THE EMBRYOTOXIC AND TERATOGENIC EFFECTS OF DIELDRIN, MERCURIC CHLORIDE, AND FENITROTHION ON DEVELOPING CHICK (*GALLUS GALLUS DOMESTICUS*) EMBRYOS.

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A Thesis

in

The Department

of

Biological Sciences

Presented in Partial Fulfillment of the Requirements
for the degree of Master of Science at
Concordia University
Montreal, Quebec, Canada

December 1982

ABSTRACT

THE EMBRYOTOXIC AND TERATOGENIC EFFECTS OF DIELDRIN, MERCURIC CHLORIDE, AND FENITROTHION ON DEVELOPING CHICK (GALLUS GALLUS DOMESTICUS) EMBRYOS

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A multiple toxicity study using dieldrin, mercuric chloride and fenitrothion was performed on White Rock chicken (Gallus gallus domesticus) embryos. The toxicants were administered to the embryo via two routes: injection into either the air space (McLaughlin et al., 1963) or yolk sac, just beneath the blastodisc (after Wyttenbach et al., 1981). The air injection technique was found to be unsuitable for these studies, due to delayed exposure of toxicants to the embryo.

Dosages encompassed environmental levels, and ranges of each were as follows: dieldrin, 10-62.4 µg./egg; fenitrothion, 40-160 µg./egg; mercuric chloride, 16-160 µg. Hg/ egg. Binary and tertiary mixtures of these toxicants, each slightly above or at subthreshold concentrations, were also tested.

Toxic and teratogenic effects were evaluated at 5 and 21 days of development. Uncontrollable external factors tended to mask toxic and teratogenic effects in 21-day studies, resulting in little or no correlation between dose and either mortality or incidence of

terata. Such difficulties were not encountered in 5-day studies.

Positive correlation between dose and lethality was observed for each of the three toxicants, while this was not the case for dose versus percent incidence of terata. A combination of lethality and incidence of terata yielded good dose-response relationships for all three toxicants, with median effective doses (ED_{50}) being 43, 55, 151 µg./egg for dieldrin, mercury and fenitrothion respectively.

Additive and supra-additive interactions were observed with all binary mixtures; however, no dose-response relationships were established. The augmentation of response was particularly significant for tertiary mixtures, especially for those comprised of sub-threshold concentrations of toxicants.

Various approaches of quantifying teratogenic effects are also discussed.

ACKNOWLEDGEMENTS

Many people helped in assorted ways throughout this study, and each individual's contribution is greatly appreciated. The complete list is too lengthy to present here, but to a few people, special thanks are due.

First, I would like to express my gratitude to Dr. Perry Anderson for his guidance, patience, support, and friendship.

I am also very grateful to my friends and fellow graduate students, particularly the following: Dan Cyr, Lori Whittaker, Ken McDonald, Luisa De Marte, Masaaki Sawada, François Chabot, Pierre Raymond, Gonum Reddy, Mike Gregory, Deborah Stedman, and Irene Menaggia; their help and encouragement was invaluable.

I would also like to acknowledge the assistance of Prasad Aysola and Ron Harris of the Chemistry Dept., Concordia University, for their help in gas chromatographic and atomic absorption spectrophotometric analyses.

Thanks are also due to the Engineering Dept., Concordia University, for their help in the construction of the egg injector apparatus.

The help of the Biology Dept. technicians is also appreciated.

I am indebted to Michael Bozarth of the Psychology Dept., Concordia University, for his help in programming the computer, and making statistics easy!

To Judy Tilton and Elizabeth Curran, for their help

in typing this thesis; thanks a million! Thanks also to Pauline Shockness, for letting me use her typewriter.

The patience and support of my committee members, Dr. S. Ruby and Dr. H. Enesco, is also greatly appreciated.

Finally, I would like to express my sincere gratitude to my family and close friends, especially Dan, for their help, support, and encouragement throughout the course of this research.

This work was supported in part by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), and from the Dept. of Education (Fonds F.C.A.C.), Government of Quebec, awarded to Dr. P. D. Anderson.

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INTRODUCTION

Wild bird populations have frequently been the coincidental targets of toxic pollutants. Because of their trophic position in terrestrial and aquatic ecosystems, birds are vulnerable to toxicants, such as DDT, which resist degradation, bio-accumulate in tissues and biomagnify in the food chain. Furthermore, because their habitats may coincide with agricultural and forestry areas that are sprayed or otherwise treated with pesticides, they may be the immediate victims of inhalation and enteral toxicity. The consequences of such environmental toxicity have led, in many instances, to significant perturbations in bird populations and in some cases, to their virtual extinction (Borg et al., 1969).

During the last three decades, there has been significant concern for the protection of birds against anthropogenic toxicants. This has resulted in an extensive program to evaluate the potential hazards of environmental contaminants to birds. The basic approach has been to define, using laboratory test populations, the thresholds of exposure to individual agents below which no apparent deleterious effects occur; these thresholds have been used to estimate the assimilation capacity of various ecosystems. The effectiveness of these studies has been challenged,

since generally, they estimate the toxicity of singular substances, disregarding the possibility of interaction with other toxicants to which the organism may be concurrently or sequentially exposed. The necessity of assessing the effects of mixtures lies in the fact that these constituents may result in adverse effects which are more potent than the effects of each constituent individually. Furthermore, mixtures of pollutants are often encountered in the environment.

Multi-contaminant exposure may occur following simultaneous application of two or more toxicants, such as might result from the spraying of increasingly popular multi-pesticide (synergistic) formulations. Other combinations of toxicants are assimilated through the food chain. As co-existing contaminants, they may have originated from the same source; for instance, as components of complex effluents released from waste pipes or flues. Still other mixtures of pollutants may arise from different sources, but come to co-exist in a single habitat as a consequence of convergence of their respective environmental fates.

The environmental hazards of toxic mixtures have prompted research on combinations of many different compounds in recent years, and information on interactions between environmental chemicals in birds has been documented since 1973 (Kreitzer & Spann, 1973). Furthermore, management agencies have shown a

desire to use this information in setting criteria for permissible environmental levels. With multiple toxicity studies to date involving only adult organisms, there is a need to examine the developmental phases of the life cycle, which may be uniquely sensitive to multiple toxicants. It is well-known that in mammals, certain substances such as thalidomide and caffeine can be toxic to the foetus at levels which are either therapeutic or harmless to the adult. These teratogenic agents are transferred to the embryo via the placenta. Transfer of contaminants from the mother to the embryo may also occur, albeit indirectly via the yolk, in avian species. For instance, ingested contaminants may be stored in various tissues, including the ovaries of female birds (Cecil et al., 1974). There may be a period just prior to ovulation, during which large quantities of yolk are deposited, when this uptake of pollutants into eggs is significantly enhanced. The avian embryo which draws its sustenance from yolk is therefore likely to be exposed to yolk contaminants throughout the course of development. This situation is often observed with lipophilic contaminants such as chlorinated hydrocarbon insecticides.

The possibility of interactions between environmental contaminants in avian embryos is not remote. Monitoring studies have revealed significant

amounts of residues of various substances in the eggs of wild birds occupying treated areas (Fimreite & Karstad, 1971; Gilbertson, 1974). In addition, embryos may be exposed during pesticide spraying operations to toxic aerosols which penetrate the egg shell.

The present study was undertaken to explore the multiple toxicity to avian development of three pesticides: dieldrin (HEOD), fenitrothion, and inorganic mercury (as mercuric chloride). Their prevalence in nature could result in their coexistence as contaminants of birds' eggs. The first objective of this study was to study the toxicity of each agent individually. The second objective was to test whether binary and tertiary mixtures of these contaminants were additive, supra-additive, or infra-additive, according to the model of Anderson & d'Apollonia (1978). The third objective of these experiments was to examine whether or not the administration of multiple toxicants resulted in unique forms of toxicity. The fourth objective was to assess whether mixtures of these contaminants are toxic when constituents are present at their respective subthreshold concentrations.

Literature Review on Individual Toxicants

The primary objective of this study was to examine the effects of coexisting environmental contaminants upon avian embryonic development, and three pesticides commonly found as pollutants were selected. They are all widely-distributed and extensively used, and two of the three (dieldrin and mercuric chloride) are highly persistent in the environment.

Mercuric Chloride

Physico-chemical Characteristics and Environmental Sources

Mercuric chloride ($HgCl_2$; M.W. 271.5 g.) is a white crystalline powder and can be readily reduced to mercurous chloride and metallic mercury.

The major sources of environmental contamination by mercury include: chlor-alkali plant discharges; mercurial wastes from industries such as pulp and paper manufacturing; mercury catalysts from acetaldehyde and vinyl chloride manufacturing processes; mining tailings and vapors released by the mining and smelting of

mercury, tin, zinc, copper, and lead; combustion of paper products and fossil fuels; naturally-occurring deposits of mercury; and smaller amounts (as wastes) from hospitals and laboratories. Mercury emissions in 1970 in Canada totalled more than 82 tons (Charlebois, 1977). Mercuric chloride was first used for crop protection in 1891. However, since it is a general poison and is strongly phytotoxic, its use in crop protection is restricted to soil application. It has also been used, in the past, as a pesticide, insecticide, and fungicide, and is now a popular disinfectant for commercial grains and feed (Pesticide Manual, 1977).

Mercury residues in tissues

Mercury residues in a variety of avian species have been reported by several authors. Haseltine et al. (1980) collected black duck eggs (Anas rubripes) from the Atlantic Flyway area in 1978. Of the forty-nine (49) eggs analyzed, thirty-one (31) were found to contain mercury, with levels ranging from 0.07-0.34 ppm. Stendell et al. (1977) examined canvasback duck eggs (Aythya valisineria) collected in 1972-73 from numerous major breeding areas in North

America, including Nevada, Alberta, Saskatchewan, Manitoba, and North Dakota. Mercury levels were generally low (less than 1 ppm.) and were similar to previously reported data on canvasback tissues (Baskett, 1975; Vermeer & Armstrong, 1972; Kleinert & Degurse, 1972). The authors noted that the levels found were generally lower than those known to directly affect survival or reproduction of birds. However, some eggs with higher residues suggest potential harm to the reproductive success of some bird species. Dustman et al. (1972) also observed problems associated with mercury residues in several species of wild birds.

Concern over the potential dangers of mercury to avian wildlife first arose in 1955, when Swedish ornithologists noted a marked decrease in the populations of certain seed-eating birds. Smith (1973) discovered increased mercury residues in the livers and kidneys of wild birds found dead, suggesting that mercurial seed dressings were the cause of death. Berg et al. (1966) noted an increase in the mercury content of the feathers of many birds. His findings coincided with the beginning of liquid alkylmercury seed treatments.

Inorganic mercury ($HgCl_2$) is converted in the gut

of chickens into methylmercury, $(CH_3)_2Hg$, which is far more toxic (Nelson, 1971). Methylmercury readily passes through membranous barriers, including that of the central nervous system and placental membranes (EPA, 1971). Inorganic forms of mercury, on the other hand, tend to accumulate in the liver, but some mercury is deposited in fatty tissues, such as the kidney and brain (EPA, 1971).

Experimental studies with mercury have revealed that various forms of this metal may be transferred from mother to egg, where they affect development. Kuwahara (1970) administered methyl mercuric chloride and mercuric chloride subcutaneously to laying hens, and reported accumulation of approximately 60-80% of the mercury in the eggs, with methyl and inorganic mercury tending to concentrate in albumin and yolk. In a different study, quail treated with intravenous doses of methyl mercuric nitrate laid eggs containing 40-52% of the administered mercury (Backstrom, 1969).

Effects of mercury poisoning

Most of the information regarding mercury toxicity has been derived from mammalian studies. Mercury poisoning in mammals causes ataxia (uncoordinated,

unsteady movement), anemia, and behavioral disturbances. These symptoms are irreversible, and brain damage may result from extreme acute mercury intoxication.

The research focussing on mercury toxicity in birds is far less extensive, although certain effects comparable to those seen in mammals have been observed. These include neurological dysfunction, resulting from demyelination of spinal cord nerves (Borg et al., 1969; Fimreite, 1970; Fimreite & Karstad, 1971), as well as hematological changes (Thaxton et al., 1974). Other effects of mercury toxicity that are unique to birds include reduced immunological function (Thaxton & Parkhurst, 1973; Bridger, 1981) and reproductive deficiencies, such as decreased egg production, eggshell thinning, and abnormal mating behavior (Tejning, 1967; Stowesand et al., 1971; Spann et al., 1972; Thaxton & Parkhurst, 1973). In addition, mercury has also been reported to inhibit growth in birds (Fimreite, 1970; Fimreite & Karstad, 1971; Parkhurst & Thaxton, 1973). One of the few studies of mercury toxicity to developing avian embryos was performed by Birge and Roberts (1976). They injected White Plymouth Rock strain chicken eggs with methyl mercury and inorganic mercury, prior to incubation. Injections were made into the yolk via

needle track procedure (Birge & Just, 1974), and concentrations ranged from 1.00 ppb. to 50.0 ppm. Inorganic mercury was found to inhibit hatching in a dose-related fashion, with zero per cent hatch at the highest administered dose (50 ppm.) of mercury. In addition, appreciable numbers (up to 33% of the total number of treated eggs) of embryos treated with inorganic mercury exhibited major defects at hatching, including neurological effects, such as brain deficiencies and severe motor impairment, as well as absent eyes, skeletal deformities, and unabsorbed yolk sacs. The frequencies of occurrence of these terata were concentration-dependent, and were inversely correlated with survival.

Dieldrin

Physico-chemical characteristics and Environmental Sources

Dieldrin is the common name for a chlorinated hydrocarbon cyclodiene ($C_{12}H_8OCl_6$) which came into prominent use as an insecticide about 1958. It consists of greater than 85% HEOD, which is 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4,endo-5,8-dimethanonopthalene. Other registered trade names include Alvit, Dieldrite, Octalox, and Panoram-D-31 (Pesticide Dictionary, 1974). Until recently, it was widely used not only as an insecticide, but also in mixtures with fungicides as a seed dispersant and disinfectant.

It has high contact and stomach activity to most insects, and is not phytotoxic. Dieldrin also has excellent residual activity, being highly stable and lipophilic, and it is known to accumulate in the environment, as well as in plant and animal tissues. Studies monitoring pesticides in wildlife samples up to 1970, indicate that second only to DDT, dieldrin is the compound most often observed (Edwards, 1970). Various formulations include wettable powders (500-750 g.a.in./kg.); emulsifiable concentrates (180-

200 g./l.); dusts; granules (20-50 g.a.in./kg.); fertilizer mixtures; seed dressings; and solutions (150-200 g./l.)(Pesticide Manual, 1977; Pesticide Dictionary, 1974).

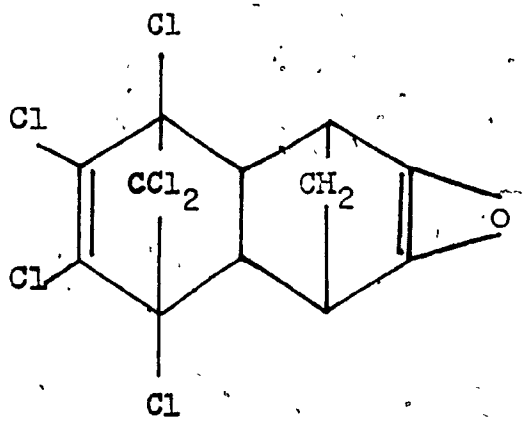


Figure 1. The structure of dieldrin (Melnikov, 1971).

Residues and Accumulation

Significant dieldrin residues in humans (Curley et al., 1973), mammals (Clark & Lamont, 1976; Davidson, 1970; Diechmann et al., 1968; Iatropoulos et al., 1975), and milk and dairy products (Wedberg et al., 1978) have been reported. Various workers have also

studied dieldrin accumulation and residues in avian species (Beardmore & Nobel, 1976; Davison et al., 1970; Seifert et al., 1968; Robinson et al., 1967a,b; Klaas et al., 1980; Haseltine et al., 1980; Koeman et al., 1967; Gannon et al., 1959; Kan et al., 1978) resulting from either environmental or experimental exposure. Seigel et al. (1978) fed 1.40 ppm. dieldrin in the feed of chicks to two (2) different breeds, White Leghorn and White Rock, to determine whether or not there were breed differences in dieldrin accumulation and depletion in growing chicks. Although significantly more dieldrin was concentrated in body fat by the White Leghorn breed up to approximately eight (8) weeks of treatment, by twelve (12) weeks there was no significant difference between the breeds. In addition, whole body loads of dieldrin were greater in White Leghorn than in White Rock, although breed differences were less than those between fat concentrations of dieldrin in the two breeds. Following removal of dieldrin from the diet, initial rates of depletion of dieldrin from fat tissue or whole body were more rapid in the White Leghorn than in the White Rock, but these differences were not significant by two weeks.

Studies of dieldrin levels in avian embryos and eggs have also been done. Guthrie and Donaldson (1970) performed studies on the distribution of dieldrin in,

and its toxicity to, developing chick embryos. Dieldrin was observed to be distributed rapidly throughout all developing tissues and organs by day 5 of incubation, and the concentrations of dieldrin seemed to parallel lipid content.

Koeman et al. (1967) injected dieldrin, at doses up to 1 mg. per egg, into chicken eggs prior to incubation. Distribution of dieldrin was almost complete after five (5) days of incubation, at which time the concentrations of dieldrin would be comparable to those of eggs receiving dieldrin from hens which had laid them. No teratogenic effects upon the embryos were reported in this study. However, at a concentration of 257 mg. dieldrin/kg., embryo mortality was 38%. These authors concluded that concentrations of dieldrin greater than those naturally occurring in eggs (to date) were not appreciably toxic. Hatched chicks from treated eggs were either starved or fed post-hatch, to determine the effects of absorption of contaminated yolk. It was concluded that absorption of the yolk following hatching caused poisoning of the young birds by residual dieldrin in the yolk, even at residue levels that did not affect hatching.

Toxic Effects of Dieldrin

Dieldrin is thought to act primarily as a neurotoxin, and is known to act at nerve synapses rather than along the nerve processes of insects and other animals (Brown, 1978). However, little is known concerning the specific mode of action of dieldrin, although Brown (1978) suggested that it may act by forming charge-transfer complexes within presynaptic membranes, affecting the release of neurotransmitter. Studies by Calhoun (1960) indicate that dieldrin is not a cholinesterase inhibitor. However, O'Brien (1967) demonstrated that dieldrin-poisoned insects exhibited increased levels of acetylcholine, probably as a result of nervous hyperactivity and/or reduced cholinesterase levels. Shankland and Schroeder (1973) suggested that dieldrin acts to promote the release and rupture of the presynaptic vesicles storing the acetylcholine. Difficulties in determining the mode of action of dieldrin relate to dieldrin-resistance in certain species, and differences in rates of penetration of dieldrin through various tissues (O'Brien et al., 1974).

Teratogenic studies involving dieldrin include those by Ottolenghi (1973) on effects in rats and mice, and by Dix et al. (1977) and Chernoff et al, (1975) in

mice. While acute and chronic toxicity testing of dieldrin in birds has been fairly well reported, there is no evidence of any teratogenic studies involving dieldrin in avian species.

Fowler et al. (1971) found that high levels of dieldrin did not significantly affect hatchability and chick survival in samples taken from breeding populations of common and purple gallinules. These levels were apparently ineffective, confirming the results of Causey et al. (1968). Furthermore, no correlation was found between shell thickness and dieldrin contamination. In contrast to this, Porter and Weimeyer (1969) demonstrated that dieldrin and DDT cause the production of significantly thinner shells in sparrow hawks. Enderson and Berger (1970) also found that dieldrin application or treatment resulted in the reduction of egg shell quality in prairie falcons. Graber (1965) reported poor hatchability and low survival of chicks of dieldrin-contaminated red-winged blackbirds. In addition, substantial dieldrin residues in the eggs of these blackbirds were thought to cause unusual behavior, deserted nests, and very poor breeding success. Thus it is apparent that the toxicity and effects of dieldrin may vary widely between species of birds, probably due, at least in part, to differences in diet and in feeding habits.

Fenitrothion

Physico-chemical characteristics and Environmental Sources

Fenitrothion (o,o-dimethyl-o-(4-nitro-3-methylphenyl) phosphorothioate) is an organophosphate insecticide which has been, and continues to be, widely used in aerial spray programs in Eastern Canada for the control of the spruce budworm (Choristoneura fumiferana, Clem.).

