JOURNAL OF THE AMERICAN COLLEGE OF TOXICOLOGY
Volume 4, Number 4, 1985
Mary Ann Liebert, Inc., Publishers

Pattern of Response of Intact *Drosophila* to Known Teratogens

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ABSTRACT

***Drosophila*, as the test organism, was used to assess 17 chemicals. The teratogenic potential of 15 of these chemicals is well established from animal studies or human epidemiology. The test involves examination of adult flies following treatment during larval stages of development. Flies are examined for abnormal external morphology. The incidence of abnormalities in treated and control populations is compared using the Chi-square test. All 17 chemicals were active to varying degrees in the test system. Most chemicals produced a unique response yielding individual patterns of abnormalities. These results suggest that *Drosophila* may have the potential to become a valuable teratogen screen, but further, more rigorous examination—particularly with nonteratogens—is required.**

INTRODUCTION

TERATOGENIC EVALUATION of a chemical using mammalian species is expensive and time consuming and requires specially trained personnel. With the large number of chemicals that lack teratological information, screening systems are needed to provide initial information and to help establish priorities for more detailed evaluation of teratogenic potential. Ideally, a screen should be simple, rapid, inexpensive, and provide reliable data.

Wilson[1] stated that the essential features of a teratology screening test organism should include the capability of producing "large numbers" and that the test be "easily performed and readily interpreted." He further suggested that "among the desirable features was that the test be built around an intact organism capable of absorbing, circulating and excreting chemicals." *Drosophila* is particularly noteworthy in this regard. In addition, *Drosophila* possesses metabolic capabilities (mixed function oxidase activities mediated by cytochrome P-450) similar to mammals.[2] *Drosophila* has been studied, to a limited degree, for morphological alterations of adults following larval treatment with vinblastine,[3] 5-bromodeoxyuridine,[4] and thymidine.[5] Vinblastine and 5-bromodeoxyuridine (a halogenated analog of thymidine) are both teratogenic in laboratory species.[6] Vinblastine induced eye deformities and a reduction in size of antennae, wings, halteres, and the thorax. 5-Bromodeoxyuridine caused deformities of the mouthparts and rear legs and reduced the size of the

Presented at the Fourth Annual Meeting of the American College of Toxicology, November 30–December 2, 1983, Arlington, Virginia.

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wings. Thymidine induced wing notches or cuts, extra bristles, abdominal irregularities, and leg malformations.

We have been developing a system using *Drosophila* to screen for teratogenic agents.[7] The current paper presents the methodology of this system together with the results of testing 17 chemicals. Included in these 17 are 15 known mammalian teratogens, one nonteratogen, and one weak teratogen. Each of the 17 chemicals showed varying degrees of activity in this test system. It must be emphasized that these are preliminary findings using a test system in the early stages of development. Before any method can be accepted as a valid teratogen screen, rigorous validation testing must be performed on a large number and great variety of chemicals comprised of teratogens and nonteratogens.

The purpose of this paper is to present preliminary data that reveal a dramatic response of *Drosophila melanogaster* following treatment with known teratogens. It is also our purpose to inform the reader of our plan to develop this test system and to encourage others to join in this effort, especially those researchers who enjoy current expertise with the fruit fly in the mutation and/or genetics fields.

MATERIALS AND METHODS

The method involved treating the fly larvae over their entire morphogenic cycle by adding the test chemical to the culture medium. Eggs were deposited by untreated females on the surface of the medium and hatched, and the emerging larvae immediately began to feed. After several days these larvae metamorphosed into adult flies. Each fly was systematically examined for morphological abnormalities. The incidence of abnormalities among exposed flies was compared to the incidence in control flies using the Chi-square test.

Table 1 presents the chemicals tested and provides examples of species affected, along with the known human teratogenic potential, if available. Laboratory animal investigations and a few epidemiological surveys have shown varying degrees of teratogenicity in 16 of the 17 chemicals tested. Colchicine was purchased from the Eli Lilly Corporation, 2-ethoxyethanol from the Aldrich Chemican Company, and ethanol from the Veterans Administration Supply Depot. The remaining chemicals were purchased from the Sigma Chemical Company. All chemicals were of the purest available grade from each vendor.

