard Deviation
	1	2	3		
STRAIN TA98					
DMSO (55)	40	35	31	35	4.5
2-AA (2.5µg)	2548	2486	2529	2521	31.8
0.10	79	157	919	385	464.1
0.30	30	326	29	128	171.2
1.0	T	T	3 (S)	3 (T)	0.0
3.0	T	T	T	T	-
5.0	T	T	T	T	-
STRAIN TA100					
DMSO (55)	168	140	161	156	14.6
2-AA (2.5µg)	1902	1702	1500	1701	201.0
0.10	238	221	258	239	18.5
0.30	176	191	206	191	15.0
1.0	115	52 (S)	126	98	39.9
3.0	38 (S)	22 (S)	T	30 (T)	9.8
5.0	T	T	T	T	-
STRAIN TA1535					
DMSO (55)	21	18	22	20	2.1
2-AA (2.5µg)	179	164	200	181	18.1
0.10	12	17	17	15	2.9
0.30	7	5	3	5 (T)	2.0
1.0	0 (S)	T	T	0 (T)	0.0
3.0	T	T	T	T	-
5.0	T	T	T	T	-
STRAIN TA1537					
DMSO (55)	17	12	15	15	2.5
2-AA (2.5µg)	228	274	221	241	28.8
0.10	18	30	23	24	6.0
0.30	4 (S)	8 (S)	5 (S)	6 (T)	2.1
1.0	T	T	T	T	-
3.0	T	T	T	T	-
5.0	T	T	T	T	-
STRAIN TA1538					
DMSO (55)	22	25	20	22	2.5
2-AA (2.5µg)	1521	1097	1005	1208	275.2
0.10	25	24	19	23	3.2
0.30	22	13	16	17	4.6
1.0	11	13	21	15	5.3
3.0	T	T	T	T	-
5.0	T	T	T	T	-

T: Toxic; absence of background lawn, or mean number colonies < 1/2 solvent control value.
 S: Sparse growth of background lawn.
 2-AA: 2-Aminanthracene; DMSO: Dimethylsulfoxide.

TABLE 4
 THIO HEXAFLUOROPHOSPHATE SALT (THIO PF6 SALT): MUTAGENIC POTENTIAL
 IN THE SALMONELLA/MICROSOME (AMES) ASSAY

RESULTS OF THE SALMONELLA/REVERSE MUTATION ASSAY
 TEST 2 WITHOUT S9

Test Substance (mg/plate)	Plate Counts			Mean	Standard Deviation
	1	2	3		
STRAIN TA98					
DMSO (55)	19	19	14	17	2.9
4-NPD (0.01)	462	416	415	431	26.9
0.01	13	149	68	77	68.4
0.03	233	29	29	97	117.8
0.10	48	52	298	133	143.2
0.30	46	12	13	24	19.3
1.0	T	T	T	T	-
STRAIN TA100					
DMSO (55)	64	57	66	62	4.7
NaN ₃ (0.01)	931	1065	1040	1012	71.3
0.01	62	64	62	63	1.2
0.03	100	86	84	90	8.7
0.10	109	137	127	124	14.2
0.30	65	87	74	75	11.1
1.0	51 (S)	62 (S)	50 (S)	54	6.7
STRAIN TA1535					
DMSO (55)	9	7	10	9	1.5
NaN ₃ (0.01)	829	901	988	906	79.6
0.01	7	6	2	5	2.6
0.03	11	9	8	9	1.5
0.10	7	7	10	8	1.7
0.30	3 (S)	2 (S)	3 (S)	3 (T)	0.6
1.0	T	T	T	T	-
STRAIN TA1537					
DMSO (55)	2	4	5	4	1.5
9-AA (0.06)	128	363	190	227	121.8
0.01	10	3	5	6	3.6
0.03	3	4	6	4	1.5
0.10	9	5	3	6	3.1
0.30	1	1	1 (S)	1 (T)	0.0
1.0	T	T	T	T	-
STRAIN TA1538					
DMSO (55)	10	17	8	12	4.7
4-NPD (0.01)	128	108	97	111	15.7
0.01	8	10	15	11	3.6
0.03	10	5	13	9	4.0
0.10	10	5	13	9	4.0
0.30	12	8	11	10	2.1
1.0	3 (S)	1 (S)	T	2 (T)	1.2

T: Toxic; absence of background lawn, or mean number of colonies < 1/2 solvent control value.
 S: Sparse growth of background lawn.
 4-NPD: 4-Nitro-o-phenylenediamine; NaN₃: Sodium azide; 9-AA: 9-Aminoacridine;
 DMSO: Dimethylsulfoxide.

