

Report Title: Drosophila Melanogaster Sex-Linked Recessive Lethal Assay of Octopirox, G0539.03

Test Type: Genotoxicity Study

Conducting Laboratory and Location: Zoology Dept., University of Wisconsin, Madison, WI

Test Substance(s): G0539.03 – OP in ethanol

Species: Drosophila Melanogaster male

Test Conditions: Drosophila Melanogaster males exposed by feeding or injection at a concentration of 1000 ppm in aqueous 10% ethanol and 5% sucrose.

Results: Does not induce mutations in the post-meiotic germ cells of Drosophila melanogaster males when administered by feeding or by injection.

Study#: UWIS TXAS-123

Report Date: 11/19/87

QA Statement/GLP compliance: Yes

Accession #: 34137

DRAFT

DROSOPHILA MELANOGASTER SEX-LINKED RECESSIVE LETHAL
ASSAY OF OCTOPIROX, G0539.03

Sponsor

The Procter & Gamble Company
- 11511 Reed Hartman Highway
Room No. HB-2D31
Cincinnati, OH 45241

Testing Laboratory

Zoology Department
University of Wisconsin
1117 W. Johnson Street
Madison, WI 53706

Testing Laboratory Project No. 123

Study conducted March 3, 1987 - November 4, 1987

Report prepared by

Ruby Valencia

Study Director, Ruby Valencia, Ph.D.

Date

11/17/87

Report inspected by

Mary Possin

Quality Assurance Officer, Mary Possin

Date

11/19/87

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UNIVERSITY OF WISCONSIN-MADISON
ZOOLOGY RESEARCH BUILDING

QUALITY ASSURANCE REPORT

SPONSOR: The Procter & Gamble Co.

TEST SUBSTANCES: Octopirox no. G0539.03

FINDINGS:

An initial inspection of the facility was performed on 4/6/87 and showed that it was prepared to conduct the study according to provisions of the study protocol, laboratory Standard Operating Procedures, and GLP's.

Subsequent periodic inspections of all crucial phases of the study were also conducted. They were: dosage and sample preparation-adult injection (6/18/87), dosage and sample preparation-adult feeding (3/10/87), exposure by injection (6/18/87), exposure by feeding (3/10/87), P1 matings (6/10/87), F1 matings (5/20/87), F2 scoring (5/19/87), and stock maintenance (9/28/87). These inspections indicated that all procedural and operational aspects of the study were carried out correctly.

The final inspection and study review conducted on 11/18/87 and 11/19/87 showed that the quality and integrity of the study have been maintained throughout it's course. Testing facility operation was in compliance with protocol, SOP's, and GLP's. The final report accurately reflects test observations and data.

DATE: 11/19/87

QUALITY ASSURANCE OFFICER:

Mary Passin

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SUMMARY

The chemical Octopirox (G0539.03) was tested for mutagenic activity (the induction of sex-linked recessive lethal mutations) in Drosophila melanogaster males exposed by feeding and by injection.

The males were fed for 3 days on the test material at a concentration of 1,000 ppm in aqueous 10% ethanol and 5% sucrose. Ingestion was good. The material was not toxic at these concentrations and no male sterility was induced.

Males were injected with the test material at a concentration of 500 ppm in 5% ethanol and 0.7% NaCl. Induced mortality averaged 26% for the three experiments and about 15% sterility was induced..

A standard genetic scheme (Basc females x Canton-S wild type males) was employed and germ cells which were post-meiotic at the time of exposure were tested for lethal mutations.

The sex-linked recessive lethal results (below) show no difference between the treated samples and the negative controls. All frequencies are well within the range of our recent historical control values.

Octopirox, 1,000 ppm, fed	6/6521	(0.092%)
Feeding controls	4/5515	(0.073%)
DMN, 100 ppm, fed	37/845	(4.38%)
Octopirox, 500 ppm, injected	7/6007	(0.117%)
Injection controls	8/8334	(0.096%)
DMN, 500 ppm, injected	91/1712	(5.32%)

It is concluded that Octopirox does not induce mutations in the post-meiotic germ cells of Drosophila melanogaster males when administered by feeding or by injection.

1. Objective of the study

The purpose of this study was to evaluate the ability of the test article to induce recessive lethal mutations in the post-meiotic germ cells of Drosophila melanogaster males treated by feeding and by injection.

2. Chemical identification and characterization

2.1 Test chemical

The test substance is a white powder, identified by the sponsor as Octopirox, no. G0539.03. The stability of the test article under experimental conditions was not determined by the testing facility.

2.2 Positive control chemical

Dimethylnitrosamine (DMN) was used. The sample was aliquot 022920, 99+% pure, provided by Radian Corporation, Austin, Texas. It is a yellow liquid and is water soluble. It is highly toxic and carcinogenic and is a potent mutagen.

3. Range-finding tests

The material dissolved well in ethanol and remained in solution when diluted with aqueous 5% sucrose or with 0.7% NaCl to the concentrations of compound and of ethanol desired.