Wild-type Oregon-R flies (derived from stock purchased from the Carolina Biological Supply Co.) were maintained in mass cultures from which virgin males and virgin females were selected. They were examined for morphological defects, and only those free of defects were selected. A pair (male and female) of 2- to 4-day-old virgin flies was placed into a cotton-stoppered shell vial (Kimble No. 60930L-8) containing the *Drosophila* medium. The medium in each vial was composed of 7.5 ml of test chemical stock solution added directly to 2.5 g of a dry, instant *Drosophila* medium (Carolina Biological Supply Co., formula 4-24-Plain). The stock solutions were prepared on the day the flies were introduced into the vials, using a range of concentrations in distilled water. To increase solubility, several chemicals (chlorambucil, coumarin, salicylic acid) were dissolved in 0.25 ml ethanol, then brought to the 7.5 ml volume with distilled water. After adding the stock solution to the dry medium, the surface was seeded with yeast as a food source for the parent flies. Three vials were prepared for each concentration of the chemical. (Hydroxyurea, diphenylhydantoin, and diethylstilbestrol were tested using 3 pairs of flies in a single vial at each concentration. These were the first chemicals tested. The method was modified, using 3 vials per concentration and 1 pair of flies per vial for the remaining chemicals to rectify problems that may arise using a single vial, e.g., mold contamination.) The vials were maintained in incubators that provided a 12-hour light/dark cycle, a temperature of 25°C, and 60% relative humidity. After 5 days, the parent flies were removed. The larvae developed through 3 larval stages before pupating on the upper portion of the vial on the seventh or eighth day following vial preparation. On the ninth or tenth day, adult flies began to eclose (emerge) from the puparium. Emerging flies were scored for morphological abnormalities on a daily

TABLE 1. EXAMPLES WHERE TERATOGENICITY HAS BEEN DEMONSTRATED FOLLOWING MATERNAL TREATMENT USING THE 17 TEST CHEMICALS

Chemical	*Species Affected*	*Reference*
6-Aminonicotinamide	Mouse	15
	Rat	16
	Rabbit	17
	Hamster	18
	Monkey	19
Caffeine	Mouse	20
	Rat	21,22
	Rabbit	23
Chlorambucil	Mouse	24
	Rat	25,26
	Human	27
Colchicine[a]	Rabbit	28[a]
	Human	10[a]
Coumarin	None	6
Cyclophosphamide	Mouse	29
	Rat	30
	Rabbit	31
	Monkey	32
	Human	33
Diethylstilbestrol	Mouse	34–37
	Rat	38,39
	Hamster	40
	Monkey	41
	Human	42,43
Diphenylhydantoin	Mouse	44,45
	Rat	46
	Rabbit	47
	Human	48,49
Ethanol	Mouse	50
	Rat	51
	Human	52–54
2-Ethoxyethanol	Rat	55
	Rabbit	55
5-Fluorouracil	Mouse	56
	Rat	25
	Hamster	57
	Monkey	58
	Human	59
Hexachlorophene	Rat	60
	Rabbit	60
Hydroxyurea	Mouse	61
	Rat	62–64
	Rabbit	65
	Hamster	66
	Monkey	67

TABLE 1. (CONTINUED)

Chemical	*Species Affected*	*Reference*
Lithium carbonate	Mouse	68
	Human	69
Methotrexate	Mouse	70
	Rat	71,72
	Rabbit	72
	Cat	73
	Monkey	71
	Human	74
Salicylic acid	Rat	75
Vincristine sulfate	Mouse	76,77
	Rat	78
	Hamster	79
	Monkey	19
	Human	80

[a]Colchicine commonly induces abortion but has been shown to be only weakly teratogenic in laboratory species. Probably due to its ability to inhibit mitosis, colchicine has been causally linked with Down's syndrome.

basis for 10 days following the first eclosure in each vial. Vials were discarded after scoring on the tenth day.