TABLE 5
 THIO HEXAFLUOROPHOSPHATE SALT (THIO PF6 SALT): MUTAGENIC POTENTIAL
 IN THE SALMONELLA/MICROSOME (AMES) ASSAY

RESULTS OF THE SALMONELLA/REVERSE MUTATION ASSAY
 TEST 2 WITH S9

Test Substance (mg/plate)	Plate Counts			Mean	Standard Deviation
	1	2	3		
STRAIN TA98					
DMSO (55)	24	20	21	22	2.1
2-AA (2.5µg)	1966	1329	2128	1808	422.4
0.01	35	71	329	145	160.4
0.03	417	51	33	167	216.7
0.10	99	815	73	329	421.1
0.30	30	34	190	85	91.2
1.0	T	T	T	T	-
STRAIN TA100					
DMSO (55)	49	89	102	80	27.6
2-AA (2.5µg)	1565	1490	1723	1593	118.9
0.01	106	164	93	121	37.8
0.03	125	179	129	144	30.1
0.10	144	110	140	131	18.6
0.30	112	139	116	122	14.6
1.0	85	108	98	97	11.5
STRAIN TA1535					
DMSO (55)	8	8	8	8	0.0
2-AA (2.5µg)	109	101	134	115	17.2
0.01	8	14	10	11	3.1
0.03	10	10	4	8	3.5
0.10	5	4	10	6	3.2
0.30	4 (S)	0 (S)	5 (S)	3 (T)	2.6
1.0	T	T	T	T	-
STRAIN TA1537					
DMSO (55)	6	4	6	5	1.2
2-AA (2.5µg)	125	140	144	136	10.0
0.01	17	4	5	9	7.2
0.03	7	7	21	12	8.1
0.10	21	18	11	17	5.1
0.30	10 (S)	4 (S)	3 (S)	6	3.8
1.0	T	T	T	T	-
STRAIN TA1538					
DMSO (55)	14	19	14	16	2.9
2-AA (2.5µg)	967	1004	977	983	19.1
0.01	13	22	19	18	4.6
0.03	17	19	C	18	1.2
0.10	8	10	20	13	6.4
0.30	17	15	20	17	2.5
1.0	11	17	7	12	5.0

T: Toxic; absence of background lawn, or mean number of colonies < 1/2 solvent control value.
 S: Sparse growth of background lawn.
 2-AA: 2-Aminoanthracene C: Contaminated; DMSO: Dimethylsulfoxide.

Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential in the
Salmonella/Microsome (Ames) Assay

QUALITY ASSURANCE UNIT INSPECTION SUMMARY

<u>Inspection Date(s)</u>	<u>Inspection Type</u>	<u>Date QAU Report Issued To</u>	
		<u>Study Director</u>	<u>Management</u>
09-20-93 to 09-21-93	PROTOCOL	09-21-93	09-21-93
12-01-93	EVENT-DOSING	12-01-93	12-01-93
01-24-94	RAW DATA, REPORT	01-24-94	03-22-94
03-22-94	ARCHIVES	03-22-94	03-22-94



 Linda J. Calisti, Manager
 Good Laboratory Practices/Quality Assurance

3/22/94
 Date

Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential
in the Salmonella/Microsome (Ames) Assay

Protocol

(9 Pages)



BUSHY RUN RESEARCH CENTER

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PROTOCOL

TITLE: Thio Hexafluorophosphate Salt (Thio PF6 Salt): Mutagenic Potential
in the Salmonella/Microsome (Ames) Assay

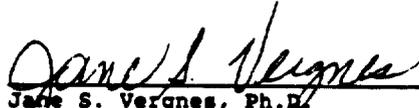
BRRC PROJECT ID: 93U1322

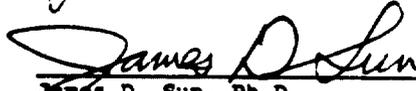
SPONSOR: Solvents and Coatings Materials Division
Union Carbide Corporation
39 Old Ridgebury Road
Danbury, CT 06817-0001

TESTING FACILITY: Bushy Run Research Center (BRRC)
Union Carbide Corporation
6702 Mellon Road
Export, PA 15632-8902

Reviewed and Approved by:

Bushy Run Research Center:

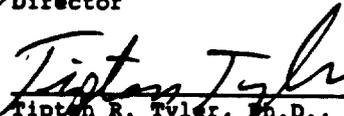
 9/14/93
Jane S. Vergnes, Ph.D. Date
Study Director

 9-16-93
James D. Sun, Ph.D. Date
Associate Director

 9/21/93
Linda J. Calisti, P.S. Date
Manager, Good Laboratory
Practices/Quality Assurance

 9/22/93
John P. Van Miller, Ph.D., DABT Date
Director

Union Carbide Corporation:

 9/30/93
Tipton R. Tyler, Ph.D., DABT Date
Associate Director of Applied Toxicology

Division:

 10-12-93
Richard C. Wise Date
Manager of Product Safety

Union Carbide Chemicals and Plastics Company Inc.
Excellence Through Quality



OBJECTIVE

The objective of this study is to assess the mutagenic potential of Thio Hexafluorophosphate Salt (Thio PF6 Salt) in the Salmonella/microsome mutation assay.

Design and Basis for the Study

Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538 will be used for the mutagenicity assay. Five dose levels of the test substance, spaced at approximately half-log intervals, will be selected for treatments both in the absence and in the presence of a rat liver S9 metabolic activation system. All 5 strains will be treated in triplicate with the vehicle control substance, an appropriate positive control substance, and 5 dose levels of the test substance using the plate incorporation method. Treated cultures will be incubated at approximately 37°C for 48-72 hours. Two independent assays will be performed.

The portions of this study conducted at BRRC will be in compliance with the following guidelines and standards:

U.S. Environmental Protection Agency (EPA), Toxic Substances Control Act (TSCA) Good Laboratory Practice (GLP) Standards, 40 CFR Part 792.

Organisation for Economic Co-operation and Development (OECD) (1981).
OECD Principles of Good Laboratory Practice, C(81)30(Final).

PERSONNEL

All personnel who participate in the conduct of the study will be documented in the raw data.

PROJECT DATES

Proposed Starting Dates of Test Substance Administration

Test 1	October 6, 1993
Test 2	October 13, 1993

Proposed Completion Dates

Test 1	October 8, 1993
Test 2	October 15, 1993

<u>Proposed Date for Submission of Draft Final Report</u>	November 12, 1993
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METHODSTest Substance

Product Name Thio Hexafluorophosphate Salt

Chemical Name Diphenyl [4-(phenylthio)phenyl]sulfonium hexafluorophosphate

CAS Registry Number 68156-13-8

Source Union Carbide Corporation, Bound Brook, NJ

Sponsor Identification Number 18687-35

BRRC Sample Number 56-353

Description White crystals according to the Material Safety Data Sheet (MSDS).

Purity Purity information will be provided by the Sponsor and documented in the raw data and final report.

Stability The test substance is considered to be stable at room temperature for the duration of the study.

Storage Conditions The test substance will be stored under well-ventilated conditions at ambient temperature in an appropriate storage area.

Quantity A two ounce amber bottle (gross weight 84.81 g) of the test substance was received for the proposed study [ies]. After the assigned study [ies] has been completed, all unused test substance will be returned to the Sponsor.

Reserve Sample A reserve sample will not be retained at BRRC.

Safety An MSDS supplied by the Sponsor will be reviewed by all relevant personnel before their participation in the study. This review will be documented. Normal precautions for untested substances will be used. These procedures include the use of disposable Tyvek® or plastic coats or smocks and disposable gloves. Eye protection will include the use of safety glasses at all times. All manipulations of the test substance will be performed in an approved safety hood. Access to the laboratory will be restricted to essential personnel while testing is in progress.

Bacterial Cultures

Strains	<u>Salmonella</u> strains TA98, TA100, TA1535, TA1537, and TA1538
Supplier	Dr. Bruce N. Ames, University of California, Berkeley, CA
Rationale	<u>Salmonella</u> strains TA98, TA100, TA1535, and TA1537 are the strains of choice for this assay. Strain TA1538 has been selected because it occasionally detects mutagens that strain TA98 does not.
Culture Conditions	<p>The test strains are stored as frozen broth cultures at -80°C. Daily working cultures are prepared fresh for each test day by inoculating bacteria from frozen working cultures into nutrient broth. Cultures are incubated overnight (10-15 hours) at 37°C with gentle agitation. The daily working cultures are kept on ice throughout the test day.</p> <p>A bacterial titer will be determined by plating serial dilutions of the daily working cultures on nutrient agar plates and counting the resultant colonies approximately 24-48 hours after plating. The integrity of the genetic markers in each bacterial strain will be verified each time frozen permanent stock cultures or frozen working cultures are prepared.</p>