Rates of application of fenitrothion have ranged from as low as 2 oz./acre (138 g./hectare) in commercial spraying to as high as 18 oz./acre (1238 g./hectare) applied experimentally (Buckner, 1967). Fenitrothion was applied at dosages between 2-6 oz./acre (138-413 g./hectare) in Eastern Canadian forests in 1973 (NRCC, 1975). The recommended dose for commercial spraying to control forest pests was 2-4 oz./acre (138-275 g/hectare) in 1967, although up to 8 oz./acre (550 g./hectare) has been used (Ecobichon, 1982; Pearce, 1968). In studies evaluating the impact of fenitrothion spraying on forest birds, Buckner (1967) reports that there remains uncertainty as to

whether the toxicity of fenitrothion to birds is primarily oral or respiratory. Due to several environmental factors such as windspeed, droplet size, and dosage application, the levels of fenitrothion to which forest birds may be exposed are highly variable. However, Pearce (1974) determined residue levels in samples of both dead and apparently healthy birds in areas sprayed with 3 oz./acre (206 g./hectare). A variety of species were examined, and fenitrothion residues ranged from only 0.183-5.22 mg./kg. fresh weight (Pearce, 1974; NRCC, 1975).

Fenitrothion is generally rapidly hydrolyzed in most animals; homeotherms metabolize fenitrothion to several terminal residues, while it is degraded primarily to aminofenitrothion by aquatic and soil micro-organisms. Fenitrothion is also susceptible to UV photo-induced degradation as well as chemical hydrolysis in water. Yule & Duffy (1972) found that under operational conditions (275 g./hectare), up to 85% of the fenitrothion was decomposed within two weeks of application, although about 10% of the initial spray persisted for nearly one year. Yule (1974) also demonstrated that fenitrothion may accumulate in foliage proportionately to the total dose and number of annual applications. In general, however, fenitrothion is relatively short-lived (up to a few weeks) in soil

(Yule & Duffy 1972) and in water (a few hours to a few days; Kingsbury, 1973).

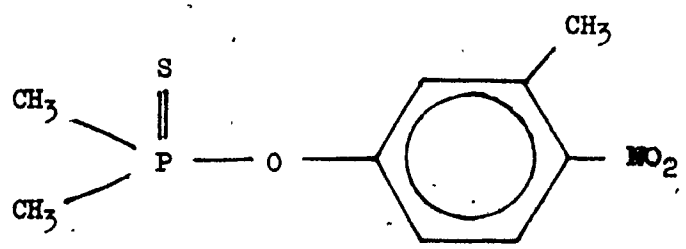


Fig. 2 The structure of fenitrothion (NRCC, 1975).

Toxic Effects of Fenitrothion

Being a cholinomimetic compound, fenitrothion acts as an anti-cholinesterase organophosphate (Durham et al., 1982; Fleming, 1981). This effect apparently has resulted in numerous malformations in avian embryos, including lordosis, scoliosis, micromelia and abnormal feathering, wry neck, and other distortions (Lutz-Ostertag & Bruel, 1981; Moscioni et al., 1977 Seifert & Casida, 1978; Roger, 1969; Meiniel, 1977). The only other known published report of teratogenic

effects of fenitrothion in chick embryos is that of Paul and Vadlamudi (1976). Injection of 0.1 ml. of 0.1,1,5,10,20 or 30% fenitrothion (in distilled water) into the yolk space of chick eggs on one day between the fourth to the twelfth day of incubation resulted in significant embryonic mortality. Dwarfism, curled toe, leg weakness, and abnormal gait (in hatched chicks) were also noted. An important observation by these researchers was that treatment during the later stages (days 8-12) of incubation produced lower toxicity than injections earlier in embryonic development. These results were confirmed in a pilot study, in which fenitrothion was injected on day 7 of incubation. The embryos were opened on day 14 and examined; no developmental or morphological effects were observed in any of the treated embryos.

Spraying often coincides with the breeding season of many birds, thus exposing both adults and eggs to fenitrothion. Buckner (1975) discussed forest spray programs, including those with fenitrothion, in Canada, and examined their impact on small forest birds. From the published data and his own studies, Buckner concluded that, compared to other compounds in use at that time, fenitrothion was of relatively low toxicity

to birds, the data being supplied by Sumitomo Chemical, Ltd. (Japan), which manufactures fenitrothion. Oral LD50's for a variety of avian species range from 27 mg./kg. (bobwhite quail) to 1190 mg./kg. (mallard ducks).

Effects on Reproduction

Few studies have examined the impact of fenitrothion upon reproduction in birds. At application rates of 138 g./hectare and greater, behavioral changes, reduced singing, and reduced movements have been detected. Mortality is observed at levels above 275 g./hectare in adult birds which inhabit the crown canopy; while adult mortality is dramatically increased at application rates of 550 g./hectare and greater. Birds inhabiting areas other than the crown canopy are also affected (NRCC, 1975,1977). Furthermore, Buckner & Ray (1973) noted a severe decrease of crown canopy birds in a fenitrothion-treated area of northwestern New Brunswick. The need for additional studies regarding the effects of fenitrothion on bird populations and reproduction was also emphasized in these reports.

MATERIALS AND METHODS

Test Organisms

Fertilized chicken eggs (Gallus gallus domesticus, White Rock variety) were obtained from a local commercial hatchery (Couvoir de Laval, Ville des Laurentides, Quebec, Canada) on the same day that each experiment commenced. A total of 2220 eggs were purchased for this study. Eggs were inspected upon arrival, and any cracked or malformed eggs were discarded.

Controls

Eggs were randomly divided into lots of 10-25 eggs. Four groups of controls were included in each trial: untreated; punctured only; injected with the carrier, propylene glycol (1,2-propanediol; Mallinkrodt Chem. Works, Toronto); and injected with an equal volume (0.10 mls.) of 0.9% saline.

Solutions of experimental compounds were made using propylene glycol as the vehicle, and a volume of 0.10 mls. of each solution was injected.

The importance of choosing the proper solvent for toxicity and teratogenicity studies has been previously reported (Swartz, 1980; Gebhardt, 1968). Modifications of the toxicity of certain compounds in different solvents, possibly leading to erroneous conclusions, has also been demonstrated (Swartz, 1980; Walker, 1967). Such modifications are thought to be due to the formation of plugs or deposits within the yolk, uneven dispersion and distribution of the test agent throughout the yolk, and immiscibility of the test compound with the yolk. The behavior of test agents within the yolk is apparently due, at least in part, to the definite stratified arrangement of white and yellow yolk in the chicken egg (Fig. 3; Walker, 1967).

In his studies, Gebhardt (1968) showed that propylene glycol (propanediol-1,2) injected into chicken eggs on the fourth day of incubation at volumes ranging from 0.05-0.2 mls. may be teratogenic. Under these conditions, 40% of the propylene glycol-treated embryos exhibited dorsal liquid-containing cysts. However, this anomaly did not occur if injections were made earlier in incubation. In the present study, propylene glycol

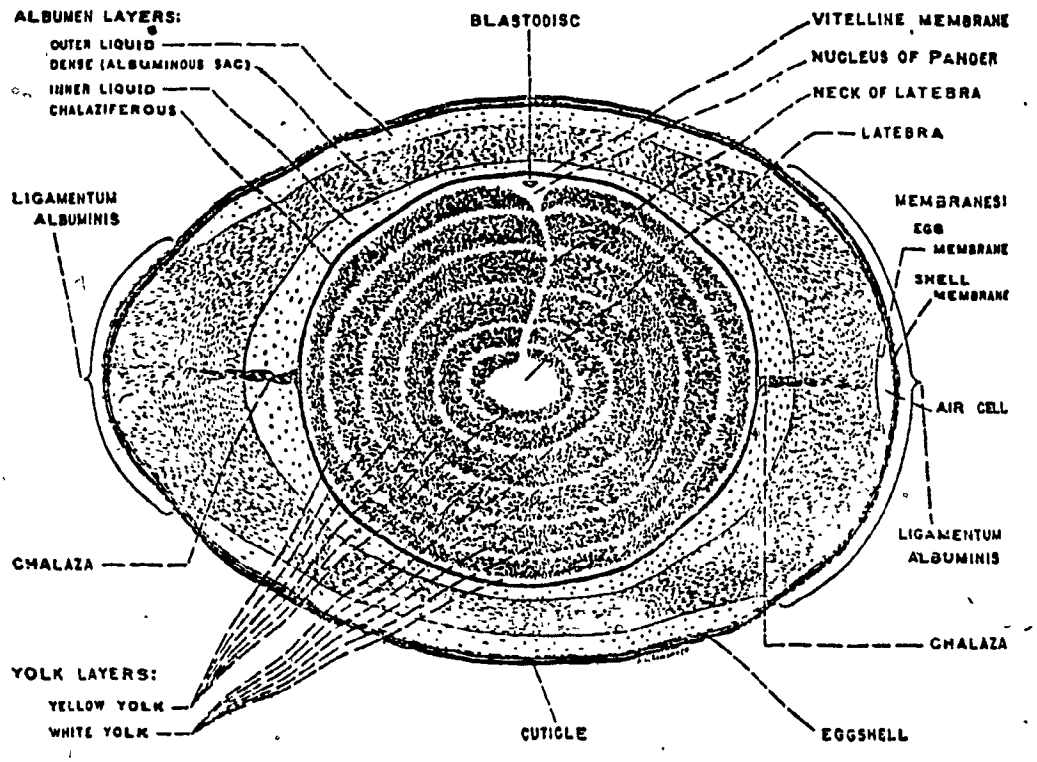


Fig. 3. Structure of a chicken (Gallus gallus domesticus) egg, showing a section through the long axis (after Romanoff & Romanoff, 1949).

was injected at an earlier time (about 4 hours of incubation) than in Gebhardt's experiments, and controls injected with propylene glycol did not display the cysts recorded by Gebhardt.

Chemicals

The dose range of each compound tested was as follows: dieldrin, 1-62.4 µg./egg injected; fenitrothion, 1-160 µg./egg injected; and mercuric chloride, 1-160 µg./egg. The average weight of eggs was 63.1 ± 5.1 g. and the average weights of yolk and albumin were 20.9 ± 1.9 and 33.3 ± 3.9 g., respectively. The abbreviations for the injected toxicants that will be used herein are listed below:

HEOD - dieldrin

F - fenitrothion

H- Mercury (as mercuric chloride)

DF - binary mixtures of dieldrin and fenitrothion

DH - binary mixtures of dieldrin and mercuric chloride

DFH - tertiary mixtures of dieldrin, fenitrothion, and mercuric chloride

Dieldrin was obtained from the Shell Oil Co., and had a purity of 97%. Fenitrothion, at a purity of 99.4%, was provided by the Sumitomo Chemical Co. (Japan). Mercuric chloride (reagent grade, A.C.S. certified) was purchased from Fisher Scientific Co.

Methods of Injection

Certain contaminants, such as DDT and dieldrin, are persistent and known to accumulate in the environment (Causey et al., 1968). Since one of the objectives of these experiments was to examine the effects of toxicants that may have been deposited in the yolk during vitellogenesis, availability of the toxicant during the initial stages of embryonic development was desirable.

The first method of injection, in accordance with that of Wyttenbach et al.(1981), was designed to

mimic this yolk deposition. Following inspection, the remaining eggs were randomly divided into test lots, wiped clean with 70% ethanol, labelled according to assigned treatment, and immediately incubated on flat open-bottomed screen trays for about four hours. During this incubation period, the yolk sac rotates so that the pole at which the blastoderm is located comes to lie directly beneath the upper surface of the egg. Eggs were carefully removed and held in the same orientation as in the incubator. While held in this position, eggs were candled to identify the exact location of the blastoderm beneath the upper surface. This location was marked on the shell. The eggs were once again wiped clean with 70% ethanol and with the blastodisc marker upright and centered, the egg was carefully affixed to the bottom of the vacuum tube of the egg injector apparatus (Fig. 4). The egg was held firmly in place by vacuum suction. A hand drill (270 Dremel Moto-tool, Pascal's, Montreal, Canada), equipped with a 0.63 mm. carbide bit and firmly attached to a sliding carriage, was moved into position directly beneath the egg and the center of the vacuum tube. The vacuum tube carriage was lowered to the point where the rotating bit just pierced the egg shell. The bit

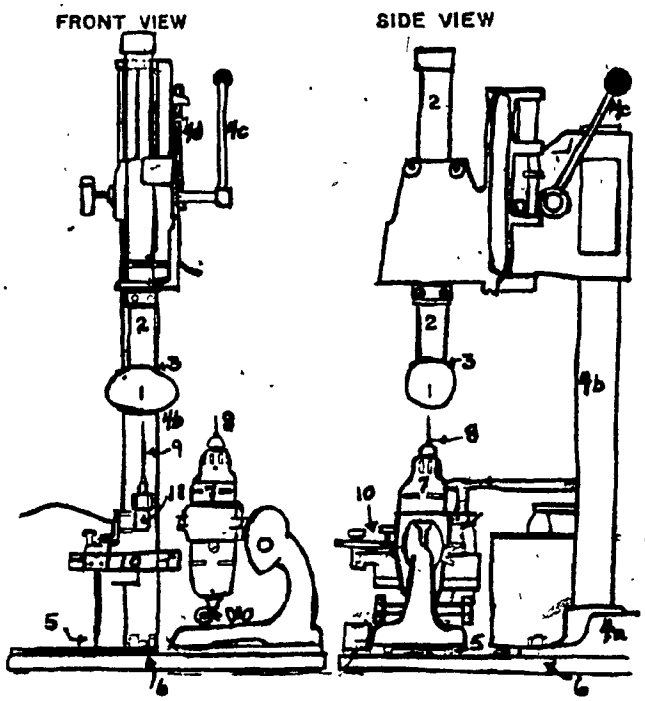


Fig. 4. Egg injector apparatus (modified from Kitos et al., 1981).

Legend:

1. Egg
2. Polyvinylchloride (PVC) tube, 25 mm. o.d., connected at the upper end to an ordinary vacuum cleaner.
3. Rubber "O" ring, contoured to the shape of the egg and glued to the end of the PVC tube.
4. Drill press stand
   (a) base
   (b) shaft
   (c) handle for raising and lowering the PVC tube and egg.
   (d) adjustable stop for maximum downward stroke
5. Precision carriage, capable of back and forth motion on a track.
6. Precision drill stand (Pascal's, Montreal, Canada).
7. Drill (Dremel Moto-tool Model 270; Pascal's, Montreal, Canada).
8. Carbide drill bit (size No. 63, 0.0355 in. diam., 0.9 mm.).
9. A 1.5 in. (38 mm.), 21-guage hypodermic needle.
10. Adjustable stage, obtained from a microscope.
11. Connector which attaches the hypodermic needle to the adjustable stage (no. 10).

was cleaned with 70% ethanol before each use. The vacuum tube carriage was returned to its resting position. Using sterilized equipment, a 21 or 22G, 1.5 inch needle attached to a graduated 1 cc. syringe was inserted vertically through the drill hole into the yolk sac for the distance pre-determined to bring the tip of the needle to just beneath the blastodisc (Fig. 5). Solutions were injected at this point, the needle carefully withdrawn, and the hole was immediately sealed with paraffin. Dose, batch, and time of injection information were recorded, and the eggs were returned immediately to the incubator.

The second method of injection was designed to mimic aerosol deposition of environmental contaminants, following their penetration through the shell. In this method, selected eggs were wiped clean with 70% ethanol, and randomly distributed into test lots. Eggs were then labelled accordingly. Prior to incubation, eggs were injected via the air space in accordance with the technique described by Hadani & Egyed (1967), Gilani & Chatzinoff (1981), Kankaanpaa et al. (1979). A hole was made into the blunt end of the egg using a 18 or 20G needle. Another needle of smaller gauge (21G) and attached to a graduated 1 cc. syringe was inserted through the

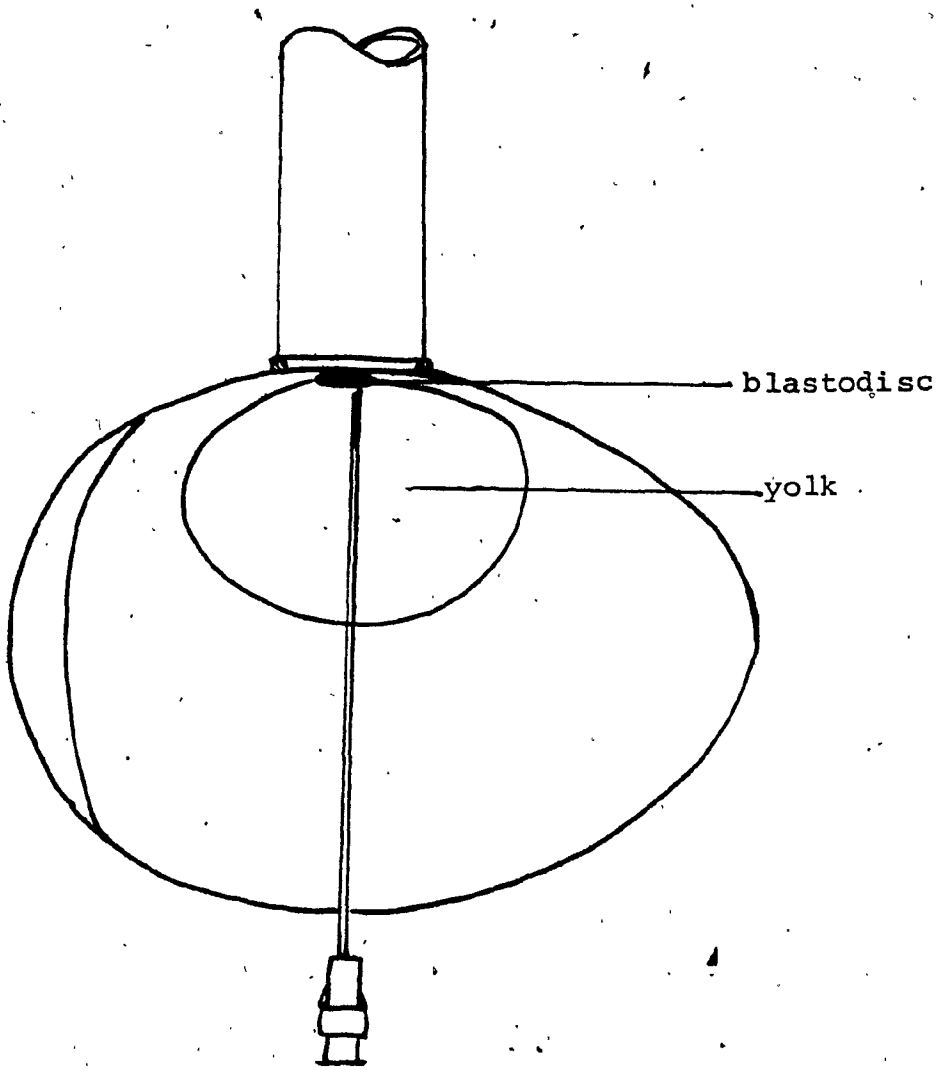


Fig. 5. Location of injection into the yolk sac of chicken eggs (after Kitos et al., 1981).

hole into the air sac at an angle of approximately 45°. Sterilized solutions were injected at this point. The needle was quickly removed, and melted paraffin (Fisher Co.) was applied to seal the hole.

Time of Injection

The time at which an acute injection is made during the course of development may be a significant variable in teratogenic studies. Injections have been performed prior to incubation (Gilani & Chatzinoff, 1981; Verrett et al., 1980; McLaughlin et al., 1963); after four (4) hours of incubation (Wyttenbach et al., 1981); and at 1-12 days of incubation (Gilani & Chatzinoff, 1981; Verrett et al., 1980; Kankaanpaa et at., 1979; Paul & Vadlamudi, 1976; Karnofsky, 1965). Several authors (Verrett et al., 1980; Gebhardt, 1965; Karnofsky, 1965) have reported enhanced teratogenic effects when eggs are injected at about 96 hours (4 days) of incubation. In this study, injection of eggs was performed prior to or within four hours of incubation.

Incubation of Eggs

Following injection, the eggs were incubated in a forced-draft incubator (Brower Co., Inc.) at standard

temperature (99.75°F; 38°C) and humidity (50-60% rel. hum.; 84-86°F, wet bulb thermometer). Humidity was decreased slightly three days prior to hatch. Eggs were turned six times daily. After the second day of incubation, eggs were candled daily. Any dead or unfertilized eggs detected by candling were removed, opened, and the contents were examined. If embryos were present, they were examined for any morphological defects, staged according to the criteria of Hamburger and Hamilton (Lillie & Hamilton, 1952), and preserved in Bouin's fixative.

Pre-hatch Test Periods and Toxicity Measurements

Quantal response determinations were based on observations recorded following 5 days of development. In these trials, eggs were opened at day 5 of incubation and examined for embryotoxic and teratogenic effects. Embryotoxic effects are those associated with the disruption of life-sustaining metabolism. They may be observed as alterations in bodily activity, general growth processes, and mortality. Teratogenic effects are associated with the disruption of normal developmental pathways, whereby distortions in form, known as terata (monstronsities), occur.

