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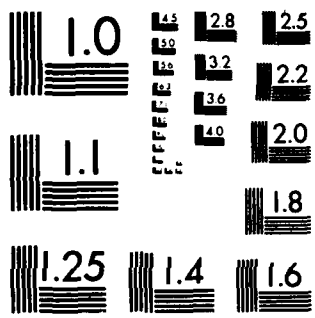

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INSTITUTE REPORT NO. 149

MUTAGENIC POTENTIAL OF THE HOLSTON COMPOUNDS:

Virgin DMSO
DMSO Recycle Solvent
DMSO Evaporator Sludge

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and
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TOXICOLOGY GROUP,
DIVISION OF RESEARCH SUPPORT

JUNE 1983

Toxicology Series 57

LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

Mutagenic Potential of the Holston Compounds: Virgin DMSO, DMSO Recycle Solvent, DMSO Evaporator Sludge (Toxicology Series 57)--Sauers et al

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7 Feb 83

(Signature and date)

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7. AUTHOR(s): Leonard J. Sauers, MS, SP5 Thomas P. Kellner, BA, SP4 John T. Fruin, DVM, PhD, COL VC	8. CONTRACT OR GRANT NUMBER(s)
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Mutagenicity, Toxicology, Ames Assay, Holston Compounds, Virgin DMSO, DMSO Recycle Solvent, DMSO Evaporator Sludge, Frameshift Mutagen, Dimethyl Sulfoxide

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The mutagenic potential of the Holston Compounds (Virgin DMSO*, DMSO Recycle Solvent, and DMSO Evaporator Sludge) was assessed by using the Ames Salmonella/Mammalian Microsome Mutagenicity Assay. Tester strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 were exposed to doses ranging from 0.1 ml of a 100% to 0.1 ml of a 0.032% solution. Negative mutagenic responses were observed for the Virgin DMSO and the DMSO Recycle Solvent. Mutagenic potential was observed for the DMSO Evaporator Sludge.

*DMSO = Dimethyl Sulfoxide

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

ABSTRACT

The mutagenic potential of the Holston Compounds (Virgin DMSO* DMSO Recycle Solvent, and DMSO Evaporator Sludge) was assessed by using the Ames Salmonella/Mammalian Microsome Mutagenicity Assay. Tester strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 were exposed to doses ranging from 0.1 ml of a 100% to 0.1 ml of a 0.032% solution. Negative mutagenic responses were observed for the Virgin DMSO and the DMSO Recycle Solvent. Mutagenic potential was observed for the DMSO Evaporator Sludge.

*DMSO = Dimethyl Sulfoxide

KEY WORDS: Mutagenicity, Toxicology, Ames Assay, Holston Compounds, Virgin DMSO, DMSO Recycle Solvent, DMSO Evaporator Sludge, Frameshift Mutagen, Dimethyl Sulfoxide

PREFACE

TYPE REPORT: Ames Assay GLP Study Report

TESTING FACILITY: US Army Medical Research and Development Command
Letterman Army Institute of Research
Presidio of San Francisco, CA 94129

SPONSOR: US Army Medical Research and Development Command
US Army Medical Bioengineering Research
and Development Laboratory
Fort Detrick, Frederick, MD 21701

PROJECT: DMSO Recrystallization Solution
TL01

GLP STUDY NUMBER: 83001

STUDY DIRECTOR: COL John T. Fruin, DVM, PhD, VC
Diplomate, American College of
Veterinary Preventive Medicine

PRINCIPAL INVESTIGATOR: SP5 Leonard J. Sauers, MS

REPORT AND DATA MANAGEMENT: A copy of the final report, study protocols, raw data, retired SOPs, chemical data, and an aliquot of each test compound will be retained in the LAIR Archives.

TEST SUBSTANCE: The Holston Compounds (Virgin DMSO, DMSO Recycle Solvent, and DMSO Evaporator Sludge).

INCLUSIVE STUDY DATES: 3 January - 20 March 1983

OBJECTIVE: To determine the mutagenic potential of the Holston Compounds using the Ames Assay. Tester strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 were used. The plate incorporation method was followed. The test substances was diluted in reagent grade dimethyl sulfoxide (DMSO) and this diluent was checked for sterility.

ACKNOWLEDGMENTS

The authors wish to thank SP4 Larry Mullen, BS and John Dacey for their assistance in performing the research.

Signatures of Principal Scientists involved
in the Study

We, the undersigned, believe the study number 83001 described in this report to be scientifically sound and the results and interpretation to be valid. The study was conducted to comply, to the best of our ability, with the Good Laboratory Practice Regulations outlined by the Food and Drug Administration.

Leonard J. Sauers 25 Apr 83
LEONARD J. SAUERS, MS / DATE
SP5, USA
Principal Investigator

John T Fruin 25 Apr 83
JOHN T. FRUIN, DVM, PhD / DATE
COL, VC
Study Director

Thomas P. Kellner / 25 Apr 83
THOMAS P. KELLNER, BA / DATE
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Toxicology Group

DEPARTMENT OF THE ARMY
LETTERMAN ARMY INSTITUTE OF RESEARCH
PRESIDIO OF SAN FRANCISCO, CALIFORNIA 94129

REPLY TO
ATTENTION OF:
SGRD-ULZ-QA

6 May 1983

MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance

I hereby certify that in relation to LAIR GLP study 83001 the following inspections were made:

12 Jan 83
26 Jan 83
27 Jan 83
16 Mar 83
19 Mar 83

The report and raw data for this study were audited on 5 May 1983.

Routine inspections with no adverse findings are reported quarterly, thus these inspections are also included in the April 83 report to management and the Study Director.

NELSON R. POWERS, Ph.D.
CPT, MSC
Quality Assurance Officer

TABLE OF CONTENTS

MUTAGENIC POTENTIAL OF THE HOLSONT COMPOUNDS: Virgin DMSO, DMSO Recycle Solvent, DMSO Evaporator Sludge--Sauers et al

Rationale for using the Ames Assay

The Ames Salmonella/Mammalian Microsome Mutagenicity Test is one of a standard bank of tests used by our laboratory for the assessment of the mutagenic potential of a test substance. It is a short-term screening assay, which we use for the prediction of potential mutagenic agents in mammals. It is inexpensive when compared to in vivo tests, yet is highly predictive and reliable in its ability to detect mutagenic activity and therefore carcinogenic probability (1). It relies on basic genetic principles and allows for the incorporation of a mammalian microsomal enzyme system to increase sensitivity through enzymatically altering the test substance into an active metabolite. It has proven highly effective in assessing human risk (1).

Description of Test (Rationale for the selection of strains)

The test was developed by Bruce Ames, Ph.D. from the University of California-Berkeley. The test involves the use of several different genetically altered strains of Salmonella typhimurium, each with a specific mutation in the histidine operon (2). The test substance demonstrates mutagenic potential if it is able to revert the mutation in the bacterial histidine operon to the wild type and reestablish prototrophic growth within the test strain. This reversion also can occur spontaneously due to a random mutational event. If, after adding a test substance, the number of revertants is significantly greater than the spontaneous reversion rate, then the test substance physically altered the locus involved in the operon's mutation and is able to induce point mutations (2).

In order to increase the sensitivity of the test system, other mutations in the Salmonella are used (2). To insure a higher probability of uptake of test substance, the genome for the lipopolysaccharide layer (LP) is mutated and, therefore, larger molecules are allowed to enter the bacteria. Each strain has another induced mutation which causes loss of excision repair mechanisms. A

mammalian microsomal enzyme system is incorporated since many chemicals are not by themselves mutagenic but have to be activated by an enzymatic process. These microsomal enzymes are obtained from livers of rats induced with Aroclor 1254; the enzymes allow for the expression of the metabolites which would occur in the mammalian system. This activated rat liver microsomal enzyme homogenate is termed S-9.

Description of Strains (History of the strains used method to monitor the integrity of the organisms, and data pertaining to current and historical control and spontaneous reversion rates)

The test consists of using five different strains of Salmonella typhimurium that are unable to grow in absence of histidine because of a specific mutation in the histidine operon. This histidine requirement is verified by attempting to grow the tester strains on minimal glucose agar (MGA) plates, both with and without histidine. The dependence on this amino acid is shown when growth occurs only in its presence. The plasmids in strains TA 98 and TA 100 contain an ampicillin resistant R factor. Strains deficient in this plasmid demonstrate a zone of inhibition around an ampicillin impregnated disc. The alteration of the LP layer allows uptake by the Salmonella of larger molecules. If a crystal violet impregnated disc is placed onto a plate containing any one of the bacterial strains, a zone of growth inhibition will occur because the LP layer is altered. The absence of excision repair mechanisms can be determined by using ultraviolet (UV) light. These mechanisms function primarily by repairing photodimers between pyrimidine bases. Exposure of bacteria to UV light will activate the formation of these dimers and cause cell lethality, since excision of these photodimers can not be made. The genetic mutation resulting in UV sensitivity also induces a dependence by the Salmonella to biotin. Therefore, this vitamin must be added. In order to prove that the bacteria are responsive to the mutation process, positive controls are run with known mutagens. If after exposure to the positive control substance, a revertant count is obtained which is greater than twice the spontaneous reversion rate, then the bacteria are adequately responsive. Sterility controls are performed to determine the presence of contamination. Sterility of the test compound is also confirmed in each first dilution. Verification of the tester strains occurs simultaneously with the running of each assay. The value of the spontaneous reversion rate is obtained by using the same inoculum of bacteria that is used in the assay (3).

