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THE MUTAGENIC POTENTIAL OF: 4-NITROPHENYL DIPHENYL
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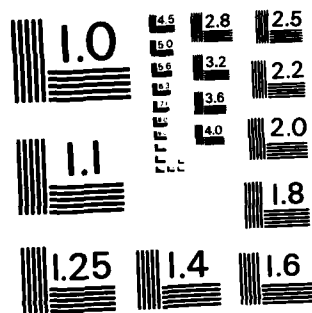
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INSTITUTE REPORT NO. 141

MUTAGENIC POTENTIAL OF:

4-nitrophenyl diphenyl phosphinate
using the *Drosophila melanogaster* sex-linked recessive lethal test

NELSON R. POWERS, PhD, CPT MS
and
PAUL WARING, BS

TOXICOLOGY GROUP,
DIVISION OF RESEARCH SUPPORT

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Toxicology Series 45

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Mutagenic Potential of: 4-Nitrophenyl Diphenyl Phosphinate using the Drosophila melanogaster Sex-linked Recessive Lethal Test--Powers and Waring

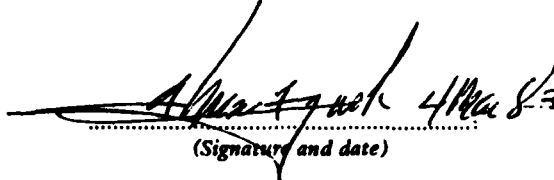
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The compound 4-nitrophenyl diphenyl phosphinate (TA016) is being con- sidered as a prophylactic agent in anticholinesterase poisoning. It was tested for mutagenic activity using <u>Drosophila melanogaster</u> Sex-linked Recessive Lethal Assay. After 72-hour feeding exposures to 0.35, 0.47, and 0.51 mM, TA016 appeared non-mutagenic.		

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ABSTRACT

The compound, 4-nitrophenyl diphenyl phosphinate (TA016) is being considered as a prophylactic agent in anticholinesterase poisoning. It was tested for mutagenic activity using Drosophila melanogaster Sex-linked Recessive Lethal Assay. After a 72-hour feeding exposure to 0.35, 0.47 and 0.51 mM, TA016 appeared non-mutagenic.

KEY WORDS: Mutagenicity; Toxicology; Sex-Linked Recessive Lethal Assay; Drosophila melanogaster; 4-nitrophenyl diphenyl phosphinate.



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PREFACE

TYPE REPORT: Drosophila melanogaster Sex Linked Recessive Lethal Assay

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

SPONSOR: U.S. Army Medical Research and Development Command,
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Aberdeen Proving Grounds, Aberdeen, MD 21005

PROJECT/WORK UNIT/APC: 35162772A875 Medical Defense Against Chemical Agents, WU 304 Toxicity Testing of Phosphinate Compounds, APC TLO4

GLP STUDY NUMBER: 81030

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

PRINCIPAL INVESTIGATOR: CPT Nelson R. Powers, PhD, MS

CO-PRINCIPAL INVESTIGATOR: Paul P. Waring, BS

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

TEST SUBSTANCE: 4-nitrophenyl diphenyl phosphinate (TA016).

INCLUSIVE STUDY DATES: 18 September 1981 - 14 July 1982.

OBJECTIVE: The purpose of this study was to assess the mutagenic potential of the organophosphinate compound, 4-nitrophenyl diphenyl phosphinate, using Drosophila melanogaster in the sex-linked recessive lethal assay.

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ACKNOWLEDGMENTS

The author wishes to thank SP5 Kincannon, BS; SP4 Mullen, BS; and SP4 Rodriguez, BS, for their assistance in performing the research.

The present generation is only a caretaker of the human genome of future generations.

Malling and Vakovic, 1978

Signatures of Principal Scientists
Involved In the Study

We the undersigned, believe the GLP Study numbered 81030, 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 for Nonclinical Laboratory Studies as outlined by the Food and Drug Administration.