Preliminary studies were performed to determine concentrations to be used in each screening test. In the range-finding tests 4 to 11 concentrations, using 1 to 3 vials per concentration, were prepared for each chemical. Vials were inspected daily for reduced number of emerging adults. Based upon these observations as compared to control vials, concentrations were selected that ranged from clearly toxic, i.e., that reduced the number of flies eclosing due to larval and/or pupal death, to nontoxic. Four to eight concentrations were selected for each screening test.

Scoring began by carefully inverting the vial and dumping all adult flies onto a screened platform[8] through which a continuous flow of CO_2 anesthetized (immobilized) the flies. The flies and their individual body parts were examined under a binocular dissecting microscope at 25× for size, shape, and color. In addition, body part alignment, extraneous tissue growth, and extra or missing parts were recorded. Figure 1 illustrates the external morphology of the adult fruit fly. Body parts examined in the scoring procedure are emphasized. Table 2 outlines the flow of the scoring procedure. Each fly was placed on its ventral surface and examined, starting with the head, followed by the thorax, wings, and abdomen. The fly was turned onto each side for scoring lateral structures. Finally, the ventral structures were examined with the fly turned on its dorsal surface.

There is a purposeful redundancy in the scoring approach, e.g., the eye is inspected 3 times (dorsally, ventrally, and laterally). Otherwise, small but significant malformations might remain hidden if not viewed 3 dimensionally. The time required for scoring individual flies varies with the number of defects observed; experience in this laboratory shows that 1 minute per fly is average.

RESULTS

Due to toxicity effects, there was great variability in the number of flies scored at each concentration. Results at each concentration were individually compared to the concurrent control value for

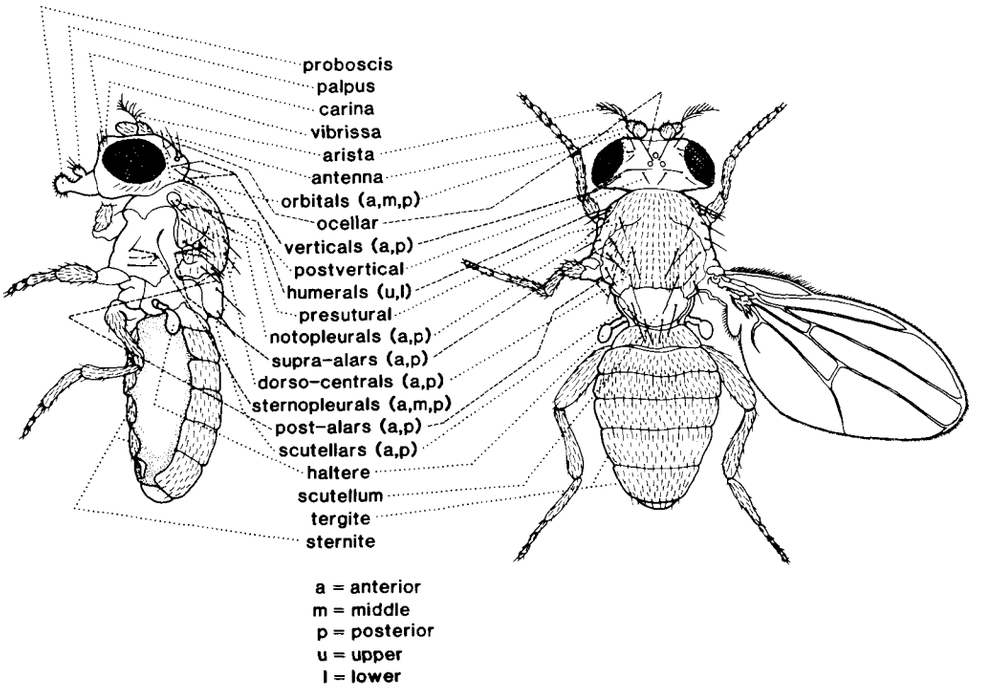

FIG. 1. External morphology of the adult fruit fly.

each chemical. An increased incidence of 14 kinds of abnormalities occurred in flies that were treated with the test chemicals (Table 3).