A preliminary study using ¹⁴C-leucine was conducted by Dr. Ed Thompson to determine the concentration of the test material giving optimum ingestion. This proved to be 1 mg/ml (1000 ppm) but there was high mortality, presumed to be due at least in part to the flies becoming wet and sticking to the vials. (See addendum to protocol, attached.) We were able to avoid this loss of flies and the material was not toxic. Thus 1000 ppm of Octopirox in 10% ethanol and 5% sucrose was used for the feeding experiments.

For injection the ethanol concentration was reduced to 5% because the flies' tolerance to ethanol is less by injection. The concentration of Octopirox was also lowered to 500 ppm due to high toxicity at 1000 ppm.

4. Method of administration of the chemical

4.1 Feeding

Adult males (8-30 hours old and previously starved for approximately 4 hours) were treated in groups of 15 in shell vials (23 mm by 90 mm) plugged with rayon fiber balls. The base of each vial was covered with two discs of glass fiber filter material (Scientific Products, Grade 141) on which 1.0 ml of feeding solution was pipetted. The males were transferred to new treatment vials with freshly-prepared feeding solution daily for 3 days. At the end of each 24-hour treatment period, the number of dead flies was noted. Negative control flies were fed on solvent alone by the same procedure. Exposures were performed at room temperature (approximately 22°C).

Positive control feedings (DMN @ 100 ppm) were for approximately 24 hours only since 72-hour treatments had been found to induce high post-mating mortality and male sterility. It was also known from experience with DMN that a 24-hour exposure yielded a high frequency of mutations. The males were of the same age and from the same collection batch as the males treated with the test material.

4.2 Injection

Two-three day old males were injected, using a glass needle attached by polyethylene tubing to a Hamilton 5 μ l syringe, the plunger of which was pushed by a micrometer. Approximately 0.3 μ l of test or control solution was injected into the body of the etherized fly at the base of a wing. The injected males were then held on sucrose-agar medium for about one day to observe recovery before mating. DMN @ 500 ppm was the positive control.

5. Genetic testing procedure

The Drosophila melanogaster stocks used are laboratory lines in existence for many years. They have been distributed and used in many laboratories. The Canton-S wild type stock (from which males were taken for exposure) was a line in use in this UW laboratory for some 20 years and the "Basc" stock (which supplied the females for the parental matings) was obtained from Brown University in 1986. The stocks are transferred weekly and observed for genetic purity (intact phenotypic markers).

The culture medium for the stocks as well as for the test matings is a standard Drosophila culture medium. The original formula is described in Drosophila Information Service, No. 34, p. 117, 1960. Our modified formula is as follows:

Water	4500 ml
Agar	72 gm
Molasses (Grandma's unsulfured)	500 ml
Cornmeal	500 gm
Dried brewer's yeast	200 gm
Propionic acid (mold retardant)	12.5 ml
Propionic acid-resistant live yeast (sprayed on surface of cooled medium)	

The mixture is cooked at 190°F. A log book is maintained with all details of preparation of each batch of medium.

Males were mated for the genetic test at the age of 3-4 days. Treated and control P1 males (of the Canton-S wild-type stock) were mated singly to 3 virgin females of the balancer stock "Basc", whose X chromosomes carry inversions and are marked with genes for apricot eye (w^a) and Bar eye (B). The females were 3 to 10 days old when mated. See crossing scheme (Fig. 1). Each male was transferred after 3 days to 3 new virgin females. The fertilized females of brood 1 were kept in the culture vial for 4 more days and then discarded. This transfer process was repeated two more times but the time the males were in broods 2 and 3 was 2 days each. The males were then discarded. Thus, only meiotic and post-meiotic germ cells were tested.

F1 females (heterozygous for the treated X and the balancer X) were mated individually to brothers. An attempt was made to mate 33 females (but no more) from each P1 male per brood, for a total of 99 chromosomes tested from each male. Due to post-mating mortality and other culture failures or impairments, the number tested was sometimes less than 99 per male.

The F2 cultures were observed when fully hatched (at 11-15 days after the mating) for the presence (non-lethal) or absence (lethal) of wild type males. Suspected lethal cases were retested by remating heterozygous females and observing the F3.

All stocks and F1 and F2 matings were kept at room temperature, approximately 22°C. Photoperiod was not controlled.

Each experiment was identified by a unique numbering sequence, a code letter and a unique color used in the labels and the data sheets. Each treated or control male was numbered individually, with the number written on the glass vial with colored wax. In later generations, the family of each P1 male was identified with index cards attached to the culture vials.

6. Results

6.1 Data presentation

Tables 1 and 2 show the recessive lethal test data per brood, with the respective concurrent negative and positive controls. The tables also show whether the lethals occurred singly or as multiples (see below).

6.2 Statistical analyses

When an individual P1 male produces more than 1 lethal among his progeny, the group of lethals can be called a "multiple". A multiple may result from many independent mutations in the treated post-meiotic germ cells (as is the case with a potent mutagen) or from a single spontaneous mutation in an untreated gonial cell, which then multiplies to produce a "cluster" of sperm cells carrying identical recessive lethal mutations. Thus each multiple must be subjected to analysis to determine whether or not it could be considered a "cluster". The statistical test (Margolin, personal communication) is based on a cumulative Poisson distribution. Only one multiple of 4, in experiment 356D, was determined to be a cluster.