Strains were obtained directly from Dr. Ames, University of California-Berkeley, propagated and then maintained at $-80^{o}C$ in our laboratory. Before any substance was tested, quality controls were performed on the bacterial strains to establish the presence of their special features and also to determine the spontaneous reversion rate (2). Records are maintained of all the data to determine if deviations from the set trends have occurred. These records are kept in the archives of the Quality Assurance Unit.

In this series of tests for the detection of mutagenic potential of different agents, we compared the spontaneous reversion values with our own historical values and those cited by Ames et al (2). Our conclusions are based on the spontaneous reversion rate compared to the experimentally induced rate of mutation. When operating effectively, these strains detect substances that cause base pair mutations (TA 1535, TA 100) and frameshift mutations (TA 1537, TA 1538, and TA 98).

Objective of Study

The objective of the study is to determine the mutagenic potential of the Holston Compounds using the Ames Assay. Tester strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538 were used. The plate incorporation method was followed. The test substances were diluted in reagent grade dimethyl sulfoxide (DMSO) and this diluent was checked for sterility.

METHODS (3)

Rationale for Dosage Levels and Dose Response Tabulations

To insure readable and reliable results, a sublethal concentration of the test substance had to be determined. This toxicity level was found by using MGA plates, various concentrations of the substance, and approximately 10^8 cells of TA 100 per plate, unless otherwise specified. Top agar containing trace amounts of histidine and biotin were placed on MGA plates. TA 100 was used because it is the most sensitive strain. Strain verification was confirmed on the bacteria, along with a determination of the spontaneous reversion rate. After incubation, the growth was observed on the plates. (The auxotrophic Salmonella will replicate a few times and potentially express a mutation. When the histidine and biotin supplies are exhausted, only those bacteria that reverted to the prototrophic phenotype will continue to reproduce and form macrocolonies; the remainder of the bacteria comprises the background lawn. The minimum toxic level is defined as the lowest serial dilution at which decreased macrocolony formation, below that of the spontaneous revertant rate, and an observable reduction in the density of the background lawn occurs.) A maximum dose of 1 mg/plate is used when no toxicity is observed. The densities were recorded as normal, slight, and no growth.

Test Format

After we validated our bacterial strains and determined the optimal dosage of the test substance, we began the Ames Assay. In the actual experiment, 0.1 ml of the particular strain of Salmonella (10^8 cells) and the specific dilutions of the test substance were added to 2 ml of molten top agar, which contained trace amounts of histidine and biotin. Since survival is better from cultures which have just passed the log phase, the Salmonella strains are used 16 hours

(maximum) after initial inoculation into nutrient broth. The dose of the test substance spanned a 1000-fold, decreasing from the minimum toxic level by a dilution factor of 5. All the substances were tested with and without S-9 microsome fraction. The optimal titer of the S-9 was determined and 0.5 ml was added to the molten top agar. After all the ingredients were added, the top agar was mixed, then overlaid on minimum glucose agar plates. These plates contained 2% glucose and Vogel Bonner "E" Concentrate (4). The water used in this medium and all reagents came from a polymetric system. Plates were incubated, upside down in the dark at 37° C for 48 hours. Plates were prepared in triplicate and the average revertant counts were recorded. The corresponding number of revertants obtained was compared to the number of spontaneous revertants; the conclusions were recorded statistically. A correlated dose response is considered necessary to declare a substance as a mutagen. Commoner (5), in his report, "Reliablilty of Bacterial Mutagenesis Techniques to Distinguish Carcinogenic and Non-Carcinogenic Chemical," and McCann et al (1) in their paper, "Detection of Carcinogens as Mutagen in the Salmonella/Mammalian Microsome Mutagenicity Test: Assay of over 300 Chemicals," have concurred on the test's ability to detect mutagenic potential.

Statistical Analysis

Quantitative evaluation was ascertained by the method of Ames et al (2). They assumed that a compound which causes twice the spontaneous reversion rate and a correlated dose response is mutagenic.

Chemical Analysis

Information on the chemical analysis on the Holston Compounds appears in Appendix A. The stability of the Holston Compounds under these test conditions has not been determined but assumed to be stable at room temperature.

RESULTS AND DISCUSSION

On 19 January 1983, the toxicity level determination was performed for the Virgin DMSO, DMSO Evaporator Sludge, and DMSO Recycle Solvent. For this experiment, all sterility, strain verification, and negative controls were normal (Appendix B, Table 1). Other results appear in Tables 2-12, Appendix B and Figures 1-6, Appendix C. No toxicity was observed after exposure of the tester strains to the compounds at the highest dose used (0.1 ml of a 100% solution) (Tables 2-4). It was observed that the DMSO Recycle Solvent precipitated when added to the top agar. The DMSO Recycle Solvent has a low solubility in water, the major component of the top agar solution. It is speculated that only about 0.5% of the solvent went into solution (personal communication, Thomas Kawakami, PhD, Laboratory for Energy-Related Health Research, 28 January 1983).

On 26 January 1983, the Ames Assay was performed on the test substances. In this assay, normal results were observed for all sterility and strain verification controls (Table 5). Normal results were also observed for all positive and negative controls (Table 6). Following exposure of the bacteria to the Virgin DMSO and DMSO Recycle Solvent, no incidences of mutagenicity were observed (Tables 7,8). Following exposure of the bacteria to the DMSO Evaporator Sludge, a doubling of the spontaneous reversion rate was induced at the 100% solution level in TA 98, TA 1537, and TA 1538. Increased reversion counts were seen for TA 100 and TA 1535 (Table 9). No evidence of mutagenicity was observed at the 20% solution level. For a positive mutagenic response in the Ames Assay, a test compound must induce a doubling of the spontaneous reversion rate and a correlated dose response. It was speculated that components in the DMSO Evaporator Sludge were mutagenic, but were in such small concentrations that a dose response could not be seen because of the wide difference between dilutions. A second Ames Assay was performed on 17 March 1983, using 0.1 ml per plate volumes of 100%, 80%, 60%, 40%, 20%, and 1% solutions of the DMSO Evaporator Sludge. For this experiment, all strain verifications and sterility controls were normal (Table 10). Normal results were observed for all positive and negative controls (Table 11). Following exposure of the bacteria to the DMSO Evaporator Sludge, mutagenic responses were seen for TA 98 at the 100%, 80%, and 60% solutions without S-9, and at the 100% and 80% solutions with S-9. A positive response was seen for TA 1537 at the 100%, 80%, and 60% solution with and without S-9, and at the 40% dose level with S-9. Mutagenicity was evident for TA 1538 at the 100% through 40% solution with and without S-9, and at the 20% solution without S-9 (Table 12). For each of the strains exhibiting a mutagenic response, a graph has been constructed to illustrate the correlated dose response (Figures 1-6). A deviation from a definitive dose response is observed for TA 1537. This can be attributed to the insensitivity of this particular strain and the closely spaced dilutions.

CONCLUSION

On the basis of the Ames Assay, the Virgin DMSO and DMSO Recycle Solvent are not mutagenic at the levels tested. The DMSO Evaporator Sludge shows characteristics of a frameshift mutagen at the levels tested but does not require the presence of metabolic activation for mutagenic induction.

Since the DMSO Recycle Solvent percipitated in the top agar, it is plausible that components within the solution are mutagenic but do not exhibit a response, since they were in such small concentrations when exposed to the Salmonella.

RECOMMENDATION

Components of the DMSO Evaporator Sludge should be identified and tested to determine the cause of the mutagenic response.

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2. Ames BN, McCann J, Yamasaki E. Methods for detection of carcinogens and mutagens with Salmonella/mammalian microsome mutagenicity test. Mutation Res 1975;31:347-364.

3. LAIR SOP OP-STX-1, Ames Salmonella/mammalian microsome mutagenicity test.

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

5. Commoner B. Reliability of the bacterial mutagenesis techniques to distinguish carcinogenic and non-carcinogenic chemicals. EPA 600/1 76-002, 1976.