Eye defects were induced in flies treated with vincristine sulfate, 5-fluorouracil, and methotrexate. Vincristine caused a bulging or rippling of the eye tissue. 5-Fluorouracil reduced the size of the eye. Methotrexate disrupted the anterior half of the eye surface.

Thoracic defects were induced in flies treated with caffeine, hexachlorophene, lithium carbonate, and vincristine sulfate. Humeral knobs (small growths protruding from the humeral bristle site) occurred in flies treated with caffeine, hexachlorophene, and lithium carbonate. Vincristine sulfate disrupted large areas of tissue; malformed bristles were associated with this effect.

Leg defects were induced in flies treated with 6-aminonicotinamide and methotrexate. 6-Aminonicotinamide produced twisted legs, usually involving a single posterior leg, whereas methotrexate caused a stunting of all 6 legs.

Wing notches were found in flies treated with 2-ethoxyethanol, coumarin, and methotrexate. The incidence of missing halteres (wing counterbalance organs) was increased in flies treated with vincristine sulfate.

Abdominal irregularities occurred in flies treated with salicylic acid, ethanol, and lithium carbonate. Salicylic acid and ethanol caused an overlapping or disruption of the dorsal segmentation (tergites). Lithium carbonate treatment was associated with a twisted appearance of the distal tergite (containing the genitalia) up to 180°; no sex specificity was found.

The size of the large bristles (macrosetae) was significantly reduced to at least one half of the normal size in flies treated with hydroxyurea, diphenylhydantoin, diethylstilbestrol, 2-ethoxyethanol, cyclophosphamide, colchicine, vincristine sulfate, chlorambucil, caffeine, 5-fluorouracil, and li-

TABLE 2. SCORING OUTLINE

Surface Examined—Body Part

Dorsal
- I. Head
  - A. Antennae/aristae—L and R[a]
  - B. Eyes (border/interior)—L and R
  - C. Bristles (individual)—L and R
    1. Postvertical—single
    2. Verticals—(a,p)
    3. Ocellars—single
    4. Orbitals—(a,m,p)[b]
- II. Thorax
  - A. Microsetae (hairs)—pattern only
  - B. Macrosetae (bristles)—individual—L and R
    1. Scutellars—(a,p)
    2. Dorsocentrals—(a,p)
    3. Postalars—(a,p)
    4. Supraalars—(a,p)
    5. Notopleurals—(a,p)
    6. Presutural—single
  - C. Wings (margin/veins)—L and R
- III. Abdomen
  - Tergites

Lateral
- I. Head
  - A. Antennae/aristae—L and R
  - B. Eyes (border/interior)—L and R
- II. Thorax
  - A. Bristles (individual)—L and R
    1. Humerals (u,l)[c]
    2. Sternopleurals (a,m,p)
  - B. Halteres—L and R

Ventral
- I. Head
  - A. Antennae/aristae—L and R
  - B. Eyes (border/interior)—L and R
  - C. Proboscis (labellum)
  - D. Palpae—L and R
  - E. Vibrissae/carina—L and R
- II. Thorax
  - Legs
- III. Abdomen
  - A. Sternites
  - B. Genitalia

[a]L and R, left and right.
[b](a,m,p), anterior, middle, posterior.
[c](u,l), upper, lower.