The recessive lethal data were evaluated by comparing the overall mutation frequency of the treated group with the overall mutation frequency of concurrent negative controls, by applying the "Normal Test" (Margolin et al., 1983). For the feeding experiments, $P = 0.36$ and for the injection experiments $P = 0.35$. All the values are well within the range of recent historical controls in this laboratory.

7. Conclusion

It is concluded that Octopirox, when fed to or injected into Drosophila melanogaster males, does not induce sex-linked recessive lethals in the post-meiotic germ cells.

8. Data storage

Copies of all raw data will be sent to the sponsor along with this report. The original raw data, data summaries and reports will be kept on file in the testing laboratory.

9. References

Margolin, Barry H., Bruce J. Collings and James M. Mason (1983)
Statistical analysis and sample size determinations for mutagenicity experiments with binomial responses. Environ. Mut. Vol. 5, No. 5, pp. 705-716.

10. Compliance

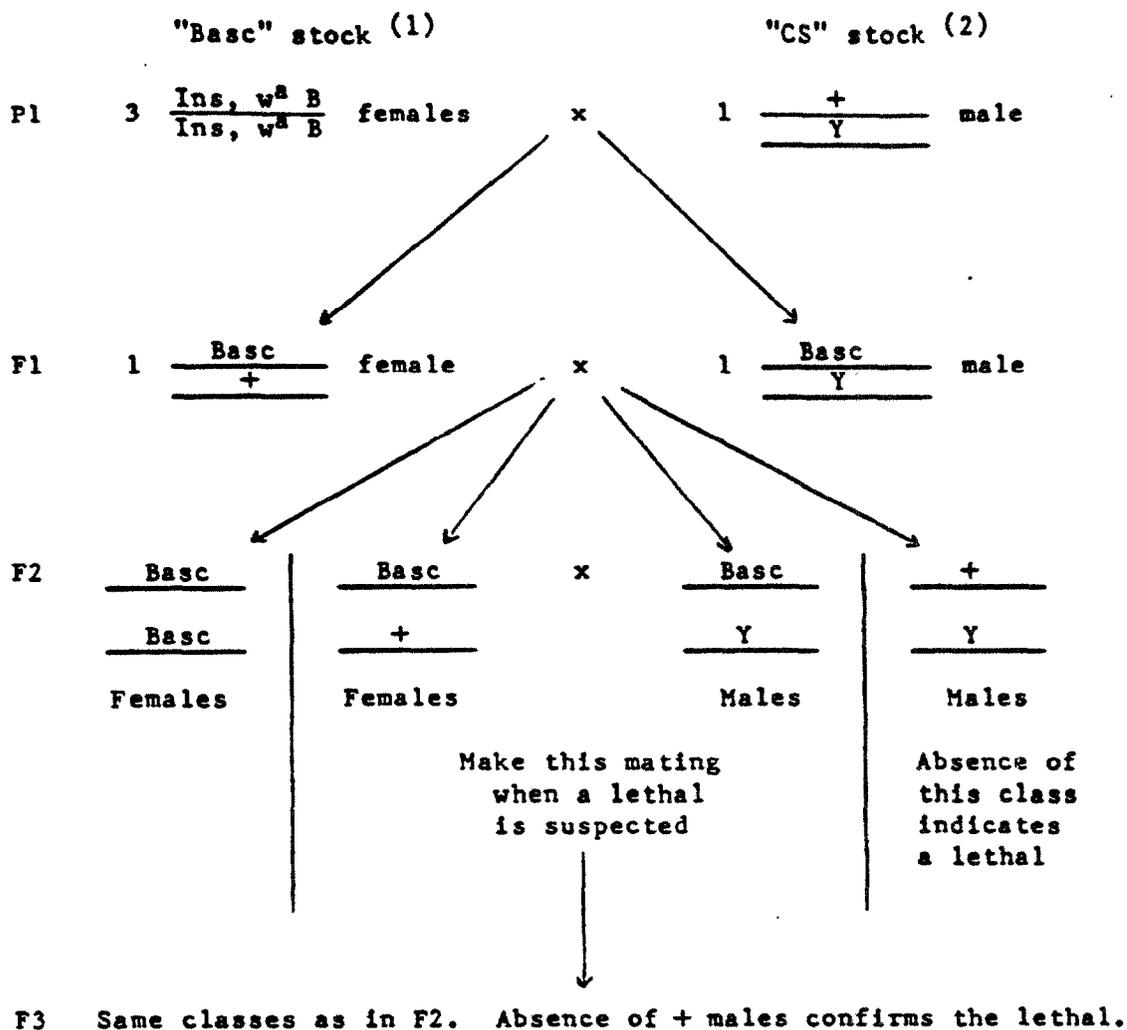
In the conduct of this study, the laboratory adhered to the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

11. Personnel

In addition to the study director, four Research Specialists were involved in the study:

Dana Possin
Anne Kenney
Dorothy Brar
Carmen Murach

A varying number of undergraduate students did routine work. Training records are on file.



(1) "Basc" is an X chromosome with a complex of inversions and the phenotypic markers (i.e. gene mutations) apricot eye (w^a) and Bar eye (B). The markers identify the chromosome and the inversions prevent crossing over with the treated + chromosome.