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APPENDICES

Toxicity Test Sample Composition[a]

Concentration by HPLC, g/1

Sample	RDX[b]	HMX[c]	TAX[d]	SEX[e]	%H_2O	%DMSO
Virgin DMSO[f]	0	0	0	0	0.63[h]	99.37
DMSO Recycle Solvent[g]	24.188	39.542	0.263	0	35.48[i]	58.64[j]
DMSO Evaporator Sludge[f]	0.548	0.942	3.521	0	5.35[i]	94.19[j]

Calculated Data In Weight Percent[a]

Sample	RDX	HMX	TAX	SEX	H_2O	DMSO
Virgin DMSO	0	0	0	0	0.63	99.37
DMSO Recycle Solvent	2.22	3.64	0.02	0	35.48	58.64
DMSO Evaporator Sludge	0.05	0.09	0.32	0	5.35	94.19

[a] Data supplied by sponsor

[b] RDX: Cyclotrimethylenetrinitramine

[c] HMX: Cyclotetramethylenetetranitramine

[d] TAX: 1-Acetylhexahydro-3,5-Dinitro-1,3,5-Triazine

[e] SEX: Octahydro-1-(N)-Acetyl-3,5,7-Trinitro-1,3,5,7-Tetrazine

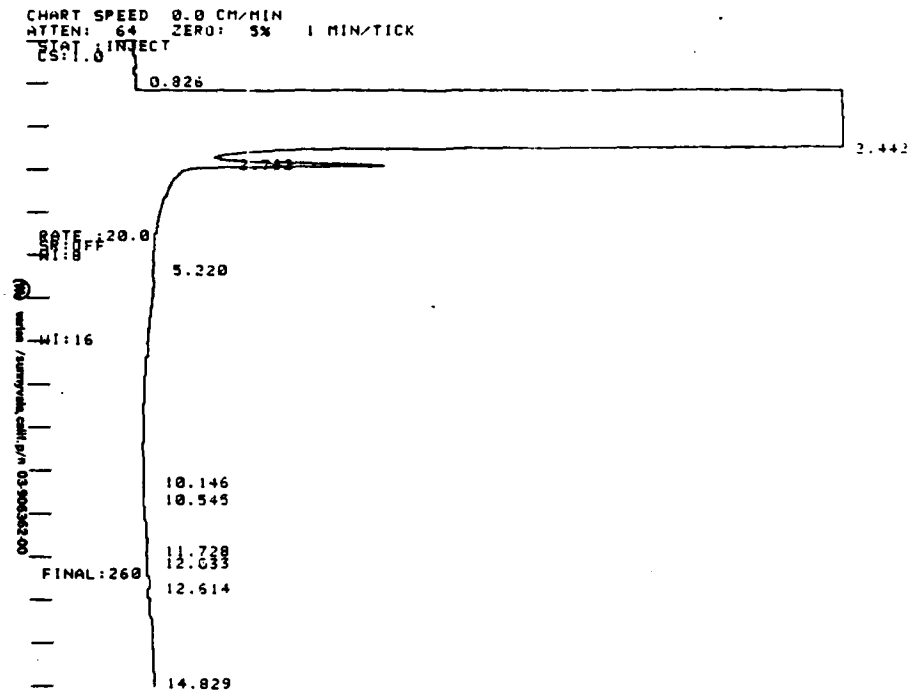
[f] At ambient temperature.

[g] Analysis of equilibrium liquid at 40°C.

[h] By Karl Fisher

[i] Water content calculated by difference.

[j] DMSO content by gas chromatography using virgin DMSO sample as the standard.



TITLE: 10:28 31 MAR 83

CHANNEL NO: 1 SAMPLE: "SLUDGE" METHOD: THIOLS

PEAK NO	PEAK NAME	RESULT AREA %	TIME (MIN)	TIME OFFSET	AREA COUNTS	SEP CODE	WI/2 (SEC)
1		65.3886	5.220		8169	BB	? 15.60
2		34.6114	11.728		4324	VV	? 27.00
TOTALS:		100.0000			12493		

DETECTED PKS: 13 REJECTED PKS: 11

MULTIPLIER: 1.00000

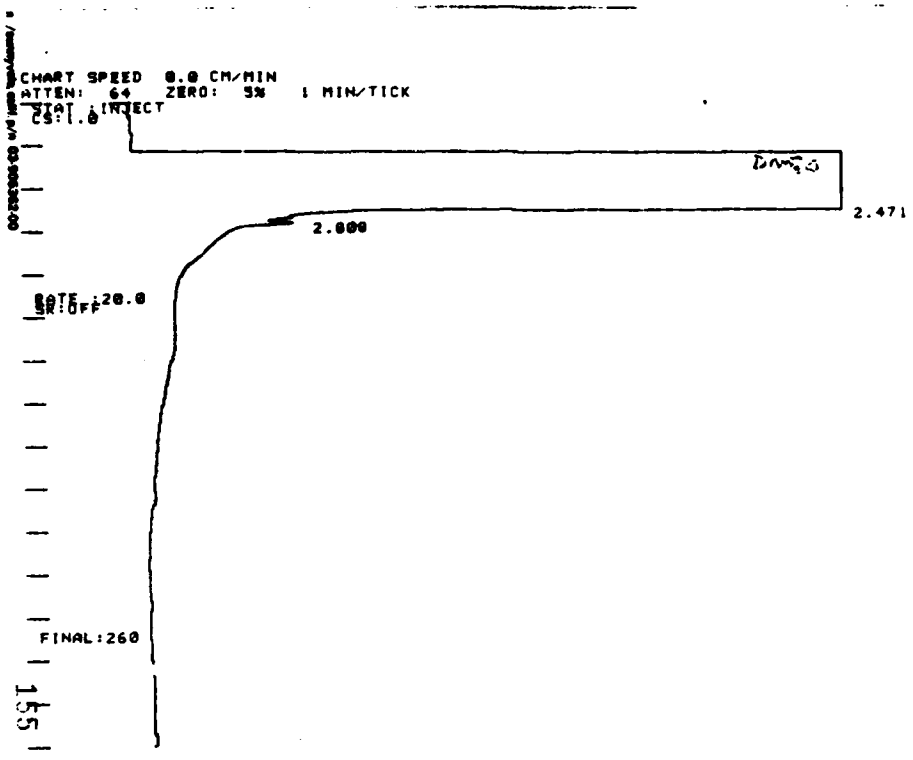
NOISE: 0.0 OFFSET: -0

SAVED FILE: RDX006

NOTES:
COL: 2 M GLASS - 5% OV-17, 80-100 MESH
CARRIER: NITROGEN - 20 ML/MIN
INJ: 150° C DET: 270° C
TEMP PROG: 100° TO 260° C @ 20°C/MIN
SOLVENT: DMSO SAMP SIZE: 0.5 UL
DETECTOR: FID - SENSITIVITY: 10-10
RUN LENGTH: 15 MINUTES

Gas Chromatograph Analysis of DMSO Evaporator Sludge

APPENDIX A (cont.)



TITLE: 9:26 31 MAR 82

CHANNEL NO: 1 SAMPLE: DMSO METHOD: THIOLS

PEAK NO	PEAK NAME	RESULT AREA %	TIME (MIN)	TIME OFFSET	AREA COUNTS	SEP CODE	W1/2 (SEC)
TOTALS:		0.0000			0		

DETECTED PKS: 4 REJECTED PKS: 4

MULTIPLIER: 1.00000

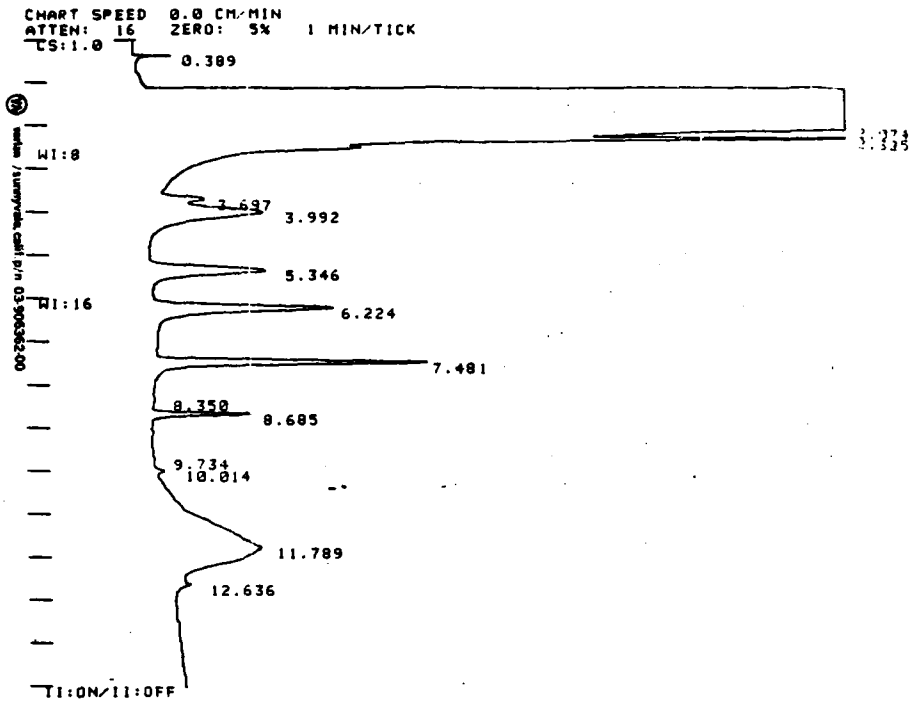
NOISE: 2.4 OFFSET: -4

SAVED FILE: RDX003

ERRORS:
NO PEAKS

NOTES:
COL: 2 M GLASS - 5% OV-17, 80-100 MESH
CARRIER: NITROGEN - 20 ML/MIN
INJ: 150° C DET: 270° C
TEMP PROG: 100° TO 260° C @ 20°C/MIN
SOLVENT: DMSO SAMP SIZE 1UL
DETECTOR: FID - SENSITIVITY: 10-10
RUN LENGTH: 15 MINUTES