Administration of Test Substance

Metabolic Activation	S9 liver homogenate, prepared from Aroclor 1254-induced rats, is purchased from Microbiological Associates. The complete S9 metabolic activation system contains the following: 8 mM MgCl ₂ , 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9. Fresh S9 mix is prepared each day of testing and kept at 0-4°C.
Vehicle Control Substances	The preferred vehicle control substances are water (CAS No. 7732-18-5), dimethyl sulfoxide (CAS No. 67-68-5), ethanol (CAS No. 64-17-5), methanol (CAS No. 67-56-1), and acetone (CAS No. 67-64-1). Other substances may be used as long as they are compatible with the test substance, noncytotoxic, and nonmutagenic in this test system. The chosen vehicle control substance will be documented in the raw data.

**Positive Control
Substances**

Both activation-dependent and activation-independent positive controls will be used. The activation-independent controls are 4-nitro-o-phenylenediamine (CAS No. 99-56-9) for TA98 and TA1538, sodium azide (CAS No. 26628-22-8) for TA100 and TA1535, and 9-aminoacridine (CAS No. 90-45-9) for TA1537. The activation-dependent control is 2-aminoanthracene (CAS No. 613-13-8) for all strains. The appropriate concentrations are determined from prior dose-response experiments with these substances. Analyses for stability, homogeneity, and concentration of the positive control substances in dosing solutions will not be performed.

**Preparation of
Dosing Solutions**

Dosing solutions will be prepared by mixing appropriate amounts of the test substance (g) (uncorrected for percent active ingredient) and vehicle. The concentration of the test substance in dosing solutions will be verified gravimetrically. Mixing procedures and storage conditions will be specified in the raw data and final report. Dosing solutions will be prepared on the day they are used and will be discarded by the end of that day. Analyses for stability, homogeneity, and concentration of the test substance in dosing solutions will not be performed.

**Preliminary
Toxicity
Testing**

Five to 10 dose levels of the test substance will be tested on strain TA100 with and without S9 metabolic activation to evaluate its cytotoxicity. Generally, the maximum dose tested in the cytotoxicity assay will be 10 mg/plate. However, if a solution, suspension, or emulsion cannot be prepared at the maximum dose level, then a lower dose level will be chosen. A 2 ml volume of complete top agar (6 g/l agar, 5 g/l NaCl, 0.05 mM L-histidine and 0.05 mM D-biotin) will be added to the required number of sterile tubes. A 100 µl aliquot of strain TA100 will be added to each tube followed by an aliquot of the vehicle control substance or the appropriate dilution of the test substance. Then, either 0.5 ml of S9 mixture or 0.5 ml of phosphate buffered saline (PBS) will be added for testing with or without metabolic activation. The mixture will be vortexed briefly and poured onto a Vogel-Bonner Medium E (VBE) agar plate. The top agar will be allowed to harden and then the plates will be incubated at 37°C for at least 48 hours. The plates will be examined to determine the condition of their background lawns. Lawns will be recorded as either confluent, sparse, or absent. Confluence indicates no toxicity, sparse growth indicates moderate toxicity, and no growth indicates extreme toxicity. Revertant colonies will be counted

either manually or with an Artek Model No. 880 colony counter. A significant ($\geq 50\%$) reduction in the number of revertant colonies/plate will be considered a toxic effect.

Mutagenicity Testing

A minimum of 5 dose levels, spaced approximately at half-log intervals, will be tested. The highest dose level will be selected to produce either a significant ($\geq 50\%$) reduction in the number of revertant colonies as compared to the vehicle control or a significant inhibition of background lawn growth (sparse or absent lawns). The highest dose level for noncytotoxic test substances will be 10 mg/plate unless the test substance cannot be kept in solution, suspension or emulsion at that dose level. At least 2 dose levels should not be cytotoxic. To a sterile tube containing 2 ml of complete top agar, a 100 μ l aliquot of the appropriate bacterial culture will be added followed by 50 μ l of the appropriate control, 50 μ l of vehicle, or 50 μ l of test substance solution. Either 0.5 ml of S9 mix or 0.5 ml of PBS will be added for tests with or without metabolic activation. The top agar mixture will be vortexed briefly and then poured onto a VBE plate. Sterility testing will be performed on the PBS, the S9 mix, all vehicles, and the highest concentration of test substance. The plates will be transferred to a darkened 37°C incubator after hardening and incubated for 48-72 hours.