Upon opening eggs, all specimens were described according to stage of development, degree of normality, and where relevant, the nature of deformities. In addition, measurements of crown-rump and third toe lengths were taken using a caliper. Samples of unsually deformed and control embryos were photomicrographed, using a Wilde stereomicroscope with a Zeiss camera attached. Upon photographing, these embryos were preserved in Bouin's fixative.

In other trials, test lots of eggs were incubated to hatch. At specific times throughout incubation, test lots were subsampled and examined as described above for any developmental anomalies, deaths, or other embryotoxic effects. All information concerning numbers and kind of effects, and incubation period were recorded. Following examination and photomicrography, embryos were preserved in Bouin's fixative.

Criteria for Terata

The criteria for death varied depending on the stage of development. These included tissue opaqueness in early embryos (fertilization to 3 days); lack of visible heartbeat for a period of 60 sec. in 3-5 day embryos; and haemorrhages and dark, clotted blood rings in the vitelline and chorio-

allantoic circulation of 6-17 day embryos. Lack of neuromuscular response to pricking by a needle was used to characterize death in older embryos (18-21 days of incubation).

Judgements on the extent and kind of terata were made by first relating each specimen to a similarly-sized embryo as defined in Hamilton and Lillie's (1952) index of development. Once the specimen was indexed according to size, the visible features of the specimen were compared to those expected at this growth stage for normal development.

Post-hatch Studies.

Within each test lot, certain eggs were incubated to term (21 days). Hatching times and success ratios were recorded. Hatched chicks were weighed, banded, and held in a standard chick brooder (Sargent-Welch). Chicks were able to drink from water troughs at will, and were fed with chick starter (Purina Co.) ad libitum. At one day of age, the hatched chicks underwent examination for external skeletal and other morphological abnormalities. Any defects were qualified and quantified, where possible.

Neuromotor Dysfunction Tests

Tests were performed on all one-day old hatched chicks. Each chick was subjected to two quantitative tests, one for balance and one for righting ability, (adapted from Alder & Zbinider, 1977). In the balance test, the chick was placed in the center of a platform which could be manually rotated around a horizontal axis (Fig. 6). When the chick was seen to be settled, the board was gradually tilted to one side. The degree of tilt at which the chick hopped or fell from the platform was determined by measuring the angle of the board with a protractor affixed to one of the stands which supported the platform. Pilot studies showed that rate of tilt, if performed gradually, was not a factor governing the "departure angle". Following the first measurement, the procedure was repeated in the opposite direction. Results were grouped according to treatment regimes. Abnormal balancing ability was defined as any "departure angle" less than that angle corresponding to the standard error limit of the mean "departure angle" of controls.

The second test examined the righting reflex of one-day old hatched chicks. Each chick was placed on its back on a flat table. The hand holding the chick in place was quickly removed, and the time required for the individual to flip over and stand upright was

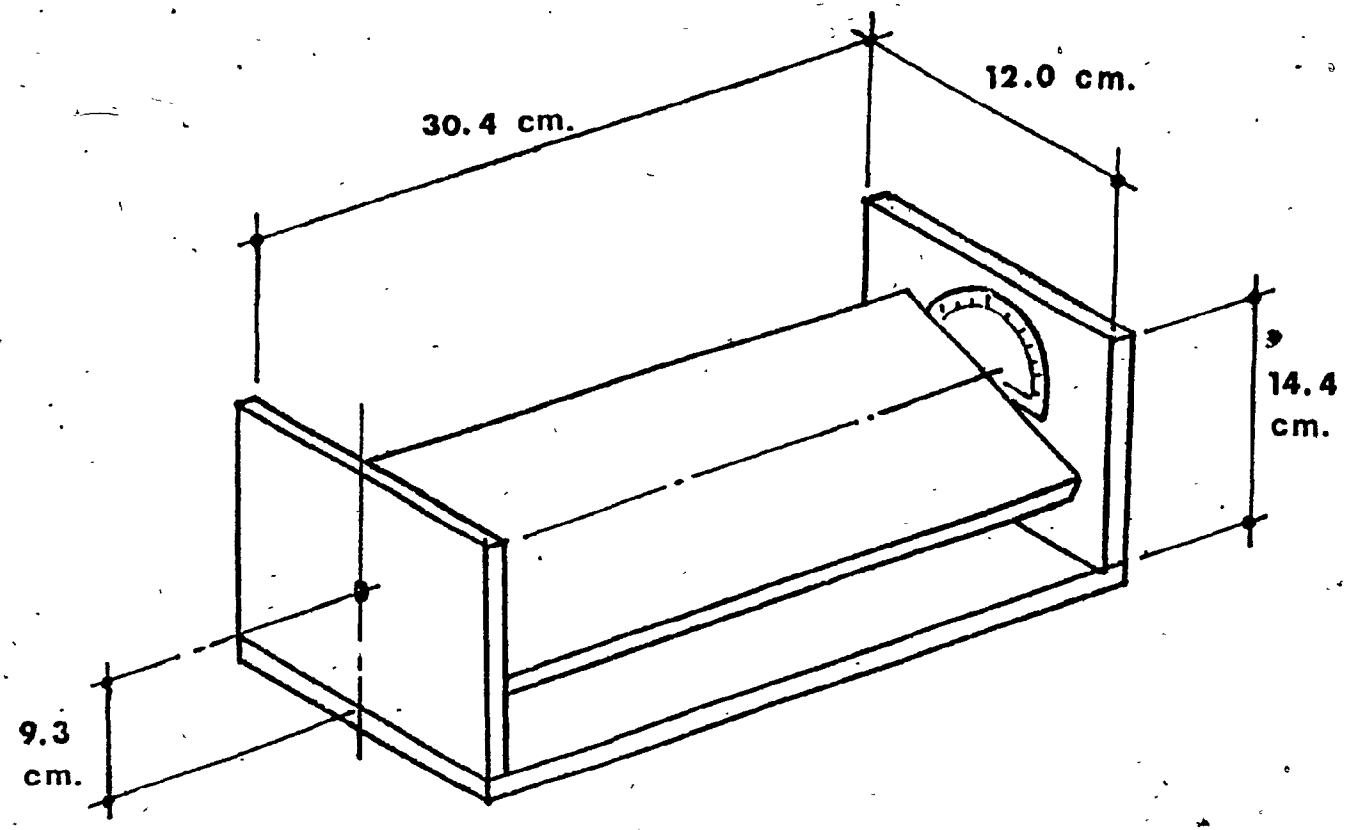


Fig. 6. Tilting board apparatus, used in balance test for the detection of neuromotor dysfunction and impaired balancing ability.

recorded with a stopwatch. If a chick did not succeed in righting itself within thirty seconds, a righting period sufficient for all controls, then it was judged to have an impaired neuromotor ability.

Chemical Analyses and Hepatosomatic Index

Following neuromotor tests, chicks were held in brooders for an additional 6 days, at which time a randomly selected subsample was sacrificed in CO_2 chambers, weighed, eviscerated, and fixed in 95% ethanol. Livers were separated from the viscera, blotted dry, weighed, and preserved in Bouin's solution. The liver was subsequently prepared and analyzed according to recommended E.P.A. techniques by electron-capture gas chromatography and atomic absorption spectrophotometry respectively. Liver samples taken from a subsample of one-day old hatched chicks were similarly prepared and analyzed. The ratio of liver to body weight (the hepatosomatic index) was determined for each individual in these subsamples.

Skeletal Preparation

A proportion of seven-day old hatched chicks were sacrificed for whole skeletal examination.

Their tissues were cleared and skeletons were stained with Alizarin Red S, according to Dawson (1926), with the modifications of Burdi & Flecker (1968). However, it was later found that improved staining resulted when Russel's staining procedure was used (1973). Some skeletons which had already been fixed in 95% ethanol were not re-fixed in FAA (formalin-acetic acid-alcohol) solution but were washed in water and submitted to remainder of the Russel protocol. Prepared skeletons were then examined for any skeletal deformities.

T.A. Method for Evaluating Terata and Mortality

It is assumed that death during embryonic development may be the consequence of two distinctly different forms of lethal toxicity: one, in which a toxicant causes dysfunction to general metabolic and life-supporting systems, such as intermediary metabolism, and the other, in which a toxicant causes a disruption in the differentiation pathways in development. The first mode of lethal action is defined as embryotoxicity; the second is referred to as teratogenicity. A method was developed to explore whether the two forms of lethal events could be

distinguished, based on data collected in yolk-injected eggs following five days of incubation. This method employed a proportionality formula called the T.A. formula, which attributes a percentage of the total number of deaths in a test lot corresponding to the average control mortality as being the result of embryotoxicity. The difference between control and total mortality was treated as being the consequence of teratogenicity. The general equation of this formula is:

$$T.A. = 1 - (1-P_1)\ (1-P_2)\ (1-P_3), \quad (1)$$

where T.A. = proportion (%) of embryos not available for expression of terata

P_1 = proportion of control embryos dead by day 5 of incubation

P_2 = proportion of control embryos exhibiting any terata

P_3 = proportion of treated embryos (in a particular dosage group) dead by Day 5 of incubation.

The principle behind this formula is to determine the total number of embryos not available

for expression of a compound's potential teratogenic effects. Values for control mortality (P_1), as well as for any controls exhibiting terata (P_2), and mortality in treated eggs (P_3) are all expressed as proportions or percentages. Each of these variables is subtracted from 1 (or 100%) and the resulting values are then multiplied. The assumption behind this operation is that these three variables (P_1,P_2,P_3) are independent of one another; i.e., the frequency of occurrence of one variable does not affect the frequency of occurrence of either of the other two variables. The product of this multiplication is then subtracted from 1 (100%), resulting in the T.A. (total affected) value, which represents the proportion of the total number of treated eggs in a particular group that is unable to exhibit terata. This T.A. value is then multiplied by the total number of eggs in that group (Q), to obtain the actual number of eggs (U) not available for expression of potential teratogenic effects. This number (U) is subtracted from the total number of eggs in that group (N), to yield N', the total number of viable and potentially able to be affected embryos. The number of embryos exhibiting terata (A) divided by the total number of viable embryos (N')

yields the proportion of embryos affected (A'). This quotient (A') is then multiplied by 100 in order to express the results on a percentage basis. Thus, the entire procedure may be summed up in the following expression:

$$A' = A/(N' - (T.A. * Q)) \qquad (2)$$

Teratogenic Index Evaluation

A teratogenic index, modified from Schom & Abbott (1978), was developed to permit a comparison of terata between agents and treatments. The index was based on the assumption that the earlier the teratogenic effect, the more profound the consequences (Table 1). Each of the anomalies was assigned a value, depending upon the temporal development of that particular feature, with respect to the entire developmental process of the chick. For example, since the notochord is among the first things to develop during gastrulation, notochord (spinal cord) distortions, such as abnormal twisting or incomplete closure, were evaluated at 6 points. Development of the eye (lens, optic cup, etc.) occurs at slightly later stages (about 33-36 hours' incubation); hence, reduced or abnormal eye

Table 1. Assigned values for sublethal effects (embryonic terata) used in calculations of TI (teratogenic index).

Criterion	Assigned Value
Death within first 24 hours of incubation	8
Spinal curvature abnormalities	6
Reduced eye development or abnormalities	5
Circulatory disturbances or abnormalities	4
Skeletal and muscular distortions	3
Epidermal abnormalities (feather discoloration)	2
Abnormal torsion (position of embryo on yolk sac)	1

development was given a value of 5 points. The values for all of the observed terata were then summated and divided by the total number of embryos tested at that particular dose level. When the teratogenic index was initially applied to the data, early death was not considered as a teratogenic effect. However, in subsequent applications of the TI to each set of data, a value of 8 points was assigned to early death (death within the first 24 hours of incubation).

Data Analysis

All data were corrected for control response according to the Abbott formula (Eq. 3), and significant differences ($p < 0.05$) between controls and experimentally-treated groups were tested using Student's t-test (Sokal & Rohlf, 1979).

$$P = P(c) + P(o)/100 - P(c), \quad (3)$$

where P = corrected % response

P(c) = per cent response in controls

P(o) = per cent response in treated groups

Percent mortality at 5 days' incubation was calculated for each test lot. These values were then

converted to probit mortality (Bliss, 1936) and a dose-response relationship, described by a linear function (Equation 2), was then derived in accordance with Finney's (1971) probit analysis procedure.

$$Y = a + b \log C, \qquad (4)$$

where Y = probit of % mortality

a = intercept

C = concentration of toxicant

b = slope of the dose-response curve.

In this procedure, a series of lines are fitted to the coordinates until a maximum likelihood line is determined by chi-square testing. Probit lines were computed for data representing both the total sum of effects (mortality + terata) and simply terata, in each experimental lot.

Multiple Toxicity Models

The different types of interaction which may occur among constituents of a mixture have been described by a varity of mathematical approaches, including those of Bliss (1939); Plackett & Hewlett (1948,1952,1963,1967); Hewlett & Plackett

(1952,1957,1959,1964); Finney (1971); Anderson & d'Apollonia (1978). These models are generally based upon pharmacological principles and distinguish between the action of components of a mixture upon the same type of physiological receptor, and upon different types or receptors. The models used in the present study are the concentration-addition model and the response-addition model, based on those published by Bliss (1939).

The dose-response curves constructed for dose versus total % affected (day 5 mortality + % affected with terata) were compared with respect to slope. If the slopes of the lines appeared to be similar, toxicants were assumed to be similarly acting and their lines constrained to parallelism using a computer program (Tallarida & Murray, 1981) based on the procedure described by Finney (1971). The goodness of fit of these parallel lines to the coordinates was tested using the chi-square test (Tallarida & Murray, 1981; Finney, 1971). The median effective concentration (ED_{50}) for each pesticide was then computed from the parallel regression dose-response lines. The relative potency factors were then derived by dividing the ED_{50} value of the less toxic compound by the ED_{50} value of the more toxic substance. Relative potency factors were used to convert the concentration of less potent

constituents of a mixture into equivalently effective doses of the more potent compound. For instance, the relative potency factor resulting from compound A divided by compound B was multiplied by the actual concentration of B in a mixture of A + B, to yield the amount of B present in terms of A-equivalents. This amount was then added to the concentration of A present in the mixture to give the total concentration of A + A-equivalents present.

Concentration-Addition Model

If the dose-response curves were not significantly unparallel, according to the constraint to parallelism procedure, then the dose-response data for the test agents was tested for concentration-addition. The concentration-addition or similar joint action model assumes that different components in a mixture act on the same type of receptor in the test organism. This model also presumes that there is a similar distribution of tolerance or susceptibilities of individuals, within randomly selected test groups. Thus, the individuals most susceptible to compound "1" should also be most susceptible to compound "2", where compounds "1" and "2" are constituents of a mixture. Consequently, the slopes of quantal response curves should be similar,

(parallel), since the slopes represent the distribution in susceptibility. The predicted response of a toxicant mixture can then be expressed as:

$$Y = a_1 + b_1 \log(C_2 + C_1) \qquad (5)$$

where a = intercept of regression line for compound 1

b = slope of regression line for compound 1

C_2 = concentration of compound 2 in mixture in equivalent units

C_1 = concentration of compound 1 (Bliss, 1939).

A linear regression line is fitted to the observed response recorded for mixtures, and this line is compared to that predicted using the chi-square test. Consistent significant differences imply that the data does not comply with the concentration- addition model, and the model is rejected.

Response-Addition Model

When it is found using the above statistical test that the concentration-addition model does not fit the observed response, the results are examined in accordance with the response-addition model. Response-addition or independent joint action assumes that toxicants in a mixture act independently of one another on different receptors or critical targets to produce a common response. Unlike the concentration-addition model, the response-addition model considers the fact that susceptibilities may or may not be correlated. Consequently, the slopes of the regression lines of the discrete quantal response curves for each component need not be parallel or similar for the response-addition of components of a mixture (Anderson & d'Apollonia, 1978).

When there is no correlation (r=0) of susceptibilities to each toxicant, the predicted response is given by the relation:

$$P' = P_1 + P_2(1-P_1) \qquad (6)$$

or

$$P' = P_1 + P_2 - P_1P_2 \qquad (7)$$

where P' = proportion responding to a mixture of compounds "1" & "2"

P_1= proportion responding to compound "1" only

P_2= proportion responding to compound "2" only

The proportion responding when the susceptibilities are positively correlated (r=1) is:

$$P' = P_1; \text{ if } P_1 \geq P_2 \quad (8)$$

$$P' = P_2; \text{ if } P_2 \geq P_1 \quad (9)$$

Finally, the proportion responding when the susceptibilities are negatively correlated (r= -1) is given by the equation:

$$P' = P_1 + P_2; \text{ if } P_1 + P_2 \leq 1 \quad (10)$$

$$P' = 1 \text{ ; if } P_1 + P_2 > 1 \quad (11)$$

(Hewlett & Plackett, 1952)

It should be noted that each constituent of a mixture will theoretically contribute to the effect of that mixture only when its ambient concentration is greater than threshold. Chi-square tests are conducted to determine whether or not there are any significant differences ($p < 0.05$) between observed and expected responses. If there is a significant

difference, the model is rejected as an adequate description of the interactions occurring in the experiments.

Treatment of Non-additive Response Data

Certain physiological interactions between toxicants may lead to a greater than additive response, or synergism. These effects are determined as those falling outside the 99% confidence limits for either concentration-addition or response-addition, and they are termed supra-additive. Other interactions between toxicants may lead to a response that is less than the 99% confidence limits for the expected for concentration- or response-addition. Such results are called infra-additive and may or may not necessarily be a form of antagonism between the toxicants. No studies were conducted to investigate the mechanisms of these supra-additive and infra-additive responses.

RESULTS

Control Mortality

Average mortality in lots of untreated eggs which were incubated to hatch was 21.0 ± 4.8% (Table 2). Egg puncture and needle penetration did not cause significantly ($p < 0.05$) different mortality as compared to that of untreated controls in air space and yolk sac injection trials. The average percent mortality recorded for either propylene glycol or saline controls in air space injection experiments was not significantly different from the average death rate in untreated controls, although the incidence of mortality in one propylene glycol and one saline trial was somewhat high. However, a significantly higher ($p < 0.05$) mortality rate was observed for propylene glycol- and saline-treated eggs in 21-day yolk sac injection experiments. Furthermore, in 5-day trials, air space injections of propylene glycol caused significantly higher ($p < 0.05$) levels of mortality, while in yolk sac injection trials, both propylene glycol and saline produced a high incidence of mortality (Table 3). In the latter case, these differences from untreated controls were not significant because of the high variance between batches.

Table 2. Percent mortality at 21 days of incubation in controls of both air space and yolk sac injection experiments.

	Air Space Injection			Yolk-Sac Injection		
Controls	Trial A	Trial B	Average	Trial A	Trial B	Average
Untreated	27.7	21.1	24.4 ± 4.8	17.8	17.4	17.6 ± 0.3
Egg punc.	11.8	25.0	18.4 ± 9.3	8.3	14.3	11.3 ± 4.2
Saline	21.4	35.3	28.4 ± 9.8	38.9	43.7	41.3 ± 3.4 *
Prop. Glycol	40.0	27.8	33.9 ± 8.6	52.9	58.3	55.6 ± 3.8 *

* Significant difference ($p < 0.05$) from untreated control values.

Table 3. Day 5 percent mortality of controls in air space and yolk sac injection trials.

	Average % Mortality	
	Air Space Injections	Yolk-Sac Injections
Controls		
Untreated	5.0 ± 4.6	6.8 ± 3.6
Egg punc.	10.7 ± 11.1	2.5 ± 2.5
Saline	6.7 ± 7.2	18.6 ± 10.6
Prop. glycol	18.7 ± 1.9 *	18.1 ± 17.1

* Significant difference from untreated control ($p < 0.05$).

In untreated controls, there were occasional developmental anomalies (< 1%) other than death. The incidence of non-lethal anomalies in eggs injected with either saline or propylene glycol into the air space was no greater than that in untreated controls.

Term Studies: Qualitative and Quantitative Approaches

Critical Stages of Mortality

Time-related studies based on subsamples of controls showed that the incidence of mortality was highest at three specific periods during the course of embryonic development. These critical periods (Figs. 7-9) were at day 1 (stages 1-8), days 4-5 (stages 21-27), and days 18-21 (stages 43-45). Of these three periods, most deaths occurred during the latter (days 18-21), Table 4. These periods correspond roughly to peak mortality phases in Gallus gallus domesticus development, as reported in the literature (Hamilton & Lillie, 1952; Romanoff, 1943). There was no shift in these critical periods for mortality in experimental lots. In air space injection experiments, the incidence of mortality at each critical phase in all toxicant-treated groups was not significantly ($p < 0.05$) different from either untreated or propylene glycol controls. In contrast, all three toxicants, injected as single agents, caused significantly higher ($p < 0.05$)

Fig. 7. A comparison of percent mortality at the three critical phases of development in propylene glycol-treated groups. Average values of percent mortality were calculated from a combination of all air space or all yolk sac injection trials. Critical periods 1,2, & 3 correspond to day 1 (stages 1-8), days 4-5 (stages 21-27), and days 18-21 (stages 43-45), respectively. This notation also applies to Figs. 8-9.