Gas Chromatograph Analysis of Virgin DMSO



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RECALC
TITLE: 12:13 29 MAR 83

CHANNEL NO: 1 SAMPLE: TP013 METHOD: THIOLS

PEAK NO	PEAK NAME	RESULT AREA %	TIME (MIN)	TIME OFFSET	AREA COUNTS	SEP CODE		WI/2 (SEC)
1		12.4597	5.346		35291	BV		12.50
2		19.7905	6.224		56055	VV		10.70
3		17.5359	7.481		49669	VV		6.75
4		4.2547	8.685		12051	VB	?	3.10
5		44.8299	11.789		126977	VV	?	53.15
6		1.1294	12.636		3199	VB	?	6.40
TOTALS:		100.0000			283242			

DETECTED PKS: 15 REJECTED PKS: 9

MULTIPLIER: 1.00000

NOISE: 1.2 OFFSET: -5

NOTES:
COL: 2 M GLASS - 5% DV-17, 80-100 MESH
CARRIER: NITROGEN - 20 CC/MIN
INJ: 150° C DET: 270° C
TEMP PROG: 100° TO 260° @ 20°C/MIN
SOLVENT: DMSO ? SAMP SIZE: 1 UL
DETECTOR: FID - SENSITIVITY 10-10
RUN LENGTH: 15 MINUTES

Gas Chromatograph of DMSO Recycle Solvent

LIST OF TABLES

TABLE 1

STRAIN VERIFICATION FOR TOXICITY LEVEL DETERMINATION

Strains	Histidine Requirement	Ampicillin Resistance	UV	Sensitivity to Crystal Violet	Sterility Control	Response (1)
100	NG	G	NG	NG 18mm	NG	+
1537	NG	NG - 25mm	NG	NG 15mm	NG	+
WT	G	NA	G	NA	NA	+

STERILITY CONTROL

His-Bio Mix Initial: NG End: NG MGA Plate: NG

Top Agar Initial: NG End: NG

Diluent: NG Nutrient Broth: NG

Test Compound (a) Virgin-NG (b) Recycle-NG (c) Sludge-NG (d) NA (e) NA

G = Growth NG = No Growth NT = Not Tested NA = Not Applicable WT = Wild Type

Spontaneous Revertants: TA 100, No S-9 86, 99, 104 average = 96

(1) + = expected response - = unexpected response

Study Number: 83001 Date: 19 Jan 83 By: Sauers, Kellner

TABLE 2

TOXICITY LEVEL DETERMINATION

Substance assayed: Virgin DMSO Substance dissolved in: DMSO (reagent grade)

Study Number: 83001 Date: 19 Jan 83 Performed by: Sauers, Kellner, Mullen, Dacey

TA 100 REVERTANT PLATE COUNT

Test Compound Concentration	Plate #1	Plate #2	Plate #3	Average	Background Lawn (1)
100% solution	77	74	84	78	NL
10% solution	79	93	108	93	NL
1% solution	98	93	110	100	NL
0.1% solution	111	99	110	107	NL
0.01% solution	95	108	118	107	NL
0.001% solution	77	81	79	79	NL
0.0001% solution	70	95	89	85	NL
0.00001% solution	81	70	76	76	NL

(1) NG = No Growth ST = Slight Growth NL = Normal Lawn

TABLE 3

TOXICITY LEVEL DETERMINATION

Substance assayed: DMSO Recycle Solvent Substance dissolved in: Reagent grade DMSO

Study Number: 83001 Date: 19 Jan 83 Performed by: Sauers, Kellner, Mullen, Dacey

TA 100 REVERTANT PLATE COUNT

Test Compound Concentration	Plate #1	Plate #2	Plate #3	Average	Background Lawn (1)
100% solution	132	116	123	124	NL
10% solution	106	84	91	94	NL
1% solution	94	96	93	94	NL
0.1% solution	92	107	77	92	NL
0.01% solution	81	86	95	87	NL
0.001% solution	82	89	99	90	NL
0.0001% solution	83	97	111	97	NL
0.00001% solution	89	90	96	92	NL

(1) NG = No Growth ST = Slight Growth NL = Normal Lawn

TABLE 4

TOXICITY LEVEL DETERMINATION

Substance assayed: Evaporator Sludge Substance dissolved in: Reagent grade DMSO

Study Number: 83001 Date: 19 Jan 83 Performed by: Sauers, Kellner, Mullen, Dacey

TA 100 REVERTANT PLATE COUNT

Test Compound Concentration	Plate #1	Plate #2	Plate #3	Average	Background Lawn (1)
100% solution	153	164	159	159	NL
10% solution	117	119	89	108	NL
1% solution	105	96	106	102	NL
0.1% solution	107	87	106	100	NL
0.01% solution	88	79	88	85	NL
0.001% solution	84	99	82	88	NL
0.0001% solution	83	87	89	86	NL
0.00001% solution	83	90	94	89	NL

(1) NG = No Growth ST = Slight Growth NL = Normal Lawn

TABLE 5

STRAIN VERIFICATION CONTROL

Strains	Histidine Requirement	Ampicillin Resistance	UV	Sensitivity to Crystal Violet	Sterility Control	Response (1)
98	NG	G	NG	NG 15mm	NG	+
100	NG	G	NG	NG 20mm	NG	+
1535	NG	NA	NG	NG 15mm	NG	+
1537	NG	NG - 29mm	NG	NG 13mm	NG	+
1538	NG	NA	NG	NG 15mm	NG	+
WT	G	NA	G	NA	NA	+

STERILITY CONTROL

His-Bio Mix Initial: NG End: NG Diluent: NG

Top Agar Initial: NG End: NG MGA Plate: NG

S-9 Mix Initial: NG End: NG Nutrient Broth: NG

Test Compound (a) Virgin-NG (b) Recycle-NG (c) Sludge-NG (d) NA (e) NA (f) NA

G = Growth NG = No Growth NT = Not Tested NA = Not Applicable WT = Wild Type

Study Number: 83001 By: Sauers, Kellner

Date: 26 Jan 83

(1) + = expected response
- = unexpected response

APPENDIX B (cont.)

TABLE 6

NUMBER OF REVERTANTS/PLATE

Compd.	Amount of Compd. Added	S-9 Added	Strain No. 98	100	1535	1537	1538
AF	2 ug/plate	yes	(256,497,339) 364	(278,335,con) 307			(408,645,368) 474
BP	2 ug/plate	yes	(92, 97, 67) 85	(586,412,627) 542		(81, 86, 70) 79	(115,142,112) 123
AA	2 ug/plate	yes	(709,758,934) 800	(999,999,999) 999		(116, 68, 82) 89	(999,867,999) 955
MNNG	2 ug/plate	no		(999,999,999) 999			
	20 ug/plate	no			(999,999,999) 999		
Spontaneous Reversion Rate							
	before	yes	(26, 23, 31)	(98, 95, 96)	(20, 11,con)	(5, 4, 7)	(18, 16, 18)
	after		(36, 37, 39) 32	(128,134,134) 114	(14, 20, 13) 16	(3, 10, 10) 7	(17, 28, 28) 21
	before	no	(11, 21, 26)	(95,102, 92)	(22, 23, 19)	(7, 6, 10)	(14, 24, 12)
	after		(36, 25, 23) 24	(129,113,119) 108	(22, 22, 24) 22	(7, 8, 6) 7	(20, 14, 19) 17

con - plate value was disregarded due to contamination

999 - signifies a revertant count greater than 1000

Study Number: 83001

Date: 26 Jan 83 By: Sauers, Kellner, Mullen, Dacey

TABLE 7

NUMBER OF REVERTANTS/PLATE

Compd.	0.1 ml Solution Added	S-9 Added	Strain Number 98	100	1535	1537	1538
Virgin DMSO	100% solution	no	(26, 19, 15) 20	(88, 99, 95) 94	(22, 16, 15) 18	(6, 6, 3) 5	(14, 15, 18) 16
		yes	(28, 34, 26) 29	(122, 92,112) 109	(18, 10, 11) 13	(6, 3, 6) 5	(15, 18, 17) 17
Virgin DMSO	20% solution	no	(16, 16, 17) 16	(96,106, 95) 99	(21, 14, 20) 18	(2, 5, 10) 6	(15, 13, 14) 14
		yes	(32, 32, 24) 29	(99, 88,101) 96	(17, 16, 10) 14	(4, 6, 7) 6	(16, 13, 19) 16
Virgin DMSO	4% solution	no	(28, 23, 14) 22	(105, 89,con) 97	(19, 18, 19) 19	(11, 5, 6) 7	(11, 12, 14) 12
		yes	(23, 17, 24) 21	(89, 99,101) 96	(17, 15, 23) 18	(6, 2, 2) 3	(22, 17,con) 20
Virgin DMSO	0.8% solution	no	(21, 17, 19) 19	(102, 98, 95) 98	(20, 11, 26) 19	(5, 6, 3) 5	(11, 14, 14) 13
		yes	(29, 29, 29) 29	(104, 99,120) 108	(18, 19, 14) 17	(9, 3, 7) 6	(29, 19, 17) 22

con - plate value was disregarded due to contamination

Study Number: 83001 Date: 26 Jan 83 By: Sauers, Kellner, Mullen, Dacey

APPENDIX B (cont.)