John T. Fruin 30 Apr 83
JOHN T. FRUIN, DVM, PhD / DATE
COL, VC
Study Director

Nelson R. Powers 28 March 83
NELSON R. POWERS, PhD / DATE
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Principal Investigator

Paul P. Waring 28 Mar 83
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DEPARTMENT OF THE ARMY
LETTERMAN ARMY INSTITUTE OF RESEARCH
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REPLY TO
ATTENTION OF:

SGRD-ULZ-QA

11 February 1983

MEMORANDUM FOR RECORD

SUBJECT: Report of GLP Compliance

I hereby certify that in relation to LAIR GLP study 81030, the following inspections were made:

7 Oct 81
13 Oct 81, 1330 hours
13 Oct 81, 1530 hours
30 Oct 81
6 Nov 81
30 Nov 81
9 Dec 81
19 May 82
10 Jun 82

The report and raw data for this study were audited on 21 Jan 83.

Routine inspections with no adverse findings are reported quarterly, thus these inspections are also included in the January 1982 and July 1982 report to management and the Study Director.

JOHN C. JOHNSON
CPT (P), MSC
Quality Assurance Officer

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MUTAGENIC POTENTIAL OF: 4-nitrophenyl diphenyl phosphinate using the Drosophila melanogaster sex-linked recessive lethal test

Currently the use of organophosphinates as prophylactic agents for anticholinesterase poisoning is being investigated. Due to the potential success of these investigations and consequent widespread use of these compounds, their mutagenicity is being studied. Among the various screening programs, the Drosophila melanogaster sex-linked recessive lethal (SLRL) assay is being performed. This report contains our findings from SLRL assay studies.

Rationale for SLRL Testing

In addition to the tests for acute and chronic toxicity, evaluation of genetic damage from exposure to chemicals must be considered. A variety of tests using Drosophila are available for the detection of specific types of genetic changes. The most sensitive assay which detects the broadest range of mutations is the SLRL test (1-3). This test uses insects of a known genotype and detects lethal mutagenic changes in 800 to 1000 loci on the X-chromosome, representing 80% of the X-chromosome or 20% of the entire genome (4,5). To date, the SLRL test has been used in most of the research on mutagenicity of test substances (1,3,4).

Genetic Basis of SLRL Test

The basic mechanism of the SLRL test is that the X-chromosome of the father is passed on to the daughter; the sons receive the X-chromosomes from the mother. The recessive lethal genes located on the X-chromosomes are expressed in males in a hemizygous condition. Since the Y-chromosome does not contain the dominant wild-type alleles to suppress their manifestation, this results in death.

The SLRL test relies on the fact that among the progeny of females carrying a recessive lethal on one of her X-chromosomes (heterozygous for a recessive lethal), half of the sons will die. By the use of suitable genetic markers, the class of males carrying the X-chromosomes of treated grandfathers can easily be determined. If a lethal was induced, this class will be missing and is easily scored.

This test is described as the Basc or Muller-5 test (5,6). The assay system uses strains which prevent the crossing-over in females heterozygous for the lethal-bearing chromosome; transfer of the lethal from the paternal to the maternal X-chromosome by genetic recombination restores viability of the chromosomes under test and leads to erroneous results. Consequently, males receiving the X-chromosome survive. Since combinations of suitable inversions effectively inhibit the occurrence of crossing over, females used for the test carry two scute inversions; the left-hand part of SC¹ and the right-hand part SC⁸ covering the whole X-chromosome, and a smaller inversion In-S in the Basc and d1-49 in the mscy chromosome (5).

Description of Test

The test (7) was developed in 1948 for determining genetic changes which in hemizygous but not homozygous or heterozygous conditions kill the developing individual (egg to pre-adult stage). Such genetic factors, recessive lethals, can be induced on all chromosomes. Only two test generations are needed to detect if sex-linked recessive lethals have been induced on the X-chromosome.