(2) CS = Canton-S wild-type stock.

**GENETIC SCHEME FOR DETECTION OF SEX-LINKED
RECESSIVE LETHALS IN DROSOPHILA**

Figure 1

TABLE SEX LINKED RECESSIVE LETHAL DATA. FEEDING

Expt.	No. lethals/No. tested (% lethals)			Total tests	Lethals			Percent lethals
	Brood 1	Brood 2	Brood 3		S(1)	M(2)	Tot.	
Octopirox, 1000ppm								
344 B	0/ 966	0/1112	3/1101	3179	1	1(2)	3	0.094
348 B	3/1251	0/1181	0/ 910	3342	1	1(2)	3	0.090
Sum	3/2217(.135)	0/2293	3/2011(.149)	6521	2	2(2)	6	0.092*
Controls								
344 A	2/ 902	0/ 911	0/ 872	2685	2	0	2	0.074
348 A	0/1116	0/1123	2/ 591	2830	2	0	2	0.071
Sum	2/2018(.099)	0/2034	2/1463(.137)	5515	4	0	4	0.073
DDN, 100 ppm								
344 F	0/ 124	0/ 152	20/ 124	400	2	1(5) 1(6) 1(7)	20	5.000
348 C	8/ 212	8/ 198	1/ 35	445	2	1(2) 2(3) 1(7)	17	3.820
Sum	8/ 336 (2.38)	8/ 350 (2.29)	21/ 159 (13.21)	845	4	1(2) 2(3) 1(5) 1(6) 2(7)	37	4.379

(1) Single lethal (2) Multiple lethals. No. of multiples of this size (no. of lethals in the multiple).
 * P = 0.36

TABLE EX LINKED RECESSIVE LETHAL DATA. INJECTION.

Expt.	No. lethals/No. tested (% lethals)			Total tests	S(1)	Lethals		Tot.	Percent lethals
	Brood 1	Brood 2	Brood 3			M(2)			
<u>Octopirox, 500 ppm</u>									
350 C	0/ 850	0/ 405	0/ 204	1459	0	0		0	0
354 C	1/1448	2/ 935	1/ 915	3298	4	0		4	0.121
356 D	0/ 487	4/ 397	3/ 427	1311	3	1(4)		7	0.534
Sum	1/2785	6/1737	4/1546	6068	7	1(4)		11	0.181
Less of # 336	0/0	2/ 29	2/ 32	61	0	1(4)		4	
Corrected sum	1/2785(.036)	4/1708(.234)	2/1514(.132)	6007	7	0		7	0.117*
<u>Controls</u>									
350 D	0/ 847	0/ 393	0/ 200	1440	0	0		0	0
354 D	1/1434	2/1248	4/1069	3751	3	2(2)		7	0.187
356 E	0/1036	1/1117	0/ 990	3143	1	0		1	0.032
Sum	1/3317(.030)	3/2758(.109)	4/2259(.177)	8334	4	2(2)		8	0.096
<u>DMN, 500 ppm</u>									
350 E	6/ 199	23/ 286	22/ 245	730	0	1(3) 3(4) 3(5) 1(6) 1(7) 1(8)		51	6.986
354 E	8/ 233	3/ 61	3/ 52	346	4	1(3) 1(7)		14	4.046
356 F	1/ 277	16/ 192	9/ 167	636	3	2(5) 1(6) 1(7)		26	4.088
Sum	15/ 709(2.116)	42/ 539(7.792)	34/ 464(7.328)	1712	7	2(3) 3(4) 5(5) 2(6) 3(7) 1(8)		91	5.315

(1) Single lethal (2) Multiple lethals. No. of multiples of this size (no. of lethals in the multiple).

* P = 0.35

ZOOLOGY DEPARTMENT, UNIVERSITY OF WISCONSIN

STUDY PROTOCOL

January 1987

I. STUDY TITLE

Drosophila sex-linked recessive lethal test

II. PROJECT IDENTIFICATION

A. Sponsor's project number BY 039282

B. Testing laboratory's project number 123

III. MANAGEMENT OF STUDY

A. Sponsor's name and address

Dr. Kenneth L. Hintze
Procter & Gamble, Co.
Sharon Woods Technical Center
11511 Reed Hartman Hwy.
Cincinnati, OHIO, 45241

B. Sponsor's project officer

Ed Thompson
Miami Valley Laboratories
Procter & Gamble Co.
Cincinnati OHIO 45241

C. Testing laboratory's name and address

Zoology Department
University of Wisconsin
1117 West Johnson Street
Madison, WI 53706

D. Study director

Ruby Valencia, Ph.D.

E. Quality assurance officer

~~Germen Hurech~~ Mary Prossin

IV. TEST MATERIAL INFORMATION (to be provided by sponsor)

A. Identification

G 0539.03

B. Physical-chemical description

off-white powder

Solubilities: ~1% (w/w) in water

~10% (w/w) in acetone or ethanol

Expiration date of sample: 11/87

Storage: Room Temperature

C. Handling precautions

The test material is an irritant. Avoid dermal contact. Flush skin thoroughly if contact occurs.
with water

D. Purity (including instructions regarding expression of concentration)

99.5% ; correct to 100% active

see pg 4 for dose preparation instructions.