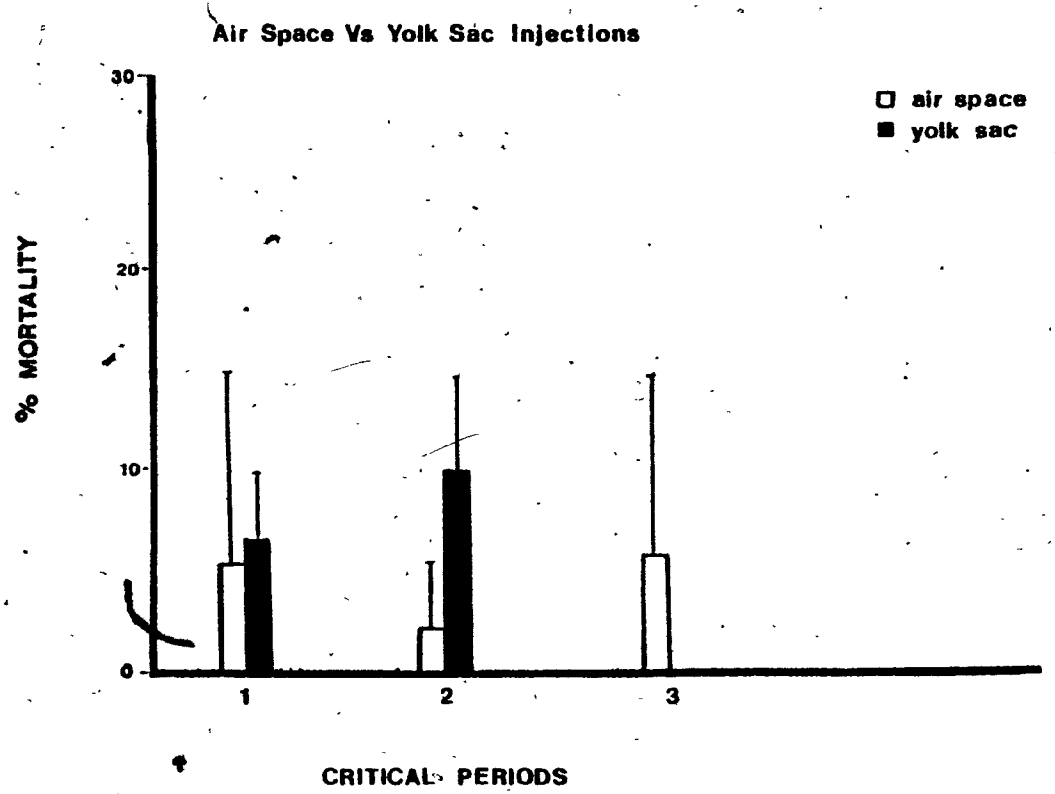

Air Space Vs Yolk Sac Injections
air space
yolk sac
% MORTALITY
30
20
10
0
1
2
3
CRITICAL PERIODS

Fig. 8. Combined data for all air space injection trials, representing percent mortality in untreated and propylene glycol controls, as well as in groups treated with a single dose of dieldrin, mercury, or fenitrothion. No multiple toxicity studies were performed in air space injection experiments.

-Combined Data – Air Space Injections

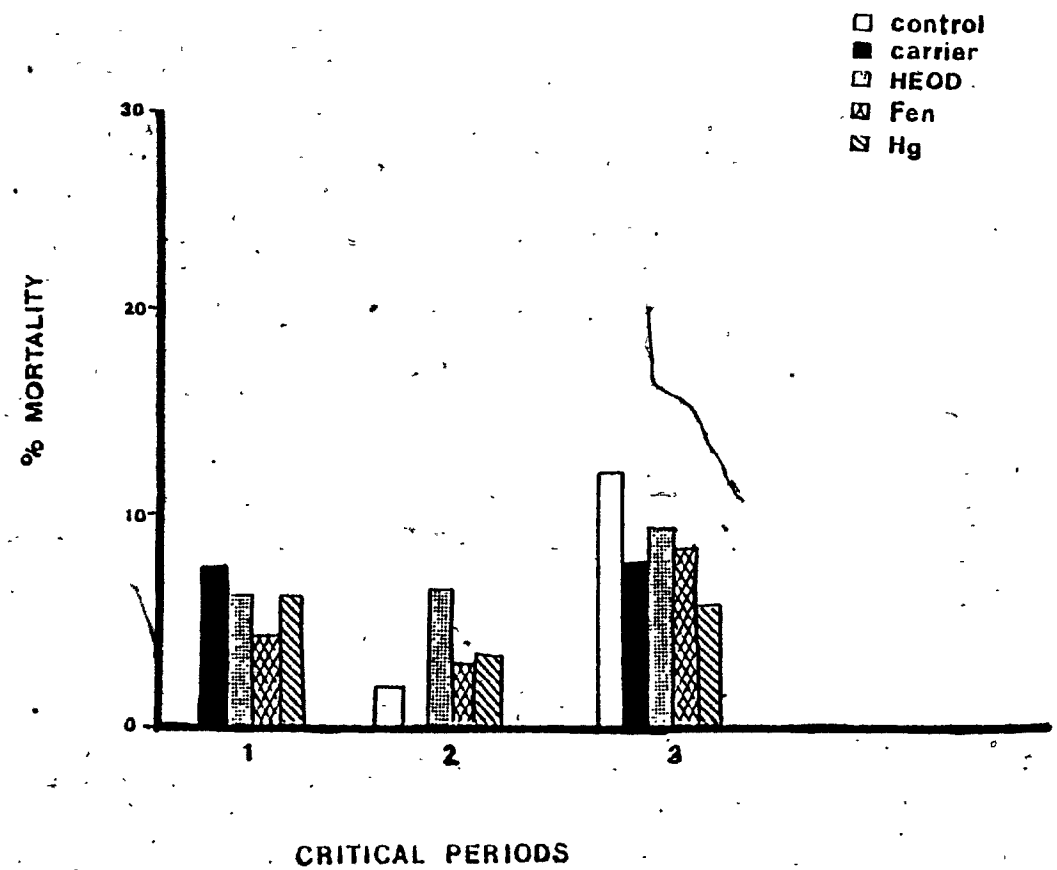


Fig. 9. Combined data for yolk sac injection trials, representing percent mortality in untreated and propylene glycol controls, as well as in groups treated with either a single dose of dieldrin, mercury, or fenitrothion, or with a single dose of a binary or tertiary mixture of these toxicants. Limited data was obtained for the third critical period, since most experiments were 5-day rather than 21-day studies.

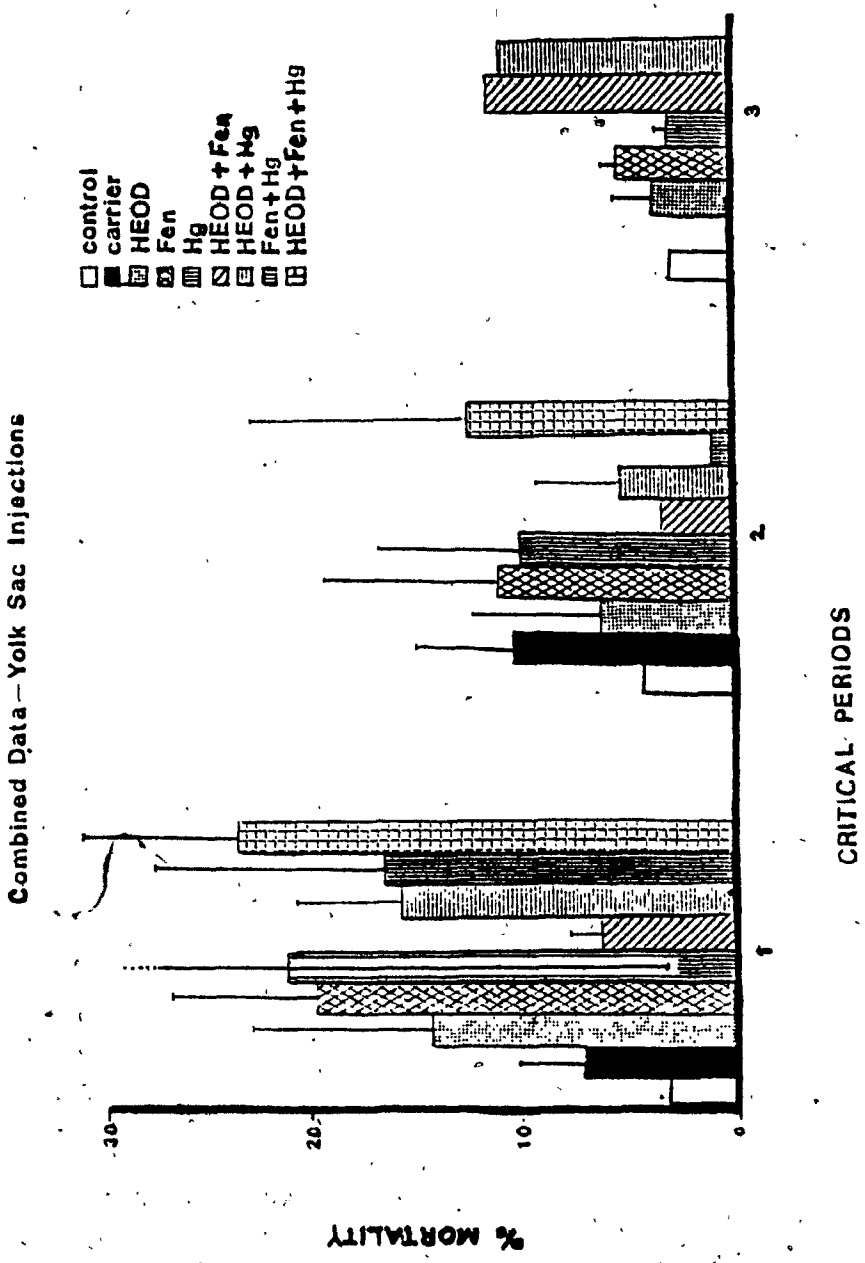

Combined Data — Yolk Sac Injections
control
carrier
HEOD
Fen
Hg
HEOD + Fen
HEOD + Hg
Fen + Hg
HEOD + Fen + Hg
% MORTALITY
30
20
10
0
1
2
3
CRITICAL PERIODS

Table 4. Percent mortality at critical periods of development in controls and toxicant treated embryos of air space injection experiments.

	Percent Mortality			
	Period I* (Day 1)	Period II (Days 4-5)	Period III (Days 18-21)	Total# Tested
Untreated	0	2.1	12.5	48
Prop. Glycol	7.9	0	7.9	38
Dieldrin	6.4	6.4	9.6	125
Fenitrothion	4.4	2.9	8.8	136
Mercury	6.7	3.3	5.8	120

*Note: No significant difference ($p < 0.05$) in % mortality between untreated or propylene glycol controls and any of the treated groups at these critical phases was observed. However, total % mortality for all toxicant-treated groups up to Day 5 or Day 21 was significantly different ($p < 0.05$).

mortality at day 1 when injected into the yolk sac, as compared to controls (Fig. 9; Table 5). All binary and tertiary mixtures containing mercury were also highly lethal at day 1 (Table 5). The mixtures resulting in the lowest incidences of mortality were binary mixtures of fenitrothion, combined with wither dieldrin or mercury. At both day 1 and days 4-5, the highest incidence of mortality amongst treatments was recorded for tertiary mixtures, although the mortality at the first critical period (day 1) only was significantly higher ($p < 0.05$) than that of untreated or propylene glycol controls.

For each toxicant, there was no linear correlation between dose and lethal response during the first and last critical stages when injections were made into the air space. Mortality appeared to be a function of dose at days 4-5 (stages 21-27) for mercury- and fenitrothion-treated eggs ($r = 0.93$ and 0.77, respectively), but no dose-related pattern was observed for dieldrin ($r = 0.41$). For this reason, data recorded for each dose was combined when represented in Figs. 7-9.

Terata and Sublethal Effects

There were no significant teratogenic or lethal effects upon development prior to day 5 observed in air space injected experimental lots. However, abnormal pigmentation (feather discoloration) as well as

Table 5. Percent mortality at critical periods of development in toxicant-treated embryos of yolk sac injection experiments. Values given represent average (mean) values ± standard deviation for each group.

	Percent Mortality		
	Critical Periods		
	I (Day 1)	II (Days 4-5)	III (Days 18-21)
Dieldrin (all doses)	13.5 ± 8.7 *	5.3 ± 6.2	3.4 ± 2.0
Fenitrothion (all doses)	18.9 ± 7.0 *	8.0 ± 8.5	5.3 ± 0.6
Mercury (all doses)	21.7 ± 17.0 *	6.0 ± 6.9	2.9 ± 0.7
DF	5.9 ± 1.6	5.5 ± 0	11.6
DH	15.4 ± 5.0 *	5.2 ± 4.2	10.8
FH	16.4 ± 11.2 *	7.0 ± 0	0
DFH	23.2 ± 7.6 *	11.7 ± 10.3	0

* Significant difference between both untreated and propylene glycol controls, and toxicant treated group ($p < 0.05$). Sample sizes range from 19-179.

Note: Percent mortality values given in this table are corrected values; i.e. corrected for any control mortality using the Abbott formula (Eq. 3).

hindlimb and digit distortions, events which arise during the latter phases of development, were observed in hatched chicks and near-term embryos (stages 43-45). Analysis of variance (ANOVA) tests showed that there was no significant ($p < 0.05$) difference in response between doses for each toxicant. Comparisons were then made on a treated versus non-treated basis. The incidence of each of these effects for hatched chicks was summed for each toxicant and its mixtures, as listed in Table 5a. More than 35% of dieldrin-treated embryos exhibited feather discoloration, while adverse effects upon limb and digit development were observed in more than 25% of these embryos. Fenitrothion and mercury both caused slightly lower incidences of feather discoloration, and slightly higher incidences of limb and digit distortions (crooked toes and feet). Tests for neuromotor dysfunction indicated that dieldrin caused significant ($p < 0.05$) effects upon righting ability, while all three toxicants exerted significant ($p < 0.05$) effects on balancing capability. Dieldrin, however, appeared to be the most potent of the three toxicants with respect to causing neuromotor dysfunction.

Less than 2% of anomalies occurred in pre-hatch controls of yolk sac injection experiments (Table 6). A variety of sublethal and teratogenic effects were seen in most lots treated with any of the toxicants.

Table 5a. Incidence of post-hatch effects in chicks hatched from eggs treated with a single dose of dieldrin, fenitrothion, or mercury, according to the air space injection procedure. Values represent mean value ± standard error for each group.

	Average Percent Incidence			
			Neuromotor Dysfunction	
	Feather Discoloration	Limb & Digit Distortions	Impaired Righting	Impaired Balance
Controls				
(all groups)	3.6 ± 2.2	3.0 ± 1.9	0	0
Dieldrin				
(all doses)	35.0 ± 9.2*	28.8 ± 7.5*	15.2 ± 4.8*	65.0 ± 11.0*
Fenitrothion				
(all doses)	26.0 ±10.2*	34.9 ± 9.1*	11.7 ± 9.9	40.0 ± 16.5*
Mercury				
(all doses)	22.7 ± 4.8*	33.9 ± 5.5*	5.9 ± 3.0	26.8 ± 8.5*

* Significant difference from controls and toxicant-treated groups ($p < 0.05$).

The three most prominent anomalies were abnormal (inverse) torsion, circulatory disturbances, and altered eye development. There was no particular structural syndrome that uniquely characterized the toxicity of any of the contaminants or their mixtures. Most defects seemed to arise from alterations of early developmental pathways. Table 6 considers the incidence of the three major pre-hatch effects in controls and in all experimental lots. There was a significant level ($p < 0.05$) of abnormal eye development caused by mercury, while both mercury and fenitrothion caused significant incidences ($p < 0.05$ and $p < 0.02$, respectively) of circulatory disturbances. None of the three toxicants, administered as single agents, caused a significant ($p < 0.05$) increase in the incidence of inverse torsion, compared to propylene glycol controls. However, the incidence of this effect in embryos treated with binary mixtures of fenitrothion and mercury was significantly different ($p < 0.02$) from both untreated and propylene glycol controls.

Subthreshold levels of all three toxicants combined in tertiary mixtures had no effects on inverse torsion that were significantly ($p < 0.05$) different from either untreated or propylene glycol controls. However, these tertiary mixtures had more profound effects than either binary mixtures or single agents

Table 6. Incidence (%) of sublethal effects (embryonic terata) of toxicants, observed in 5-day embryos in yolk sac injection experiments. Values represent mean ± standard error for each group; sample sizes ranged from 19-179.

	% Affected		
	Inverse Torsion (lying on wrong side)	Circulatory Disturbances	Abnormal Eye Development
Controls:			
Untreated	0	1.1 ± 2.1	0
P. Glycol	1.4 ± 1.6	1.3 ± 2.5	0
Dieldrin	4.3 ± 3.3	9.0 ± 6.9	4.9 ± 7.0
Fenitrothion	3.0 ± 2.5	15.9 ± 5.4***	6.4 ± 5.2
Mercury ($HgCl_2$)	0.3 ± 0.6	14.4 ± 8.2**	7.7 ± 5.1**
DF	2.1 ± 0.8*	16.4 ±10.5	13.6 ± 9.3
DH	7.1 ± 6.1	11.6 ± 3.8*	6.8 ± 7.3
FH	9.0 ± 1.3***	7.0 ± 5.2	4.3 ± 0
DFH	0	24.6 ± 0.3**	19.5 ± 11.8

Significant difference between both untreated and propylene glycol controls, and toxicant-treated group at: $p < 0.10$ *
$p < 0.05$ **
$p < 0.02$ ***

on both circulatory disturbances and abnormal eye development. Although fenitrothion and mercury, when administered individually, caused the highest incidences of circulatory disturbances, their binary mixtures were the least effective of all binary combinations with respect to this parameter. Furthermore, while mercury was the most disruptive of the three toxicants with respect to eye development, its effects in binary mixtures resulted in an even lower incidence of this anomaly. Of the three binary combinations, those of dieldrin and fenitrothion caused the highest incidence of circulatory disturbances. There was, however, no apparent consistent relationship in potency.

In yolk sac injection trials (Table 7), there was a low incidence of structural defects in feathers and limbs observed in controls, particularly propylene glycol. However, these defects were mild in degree. For example, the slight deviations from normalcy observed in the limbs of certain propylene glycol-treated specimens did not adversely affect their ability to perform righting and balance tests. All three toxicants caused considerable effects on both feather and limb and digit development. However, due to somewhat high variability and small sample size (generally 5-10/group), the differences in the incidence of each of these effects between all control and

Table 7. Incidence (%) of post-hatch terata in one-day chicks hatched from eggs treated with a single dose of dieldrin, mercury, or fenitrothion, according to the yolk sac injection procedure. Values represent mean value ± standard error.

	% Affected			
	Feather Discoloration	Limb & Digit Distortions	Neuromotor Dysfunction	
			Impaired Righting	Impaired Balance
Controls				
(all groups)	8.0 ± 0	11.5 ± 0	0	0
Dieldrin				
(all doses)	32.5 ± 7.4	16.2 ± 4.9	17.0 ± 6.1	32.2 ± 10.3
Fenitrothion				
(all doses)	22.4 ± 6.4	29.8 ± 7.1	13.6 ± 6.2	34.9 ± 9.0*
Mercury				
(all doses)	18.2 ± 3.8	26.1 ± 4.9	6.1 ± 2.3	31.8 ± 6.4**

Significant difference between controls and toxicant-treated group at $p < 0.10$ or $p < 0.05$ **.

Note: Sample sizes ranged from 9-19.

toxicant-treated groups was not statistically significant ($p < 0.05$).

While all three agents had some effect on righting ability (Table 7), the incidence of impaired righting ability was again not significantly different ($p < 0.05$) from any of the control groups. Fenitrothion and mercury caused significant ($p < 0.10$ and $p < 0.05$, respectively) effects on balancing ability, while those resulting from dieldrin treatment were not significantly different ($p < 0.10$) from controls, due to the high variation among dieldrin-treated groups.

There was no altered feather development observed in chicks treated with fenitrothion-mercury mixtures (Table 8). Binary mixtures containing dieldrin caused incidences of both feather discoloration and limb and digit distortions, with levels ranging from 16.7-25.0 percent of embryos affected. Although all binary mixtures caused relatively low incidences of impaired righting ability (8.4-25.0% affected), none of these mixtures exerted any apparent effects upon balance. In contrast, although the tertiary mixtures did not affect feather development, they caused the highest incidences of neuromotor dysfunction and limb and digit distortions.