TABLE 3. TEST RESULTS OF 17 CHEMICALS

Chemical	*Concentrations (per ml of medium)*	*Defect*[a]	*Significance*[b]
6-Aminonicotinamide	0 (Control), 2.0 mg,	D	3.0 mg $P < 0.05$
	3.0 mg, 4.0 mg, 5.0 mg,	D	4.0 mg $P < 0.001$
	7.0 mg, 8.0 mg	F	2.0 mg $P < 0.01$
		F	3.0 mg $P < 0.001$
Total number of flies examined = 714			
Caffeine	0 (Control), 0.5 mg,	B	2.0 mg $P < 0.01$
	1.0 mg, 2.0 mg, 3.0 mg,	D	0.5 mg $P < 0.01$
	4.0 mg, 5.0 mg,	D	5.0 mg $P < 0.001$
		E	2.0 mg $P < 0.05$
Total number of flies examined = 1254		K	4.0 mg $P < 0.001$
Chlorambucil	0 (Control), 1.0 mg,	B	2.5 mg $P < 0.001$
	2.5 mg, 5.0 mg, 10.0 mg		
Total number of flies examined = 705			
Colchicine	0 (Control), 0.5 μg,	B	3.0 μg $P < 0.001$
	1.0 μg, 2.0 μg, 3.0 μg		
Total number of flies examined = 896			
Coumarin	0 (Control), 60 μg,	C	90 μg $P < 0.05$
	90 μg, 120 μg, 150 μg	G	150 μg $P < 0.05$
Total number of flies examined = 1268			
Cyclophosphamide	0 (Control), 25 μg,	B	200 μg $P < 0.001$
	50 μg, 100 μg, 200 μg,	D	25 μg $P < 0.01$
		N	25 μg $P < 0.01$
Total number of flies examined = 765			
Diethylstilbestrol	0 (Control), 0.5 mg,	B	1.0 mg $P < 0.01$
	1.0 mg, 1.5 mg, 2.0 mg,	B	1.5 mg $P < 0.001$
	3.0 mg, 5.0 mg	D	5.0 mg $P < 0.05$
Total number of flies examined = 796			
Diphenylhydantoin	0 (Control), 0.5 mg,	B	0.5 mg $P < 0.001$
	1.5 mg, 2.0 mg, 3.0 mg,	D	0.5 mg $P < 0.05$
	4.0 mg, 5.0 mg	D	2.0 mg $P < 0.001$
Total number of flies examined = 363			
Ethanol	0 (Control), 40 μl,	A	60 μl $P < 0.05$
	60 μl, 80 μl, 100 μl,		
	120 μl, 140 μl		
Total number of flies = 1405			
2-Ethoxyethanol	0 (Control), 7.0 μl,	B	13.0 μl $P < 0.01$
	9.0 μl, 11.0 μl,	B	17.0 μl $P < 0.001$
	13.0 μl,		
	15.0 μl, 17.0 μl	D	13.0 μl $P < 0.05$
		D	17.0 μl $P < 0.01$
Total number of flies = 1262		G	9.0 μl $P < 0.001$
5-Fluorouracil	0 (Control), 1.0 μg,	B	1.0 μg $P < 0.01$
	2.5 μg	H	1.0 μg $P < 0.001$
Total number of flies = 486			

TABLE 3. (CONTINUED)

Chemical	*Concentrations (per ml of medium)*	*Defect*[a]	*Significance*[b]
Hexachlorophene	0 (Control), 5 mg, 10 mg, 15 mg, 20 mg, 30 mg	D	10 mg $P < 0.05$
		E	10 mg $P < 0.001$
		K	5 mg $P < 0.001$
Total number of flies = 556			
Hydroxyurea	0 (Control), 50 μg, 100 μg, 200 μg, 400 μg	B	200 μg $P < 0.001$
Total number of flies = 809			
Lithium carbonate	0 (Control), 100 μg, 200 μg, 300 μg, 400 μg, 500 μg	B	200 μg $P < 0.01$
		B	400 μg $P < 0.001$
		E	400 μg $P < 0.05$
		E	500 μg $P < 0.001$
Total number of flies = 1542		I	500 μg $P < 0.001$
		K	400 μg $P < 0.01$
		K	500 μg $P < 0.001$
Methotrexate	0 (Control), 10 μg, 20 μg, 40 μg, 60 μg, 80 μg, 100 μg, 120 μg	F	10 μg $P < 0.001$
		G	10 μg $P < 0.001$
		H	20 μg $P < 0.001$
		J	10 μg $P < 0.001$
Total number of flies = 370			
Salicylic acid	0 (Control), 0.5 mg, 1.0 mg, 1.5 mg, 2.0 mg 3.0 mg, 4.0 mg, 5.0 mg	A	1.0 mg $P < 0.001$
Total number of flies = 1273			
Vincristine sulfate	0 (Control), 5 μg, 10 μg, 20 μg	B	5 μg $P < 0.05$
		B	10 μg $P < 0.001$
		D	10 μg $P < 0.05$
Total number of flies = 1150		D	20 μg $P < 0.001$
		H	20 μg $P < 0.001$
		L	20 μg $P < 0.001$
		M	20 μg $P < 0.001$