E. Units of measurement preferred by sponsor

~~to~~ µg/ml

V. TEST SYSTEM

A. Description of test principles

The sex-linked recessive lethal test detects the occurrence of mutations in the germ line of an insect. The assay is capable of screening for forward mutations at about 800 loci on the X chromosome. This represents about 80% of the X chromosome or approximately 1/5 of the entire genome. The mutations detected are recessive and are lethal to the carrier when expressed.

B. Stocks

The Drosophila melanogaster stocks to be used are laboratory lines in existence for many years. They have been distributed all over the world and used in many laboratories. The Canton-S wild type stock (from which males will be taken for exposure) has been in use in this UW laboratory for approximately 20 years. The "Basc" stock (which will supply the females for the parental matings) was obtained in 1980 from Brown University. It has been used extensively in this UW laboratory since then.

Stocks are kept at room temperature (approximately 22°C), transferred weekly and observed for genetic purity (intact phenotypic markers).

C. Culture medium

The culture medium for the stocks as well as for the test matings is a standard Drosophila culture medium. The original formula is described in Drosophila Information Service, No. 34, P. 117, 1960. Our modified formula is as follows:

Water	4500 ml
Agar	72 gm
Molasses (Grandma's unsulfured)	500 ml
Cornmeal	500 gm
Dried brewers' yeast	200 gm
Propionic acid (mold retardant)	12.5 ml
Propionic acid-resistant live yeast (sprayed on surface of cooled medium)	

The mixture is cooked at 190°F. A log book is maintained with all details of preparation of each batch of medium.

VI. TESTING PROCEDURE

A. Route of administration

The route of administration will be chosen, based on:

1. The method or methods most appropriate for the chemical, considering its physical state, solubility, stability and volatility.
2. The route by which humans might be exposed.
3. The sponsor's request.

The routes of choice are:

1. Adult feeding. Previously starved (for 4 hours) adult males, 8-30 hours old, are treated in groups of 15 in 23 mm by 90 mm shell vials, plugged with rayon fiber balls. The base of each vial is covered with one or two discs of glass fiber filter material (SP 141) on which approximately 0.5 ml or 1.0 ml of feeding solution is pipetted. Daily for 3 days, the males are transferred to new treatment vials with freshly-prepared feeding solution. The length of exposure may be limited to 24 or 48 hours if toxicity is high and if there is ample evidence of ingestion.
2. Injection. Adult males are injected, using a glass needle attached by polyethylene tubing to a Hamilton 5 μ l syringe, the plunger of which is pushed by a micrometer buret. 0.3 μ l of solution is injected into the body of the fly. The males (2 or 3 days old when treated) are allowed to recover before mating.
3. Inhalation. Flies are treated in sealed hypovials. The amount of gas in the vial and the duration of exposure are adjusted as needed, considering toxicity, narcosis and other effects.

For this study, the route of administration will be:

MLH 2/10/67
~~Prepare as follows. Dissolve 10mg of GC551.05 in 1.0 ml of absolute ethanol and dilute to 10ml with 5% sucrose. Dilute a portion of this solution to obtain 0.3 mg/ml. Start assay using 0.3 and 1.0 mg/ml solutions.~~
 Feeding and injection - See Protocol Addendum ^{CAF} 2/10/67 (1.0 mg/ml)

B. Solubility tests (for liquids and solids)

Possible solvents, in order of preference, are: water, ethyl alcohol, Tween 80, DMSO and combinations of the above solvents. Other solvents or carriers (ex. oil or butter) may be used occasionally.

Poor solubility may limit the concentration of the compound that can be used to treat the flies.

The solvent to be used in this study will be chosen based on the chemical information supplied and the results of preliminary solubility observations.

C. Sample preparation

1. Adult feeding. The chemical is administered in 5% aqueous sucrose, plus any other solvent(s) necessary to dissolve the chemical.
2. Injection. The chemical, plus any necessary solvent(s), is administered in 0.7% aqueous NaCl.
3. Inhalation. The gas is administered in air.

D. Toxicity tests

Using the chosen solvent(s) and route of administration, a series of concentrations is administered. The exposure procedure is carried out exactly as if a genetic test were in progress. (See "A" above.) Mortality is recorded at 24-hour intervals for feeding exposure and at 24 hours following injection. Recording times for inhalation exposures vary with the duration of exposure.

E. Sterility tests

Whenever preliminary test time allows, an experiment is set up to test for induced male sterility since this effect may also limit the concentration of the compound that can be used. Males are mated and transferred as for a genetic test and the cultures observed after 1-2 weeks. If preliminary test time is not available, the genetic test is started and if male sterility is encountered, it may be necessary to lower the concentration of the compound for succeeding experiments.

F. Ingestion tests

If adult feeding is the route of administration, it will be ascertained whether the flies do ingest the feeding solution. The walls of the exposure vials are observed for excreta. In addition, behavior of the flies indicates feeding or avoidance. Whenever treatment can be continued for 72 hours, at least some ingestion is assured since flies cannot survive for 72 hours without drinking.