Growth Rate

No significant effects on growth were observed

Table 8. Incidence (%) of post-hatch effects in chicks hatched from eggs treated with a single dose of a binary or tertiary mixture of toxicants, according to the yolk sac injection procedure.

	% Affected			
			Neuromotor Dysfunction	
	Feather Discoloration	Limb & Digit Distortions	Impaired Righting	Impaired Balance
Controls				
(all groups)	8.0 ± 0	11.5 ± 0	0	0
DH (all doses)	21.5	25.0	12.5	0
DF (all doses)	16.7	25.0	8.4	0
FH (all doses)	0	25.0	25.0	0
DFH (all doses)	0	50.0	50.0	50.0

Note: Values for control groups represent mean ± standard error. Mean values could not be determined for any of the binary or tertiary mixture groups, since only one experiment involving multiple toxicant mixtures lasted 21 days (hatching day); furthermore, sample sizes in all toxicant-treated groups were < 10.

in hatched chicks from any of the toxicant-treated groups. Body weights and crown-rump measurements of a subsample of treated chicks were compared with those of controls, and no significant ($p < 0.05$) differences could be demonstrated, using the Student's t-test for grouped data (Tallerida & Murray, 1981).

Hatching Success

Hatching success appeared to be greatly reduced in certain treated groups, particularly mercury (Table 9). Data was insufficient to statistically compare hatching times between control and toxicant-treated groups; hence, there was no basis for evaluation of this variable.

Chemical Analysis

Chemical analysis of liver tissue samples from hatched chicks and from embryos which died just prior to hatching (days 18-21, stages 43-45) did not reveal any detectable levels of fenitrothion. Levels of dieldrin and mercury were generally less than 1 ppm. in all samples, suggesting that no appreciable accumulation, at least in liver tissues, occurred.

Skeletal Analysis

Skeletons were also examined for skeletal defects;

Table 9. Hatching success (%) of controls and of eggs treated with a single dose of dieldrin, fenitrothion, or mercury (as $HgCl_2$), according to the air space or yolk sac injection procedure.

	Percentage of eggs hatched	
	Air Space injection	Yolk Sac injection
Controls:		
Untreated	77.8	76.3
P. Glycol	66.7	44.4
Dieldrin (ug./egg)		
10	47.1	58.3
20	-	58.3
22.4	-	29.4
50	-	31.3
62.4	-	16.7
Fenitrothion		
40	81.2	66.7
80	-	30.8
83.5	-	37.5
107.5	-	41.2
160	-	17.6
Mercury ($HgCl_2$)		
20	77.8	22.2
27	-	37.5
40	58.3	15.4
80	-	7.7
160	-	0

the only apparent deformities were twisted spines, wry (twisted) neck, and curved and/or crooked toes (limb and digit distortions). The first two anomalies were seen in the occasional chick in all treated groups in yolk sac injection trials, but no significant differences ($p < 0.05$) between toxicant-treated and control groups with respect to percent affected was shown. The incidence of crooked feet and toes (limb and digit distortions) is given in Tables 7-8 for each treatment group. However, since no dose-response relationship could be demonstrated, the dosage groups for each type of treatment (eg. dieldrin-treated embryos) were grouped together for comparison with controls.

Five-day Incubation Studies: Quantitative and Quantal Approaches

Embryotoxic versus Teratogenic Response

At five days of incubation, terata were observed in certain controls and in all experimental lots (Table 6). However, the numbers affected per lot varied considerably depending on the treatment. An attempt was made to distinguish between those agents whose toxicity is selectively rather than coincidentally manifested in terata. The independant action formulation as described in "Materials and Methods" (p. 38) and identified by the acronym T.A. was used for this purpose. This formulation adjusts the percentage of observed teratogenic response in each test lot by removing from the total numbers per lot (1) that proportion assumed to have died from either embryotoxic or natural effects, and (2) that proportion assumed to be equivalent to the incidence of naturally-arising terata. Tables 10-12 compare the combined lethal and teratogenic responses observed for each toxicant with the selectively teratogenic response predicted by the T.A. formula. Although there was no correlation between total response observed for dieldrin and dieldrin's dose, the magnitude of teratogenic response accredited by the T.A. formula appeared to increase with increasing dose (Fig. 10).

Table 10. T.A. formula data for dieldrin (HEOD); 0.99 confidence limits + heterogeneity factor.

Log Dose	Sample Size (N)	Observed Response (probits)	Predicted Response (probits)	Fiducial Limits Lower Limit	Fiducial Limits Upper Limit
1.0	18	--*	2.3369	-3.5623	8.2361
1.079	14	4.3255	2.5829	-2.6144	7.7802
1.079	17	4.0458	2.5829	-2.6144	7.7802
1.301	20	--*	3.2720	-0.0687	6.6127
1.322	16	4.0846	3.3378	0.1587	6.5169
1.350	20	5.3585	3.4249	0.4523	6.3975
1.602	15	4.3255	4.2071	2.2778	6.1364
1.653	15	4.1584	4.3660	2.3489	6.3831
1.699	19	4.2278	4.5082	2.3230	6.6934
1.795	21	4.8996	4.8071	2.0719	7.5423

$x^2 = 144.0$
correlation coefficient = 0.46
regression line : $Y = 3.106x - 0.769$
degrees of freedom = 8

*Note: Dashed lines represent % responses of 0, for which there is no probit value.

Table 11. T.A. formula data for fenitrothion ; 0.99 confidence limits + heterogeneity factor.

Log Dose	Sample Size (N)	Observed Response (probits)	Predicted Response (probits)	Fiducial Limits Lower Limit	Upper Limit
1.602	21	3.4452	3.6359	2.6029	4.6689
1.653	14	3.7735	3.7735	2.8519	4.6951
1.903	19	4.4466	4.4460	3.9773	4.9147
1.922	20	4.7207	4.4961	4.0471	4.9451
1.949	15	4.4756	4.5706	4.1439	4.9973
2.031	16	5.2533	4.7903	4.3707	5.2099
2.031	22	4.8996	4.7903	4.3707	5.2099
2.097	14	4.7467	4.9676	4.4909	5.4443
2.204	16	4.6681	5.2562	4.6101	5.9023
2.204	22	5.5534	5.2562	4.6101	5.9023

x^2 = 11.9
degrees of freedom = 8
correlation coefficient = 0.88
regression line : Y = 2.691x - 0.676

Table 12. T.A. formula data for mercury (as $HgCl_2$) ; 0.99 confidence limits + heterogeneity factor.

Log Dose	Sample Size (N)	Observed Response (probits)	Predicted Response (probits)	Fiducial Limits Lower Limit	Upper Limit
1.204	14	4.2612	4.8170	3.2624	6.3716
1.265	20	4.3255	4.5429	3.3530	5.7328
1.301	20	5.0000	4.3794	3.3248	5.4340
1.342	17	4.6415	4.1925	3.1756	5.2094
1.431	19	5.1004	3.7909	2.4359	5.1459
1.491	15	-- *	3.5200	1.7523	5.2877
1.602	18	3.9197	3.0201	0.3583	5.6817
1.778	17	5.2019	2.2250	-1.9712	6.4212
1.903	14	-- *	1.6608	-3.6517	6.9733
2.204	18	-- *	0.3015	-7.7314	8.3344

x^2 = 39.3
degrees of freedom = 8
correlation coefficient = -0.64
regression line : Y = -4.516x + 10.254

*Note: Dashed lines represent % responses of 0, for which there is no probit value.

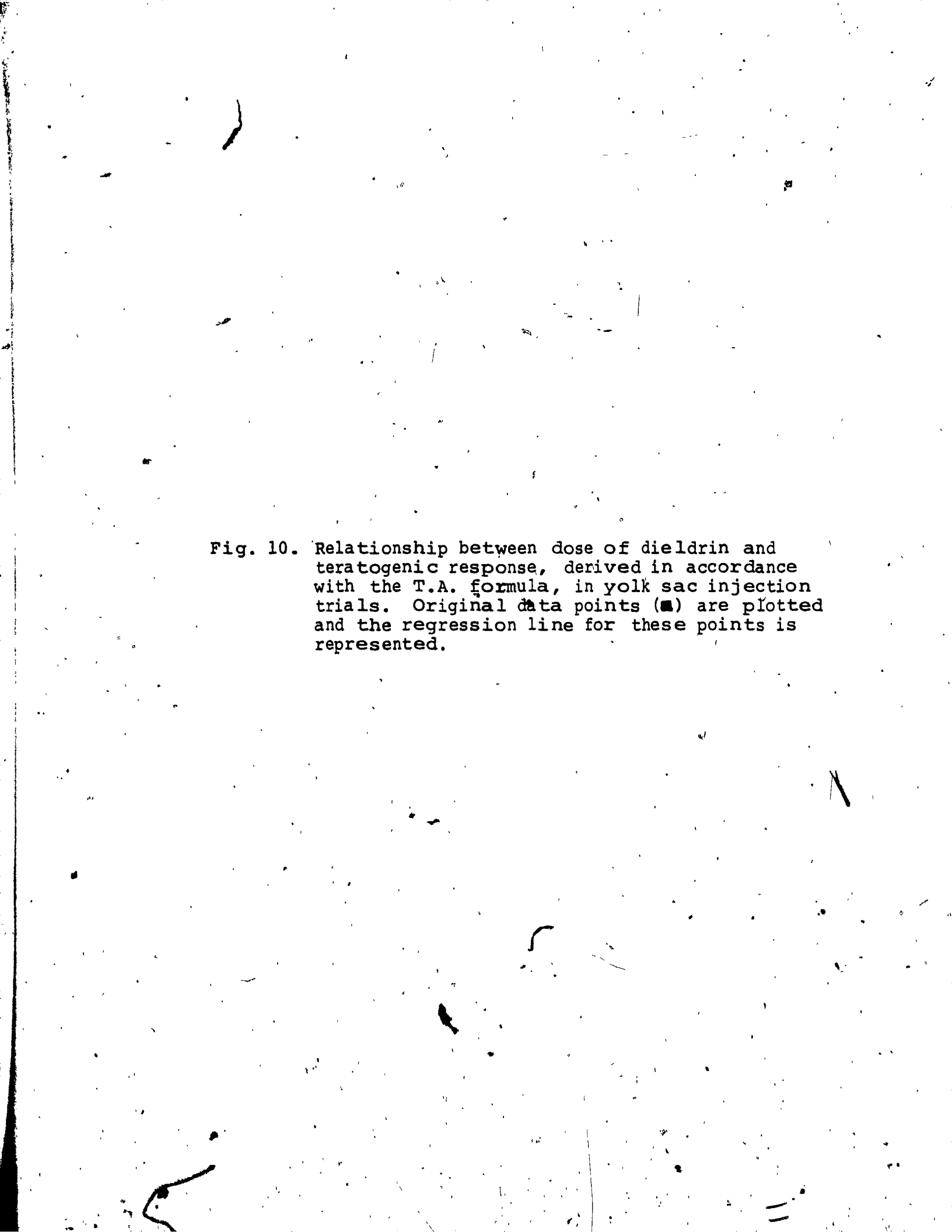
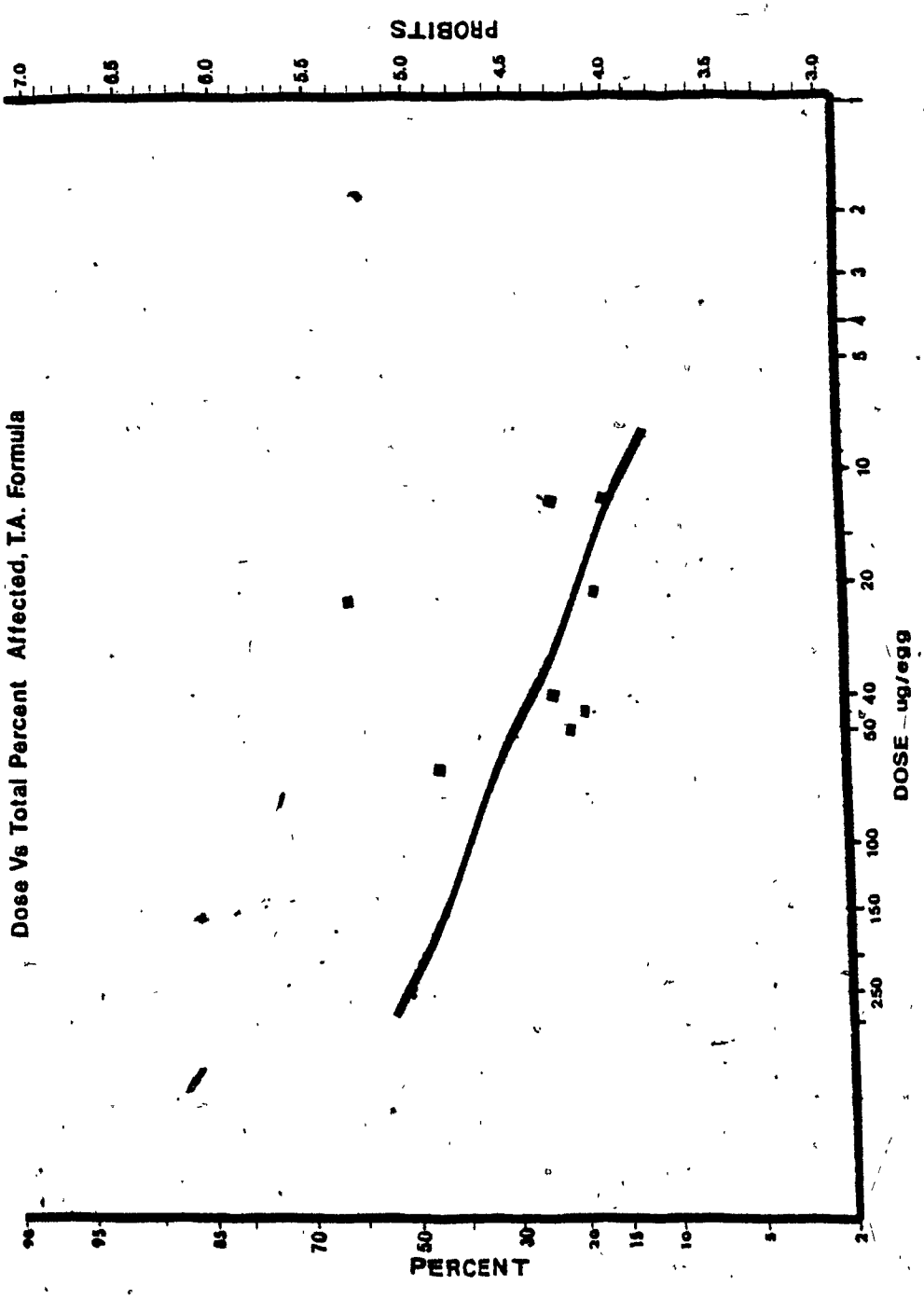


Fig. 10. Relationship between dose of dieldrin and teratogenic response, derived in accordance with the T.A. formula, in yolk sac injection trials. Original data points (■) are plotted and the regression line for these points is represented.


Dose Vs Total Percent Affected, T.A. Formula
PROBITS
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
DOSE - ug/egg
2
3
5
10
20
40
60
100
150
250
PERCENT
99
95
90
70
50
30
20
15
10
5
2

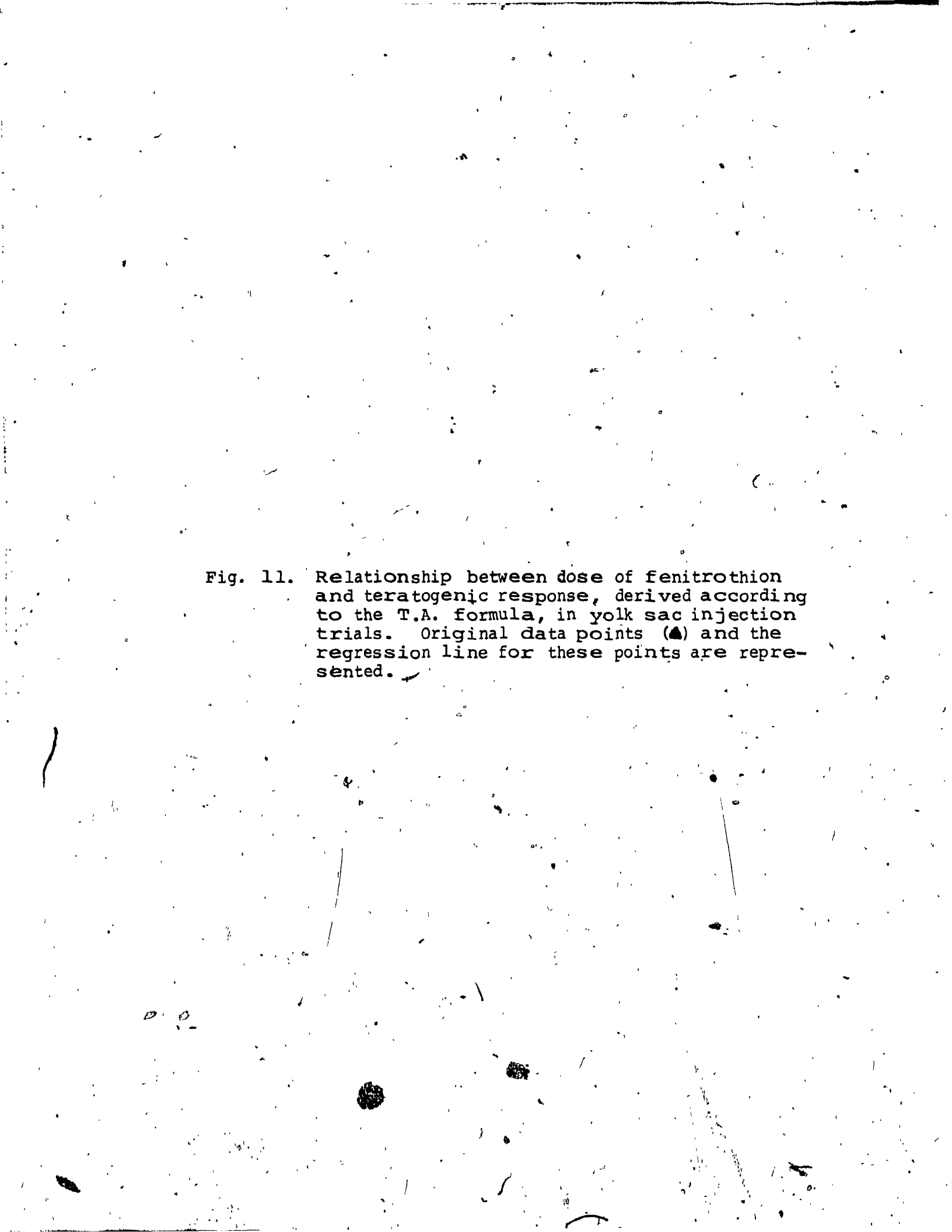


Fig. 11. Relationship between dose of fenitrothion and teratogenic response, derived according to the T.A. formula, in yolk sac injection trials. Original data points (▲) and the regression line for these points are represented.