In the SLRL test, wild-type males, normal round red eyes, (we use Canton-S (CS)) are exposed to the test materials (treated). Such an exposure will be regarded as a recessive lethal if it affects the X-chromosome. These males are mated to homozygous females (we use First Multiple Number 6 (FM6)) carrying the Basc chromosome. This chromosome is expressed as bar (narrow-shaped) eyes, white-apricot in color. The bar serves as a genetic marker in homozygous or hemizygous conditions. It is kidney-shaped in heterozygous females. The progeny of this cross now consists of females heterozygous for the treated X-chromosome, characterized by kidney-shaped, red eyes and males of the FM6 phenotype that have received their X-chromosome from their FM6 mother. Each F₁ female represents one paternal X-chromosome, treated in the male gametes. These siblings are mated to produce the F₂ generation. This generation now consists of males of two phenotypic expressions, those with round red eyes (hemizygous carrying the treated X-chromosome from the F₁ female) and bar-shaped, white-apricot eyed males (hemizygous for the Basc chromosome); and females of two phenotypic expressions, kidney-shaped, red eyed (heterozygous, carrying the treated X-chromosome from the F₁ females and the Basc chromosome) and the females that are bar-shaped, white-apricot eyed (homozygous for the Basc chromosome). This generation is inspected for the presence of males with round red eyes. If this class is missing, it can be concluded that the treated male gamete contained a recessive lethal. Thus, this test relies upon the disappearance of a whole Mendelian class (males with round red eyes).

Brooding

As part of the SLRL testing, a brooding scheme was used to sample sperm cells exposed to the test substance. This is done as chemicals often exhibit stage-specificity on different stages of germ cell development. The brooding scheme was done at intervals of 3, 2, 2 and 3 days. This insures that sperm exposed to the test material are in different stages of development: Brood 1 = mature and near-mature sperm; Brood 2 = primarily spermatids; Brood 3 = primarily meiotic stages; and Brood 4 = primarily spermatogonia. This procedure safeguards against the possibility that chemicals with more pronounced effects in earlier stages of spermatogenesis are not erroneously dismissed as false negatives.

Objective of Study

The objective is to assess the mutagenic potential of the organophosphinate compound, 4-nitrophenyl diphenyl phosphinate by using Drosophila melanogaster in the SLRL Assay.

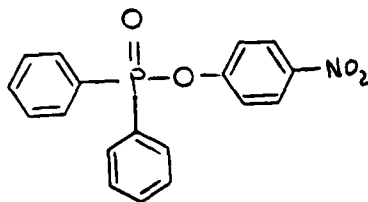
MATERIALS AND CONDITIONS

Test Substance

Chemical name: 4-nitrophenyl diphenyl phosphinate
(LAIR Code TA016)

CAS: none

Molecular structure: $C_{18}H_{14}NO_4P$



Molecular weight: 339

Vehicle

Due to the instability of TA016 when prepared in an aqueous system, a mixture of Tween 80TM, ethanol (100%), citrate buffer, (5.0 mM) and distilled water were prepared to stabilize the test

Powers—4

compound (LAIR SOP OP-STX-45, Preparation of Compounds Unstable in Water for SLRL Assay). This mixture was appropriate for consumption by test insects.

CAS: N/A

Molecular structure: N/A - mixture

Molecular weight: N/A - mixture

Analytical data appear in Appendix A.

Test Model

Insect Genus and Species: Drosophila melanogaster

Strains: Canton-S (CS), a wild-type stock, characterized by round red eyes. This stock is selected for mutagenicity studies because it has shown a relatively low constant spontaneous mutation frequency (8).

First Multiple Number 6 (FM6) is a laboratory stock containing the homozygous Basc chromosome for females and the hemizygous Basc X-chromosome for males. This strain carries the phenotypic markers for yellow body (Y), bar-shaped eye (B), and white-apricot colored eye (W), and several superimposed inversions which prevent "crossing over" (exchange of chromosome segments) with homologous non-inverted X-chromosomes.