G. Choice of exposure concentration ("Dose")

The chemical concentration that can be adequately administered may be limited by any one or more of several factors, namely solubility, toxicity, palatability or sterility. In addition, for adult feeding exposure, the dose ingested may not follow from the concentration of chemical in the feeding mixture, since flies often ingest less volume of more concentrated solutions.

Whenever possible, an LC₃₀ dose is recommended. If there is no limiting factor or if more than one dose is requested, the exposure level(s) will be discussed with the sponsor.

It should be noted that the exposure concentration is chosen on the basis of range-finding tests. The observations in the genetic test experiments may vary.

Extremely toxic substances, that must be used at very low concentrations and/or for very short exposure times, can be tested only with the understanding that a negative result is questionable.

H. Genetic test plan

1. The genetic scheme is shown in Figure 1.
2. The parental (P1) treated (or control) males are mated individually in order to identify the family descended from each. The family size is limited to no more than 100 to reduce the probability of "clusters." (See treatment of data, below.)
3. After adult treatments, three post-meiotic germ cell stages are sampled by mating each P1 male to three successive harems of three "Basc" females. The mating duration is three days in brood 1 and two days in each of broods 2 and 3. Brood 1 samples mature sperm, brood 2 samples spermatids and brood 3 includes early spermatids and spermatocytes.
4. The running time required is usually 19-21 weeks. See flow chart, Figure 2.
5. Proposed experimental design (per route of administration):

The sponsor will choose the test power desired (Margolin et al, 1983). The sample sizes to be approximated for three test powers are as follows: *use 75%*

	<u>75%</u>	<u>90%</u>	<u>95%</u>
Treated*	5000	8000	10,500
Negative control	5000	8000	10,500
Positive control	600	600	600

* Per dose point

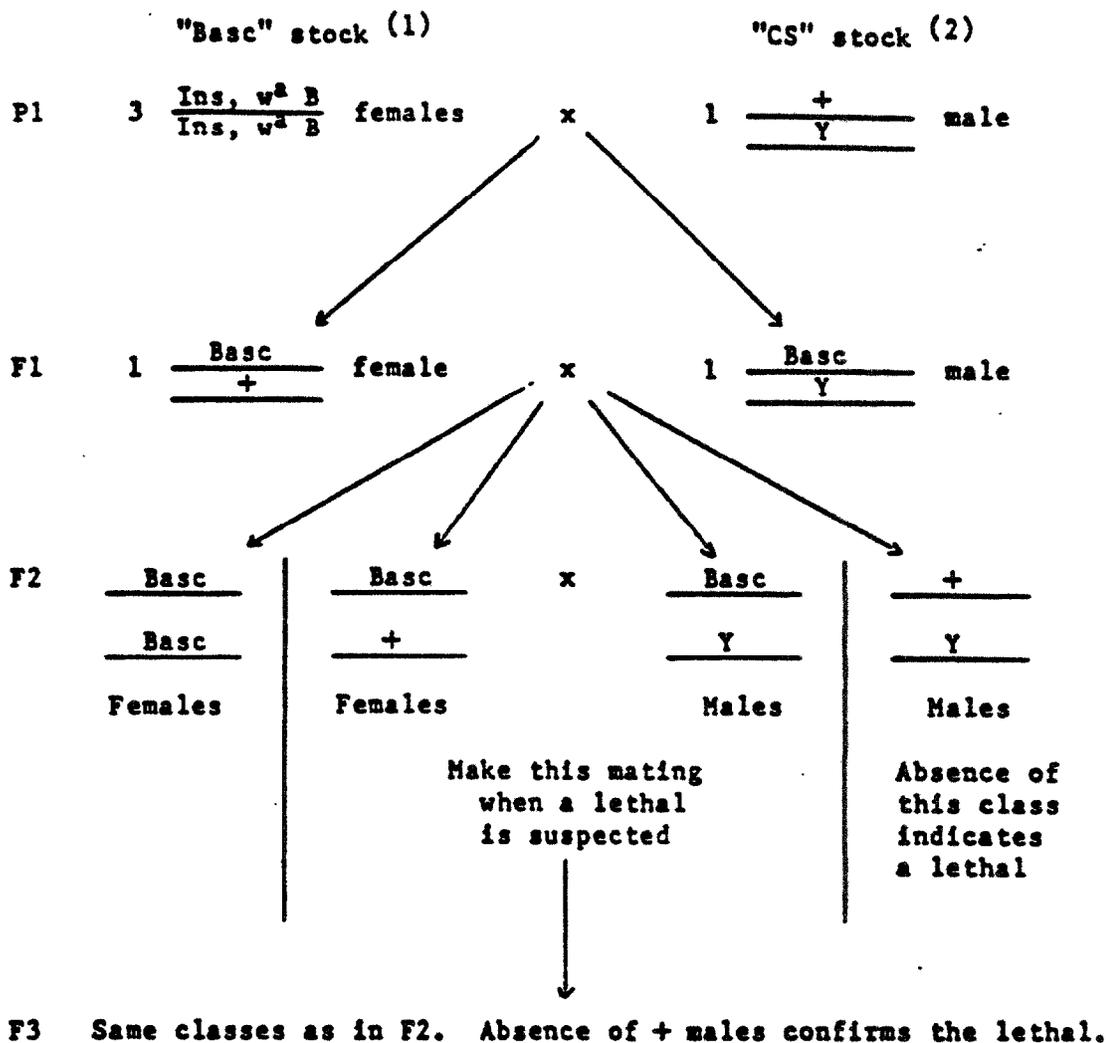
The numbers of chromosomes suggested are sufficient to detect a minimum induced mutation frequency of approximately 0.2% at a statistical significance level of $P = 0.04$ or less, given a control rate of approximately 0.1%. Lower control rates will increase the test power or allow detection of smaller induced mutation frequencies. The historical control rate in this laboratory is approximately 0.07% at this time.