TABLE 7 (cont.)

NUMBER OF REVERTANTS/PLATE

Compd.	0.1 ml Solution Added	S-9 Added	Strain Number 98	100	1535	1537	1538
Virgin DMSO	0.16% solution	no	(17, 18, 14) 16	(120,102,101) 108	(25, 24, 31) 27	(3, 7, 6) 5	(15, 18, 14) 16
		yes	(25, 32, 27) 28	(101,119,104) 108	(10, 38, 14) 21	(3, 6, 5) 5	(23, 20, 24) 22
Virgin DMSO	0.032% solution	no	(20, 19, 13) 17	(121,106, 89) 105	(20, 12, 16) 16	(4, 3, 5) 4	(9, 12, 15) 12
		yes	(31, 22, 25) 26	(86,103,127) 105	(18, 12, 14) 15	(6, 3, 4) 4	(20, 22, 24) 22

APPENDIX B (cont.)

Study Number: 83001 Date: 26 Jan 83 By: Sauers, Kellner, Mullen, Dacey

TABLE 8

NUMBER OF REVERTANTS/PLATE

Compd.	0.1 ml Solution Added	S-9 Added	Strain Number 98	100	1535	1537	1538
Recycled DMSO	100% solution	no	(20, 15, 16) 17	(116, 98, 88) 101	(12, 16, 12) 13	(6, 7, 7) 7	(13, 17, 16) 15
		yes	(18, 16, 21) 18	(101,118,104) 108	(12, 9, 11) 11	(7, 8, 4) 6	(20, 16, 12) 16
Recycled DMSO	20% solution	no	(25, 21, 25) 24	(107,107,118) 111	(14, 25, 18) 19	(11, 8, 5) 8	(22, 23, 21) 22
		yes	(34, 30, 25) 30	(111,117, 99) 109	(27, 11, 15) 18	(5, 9, 11) 8	(26, 25, 21) 24
Recycled DMSO	4% solution	no	(18, 19, 19) 19	(112,105,140) 119	(18, 18, 35) 24	(7, 5, 7) 6	(20, 16, 13) 16
		yes	(33, 34,con) 34	(114,119, 86) 106	(26, 19, 20) 22	(4, 8, 9) 7	(24, 20, 21) 22
Recycled DMSO	0.8% solution	no	(24, 26, 21) 24	(101,114,114) 110	(20, 21, 23) 21	(7, 7,con) 7	(22, 20, 21) 21
		yes	(20, 21, 29) 23	(91, 88,109) 96	(23, 21, 20) 21	(1, 4, 6) 4	(29, 20, 21) 23

con - plate value was disregarded due to contamination

Study Number: 83001 Date: 26 Jan 83 By: Sauers, Kellner, Mullen, Dacey

APPENDIX B (cont.)

TABLE 8 (cont.)

NUMBER OF REVERTANTS/PLATE

Compd.	0.1 ml Solution Added	S-9 Added	Strain Number 98	100	1535	1537	1538
Recycled DMSO	0.16% solution	no	(25, 21, 17) 21	(111, 120, 95) 109	(27, 27, 22) 25	(4, 14, 6) 8	(20, 15, 23) 19
		yes	(42, 34, 33) 36	(104, 90, 99) 98	(10, 17, 27) 18	(5, 2, 6) 4	(19, 19, 18) 19
Recycled DMSO	0.032% solution	no	(16, 19, 23) 19	(110, 99, 110) 106	(25, 22, 14) 20	(7, 4, 5) 5	(15, 15, 19) 16
		yes	(38, 18, con) 28	(123, 107, 118) 116	(13, 11, 13) 12	(4, 4, 4) 4	(21, 22, 18) 20

con - plate value was disregarded due to contamination

Study Number: 83001 Date: 26 Jan 83 By: Sauers, Kellner, Mullen, Dacey

TABLE 9

NUMBER OF REVERTANTS/PLATE

Compd.	0.1 ml Solution Added	S-9 Added	Strain Number 98	100	1535	1537	1538
Evaporator sludge	100% solution	no	(115, 84, 121) 107	(140, 137, 154) 144	(42, 27, 38) 36	(21, 27, 14) 21	(123, 103, 96) 107
		yes	(53, 58, 78) 63	(126, 159, 142) 142	(29, 26, 25) 27	(23, 21, 20) 21	(66, 55, 74) 65
Evaporator sludge	20% solution	no	(33, 29, 29) 30	(110, 109, 156) 125	(22, 22, 21) 22	(14, 5, 6) 8	(30, 21, 29) 27
		yes	(38, 33, 27) 33	(113, 86, 105) 101	(18, 15, 20) 18	(10, 5, 8) 8	(33, 29, 26) 29
Evaporator sludge	4% solution	no	(24, 20, 26) 23	(95, 87, 97) 93	(22, 19, 22) 21	(4, 3, 4) 4	(19, 12, 14) 15
		yes	(36, 22, 27) 28	(126, 120, 108) 118	(7, 15, 13) 12	(4, 5, 3) 4	(19, 23, 23) 22
Evaporator sludge	0.8% solution	no	(24, 18, 15) 19	(94, 120, 103) 106	(15, 18, 18) 17	(5, 6, 4) 5	(15, 26, 22) 21
		yes	(35, 24, con) 30	(125, 104, 111) 113	(17, 16, 12) 15	(5, 6, 9) 7	(27, 27, 15) 23

con - plate value disregarded due to contamination

Study Number: 83001 Date: 26 Jan 83 By: Sauers, Kellner, Mullen, Dacey

APPENDIX B (cont.)

TABLE 9 (cont.)

NUMBER OF REVERTANTS/PLATE

Compd.	0.1 ml Solution Added	S-9 Added	Strain Number 98	100	1535	1537	1538
Evaporator sludge	0.16% solution	no	(14, 23, 26) 21	(111,101,114) 109	(22, 22, 23) 22	(2, 5, 6) 4	(17, 20, 14) 17
		yes	(29, 21, 30) 27	(123,102,108) 111	(19, 15, 18) 17	(5, 4, 3) 4	(18, 14, 16) 16
Evaporator sludge	0.032% solution	no	(16, 18, 23) 19	(107, 98,109) 105	(27, 19, 13) 20	(7, 5, 3) 5	(38, 15, 18) 24
		yes	(39, 26, 23) 29	(125,107,125) 119	(12, 18, 23) 18	(7, 5, 8) 7	(20, 16, 18) 18

APPENDIX B (cont.)

Study Number: 83001 Date: 26 Jan 83 By: Sauers, Kellner, Mullen, Dacey

APPENDIX B (cont.)

TABLE 10

STRAIN VERIFICATION CONTROL

Strains	Histidine Requirement	Ampicillin Resistance	Sensitivity to UV	Sensitivity to Crystal Violet	Sterility Control	Response (1)
98	NG	G	NG	NG (15 mm)	NG	+
100	NG	G	NG	NG (18 mm)	NG	+
1535	NG	NA	NG	NG (16 mm)	NG	+
1537	NG	NG (28 mm)	NG	NG (13 mm)	NG	+
1538	NG	NA	NG	NG (19 mm)	NG	+
WT	NA	NA	G	G	NA	+

STERILITY CONTROL

His-Bio Mix Initial: NG End: NG Diluent: NG

Top Agar Initial: NG End: NG MGA Plate: NG

S-9 Mix Initial: NG End: NG Nutrient Broth: NG

Test Compound (a) Evaporator sludge - NG (b) NA (c) NA (d) NA (e) NA (f) NA

G = Growth NG = No Growth NT = Not Tested NA = Not Applicable WT = Wild Type

Study Number: 83001 By: Sauers, Kellner

Date: 17 Mar 83

(1) + = expected response
- = unexpected response

TABLE 11

NUMBER OF REVERTANTS/PLATE

Compd.	Amount of Compd. Added	S-9 Added	Strain No. 98	100	1535	1537	1538
AF	2 ug/plate	yes	(439,344,359) 381	(289,385,148) 274			(553,516,452) 507
BP	2 ug/plate	yes	(88,102, 76) 89	(440,422,487) 450		(74, 66, 70) 70	(97,102, 97) 99
AA	2 ug/plate	yes	(999,999,999) 999	(999,999,999) 999		(243,176,186) 202	(965,999,999) 988
MNNG	2 ug/plate	no		(999,999,999) 999			
	20 ug/plate	no			(999,999,999) 999		
Spontaneous Reversion Rate							
	before	yes	(30, 34, 39)	(97,104,111)	(8, 14, 12)	(5, 4, 3)	(21, 19, 17)
	after		(27, 26, 34) 32	(99,103,112) 104	(9, 12, 13) 11	(5, 2, 7) 4	(22, 20, 25) 21
	before	no	(17, 30, 15)	(95, 82,101)	(22, 13, 15)	(4, 4, 4)	(9, 17, 12)
	after		(14, 20, 29) 21	(104,109, 97) 98	(10, 14, 18) 15	(4, 9, 8) 6	(19, 17, 10) 14