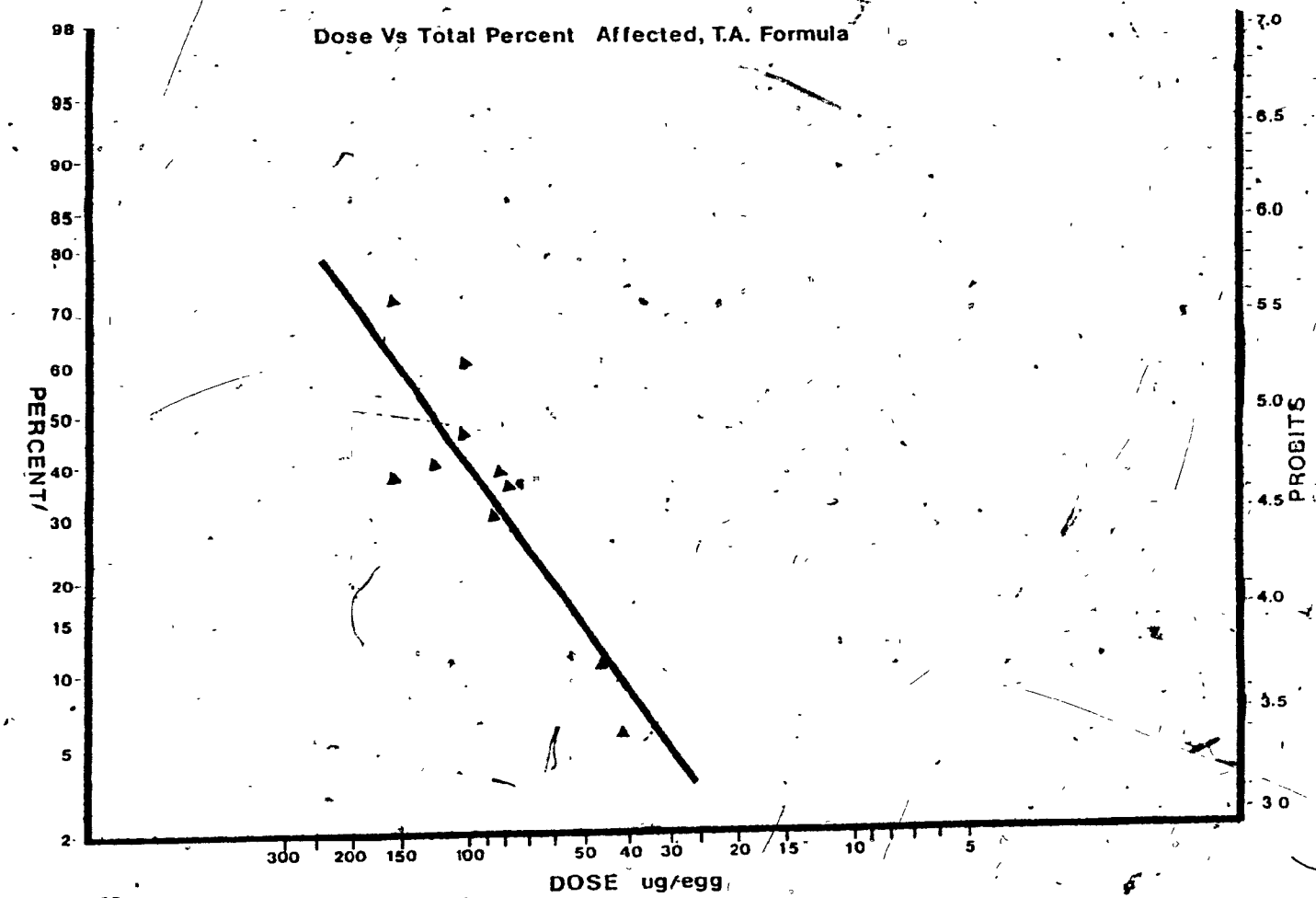
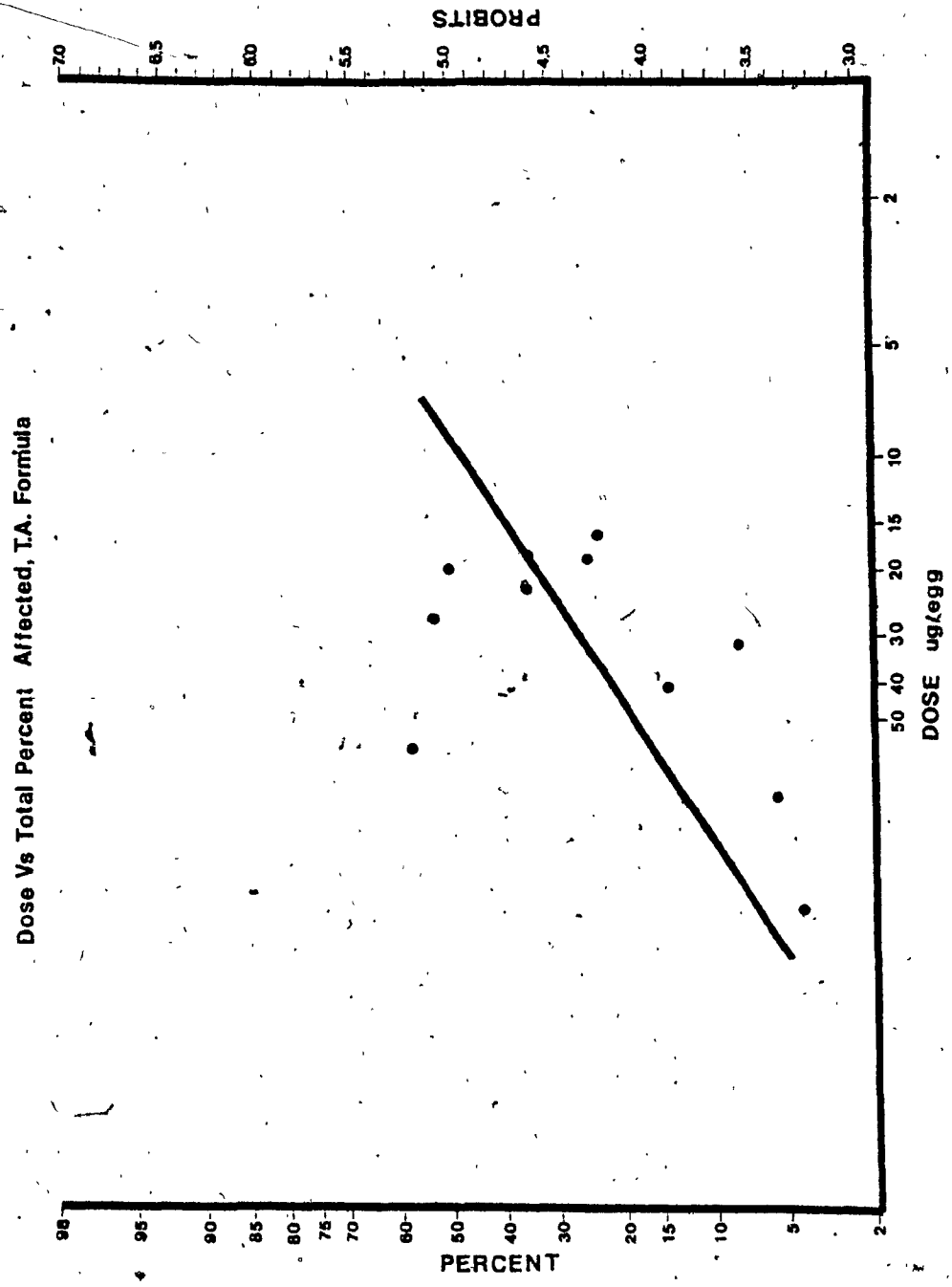

Dose Vs Total Percent Affected, T.A. Formula
PERCENT
98
95
90
85
80
70
60
50
40
30
20
15
10
5
2
PROBITS
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
300
200
150
100
50
40
30
20
15
10
5
DOSE ug/egg

Fig. 12. Relationship between dose of mercury ($HgCl_2$) and teratogenic response, derived in accordance with the T.A. formula, in yolk sac injection trials. Observed data points (●) and the regression line for these points are represented.


Dose Vs Total Percent Affected, T.A. Formula
PROBITS
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
PERCENT
99
95
90
85
80
75
70
60
50
40
30
20
15
10
5
2
DOSE ug/egg
2
5
10
15
20
30
40
50

There was little difference observed between the total response data in fenitrothion-treated test lots and the teratogenic response predicted by the T.A. formula. The similarity between predicted and observed responses was confirmed by a chi-square test (Statpak, Concordia University). Fig. 11 illustrates the high correlation of accredited teratogenic response to the dose of fenitrothion.

Although there is no apparent direct correlation between total response and dose of mercury, there would appear to be an inverse relationship between dose and mercury's accredited teratogenic effects (Table 12). Based on the assumption of the T.A. formula then, the incidence of terata in mercury-treated lots decreases with increasing dose, as illustrated in Fig. 12.

Teratogenic Index

The T.A. formula assumes that the incidence of mortality in experimental lots is due either to natural or embryotoxic events, and that the nature of the various malformations observed have equal weight in defining the teratogenicity of a toxicant. An approach called the teratogenic index (TI) was devised to evaluate the teratogenic potential of toxicants on the assumption that certain malformations are more profound than others, and that death is the consequence of severe teratogenic effects. Mortality and terata are weighted in accordance with teratogenic criteria as

defined in "Materials and Methods" (p. 42). The results of this approach are listed in Table 13, and include a consideration in which death is not considered a profound consequence of teratogenesis, and is accordingly deleted from the computations. Above the apparent threshold level of 12 µg./egg, there does not appear to be any further increase in the severity of the teratogenic effects of dieldrin with dose until a concentration of 62.4 µg./egg is reached. If death is removed as a factor in the TI formula, the threshold, which is defined by a significant ($p < 0.05$) increase in the severity of teratogenic effects above controls, occurs at a dose of 62.4 µg./egg.

If death is considered in a TI evaluation of fenitrothion, than the apparent threshold to forms of terata more severe than controls is 45 µg./egg. There is no evidence of a correlation between increased severity and dose beyond this "threshold" level. If death is not considered in the TI formula, then the threshold to severe teratogenic effects is apparently 80 µg./egg and again there is no change in severity with an increase in dose.

Unlike dieldrin or fenitrothion, the severity of teratogenic response is a reasonably linear function of the dose of mercury when death is indexed. Even at the lowest dose of 16 µg./egg) of mercury, the TI is considerably greater than that of controls. When death

Table 13. Teratogenic Index Data, with and without early mortality (within first 24 hours), for eggs treated according to the yolk-sac injection method.

Dose (ug/egg)	#Tested	Score (incl. death)	T.I.	Score (excl. death)	T.I.
Controls					
Untreated	92	33	0.36	9	0.10
Punc. only	32	8	0.25	0	0
Saline	41	28	0.68	12	0.29
Prop. glycol	74	44	0.59	12	0.16
Dieldrin					
10	18	8	0.44	0	0
12	14	26	1.86	10	0.71
12	17	42	2.47	10	0.59
20	20	24	1.20	0	0
21	16	37	2.32	5	0.31
22.4	20	72	3.60	32	1.60
40	15	29	1.93	13	0.87
45	15	34	2.27	2	0.13
50	19	45	2.37	5	0.26
62.4	21	100	4.76	36	1.71
Fenitrothion					
40	21	12	0.57	4	0.19
45	14	33	2.36	1	0.07
80	19	80	5.71	26	1.37
83.5	20	39	1.95	31	1.55
89	15	47	3.13	15	1.0
107.5	22	61	2.77	45	2.05
107.5	16	38	2.38	30	1.88
125	14	36	2.57	12	0.86
160	22	92	4.18	28	1.27
160	16	88	5.50	24	1.50
Mercury					
16	14	15	1.07	15	1.07
18.5	20	17	0.85	17	0.85
20	20	54	2.70	30	1.50
22	17	37	2.18	21	1.24
27	19	84	4.42	60	3.16
31	15	24	1.60	0	0
40	18	76	4.22	4	0.22
40	18	76	4.22	44	2.14
60	17	61	3.59	29	1.71
80	14	80	5.71	0	0
160	18	144	8.00	0	0

is removed from the index, an inverse relationship is suggested between the severity of response and the dose of mercury. This dose-response relationship for mercury corresponds to the pattern derived in accordance with the T.A. formula.

Quantal Response

Dose-response relationships were evident in yolk sac injected test lots at five days of incubation. The combined lethal and teratogenic effects were used as end-point response criteria for each toxicant. These data are compiled in Table 14. Response data were subjected to probit analysis, and linear functions for each of the three toxicants were computed (Figs. 13-15). The respective regression equations, correlation coefficients, and fiducial limits are presented in Tables 15-17.

Relative Potency Determinations

Quantal response data for each of the three toxicants were constrained to parallelism, in accordance with Finney's (1971) method, and using a computer program (Tallarida & Murray, 1981) (Table 18, Fig. 16). The median effective dose (ED_{50}) was computed from these common slope lines for each toxicant. The ED_{50} was 43, 55, and 151 µg./egg for dieldrin, mercury, and fenitrothion, respectively.

Table 14. Corrected values for combined day 5 % mortality + % affected with minor terata for controls and for eggs treated with single dose of either dieldrin, fenitrothion, or mercury, according to the yolk sac injection technique.

Dose (ug. inj.)	#Dead	#w/Terata	Total Aff.	#Tested	%Affected
untreated	6	0	6	92	6.5
punc. only	1	0	1	32	3.2
saline	8	1	9	41	21.9
prop. glycol	13	3	16	74	21.6
Dieldrin					
12	3	2	5	14	35.7
12	4	1	5	15	29.4
20	4	2	6	20	30.0
21	5	2	7	16	43.8
22.4	5	6	11	20	55.0
40	1	3	4	15	26.7
45	4	1	5	15	33.3
50	9	2	11	19	57.9
62.4	7	7	14	21	66.7
Fenitrothion					
40	0	2	2	21	9.5
45	2	2	4	14	28.6
80	0	6	6	19	31.6
83.5	3	5	8	20	40.0
89	5	2	7	15	46.7
107.5	2	3	5	16	31.3
107.5	6	6	12	22	54.5
125	2	3	5	14	35.7
160	8	5	13	16	81.3
160	13	6	19	22	86.4

Table 14 -- Continued

Dose (ug. inj.)	#Dead	#w/Terata	Total Aff.	#Tested	%Affected
Mercury					
16	0	1	1	14	7.1
18.4	0	4	4	20	20.0
20	4	2	6	20	30.0
22	2	3	5	17	29.4
27	3	7	10	19	52.6
31	4	0	4	15	26.7
40	6	8	14	18	77.8
40	10	1	11	18	61.1
60	4	6	10	17	58.8
80	11	1	12	14	85.7
160	17	1	18	18	100.0

Table 15. Dosé versus total percent affected data (Day 5) for dieldrin; 0.99 confidence limits + heterogeneity factor.

Log Dose	Sample Size (N)	Observed Response (probits)	Predicted Response (probits)	Fiducial Limits Lower limit	Upper limit
1.000	18	4.2278	3.9507	2.9386	4.9628
1.079	14	3.7184	4.0145	3.1321	4.8969
1.079	17	4.0846	4.0145	3.1321	4.8969
1.301	20	3.7735	4.1932	3.5957	4.7907
1.322	16	4.4172	4.2103	3.6295	4.7911
1.350	20	4.8236	4.2329	3.6701	4.7957
1.602	15	3.5242	4.4358	3.7929	5.0787
1.653	15	3.9636	4.4771	3.7737	5.1805
1.699	19	4.8996	4.5139	3.7487	5.2791
1.795	21	5.2019	4.5915	3.6802	5.5028

x^2 = 18.73
degrees of freedom = 8
correlation coefficient = 0.410
regression line : Y = 0.8059x + 3.1448

Table 16. Dose versus total percent affected data (Day 5) for fenitrothion; 0.99 confidence limits + heterogeneity factor.

Log dose	Sample size (N)	Observed Response (probits)	Predicted response (probits)	Fiducial Limits Lower limit	Fiducial Limits Upper limit
1.602	21	2.6737	2.8907	1.2809	4.5005
1.653	14	3.6592	3.0980	1.6598	4.5362
1.903	19	3.8736	4.1108	3.4263	4.7953
1.922	20	4.2937	4.1862	3.5436	4.8288
1.949	15	4.5323	4.2984	3.7100	4.8868
2.031	22	4.7981	4.6292	4.1161	5.1423
2.031	16	3.8250	4.6292	4.1161	5.1463
2.097	14	4.0846	4.8963	4.3309	5.4617
2.204	16	5.9542	5.3309	4.5264	6.1354
2.204	22	5.7063	5.3309	4.5264	6.1354

$X^2 = 16.15$
degrees of freedom = 8
correlation coefficient = 0.851
regression line : $Y = 4.053x - 3.6024$

Table 17. Dose versus total percent affected data (Day 5) for mercury (as $HgCl_2$) ; 0.99 confidence limits + heterogeneity factor.

Log Dose	Sample size (N)	Observed Response (probits)	Predicted Response (probits)	Fiducial Limits Lower limit	Fiducial Limits Upper limit
1.204	14	2.6737	3.0301	1.9402	4.1200
1.265	20	2.9463	3.2739	2.3055	4.2423
1.301	20	3.7735	3.4193	2.5194	4.3192
1.342	17	3.7184	3.5855	2.7591	4.4119
1.430	19	4.7467	3.9363	3.2410	4.6316
1.491	15	3.5242	4.1837	3.5528	4.8146
1.602	18	5.0000	4.6284	4.0283	5.2285
1.778	17	4.9247	5.3356	4.5576	6.1136
1.903	14	5.9154	5.8374	4.8340	6.8408
2.204	18	7.0537	7.0464	5.3881	8.7047

x^2 = 15.57
degrees of freedom = 8
correlation coefficient = 0.945
regression line : Y = 4.0164x - 1.8061

Table 18. Common slope linear functions for each toxicant, derived from individual regression lines of dose versus total % affected; lines were constrained to parallelism, using an Apple computer parallel lines program.

Regression lines for single agents (dose versus total % affected) :

Mercury ($HgCl_2$) : $Y = 4.0164x - 1.8061$ (#1)

Fenitrothion : $Y = 4.053x - 3.6024$ (#2)

Dieldrin (HEOD) : $Y = 0.8059x - 3.1448$ (#3)

Line #1 vs. #2 : degrees of freedom = 16
t-value (0.95) = 3.3
t-calculated = 0.0352

Conclusion: lines are not significantly unparallel

Line #2 vs. #3 : degrees of freedom = 16
t-value (0.95) = 3.3
t-calculated = 2.9821

Conclusion: lines are not significantly unparallel

Common slope lines

Mercury ($HgCl_2$) : $Y = 3.0165 (x - 1.552) + 4.4277$

Fenitrothion : $Y = 3.0165 (x - 1.9596) + 4.3401$

Dieldrin (HEOD) : $Y = 3.0165 (x - 1.388) + 4.2634$

Relative potency factors were calculated according to the procedures outlined in "Materials and Methods" (p. 46), and are listed in Table 19. These factors are used in the method of Anderson & d'Appolonia (1978) to assess the concentration-addition model of multiple toxicity, as seen in the following section.

Multiple Toxicity Data

The observed responses recorded for binary and tertiary mixtures of dieldrin, fenitrothion, and mercury, were compared to the response range predicted by the concentration-addition model (Material & Methods, p. 47). Data and the computations for each of these studies are compiled in Table 20. In dieldrin-fenitrothion mixtures, the concentrations of dieldrin as a constituent were converted to fenitrothion equivalents using the appropriate relative potency factor (Table 19), and observed responses were filled to the dose-response line of fenitrothion. As seen in Fig. 17, coordinates representing the dose-response data for fenitrothion-dieldrin mixtures are horizontally distributed at about the 35% response level. This distribution would suggest that at intermediate doses, dieldrin and fenitrothion are additive, and at the lowest doses, supra-additive.