Both strains are presently being reared in the insectary at Letterman Army Institute of Research. The original stock colonies were obtained from the University of Wisconsin, Madison, Wisconsin.

Diet

The diet is the standard medium used for colony rearing of D. melanogaster. Materials and instructions for its preparation are contained in LAIR SOP OP-STX-5, Drosophila Media Preparation.

Restraint

Ether for anesthesia was used only when conducting matings of F_2 and F_3 generations and for general colony maintenance.

Identification

Each CS male from the LC₅₀, 72-hour exposure (test, negative and positive control), had a unique number assigned and placed on the vial in which its progeny were produced (LAIR SOP OP-STX-8, Sex-linked Recessive Lethal (SLRL) Drosophila melanogaster Mutagenicity Test). In this manner progeny were traced back to the parental male which had been subjected to the test compound or controls.

Environmental Conditions

All studies were conducted within the insectary at a temperature of 21 ± 4 C, relative humidity of 50 ± 5%, and a photoperiod of 12 hours light and 12 hours dark. All insect colonies were reared in polypropylene bottles and SLRL testing was done in glass vials (LAIR SOP OP-STX-6, Drosophila Stock Colony Maintenance).

Dosing

Dosing was done in compliance with LAIR SOP OP-STX-7, Drosophila melanogaster Exposure Procedures and LAIR SOP OP-STX-45, Preparation of Compounds Unstable in Water for SLRL Assay, by allowing the CS strain (wild-type) male to feed upon 250 ul of various concentrations of the test chemical formulated with Tween 80™, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose. These males formed the test groups. Concurrent exposure, 250 ul of a mixture of Tween 80™, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose for CS males to feed upon were designated as negative control (spontaneous mutation frequency), and 250 ul of a mixture of 1 mM ethylmethane sulfonate with Tween 80™, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose for CS males to feed upon were designated as positive control. Ethylmethane sulfonate is a known mutagen and was used to confirm the ability of the test organism to produce SLRL mutagens (9). Dosing was done continuously for 72 hours with test compound replenishment every 24 hours for a total of 3 exposures. For test chemical exposure, a pilot toxicity study was done to establish the upper and lower limits of mortality. Based on the results of this study, a second study was conducted from which an exposure level was selected (usually the LC₅₀ level for treated males after 72 hours of exposure (4)). It was these males that were used in the SLRL testing. For TA016, groups of 100 CS males were exposed to each mean dose level (based on six replications) in this second study of 0.096, 0.233, 0.358, 0.410, 0.521, 0.572, and 0.831 mM. The male insects surviving the LC₅₀ (or as close to the LC₅₀ as possible) in each of the six replicates were selected for the SLRL assay.

Test Format

The CS males surviving the LC_{50} of the test chemical after 72 hours of exposure and those males subjected to the concurrent controls were used in the SLRL assay. Progeny of the survivors from the test chemical and negative control compound exposure were scored by mating 25 dosed, CS males (wild-type) to FM6-virgin females (Basc chromosome). This was done by placing 3 FM6 virgin females in a vial with one CS male; the vial was labelled with that male's unique number. At intervals of 3, 2, 2, and 3 days, the CS male was transferred to successive groups of 3 FM6-virgin females in vials with that male's unique number. These intervals of days corresponded to broods 1, 2, 3, and 4. This procedure was replicated 6 times. Scoring of the mutants resulting from positive control exposure was based on mating of 10 CS males for five replications and the mating of 5 CS males for the sixth replication, using the above mating scheme. After sufficient numbers of flies had emerged, a maximum of 25 (minimum of 5) kidney-shaped, red eyed F_1 females were selected at random and mated with their sibling bar-shaped, white-apricot eyed male. Each pair was placed in an individual vial and these vials, from the same unique numbered father, were placed together and labelled with that unique number for reference. After 2 to 3 weeks, the F_2 progeny were examined and scored for the absence of round red eyed males, which indicated that a lethal mutation had taken place in the treated male. To confirm this, an F_3 cross was conducted from each vial scored as a lethal mutation. This F_3 cross consisted of three F_2 females (kidney-shaped, red eyed) crossed with one bar-shaped, white-apricot eyed male. Experimental conclusions were based on the spontaneous mutation frequency (negative control) compared to the mutation frequency induced by the test chemical.