Experimental Evaluations

Data Collection

The number of bacterial colonies/plate will be counted either manually or using an Artek Model No. 880 Colony Counter and recorded. The counter will be calibrated prior to use to verify counting accuracy.

The background lawn on each plate will also be evaluated. If the background lawn on a plate is sparse, an "S" will be entered beside the colony count for that plate. If the background lawn on a plate is absent, there will be no colony count.

The mean number of revertant colonies and the standard deviation of that mean will be calculated for each dose level and bacterial strain. Sparse plates will be used to calculate the mean number of revertant colonies. Whether sparse dose levels are used in the evaluation of mutagenicity will be decided by the Study Director on a case-by-case basis. When the mean number of revertant colonies/plate is less than 50% that of the vehicle control, the dose level is labeled toxic and a "T" is entered beside the mean on the report table.

Data Interpretation

The mean spontaneous reversion frequencies for the vehicle controls should be within this laboratory's historical ranges. The positive controls should demonstrate that the test system is responsive to known mutagens. A test substance will be considered a bacterial mutagen if it produces a dose-related increase in the mean reversion frequency of at least one bacterial strain as compared to the vehicle control for that strain. Furthermore, at least one of those doses must produce a mean reversion frequency at least twice that of the vehicle control. Alternatively, a test substance will be considered a bacterial mutagen if there is a reproducible increase in the mean number of revertant colonies at a single dose level of at least 2-fold compared to the vehicle control. A test substance will not be considered a bacterial mutagen if it does not meet these criteria. Increases which are not dose related, or which cannot be reproduced, will be considered negative test results.

ALTERATION OF PROTOCOL

Alterations to this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, such change will be honored. However, it then becomes the responsibility of the Sponsor to follow such verbal change with a written verification. BRRC reserves the right to revise the protocol or deviate therefrom solely at the discretion of the Study Director if prior approval of the Sponsor cannot be obtained and the integrity of the study is considered in jeopardy. In this event, the Sponsor will be notified of the alteration as soon as possible, and documentation of the change will be the responsibility of the Study Director.

RETENTION OF RECORDS

All raw data, documentation, the protocol and any amendments, and a copy of the final report generated as a result of this study will be retained in the BRRC Archives for at least 5 years.

Following the retention period specified above, the Sponsor will be contacted and given the option of taking receipt, destroying, or arranging for other storage of the data and materials. All data and materials mentioned above will remain the sole property of the Sponsor and can be removed from BRRC at the Sponsor's discretion.

REPORTS

Draft Final Report

An unaudited draft of the final report will be submitted to the Sponsor approximately 1 month after the completion of the testing. This report will be a comprehensive report which will include all information necessary to

provide a complete and accurate description and evaluation of the test procedures and results. It will include: a summary and appropriate text discussions of the experimental design, materials and methods, and results. In addition, it will contain appendices with other pertinent information.

Final Report

The draft final report will be reviewed by the Sponsor, and comments on the report will be provided to BRRC within 4 weeks from the date of submission of the draft version. BRRC will consider these comments in preparing the final report. Assuming the Sponsor's comments are received at the specified time and no major revisions are required, BRRC will submit a final report within 8 weeks of issuance of the draft report.

The final report will be audited by the Quality Assurance Unit and contain a signed quality assurance statement. It will conform to the formatting specifications of EPA PR notice 86-5.

GOOD LABORATORY PRACTICE COMPLIANCE

BRRC, through the administration of a quality assurance program by the Good Laboratory Practice Committee and Quality Assurance Unit, assures compliance of all phases of studies conducted at BRRC with existing regulations and generally accepted good laboratory practices.

The study will be subjected to periodic inspections and the final report will be reviewed by the BRRC Quality Assurance Unit.

REFERENCES

- Ames, B. N., McCann, J., and Yamasaki, F. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Research* 31, 347-364.
- Kier, L. D., Brusick, D. J., Auletta, A. E., VonHalle, E. S., Brown, M. M., Simmon, V. F., Dunkel, V., McCann, J., Mortelmans, K., Prival, M., Rao, T. K., and Ray, V. (1986). The Salmonella typhimurium/Mammalian microsomal assay. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutation Research* 168, 69-240.
- Maron, D. M. and Ames, B. N. (1983). Revised Methods for the Salmonella mutagenicity test. *Mutation Research* 113, 173-215.