For mixtures in which mercury was a constituent, the amounts of the other agents were converted into

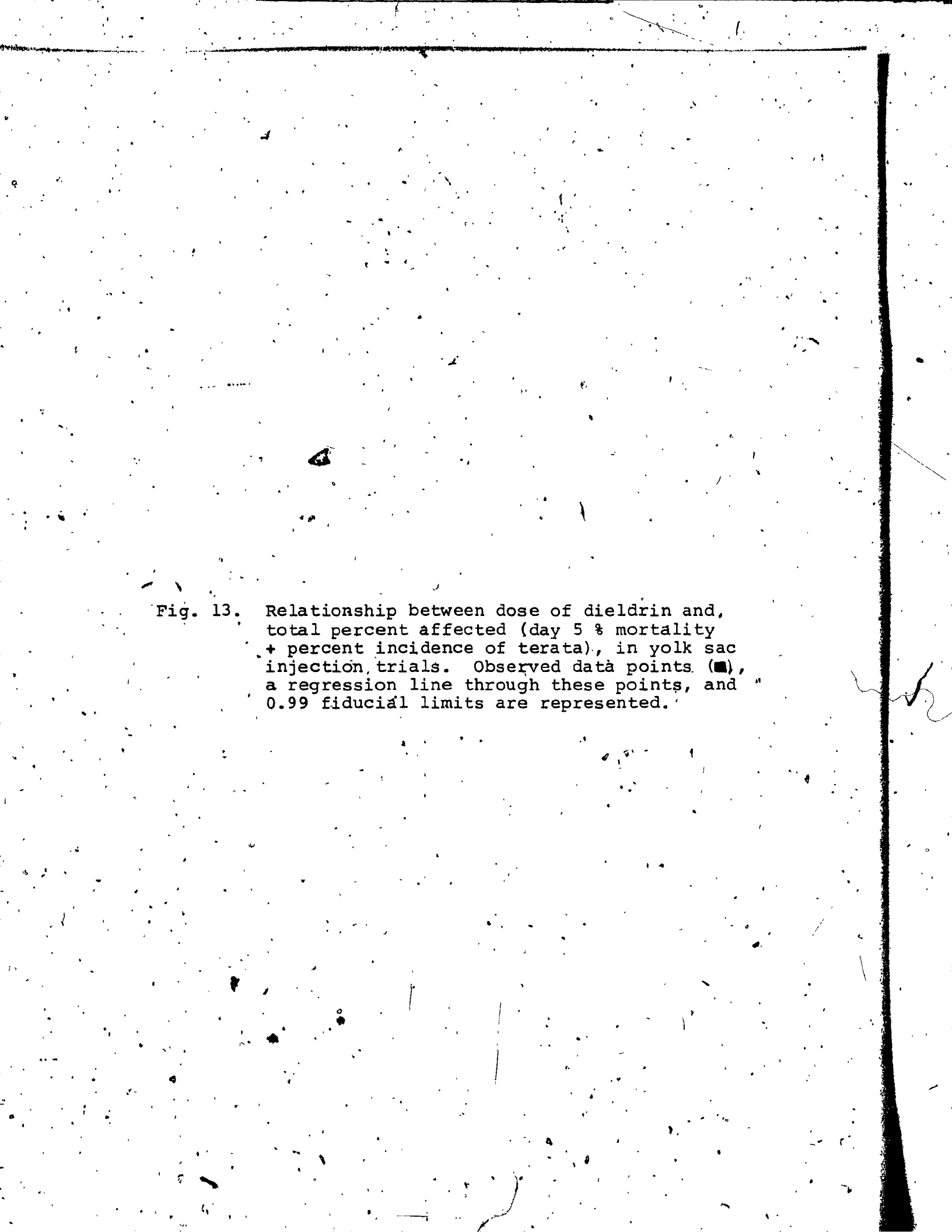
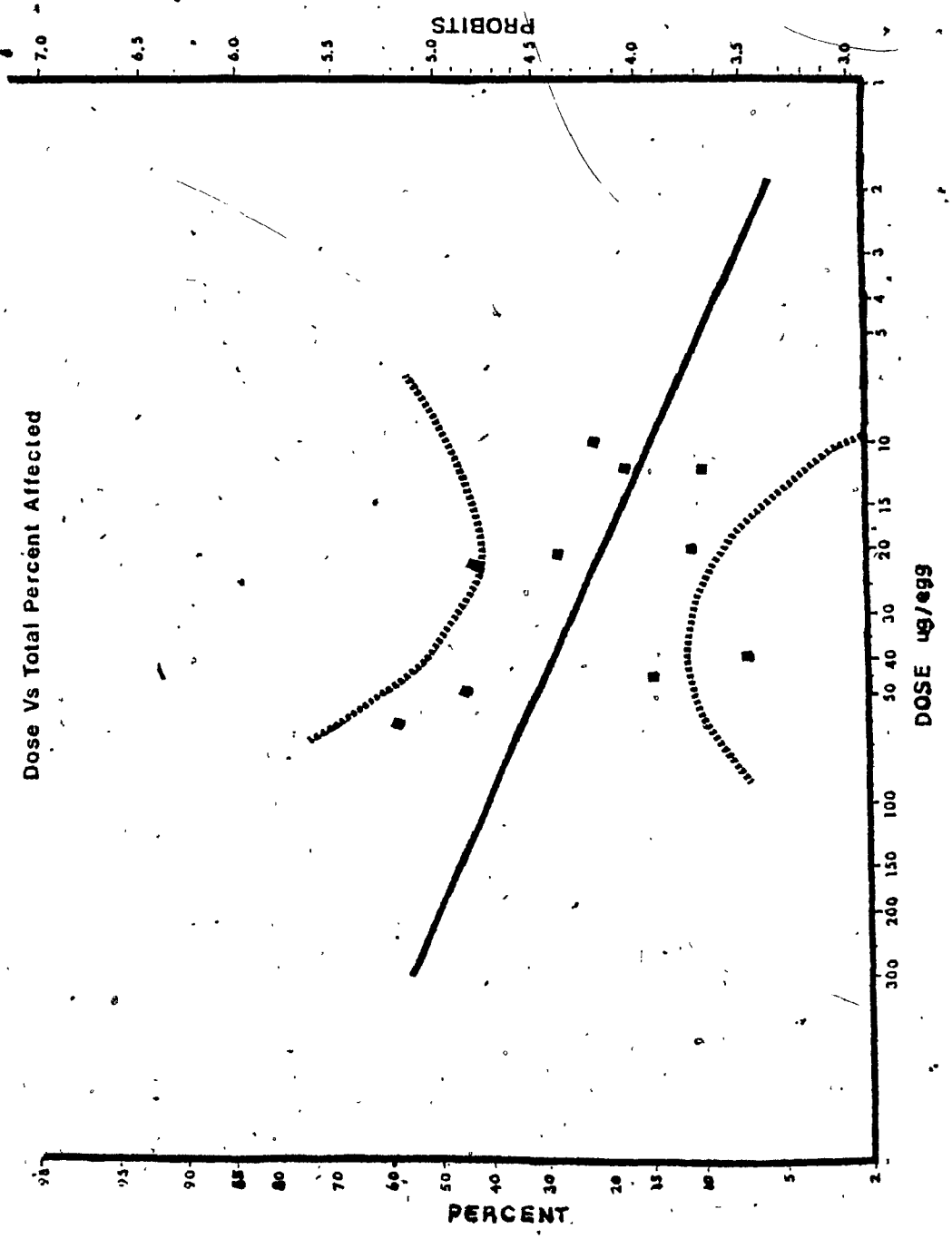


Fig. 13. Relationship between dose of dieldrin and, total percent affected (day 5 % mortality + percent incidence of terata), in yolk sac injection trials. Observed data points (■), a regression line through these points, and 0.99 fiducial limits are represented.


Dose Vs Total Percent Affected
PROBITS
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
DOSE ug/egg
1
2
3
4
5
10
15
20
30
40
50
100
150
200
300
PERCENT
90
80
70
60
50
40
30
20
10
5
2

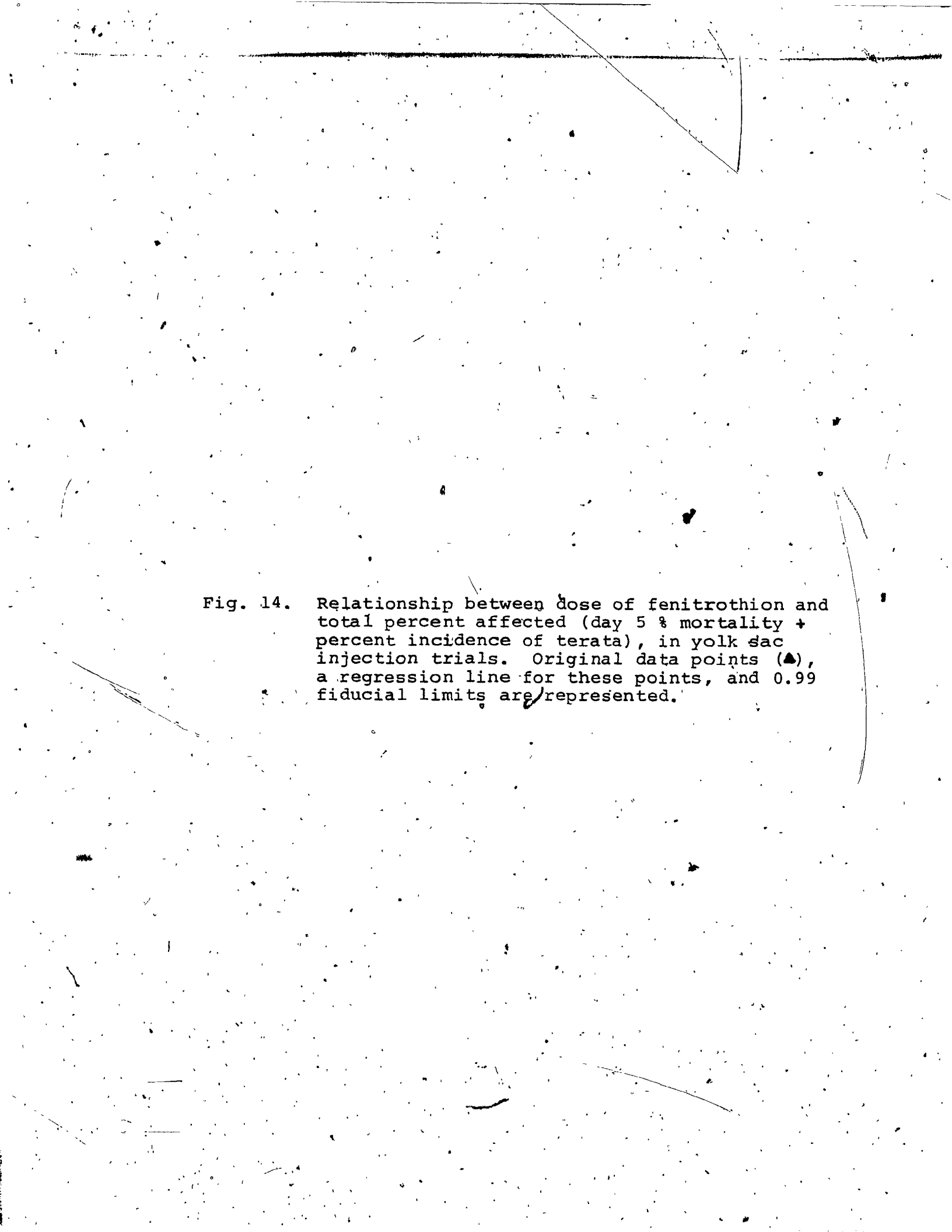
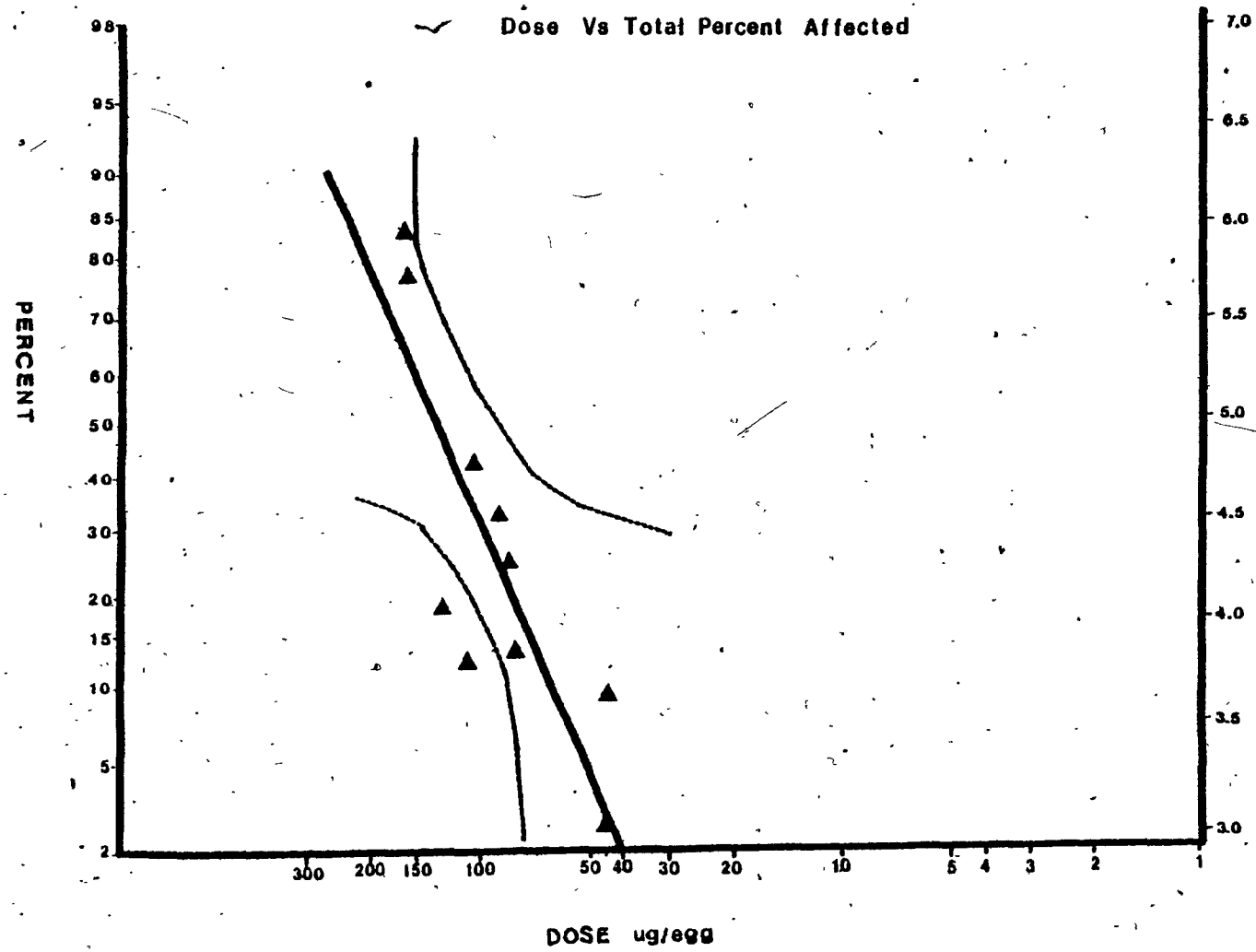


Fig. 14. Relationship between dose of fenitrothion and total percent affected (day 5 % mortality + percent incidence of terata), in yolk sac injection trials. Original data points (▲), a regression line for these points, and 0.99 fiducial limits are represented.


Dose Vs Total Percent Affected
PERCENT
98
95
90
85
80
70
60
50
40
30
20
15
10
5
2
PROBITS
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
300
200
150
100
50
40
30
20
10
5
4
3
2
1
DOSE ug/egg

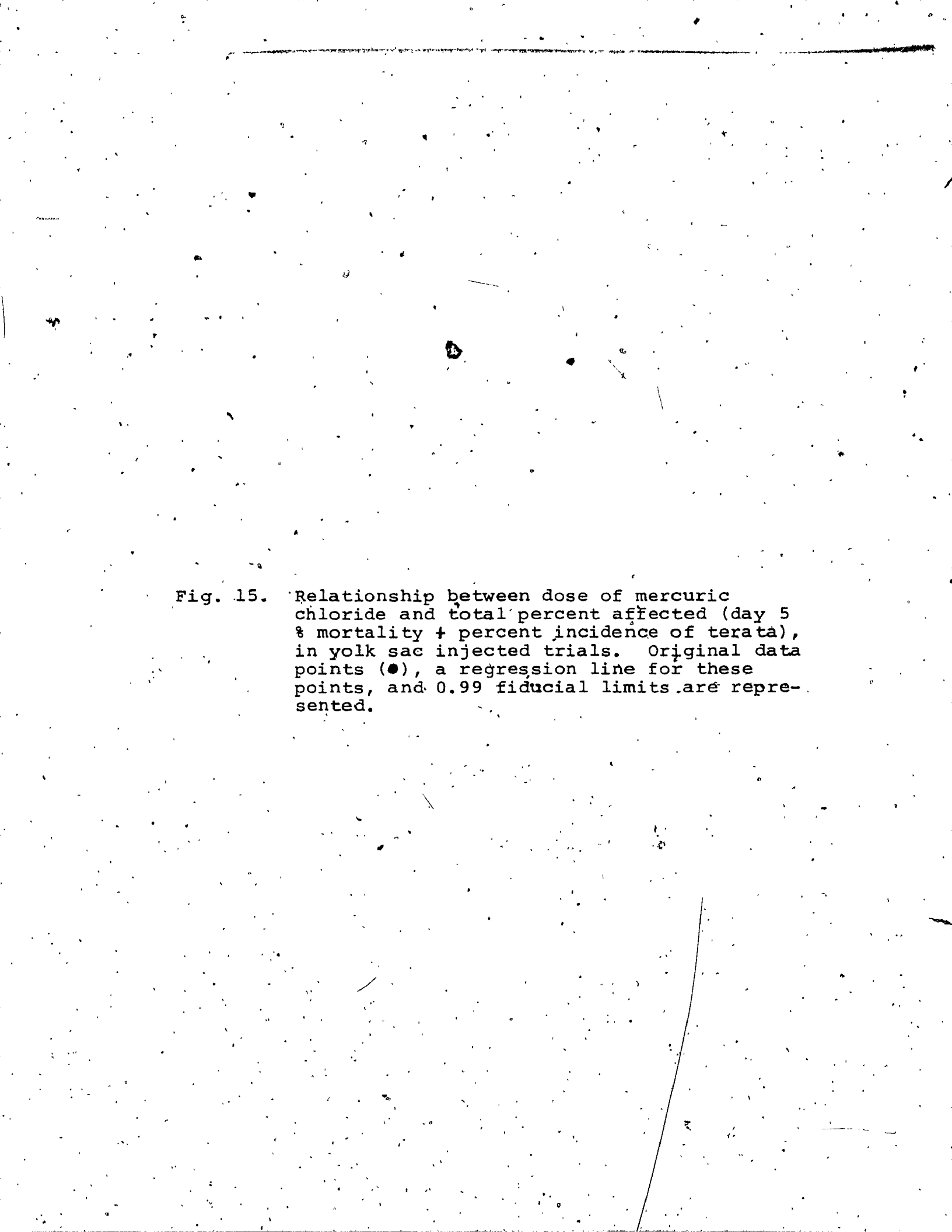
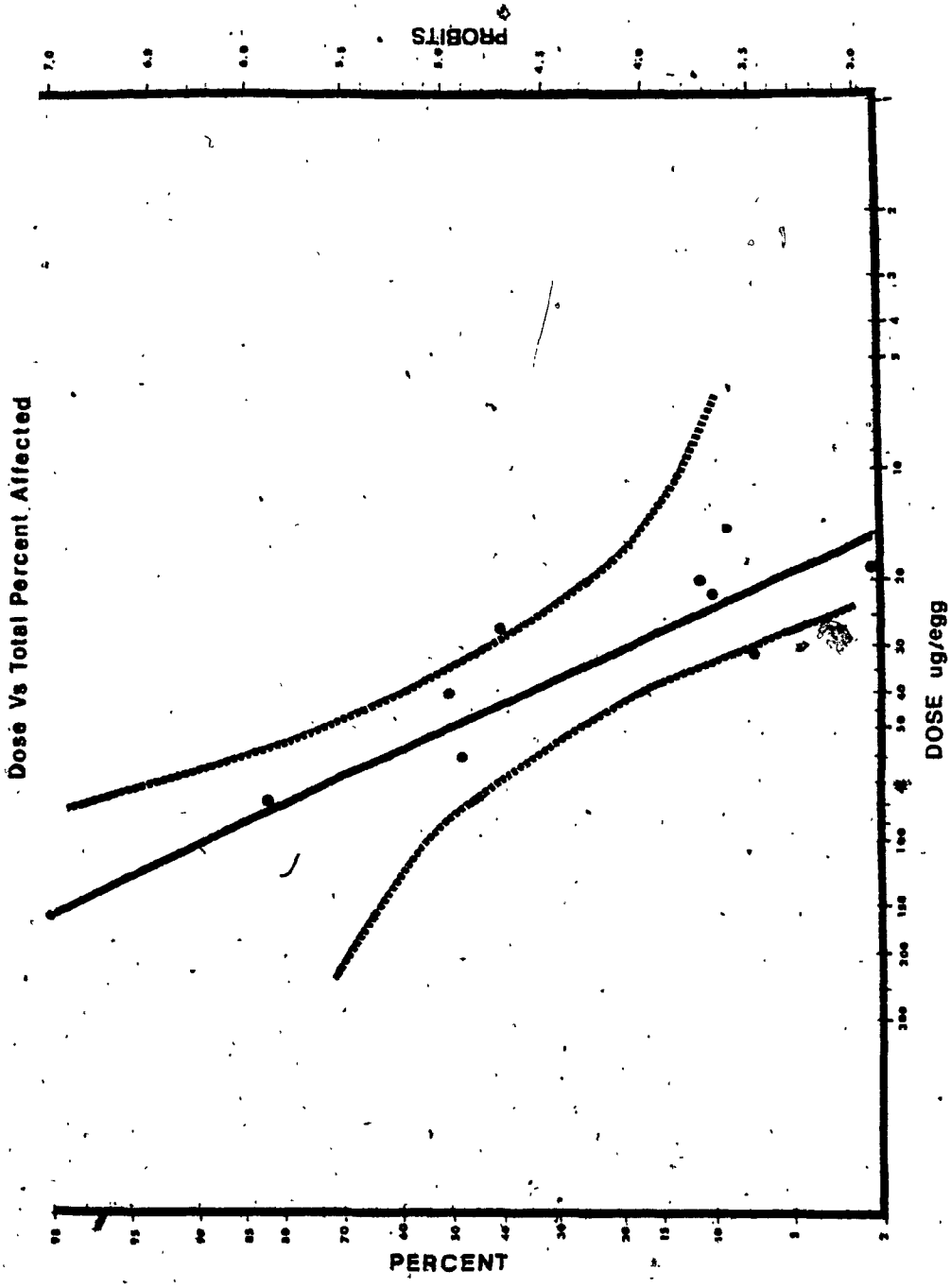


Fig. 15. Relationship between dose of mercuric chloride and total percent affected (day 5 % mortality + percent incidence of terata), in yolk sac injected trials. Original data points (●), a regression line for these points, and 0.99 fiducial limits are represented.