VII. DATA TO BE COLLECTED

Recorded during exposure: Male mortality (and ingestion if by feeding).

Recorded during the brooding: Male mortality and sterility.

Number of lethal and non-lethal chromosomes per P1 male per brood.



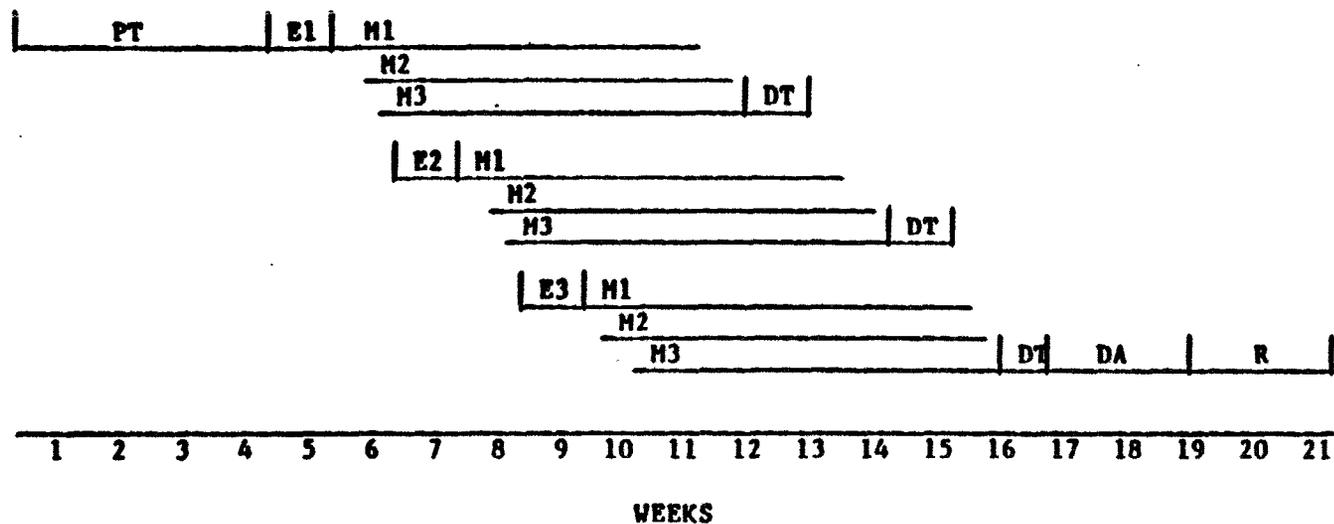
(1) "Basc" is an X chromosome with a complex of inversions and the phenotypic markers (i.e. gene mutations) apricot eye (w^a) and Bar eye (B). The markers identify the chromosome and the inversions prevent crossing over with the treated + chromosome.

(2) CS = Canton-S wild-type stock.

GENETIC SCHEME FOR DETECTION OF SEX-LINKED
RECESSIVE LETHALS IN DROSOPHILA

Figure 1

FLOW CHART FOR RECESSIVE LETHAL TEST



PT Preliminary testing

E Exposure of flies

M Mating (1,2 & 3 are sequential transfers)

DT Data tabulation

DA Data analysis

R Report preparation

Zoology Department
University of Wisconsin
Madison, WI 53706

(608) 262-2701

Figure 2

VIII. POSITIVE CONTROLS

For feeding or injection experiments, the positive control compound will be dimethylnitrosamine (DMN), a powerful mutagen requiring metabolic activation. It will be fed in 5% aqueous sucrose for one day at a concentration of 25 ppm. Injection will be done using 500 ppm in 0.7% NaCl.

For inhalation experiments, 1,2-dibromoethane (DBE) is used.

Positive controls may not be run concurrently with all experiments but will be concurrent with at least one experiment.

IX. PRESENTATION AND ANALYSIS OF RECESSIVE LETHAL DATA

- A. The data are presented as number of tests, number of lethals and percent lethals per experiment per brood and overall, along with concurrent negative controls and the positive controls. The lethals are distinguished as to whether they occurred singly or as multiples in the progeny of a single P₁ male.
- B. Lethals occurring as multiples are subjected to statistical analysis to determine the probability that they could be due to independent mutation events. If they are unlikely to be independent $P = < 0.01$, they are considered to be "clusters" of lethals descended from a single spontaneous mutation in a gonial cell. These clusters and all tests contributed by that P₁ male are removed from the data.
- C. Treated and control lethal frequencies are tested for a significant difference at the .04 level, using the normal test recommended by B.H. Margolin et al., 1983.