999 - signifies a revertant count greater than 1000

Study Number: 83001

Date: 17 Mar 83 By: Sauers, Kellner, Mullen, Dacey

TABLE 12

NUMBER OF REVERTANTS/PLATE

	Compd.	0.1 ml Solution Added	S-9 Added	Strain Number 98	100	1535	1537	1538
1	Evaporator sludge	100% solution	no	(122, 72, 183) 126	(153, 149, 144) 149	(17, 18, 26) 20	(27, 14, 23) 21	(119, 140, 131) 130
2			yes	(72, 71, 76) 73	(139, 141, 120) 133	(27, 21, 29) 26	(21, 14, 20) 18	(82, 71, 54) 69
3	Evaporator sludge	80% solution	no	(77, 41, 129) 82	(158, 119, 129) 135	(20, 19, 12) 17	(33, 18, 20) 24	(85, 107, 108) 100
4			yes	(71, 59, 65) 65	(127, 126, 130) 128	(24, 23, 22) 23	(16, 17, 15) 16	(62, 84, 65) 70
5	Evaporator sludge	60% solution	no	(59, 76, 55) 63	(112, 117, 117) 115	(14, 10, 17) 14	(14, 17, 17) 16	(54, 60, 80) 65
6			yes	(52, 50, 46) 49	(136, 116, 118) 123	(23, 18, 32) 24	(6, 12, 10) 9	(48, 54, 62) 55
7	Evaporator sludge	40% solution	no	(46, 47, 34) 42	(116, 142, 136) 131	(14, 17, 13) 15	(11, 7, 12) 10	(37, 53, 46) 45
8			yes	(36, 35, 44) 38	(106, 103, 94) 101	(22, 20, 15) 19	(14, 7, 11) 11	(51, 42, 40) 44

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APPENDIX B (cont.)

TABLE 12 (cont.)

NUMBER OF REVERTANTS/PLATE

Compd.	0.1 ml Solution Added	S-9 Added	Strain Number 98	100	1535	1537	1538
Evaporator sludge	20% solution	no	(40, 36, 39) 38	(122,117,124) 121	(14, 19, 21) 18	(6, 4, 8) 6	(34, 34, 30) 33
		yes	(31, 32, 27) 30	(127,101,116) 115	(19, 13, 14) 15	(5, 6, 5) 5	(43, 34, 45) 41
Evaporator sludge	1% solution	no	(18, 12, 14) 15	(84, 74,118) 92	(7, 17, 23) 16	(6, 4, 8) 6	(9, 13, 7) 10
		yes	(26, 33, 27) 29	(103,123, 96) 107	(7, 12, 10) 10	(7, 8, 4) 6	(13, 25, 18) 19