Historical Listing of Significant Study Events

Appendix B contains the Historical Listing of Study Events.

Statistical Analysis

Data analysis of the mutation frequency of the test compound, TA016 was compared to the spontaneous mutation frequency (negative control) by using the Fisher's Exact 2 X 2 table (10,11). This test is independent of sample size; a more conservative test, the Kastenbaum-Bowman test (12,13) dependent upon sample size was also considered. These tests were based on the number of lethal and non-lethal culture vials of the total number examined (each culture vial contains F_2 progeny, and each vial is considered as an X-chromosome) from each unique numbered male. Also vials without F_2

progeny or less than 5 progeny (F_2) were scored as failures. In addition, the mutation frequency of each of the four broods was also analyzed.

Change in Procedure During Study

Deviations were made in the following standard operating procedures: In LAIR SOP-OP-STX-45, Preparation of Compounds Unstable in Water for SLRL Assay, to prepare various concentrations of TA016 with the vehicle, various amounts of the stock TA016 with various amounts of distilled water and a constant volume of fructose and diluent were required. However, in place of the various amounts of distilled water, a "blank" (Tween 80TM, ethanol, citrate buffer, and distilled water) was substituted. This was done to aid in solubility. LAIR SOP-OP-STX-7, Drosophila melanogaster Exposure Procedures. The filter papers upon which test and control solutions were placed for feeding were washed in 150 ml of deionized water with 2-3 drops of 1 N HCL. This procedure is outlined in SOP OP-STX-68, Preparation of Glass Fiber Discs for Exposure to Unstable Compounds. This served to help further stabilize the TA016 during exposure for feeding. LAIR SOP-OP-STX-8, Sex-Linked Recessive Lethal (SLRL) Drosophila melanogaster Mutagenicity Test, requires the testing of 8,000 to 10,000 X-chromosomes. This is the sample size to be used for analysis by the Kastenbaum-Bowman test; however, as this test was not employed, this sample size was not critical. LAIR SOP-OP-STX-6, Drosophila Stock Colony Maintenance, outlines the acceptable limits of spontaneous mutations not to exceed 0.3%. In addition, other researchers (4) have defined the acceptable limits not to exceed 0.3%. Therefore, of the spontaneous mutations resulting from the six replications as well as the concurrent test in this study, those numbered 2, 3, and 4 were deleted from this study as the spontaneous mutation exceeded these limits (Appendix C, Table 2). Therefore, statistical analysis was based on replications numbered 1, 5, and 6 whose spontaneous mutations did not exceed this limit (Appendix C, Table 3).

RESULTS

Measurements of TA016 formulated with the vehicle and its stability were made with a spectrophotometer. The various concentrations (mM) of TA016 used in dosing were prepared from a stock of approximately 8 mM (theory). Based on this initial theoretical concentration, the mean theoretical concentration from the 6 replicates exposed to the test organism over the 72-hour period were 0.079, 0.239, 0.319, 0.399, 0.478, 0.585, and 0.797 mM. The initial spectrophotometer reading indicated the actual mean amounts from the six replicates were 0.096, 0.233, 0.358, 0.410, 0.521, 0.572, and 0.831 mM. Twenty-four hours after formulation, hydrolysis measurements were made for each concentration of the 6 replicates and

showed a range in hydrolysis from 1.5 to 13.8%. It is believed that these concentrations were stable enough to be used over the 72-hour exposure period.

The concentrations of TA016 producing the mean percentage mortality and standard deviation after 72 hours of exposure from which the surviving males were selected for SLRL testing are shown in Appendix C, Table 1. The 100 CS males exposed to each of the concurrent negative controls for the six replications showed a total mean mortality of 1.6%.