X. RECORDS TO BE RETAINED

All original data (or copies, if original is sent to sponsor) and copies of all reports will be retained for not less than five years after completion of the study.

XI. GOOD LABORATORY PRACTICES

This study will be conducted according to FDA Good Laboratory Practice Regulations (21 CFR 58.1-58.219, 1979).

XII. QUALITY ASSURANCE

An in-house QA Officer oversees quality assurance procedures in the laboratory and, if desired, collaborates with the sponsor's QA officer.

XIII. ALTERATIONS OF STUDY DESIGN

Alterations of this protocol may be made as the study progresses. No changes will be made without consultation with the sponsor. Any change will be documented and signed by sponsor's project officer and laboratory's study director.

XIV. REPORTS

A. Interim reports

If requested, interim oral (telephone) reports will be made to the sponsor's designated representative.

B. Final report

At the termination of the study, a final report which includes the following information will be submitted:

1. Experimental design and methods
2. Results
3. Interpretation of the results
4. Conclusions

XV. PROPOSED STUDY SCHEDULE

A. Initiation

March 3, 1987

B. Final report

Approximately 21 weeks after initiation of study.

XVI. REFERENCE

Margolin, Barry H., Bruce J. Collings and James M. Mason. (1983)
Statistical analysis and sample-size determinations for mutagenicity
experiments with binomial responses. Environ. Mutagen. 5(5): 705-716.

PROTOCOL APPROVAL

A. Study Director

By Ruby Valencia

Date 2/26/87

B. Sponsor BX Huntz

Title Division Toxicologist

Date FEB 6 1987

Study cost : \$25,080

Protocol Addendum

A preliminary dose-finding study for the feeding portion has been performed, and the data are summarized in Table 1. The results indicate that the 1.0 mg/ml solution delivers the highest effective dose. There is high mortality associated with this dose, but all deaths occurred within 24 hrs. The flies appeared to become "wet" and stuck to the vials. Normal consumption patterns at days 2 and 3 indicates the flies are not avoiding the material, and thus the mortality is probably due to the "sticky" nature of the test material.

I recommend that the 1 mg/ml dose be tried first, but if it is unsuitable, the dose should be lowered to 0.3 mg/ml.

Prepare the dosing solution as follows:

Feeding Study

1. Dissolve 10 mg of G0539.03 in 1.0 ml of absolute ethanol. The material should dissolve quickly and completely.
2. Add 9.0 ml of 5% sucrose to this solution. The solution should be clear, but will be alkaline. Do not adjust the pH as the material will precipitate.
3. Any volume may be prepared but the above proportions are critical.

Injection Study

The toxicity of G0359.03 by the injection route of administration is unknown. I would recommend preparing a 1 mg/ml solution as outlined for the feeding study except for replacing the 5% sucrose with the appropriate solvent. Do not use any phosphate buffers.

A 10% ethanol in 5% sucrose or other appropriate solvent control and the normal solvent controls should be included in the study.

EDTadd

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2/20/27

Table 1: The Uptake of *60359.03* Over a 3 Day Feeding Period, and the Mortality to the Flies

Day	Test Solution	dpm per fly	Effective dose (1)	Percent Mortality
1	10% Ethanol	911	-	24
	0.1 mg/ml	528	1.0	31
	0.3 mg/ml	579	3.3	40
	1.0 mg/ml	754	14.3	72
2	10% Ethanol	1341	-	28
	0.1 mg/ml	1451	1.0	31
	0.3 mg/ml	2214	4.6	40
	1.0 mg/ml	2303	15.9	72
3	10% Ethanol	2969	-	28
	0.1 mg/ml	3515	1.0	31
	0.3 mg/ml	3204	2.7	40
	1.0 mg/ml	2842	8.1	72

(1) Effective dose represents the relative amount of the chemical consumed. It is determined by using the 0.1 mg/ml dose as a reference, then correcting the dpm/fly values to reflect the amount of chemical consumed at the other doses relative to the 0.1 mg/ml dose. For example on day 1, the 0.1 mg/ml dosed flies contained 528 dpm/fly. The effective dose is then 528 dpm/fly divided by 528 dpm/fly, which equals 1.0. The 0.3 mg/ml dosed flies actually consumed 3 times more Octopirox for each dpm in the fly. Thus the effective dose equals the dpm/fly value (579) times 3 divided by the 0.1 mg/ml reference (528 dpm per fly), which equals 3.3. Accordingly, each dpm per fly in the 1.0 mg/ml dose represents 10 times as much uptake of Octopirox relative to the 0.1 mg/ml dose, and thus the effective dose equals 754 dpm/fly X 10 divided by 528 dpm/fly = 14.3.

PROTOCOL DEVIATIONS

1. Page 4, line 1

Males were starved for approximately 4 hours.

Reason: It is not important that the starve period be exactly 4 hours.

2. Page 9, VIII

The DMN concentration was 100 ppm rather than 25 ppm.

Reason: The change was made in order to share the experiments with another study requiring 100 ppm.

Ruby Valencia

Ruby Valencia, Study Director

Date

10/20/87