[a]Defects:
A, Abdominal irregularities (disrupted banding pattern).
B, Bristle (large hair) reduced in size.
C, Bristle missing.
D, Bristle bent (distinct angle in bristle).
E, Humeral knob (knobby growth at site of humeral bristle).
F, Leg defect (missing, extra, or twisted).
G, Wing notch.
H, Eye defect (missing tissue, misshapen).
I, Abdominal defect (distal tergite twisted or missing).
J, Multiple bristles (more than 2 at a single site).
K, Whole body reduced in size.
L, Haltere defect (missing or misshapen).
M, Large defect affecting major portion of thorax (missing tissue, tissue misshapen, and bristle anomalies).
N, Bristle doubled at a single site.

[b]Significance: Listed in this column are the lowest concentrations where significant differences were found for each defect when compared to control values. These concentrations are provided for 3 levels of probability ($P < 0.05$, $P < 0.01$, and $P < 0.001$) where appropriate.

thium carbonate. Diphenylhydantoin, diethylstilbestrol, and lithium carbonate primarily reduced the size of the notopleural bristles, whereas 2-ethoxyethanol primarily reduced the supraalar bristles. Other chemicals affected no specific bristle type.

Several chemicals altered the frequency of normally occurring large bristles. Missing bristles occurred in flies treated with coumarin. Cyclophosphamide induced double bristles (2 at a single bristle site), and methotrexate caused multiple bristle formation (3 or more at a single bristle site).

Bent bristles were induced in flies treated with diphenylhydantoin, diethylstilbestrol, 2-ethoxyethanol, cyclophosphamide, vincristine sulfate, caffeine, 6-aminonicotinamide, and hexachlorophene. The affected bristles had distinct kinks, forming a sharp ($>90°$) bend near the midpoint of the bristle. The majority of bent bristles found in flies treated with diphenylhydantoin, diethylstilbestrol, 2-ethoxyethanol, cyclophosphamide, and 6-aminonicotinamide were humeral bristles. Other chemicals affected no specific bristle type.

A reduction in the whole body size was found in flies treated with caffeine, hexachlorophene, and lithium carbonate. Flies were generally ¼ to ¾ normal size.

DISCUSSION

The intent of this report is to suggest that *Drosophila* may have the potential to serve as a tool for quickly and inexpensively obtaining information that would aid in selecting the chemicals most likely to show activity in traditional teratology investigations.

There was a wide variety of response in *Drosophila*, both across concentrations and across categories of defects. However, each of the chemicals tested elicited a unique and reproducible pattern of anomalies that generally appeared to be dose related. These observations appear to indicate that groups of cells in developing *Drosophila* differentiate in response to specific chemical effects during larval development. There are a number of distinct cellular aggregates, known as imaginal discs,[9] within the larva that, during metamorphosis, give rise to distinct adult body parts (internal and external). Thus, most defects found in the adult can be considered to be manifestations of independent toxic effects upon separate imaginal discs within the larva.