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LIST OF FIGURES

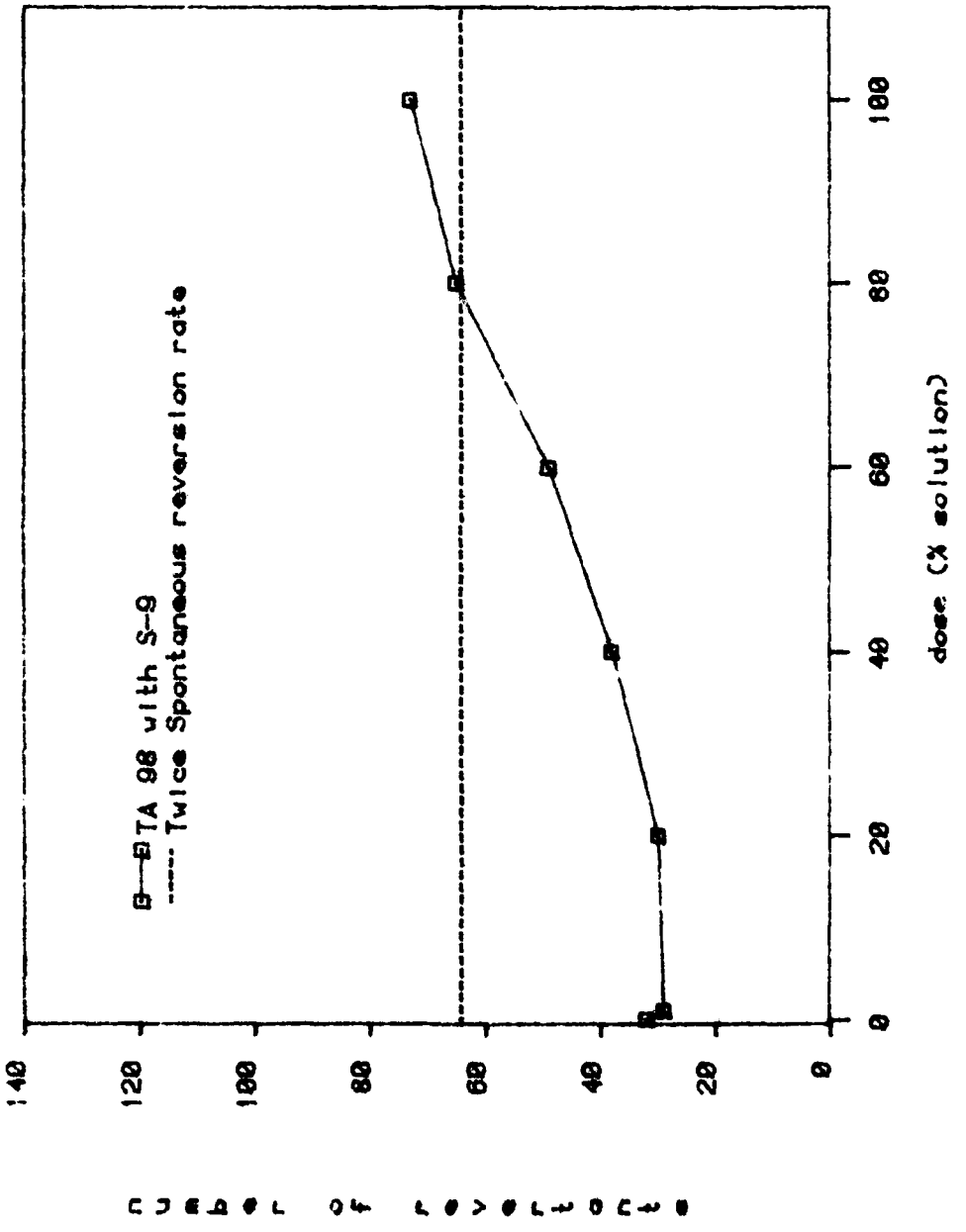


FIGURE 1

APPENDIX C

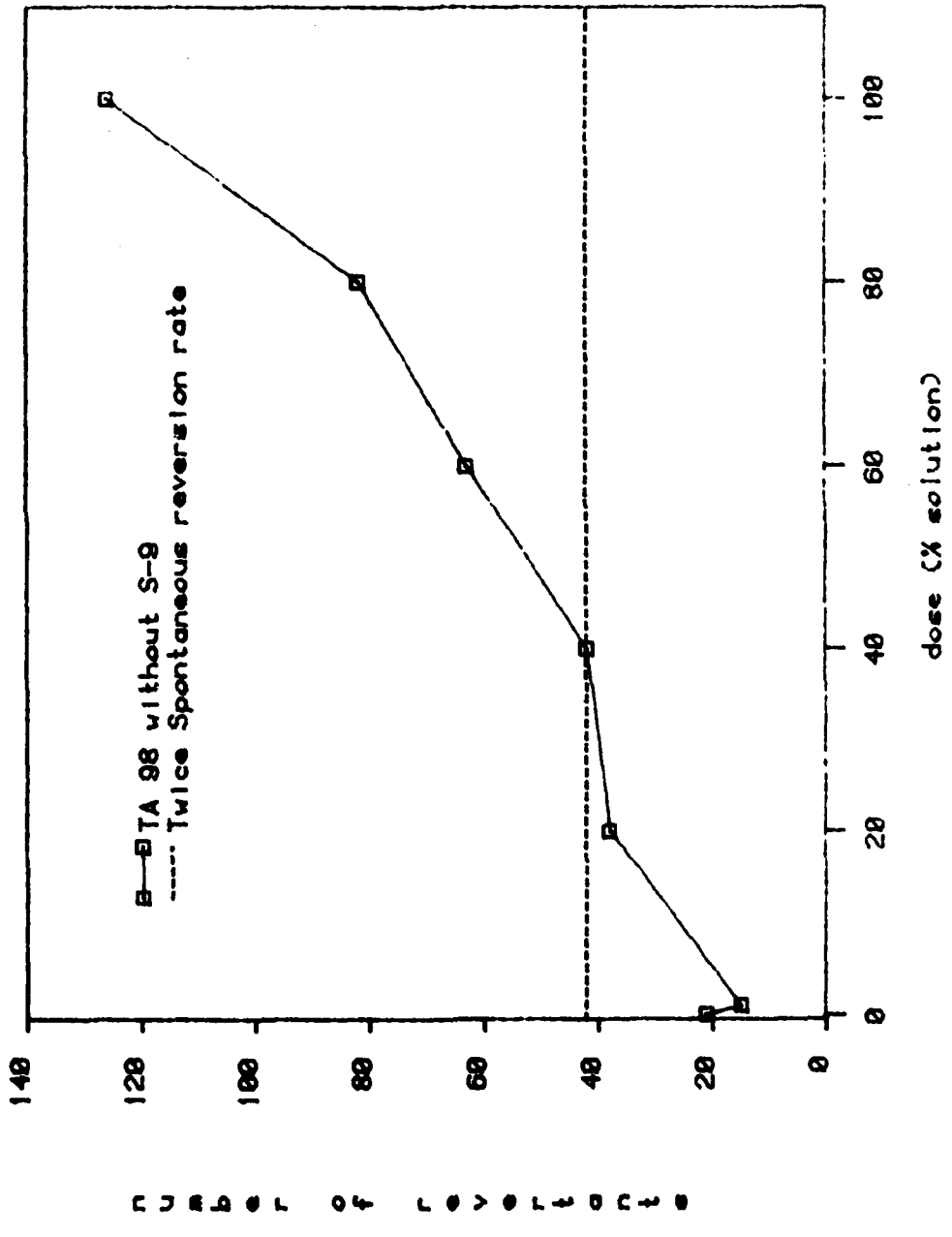

TA 98 without S-9
Twice Spontaneous reversion rate
dose (% solution)
0
20
40
60
80
100
0
20
40
60
80
100
120
140

FIGURE 2

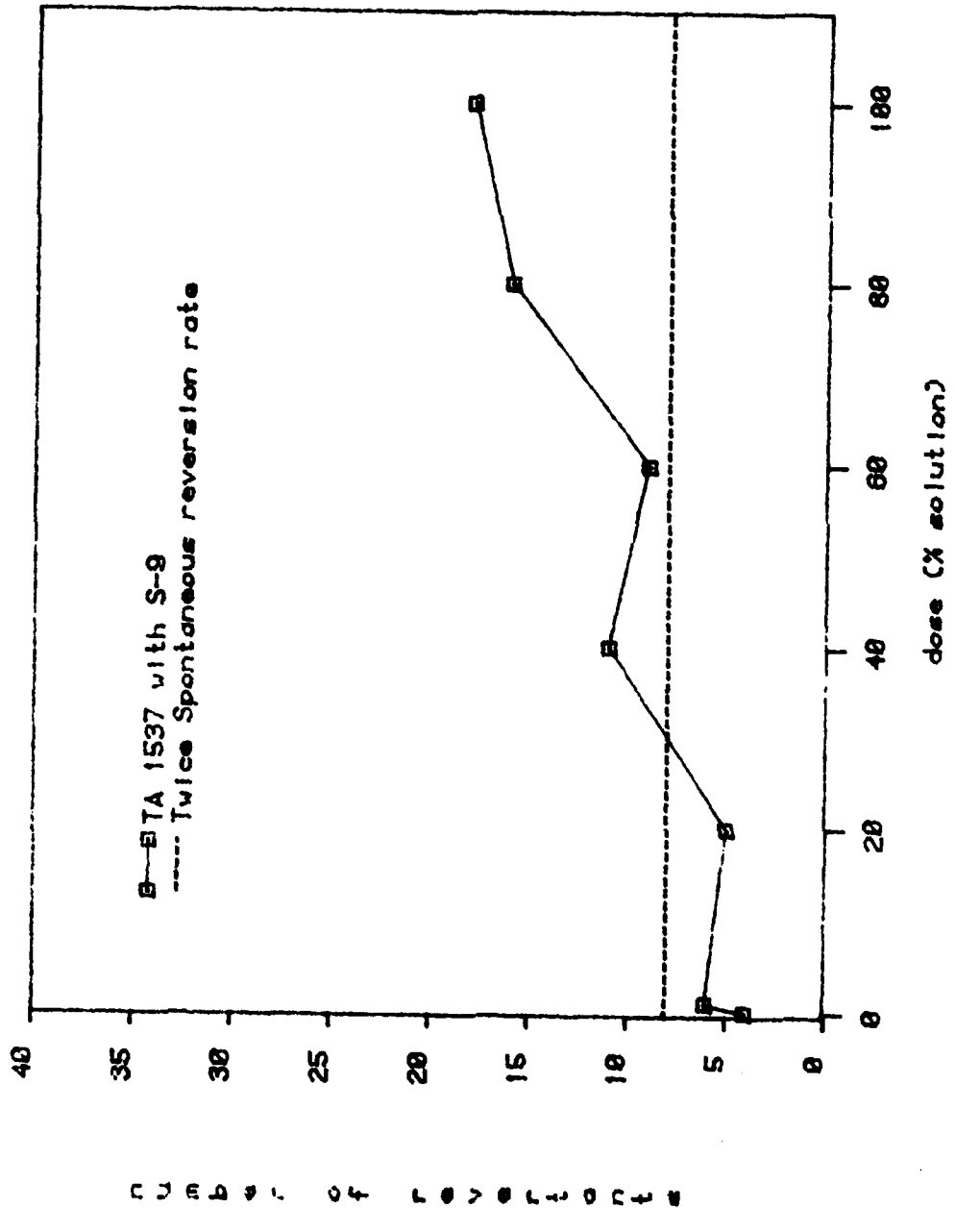
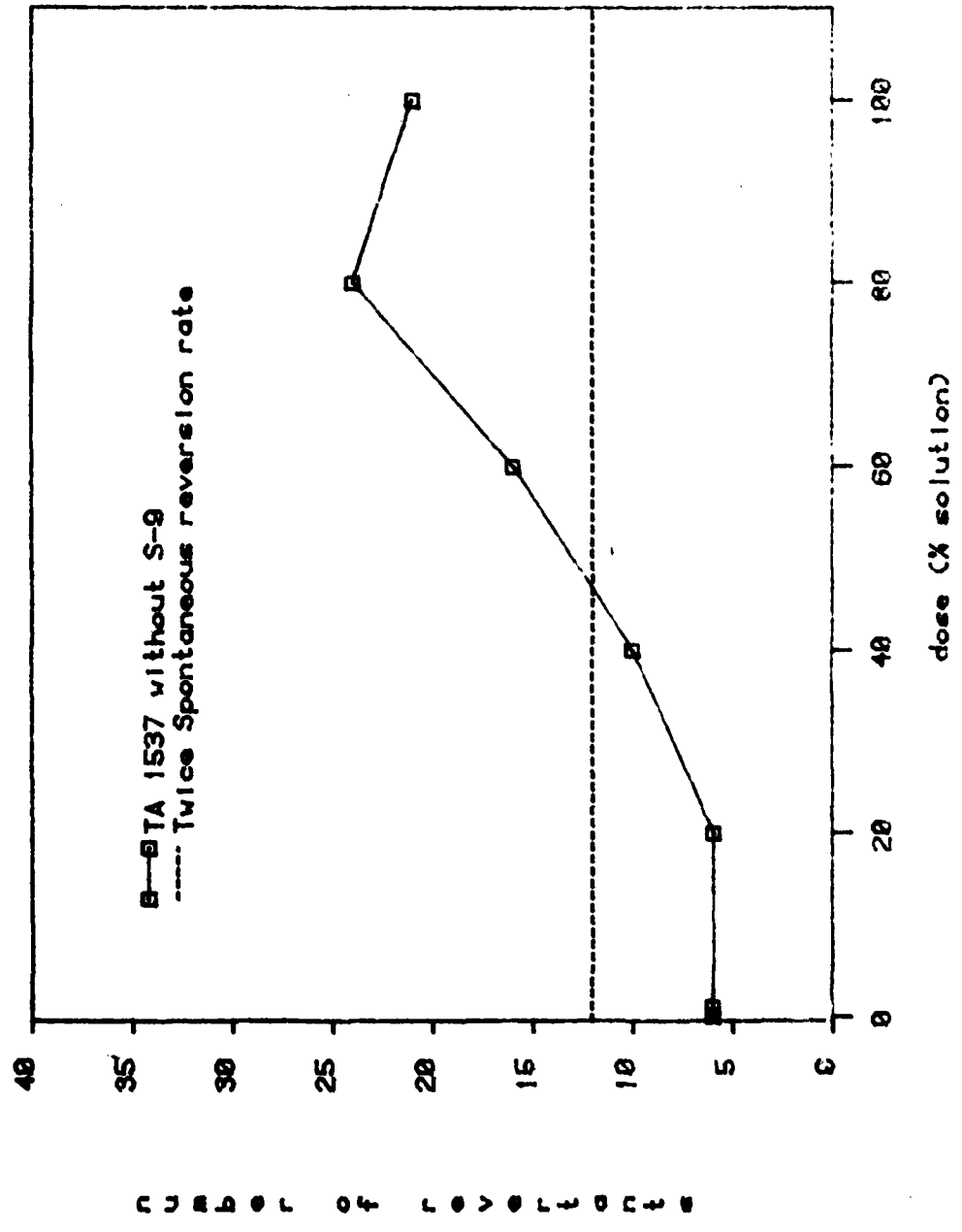


FIGURE 3

APPENDIX C (cont.)