The SLRL mutation frequency resulting from CS males exposed to the concurrent positive controls, 1 mM ethylmethane sulfonate was 15.6%.

The results of the six replications of the test compound, TA016 and concurrent negative controls are shown in Appendix C, Table 2. However, replications 2, 3, and 4 were deleted from the study, as the concurrent negative control mutation frequencies exceeded the acceptable limits. Therefore, statistical analysis was based on replications numbered 1, 5, and 6; their spontaneous mutation frequency was 0.164%, based on 6,669 X-chromosomes; while the mutation frequency resulting from the test compound, TA016, was 0.272% based on 3,665 X-chromosomes (Appendix C, Table 3). The criteria used as an indication of the compound being at least a weak mutagen is the detection of a doubling of the spontaneous mutation frequency based on the Katzenbaum-Bowman tables (12). To detect such doubling requires between 7,000 to 9,000 control and test X-chromosomes. However, the resulting sample size of 3,665 X-chromosomes from the test compound was inadequate. Therefore, the Kastenbaum-Bowman test would not be as sensitive as the Fisher's Exact test, although both tests are nearly equivalent (13). Thus, the Fisher's Exact Test (LAIR SOP OP-STX-10, Execution of Fortran V Program FEXP) based on replicated 1, 5, and 6 was used.

Compliance was made with the following standard operating procedures: LAIR SOP OP-STX-3, Positive Control Substances; LAIR SOP OP-STX-5, Drosophila Media Preparation; LAIR SOP OP-STX-6, Drosophila Stock Colony Maintenance; LAIR SOP OP-STX-7, Drosophila melanogaster Exposure Procedures; LAIR SOP OP-STX-8, Sex-Linked Recessive Lethal (SLRL) Drosophila melanogaster Mutagenicity Test.

Tabular data from this study (GLP 81030) for each male exposed to the substance, TA016, and concurrent negative and positive controls are in the archives of Letterman Army Institute of Research, Presidio of San Francisco, California.

DISCUSSION

The results of the Fisher's Exact Test, based on replications 1, 5, and 6, showed non-significant differences between the mutation frequency of the negative control and TA016 (Appendix C, Table 3); the "P" value (0.093) was non-significant at the 5% level ($p > 0.05$). This indicated non-significant differences between the mutation frequency (0.273%) due to TA016 and the spontaneous mutation frequency, negative control (0.165%). The "P" value for the extremes was 1.043. However, at the 10% level the "P" value of 0.093 is approaching this actual limit and may suggest differences ($p < 0.10$).

The results of the analysis of each brood for the three replicates, using Fisher's Exact Test, are shown in Appendix C, Table 4. Each brood corresponds to a particular stage of sperm when exposed to the test compound or negative control. The Fisher's Exact Test for each of the four broods showed non-significant differences at the 5% level ($p > 0.05$) between the mutation frequency for each brood resulting from TA016 and the spontaneous mutation frequency resulting from the concurrent negative control. The "P" values for each of the four broods, 1, 2, 3, and 4 were: 0.101, 0.425, 0.129, and 0.503, respectively.

It should be noted that the total number of culture vials examined (F_1 progeny) resulting from all six replicates of TA016 was 7,068, while the total number resulting from the negative control was 13,337. This reduction in F_1 progeny from P_1 exposure to TA016 may indicate that this compound reduces the fecundity of the organisms exposed to it.

CONCLUSION

The mutation frequency of TA016 at the concentrations tested and method of exposure was not significantly different from the concurrent negative control mutation frequency. This suggests non-mutagenic activity associated with this compound under these conditions.

RECOMMENDATION

Based on the results of the SLRL assay conducted in this study, I recommend no further mutagenicity testing of TA016. However, due to the decrease in F_1 progeny resulting from TA016, the use of an appropriate Dominant Lethal test (5) might indicate whether genetic or non-genetic damage is responsible for the reduction of X-chromosomes resulting from TA016 compared to the negative control.