It is curious that some chemicals, such as chlorambucil, hydroxyurea, and salicylic acid, caused unique defects at only 1 site, whereas others, such as caffeine, lithium carbonate, methotrexate, and vincristine sulfate, were associated with multiple anomalies. All chemicals except the 3 mentioned in the next paragraph, showed significant effects in at least 2 categories of defects or in at least 2 concentrations, and all were experimental teratogens.

Of all chemicals tested, only colchicine, coumarin, and ethanol were weak in producing a response. The only positive response to colchicine treatment was 3 of 9 flies showing a significant reduction in bristle size. In mammals, colchicine induces abortion and may cause Down's syndrome in humans but is only rarely teratogenic.[10] Only coumarin and ethanol had P values greater than 0.01 for all categories of defects at all concentrations tested. In all mammalian species tested, coumarin is not considered to be teratogenic and has not been implicated as a human teratogen.[6] Ethanol only slightly increased the incidence ($P \leq 0.05$) of abdominal irregularities, and these were seen at 2 of the lower concentrations. The meaning of this response is unclear, since this effect was not observed at higher concentrations, and the background of 7.1% abdominal irregularities in the concurrent control group was the highest for any control defect in the study. It is presumed that the fruit fly has a tolerance to ethanol, since its natural life cycle includes feeding upon and depositing eggs within decaying (fermenting) fruit, which may contain considerable levels of ethanol. These results suggest that ethanol may be a reasonable choice as an aid in solubilizing test chemicals for incorporation into the medium. In summary, the 3 weak responses occurred following treatment with 2 virtual nonteratogens (colchicine and coumarin) and with ethanol, to which the fly is presumed to be resistant.

Being in the early stages of development, the test system has no established criteria that label a result as positive or negative. Actually, at this point in test development, it is impossible to say what the criteria will be that determine the "positiveness" or "negativeness" of a specific chemical. Only by

building a sizable database consisting of a large number of flies and many chemical tests can such criteria be secured.

As is well known, almost any chemical can induce developmental toxicity if administered in large enough quantities.[11] Therefore, it will be necessary to eliminate those false-positive effects that may occur routinely following treatment with innocuous substances. Only by directly comparing effects produced in the fly with effects produced in mammalian species can this be accomplished. Initially, this *Drosophila* test will focus on screening for the induction of structural abnormalities and death of the developing organism (2 of the 4 manifestations of developmental toxicity);[12] the remaining 2—altered growth and functional deficiency—are not directly considered at present. Our first objective is to search for distinct and easily scored abnormalities, the production of which can be correlated with the production of mammalian malformations. The utility of the test system will depend on the degree of this correlation.

The economic, logistic, and biological advantages of using *Drosophila* in a teratogen screen are clear, but again, before any method can be accepted as a valid test system, a large number of compounds (including teratogens and nonteratogens) must be tested. For this reason, we plan to test up to 45 chemicals taken from a list generated by the ad hoc committee formed during the August 1981 "Consensus Workshop in In Vitro Teratogenesis Testing."[13] The test regimen is being revised to improve the range-finding studies, to provide better information on dose-response relationships, and to provide consistent group sizes for statistical analyses. In addition, concentrations causing abnormalities will be compared to concentrations causing toxicity (death of developing larvae or based upon an adult LD_{50} dose) to provide an index of the relative teratogenic potential of each chemical. Such a ratio, as suggested by Johnson,[14] may better identify chemicals that are uniquely developmental toxins.

Our specific goals in future studies will be to define the criteria for labeling results as positive or negative, to measure the frequency of false positives and false negatives, to determine the most sensitive and meaningful endpoints, and ultimately, to determine the utility of the *Drosophila* system as an intact organism for the screening of teratogens.

ACKNOWLEDGMENTS

We thank Ms. Sandra Clark (NIOSH) for her aid in the typing and compilation of this manuscript and Dr. Mark Toraason for his helpful comments in reviewing the manuscript.

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Submitted July 31, 1984
Accepted May 27, 1985