FIGURE 4



APPENDIX C (cont.)

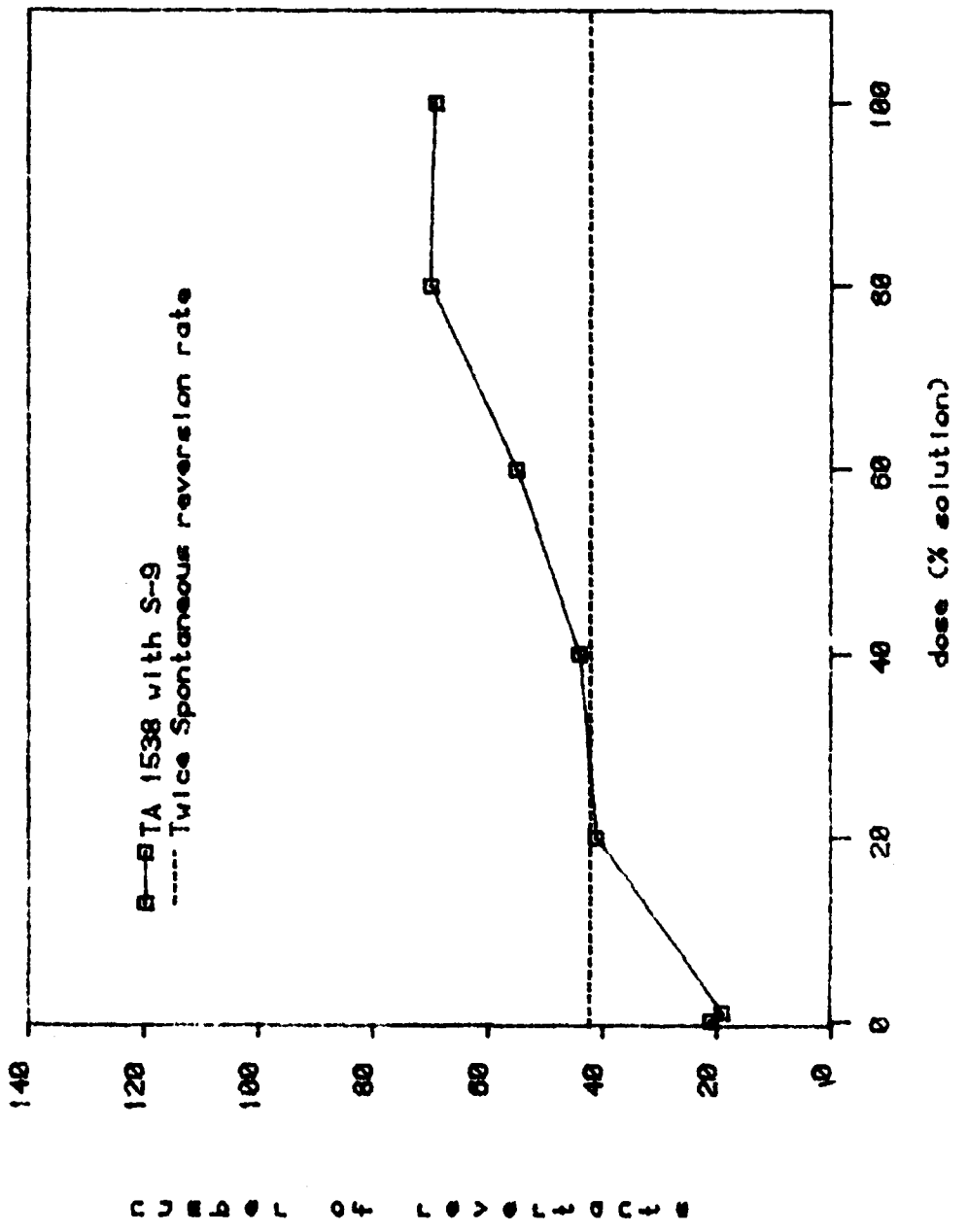

TA 1538 with S-9
Twice Spontaneous reversion rate
dose (% solution)
0
20
40
60
80
100
120
140

FIGURE 5

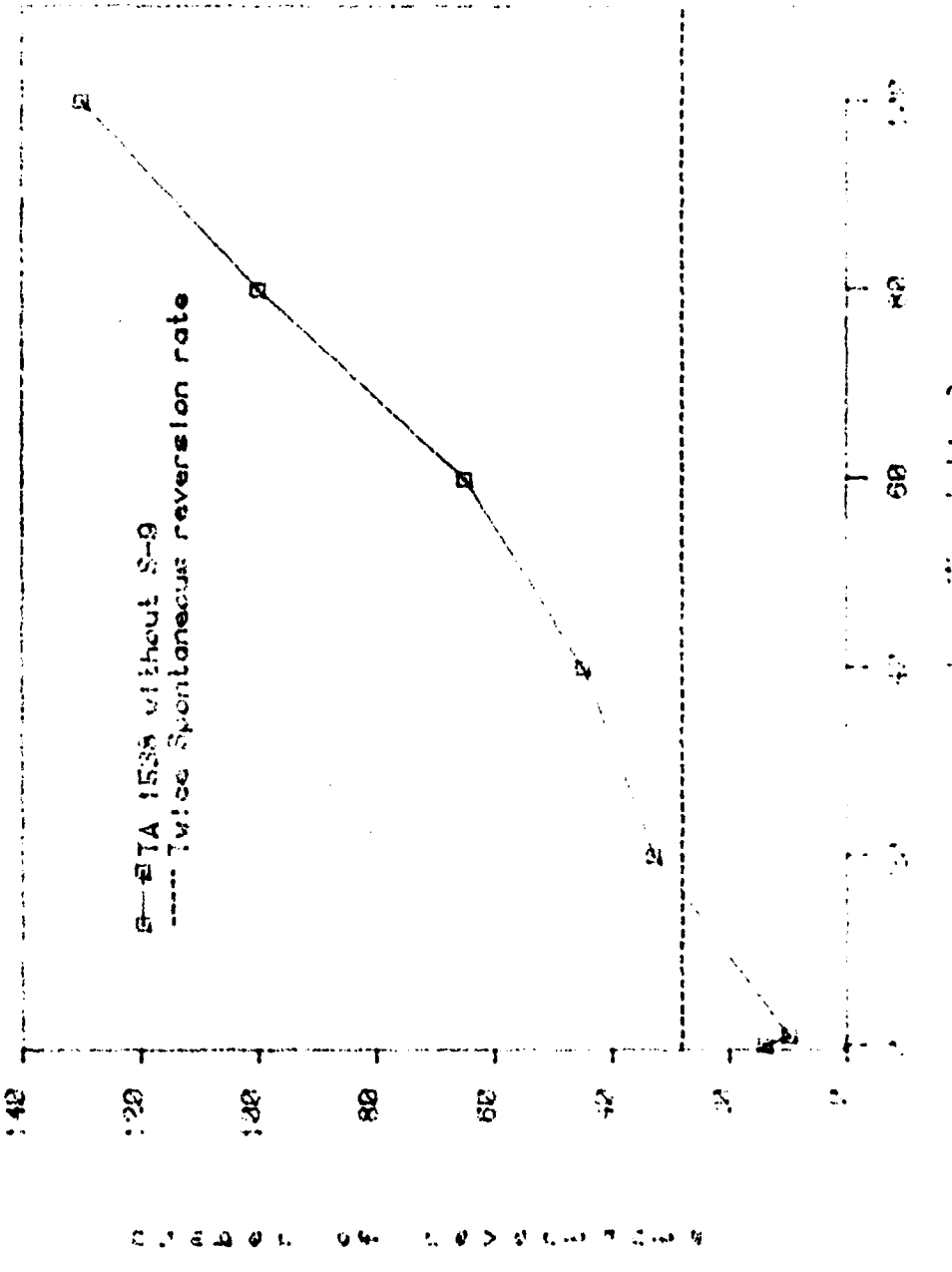


FIGURE 6

APPENDIX C (concluded)

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