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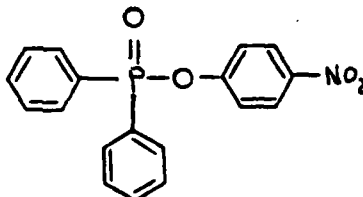
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APPENDICES

TEST SUBSTANCES - ANALYTICAL DATA

Chemical name: 4-nitrophenyl diphenyl phosphinate (LAIR Code TA016)

Molecular structure: $C_{18}H_{14}NO_4P$



pH: N/A, non-aqueous

Physical state: Solid crystals

Boiling point: N/A

Melting point: 126.5 - 127 C

Stability: Under refrigerated conditions in the absence of H₂O, Dr. Lieske (Biomedical Laboratory, Aberdeen Proving Ground, Aberdeen, MD 21005) believed the compound would remain stable for two years.

Names and percentage of contaminants: unknown.

Manufacturer: Ash Stevens
Detroit Research Park
5861 John C. Lodge Freeway
Detroit, MI 48202.

Manufacturer Lot No.: XXXIV-73-A.

This sample was kept from exposure to light, H₂O, and frozen, as required.

Chemical name: 4-nitrophenyl diphenyl phosphinate formulated with Tween 80, ethanol (100%), citrate buffer (5.0 mM) and distilled water.

The various concentrations of TA016 used in this study were formulated in the following manner:

Stock: 15 mg TA016, 1.5 ml Tween 80, 0.75 ml EtOH (100%), 1.5 ml citrate buffer (5.0 mM) and 1.5 ml distilled water.

Blank: 7.5 ml Tween 80, 3.5 ml EtOH (100%), 7.5 ml citrate buffer (5.0 mM) and 15 ml of distilled water.

APPENDIX A

Diluent: 24 ml Tween 80, 16 ml EtOH (100%), 100 ml citrate buffer (5.0 mM) and 20 ml of distilled water.

Control: 1 ml blank, 8 ml diluent and 1 ml of 10% fructose.

Various proportions of TA016 stock and blank were combined to give a final yield of 1 ml; resulting in various concentrations of TA016. This was then formulated with 8 ml of diluent and 1 ml of 10% fructose.

pH: 4.0

Physical state: liquid, clear yellow.

Boiling point: N/A.

Melting point: N/A.

Compound refractory: N/A.

Stability: Hydrolysis measurements were conducted immediately after preparation and 24 hours later on the various concentrations of TA016.

Name and percentage of contaminants: unknown.

Manufacturer: Fisher Scientific Company
Chemical Manufacturing Division
Fair Lawn, NJ 07410.

Tween 80 is a preparation of polyoxyethylene-20 and sorbitan monooleate and is approved for use in humans.

APPENDIX A (cont.)

HISTORICAL LISTING OF SIGNIFICANT STUDY EVENTS

15-18 Sep 81	The formulation of 4-nitrophenyl diphenyl phosphinate with the diluent and 1% fructose (LAIR SOP OP-STX-45, Preparation of Compounds Unstable in Water for SLRL Assay) for pilot toxicity testing to establish the dosage range after 72-hour exposure.
21 Sep 81-18 May 82*	Removal of all adult insects from CS colony and collecting of newly emerged adult males, 24 hours later for toxicity testing (LAIR SOP OP-STX-7, <u>Drosophila melanogaster</u> Exposure Procedures).
22 Sep 81-21 May 82 [†]	Selection of CS male survivors after toxicity testing and concurrent positive and negative control exposure at 72 hours from the concentration that resulted in LC ₅₀ (approximately).
25,28,30 Sep-2 Oct 81 [‡]	Each exposed CS male was crossed with 4 groups of 3 virgin FM6 females in corresponding vials labeled Broods 1, 2, 3, and 4. Replicate 1 (Run 31).
16,19,21,23 Oct 81 [‡]	Replicate 2 (Run 32).
6,9,11,13 Nov 81 [‡]	Replicate 3 (Run 33).
11,14,16,18 Dec 81 [‡]	Replicate 4 (Run 34).
17,20,22,24 Apr 82 [‡]	Replicate 5 (Run 39).
21,24,26,28 May 82 [‡]	Replicate 6 (Run 40).
9 Oct 81-14 Jul 82 [‡]	F ₂ crosses of all broods for all 6 replicates were made and scored, the F ₃ crosses were made and scored for all 6 replicates.

*The events enclosed in parentheses required two days for each replicate, e.g., (1) 21-22 Sep 81; (2) 12-13 Oct 81; (3) 2-3 Nov 81; (4) 8 Dec 81; (5) 13-14 Apr 82; (6) 17-18 May 82.

†The events enclosed in parentheses required three days for each replicate, e.g., (1) 22-25 Sep 81; (2) 13-16 Oct 81; (3) 3-6 Nov 81, (4) 8-11 Dec 81; (5) 14-17 Apr 82; (6) 18-21 May 82.

‡Dates for each of the four broods.

APPENDIX B

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APPENDIX C

Table 1

Mean Percentage Mortality for TA016*
Fed to CS Males for the SLRL Assay

Replication No.	Concentration mM	% Mortality [†] $\bar{x} \pm s.d$
1	0.35	52.0 $\pm$ 16.19
2	0.37	72.0 $\pm$ 20.98
3	0.47	45.0 $\pm$ 20.14
4	0.44	47.0 $\pm$ 29.08
5	0.51	49.9 $\pm$ 17.04
6	0.47	53.0 $\pm$ 19.47

*TA016: 4-nitrophenyl diphenyl phosphinate formulated with Tween 80, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose.

[†] Based on a sample size range from 98 to 100 CS males.

APPENDIX C (cont.)

Table 2
Sex-Linked Recessive Lethal Assay
of TA016

Compound	Replication*						Total % Mutation
	1	2	3	4	5	6	
TA016 [†]	3/1460	7/1241	4/1393	2/769	4/1324	3/882	0.326
% Mutation	0.198	0.584	0.296	0.260	0.364	0.360	
Neg. Control [‡]	5/2287	13/2247	7/2081	8/2340	1/2124	5/2258	
% Mutation	0.204	0.574	0.352	0.322	0.046	0.208	0.292

*Data are recorded as number of SLRL events/number of X-chromosomes tested. All single mutations, i.e., no "clusters" or "multiples" were detected.

[†]TA016: 4-nitrophenyl diphenyl phosphinate is formulated with Tween 80, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose.

[‡]Negative control: Tween 80, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose.

APPENDIX C (cont.)

Table 3
Sex-Linked Recessive Lethal Assay
of TA016
Using Selected Replications

Compound	Replication*						Total	% Mutation
	1	2	3	4	5	6		
TA016 [†]	3/1460	—	—	—	4/1323	3/882	10/3665	0.273
Neg. [‡] Control	5/2287	—	—	—	1/2124	5/2258	11/6669	0.165

*Data are recorded as number of SLRL events/number of X-chromosomes tested.

[†]TA016: 4-nitrophenyl diphenyl phosphinate is formulated with Tween 80, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose.

[‡]Negative control: Tween 80, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose.

APPENDIX C (cont.)

Table 4
Sex-Linked Recessive Lethal Assay
For Each Brood From
Replications 1, 5, and 6 Fed TA016

Compound	Brood*			
	1	2	3	4
TA016 [†]	8/1555	0/935	2/576	0/599
Negative Control [‡]	4/1694	2/1754	3/1760	2/1461
Fisher's Exact P Values	0.101	0.425	0.129	0.503

*Data are recorded as number of SLRL events/number of X-chromosomes tested.

[†]TA016: 4-nitrophenyl diphenyl phosphinate is formulated with Tween 80, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose

[‡]Negative control: Tween 80, ethanol (100%), citrate buffer (5.0 mM), distilled water, and 1% fructose.

APPENDIX C (concluded)

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