Dose Vs Total Percent Affected
PROBITS
PERCENT
DOSE ug/egg

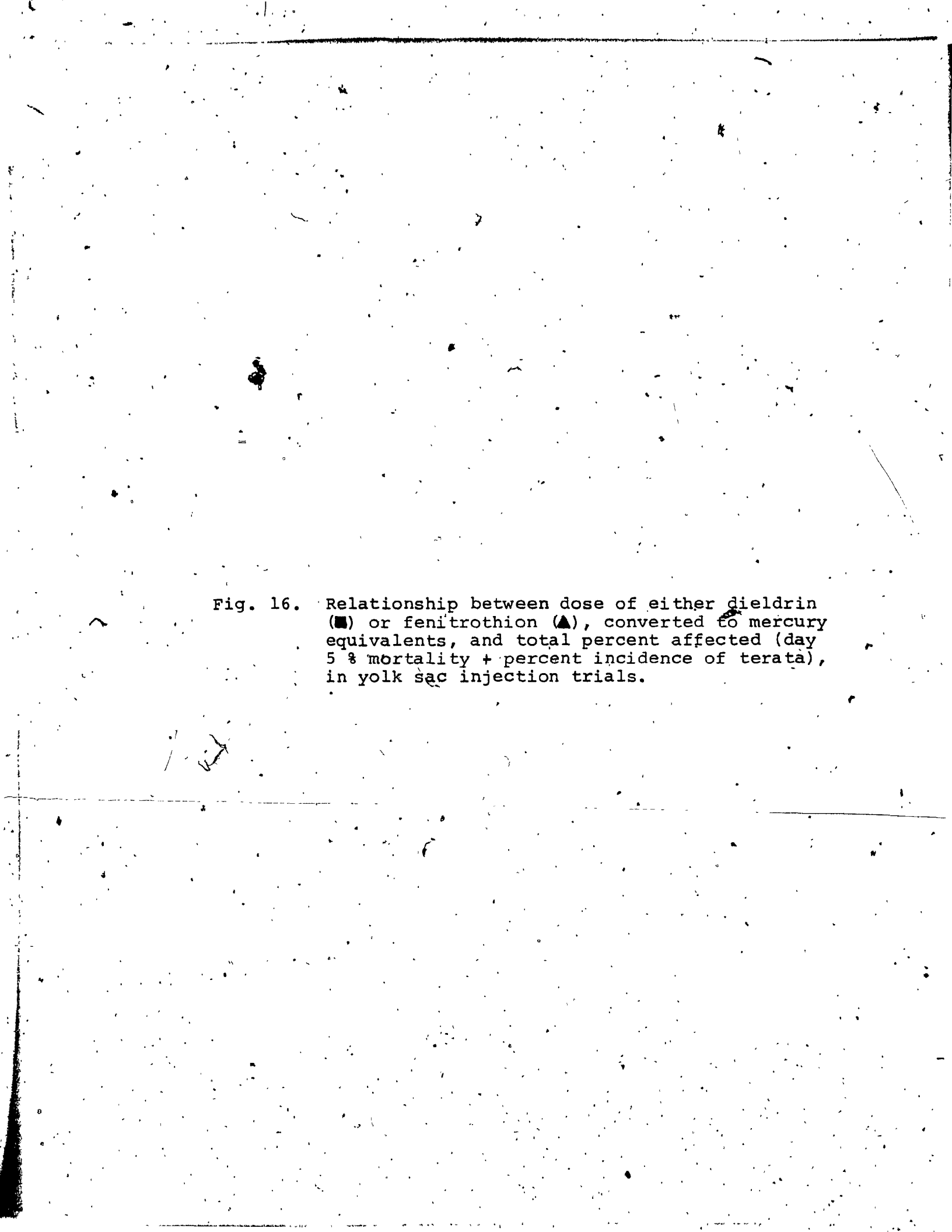
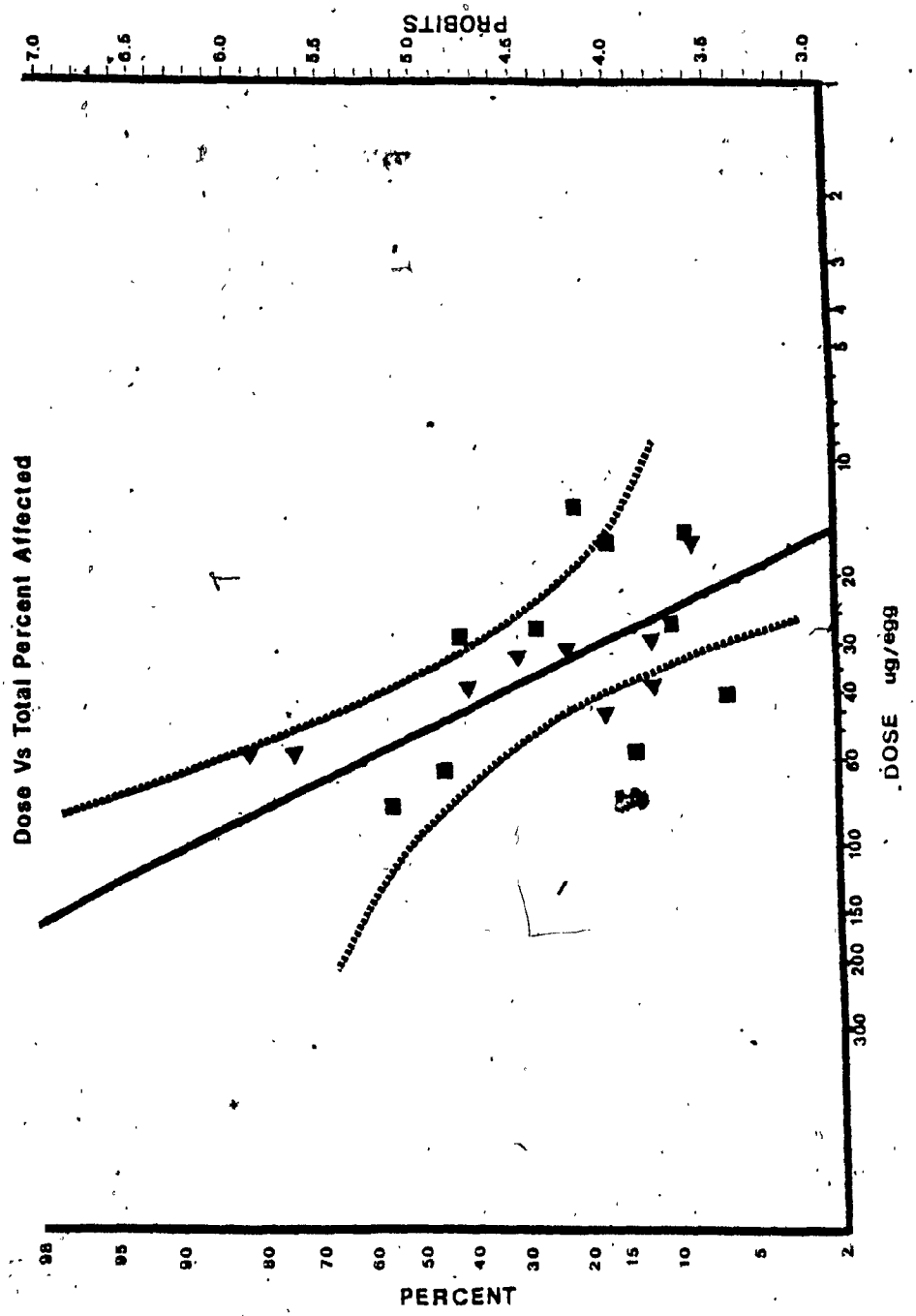


Fig. 16. Relationship between dose of either dieldrin (■) or fenitrothion (▲), converted to mercury equivalents, and total percent affected (day 5 % mortality + percent incidence of terata), in yolk sac injection trials.


Dose Vs Total Percent Affected
PROBITS
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
DOSE ug/egg
2
3
5
10
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30
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PERCENT
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Table 19. Relative potency factors (R.P.F.) derived from the median effective dose (ED_{50}) values of the three toxicants tested (dieldrin, fenitrothion, and mercuric chloride); all units are in micrograms per egg.

R.P.F. (Hg/Fen.)	=	0.364
R.P.F. (Hg./HEOD)	=	1.279
R.P.F. (Fen./HEOD)	=	3.515
R.P.F. (HEOD/Fen.)	=	0.285

Table 20. Predicted and observed percent response for binary and tertiary mixtures of toxicants according to the concentration addition model, using dose equivalent versus total percent affected data from yolk sac injection trials.

Binary Mixtures	Dose Equivalents (Hg)	Corrected Observed % Response	Predicted % Response
DH	6.9	30.0	0
	13.4	75.0	3.5
	28.0	81.8	19.0
FH	8.8	20.0	2.0
	13.9	58.3	4.0
	23.2	33.3	13.0
	(Fen)		
DF	22.8	36.4	0
	43.6	33.3	5.0
	88.5	30.8	24.0
Tertiary Mixtures	(Hg)		
DFH	14.6	50.0	4.5
	28.2	16.7	19.0
	43.2	91.7	37.0

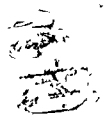
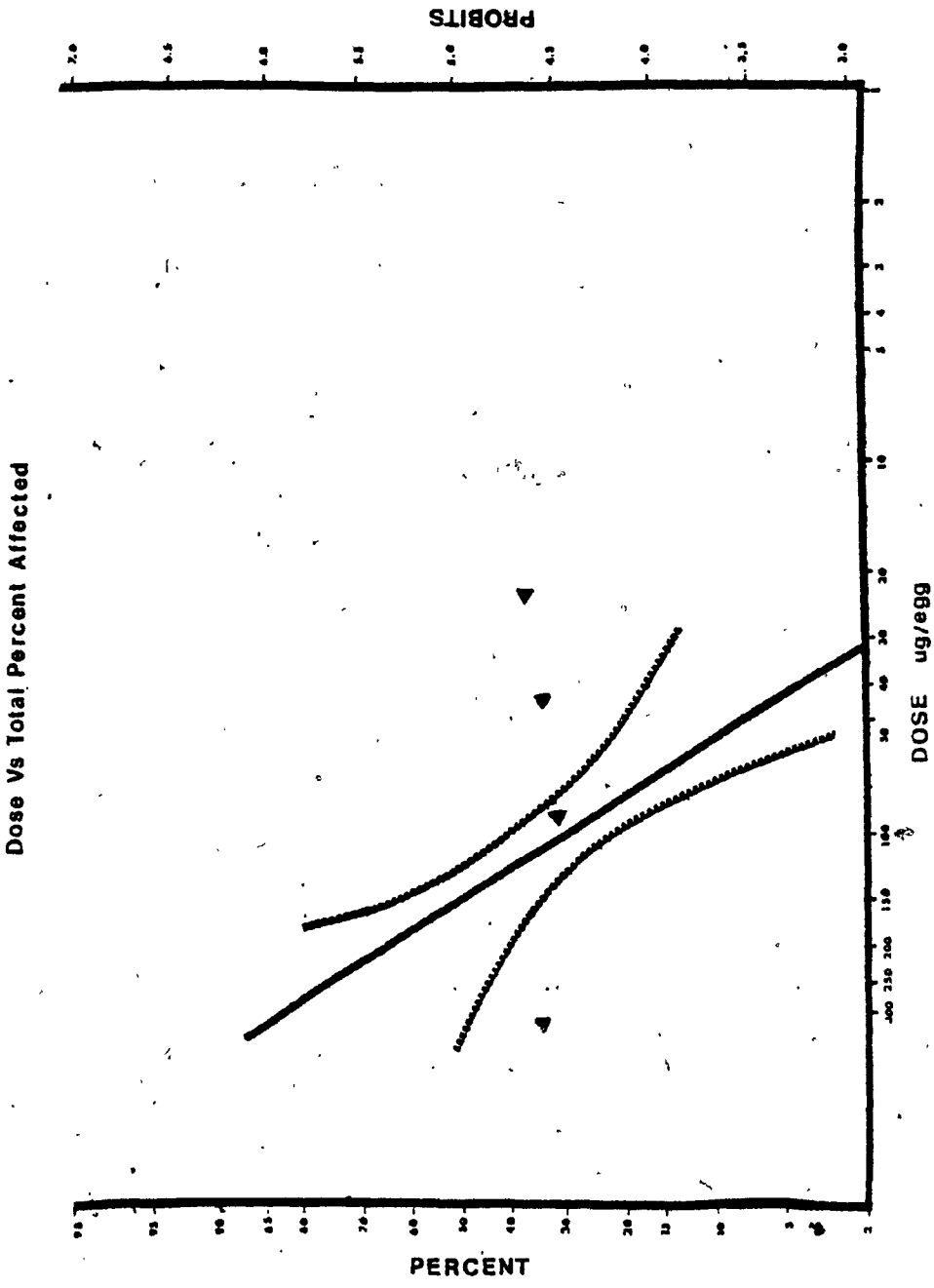


Fig. 17. Relationship between dose (in fenitrothion equivalents) of dieldrin-fenitrothion mixtures, and total percent affected (day 5 % mortality + percent incidence of terata), in yolk sac injection trials. Original data points for DF mixtures (▲) are plotted with respect to the regression line for dose of fenitrothion versus total percent affected data.

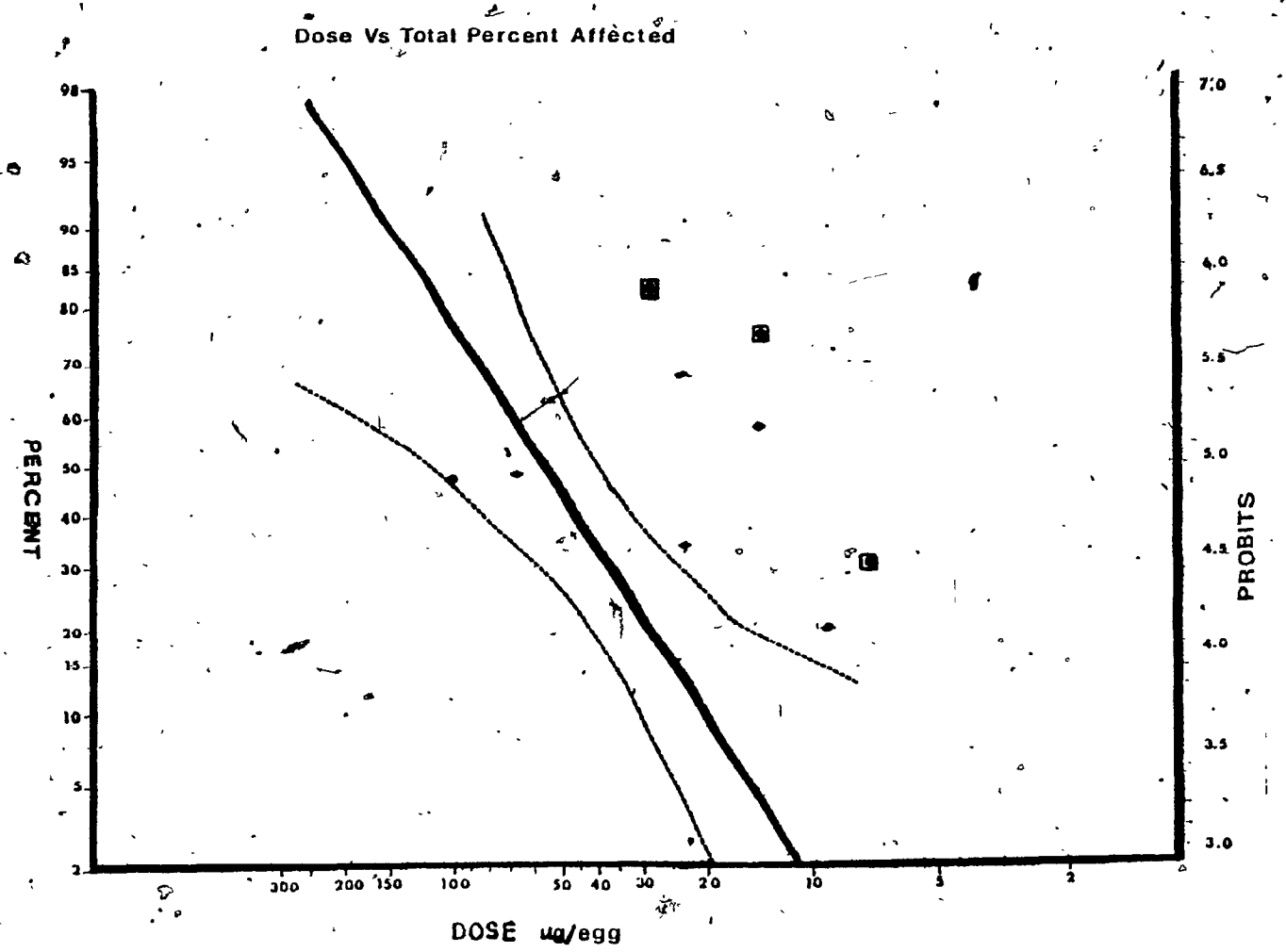

Dose Vs Total Percent Affected
PROBITS
PERCENT
DOSE ug/egg

mercury equivalents, using the appropriate relative potency factor (Table 19). The observed results were thereupon fitted to the dose-response line and its 99% fiducial limits derived for mercury alone (Fig. 19). As seen in Fig. 18, the dose-response coordinates recorded for binary mixtures of dieldrin and mercury fall outside the limits predicted for concentration-addition. The responses for these doses are all greater than expected (Table 20), and are therefore in accordance with the model of supra-addition. The magnitude of response to dieldrin-mercury mixtures appears to increase linearly with dose as mercury-equivalents. If a parallel line is fitted to these points, and the ED_{50} determined, there is approximately a six-fold increase in the potency of the binary mixture to that expected for concentration-addition. Furthermore, the lowest dose of mercury and dieldrin mixtures is a composite of sub-threshold levels of each constituent.

There is an apparent linear dose-response relationship in the distribution of coordinates for mercury-fenitrothion mixtures. The coordinates of the doses tested fall outside the concentration-addition limits and suggest supra-additivity between mercury and fenitrothion (Fig. 18).

A dichotomy exists with respect to the distribution of coordinates for tertiary mixtures of

Fig. 18. Relationship between dose (in mercury equivalents) of binary mixtures of dieldrin-mercury (■) or dieldrin-fenitrothion (◆), and total percent affected (day 5 % mortality + percent incidence of terata), in yolk sac injection trials. The coordinates for these mixtures are plotted with respect to the regression line for mercury data (p. 92, Fig. 15).


Dose Vs Total Percent Affected
PERCENT
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PROBITS
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6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
300
200
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100
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20
10
5
2
DOSE ug/egg

mercury, fenitrothion, and dieldrin, as seen in Fig. 19. One of the points falls within the range of concentration-addition, while the other two responses are judged to be supra-additive, falling to the right of the concentration-addition limits. The concentration of each of the constituents in the three doses of tertiary mixtures are either at or near the threshold of their respective dose-response curves.

Observed responses for all binary and tertiary mixtures were compared to those predicted according to the response-addition model (Tables 21-22), assuming firstly that there was no correlation ($r = 0$), secondly, that there was a negative correlation ($r = -1$), and thirdly, that there was a positive correlation ($r = 1$), in the susceptibilities of embryos to each of the toxic constituents of the mixtures. On the basis of chi-square tests applied to sets of data for each mixture, the response-addition model was rejected. However, if the highest dose of the tertiary mixtures is considered alone, and conceding that there is no correlation in susceptibility, there is an apparent similarity between the observed response and response predicted by the response-addition model.

Table 21. Predicted response for binary mixtures of toxicants according to the response addition model. Dose versus Total percent affected data.

Mixture	Compound 1 (ug)	Compound 2 (ug)	P_1(%)	P_2(%)	Obs. P(%)	Predicted P(%) r=0	r=1	r=-1
DF	2.5	14.0	6.5	0	36.4	6.5	6.6	6.5
	6.0	22.5	11.0	0	33.3	11.0	11.0	11.0
	14.5	37.5	18.0	0	30.8	18.0	18.0	18.0
DH	2.5	3.7	6.5	0	30.0	6.5	6.5	6.5
	6.0	5.8	11.0	0	75.0	11.0	11.0	11.0
	14.5	9.5	18.0	0	81.8	18.0	18.0	18.0
FH	14.0	3.7	0	0	20.0	0	0	0
	22.5	5.8	0	0	58.3	0	0	0
	37.5	9.5	0	0	33.3	0	0	0

Table 22. Predicted response for tertiary mixtures of toxicants according to the response addition model. Dose versus Total percent affected data.

Mixture	ug #1	ug #2	ug #3	P_1	P_2	P_3	Obs. P(%)	Predicted P(%) r=0	r=1	r=-1
DFH	15	4.0	4.0	0	0	0	50.0	0	0	0
	25	10	6.3	0	14.5	0	16.7	14.5	14.5	14.5
	45	12	11.5	2.8	16.5	0	91.7	18.8	16.5	19.3

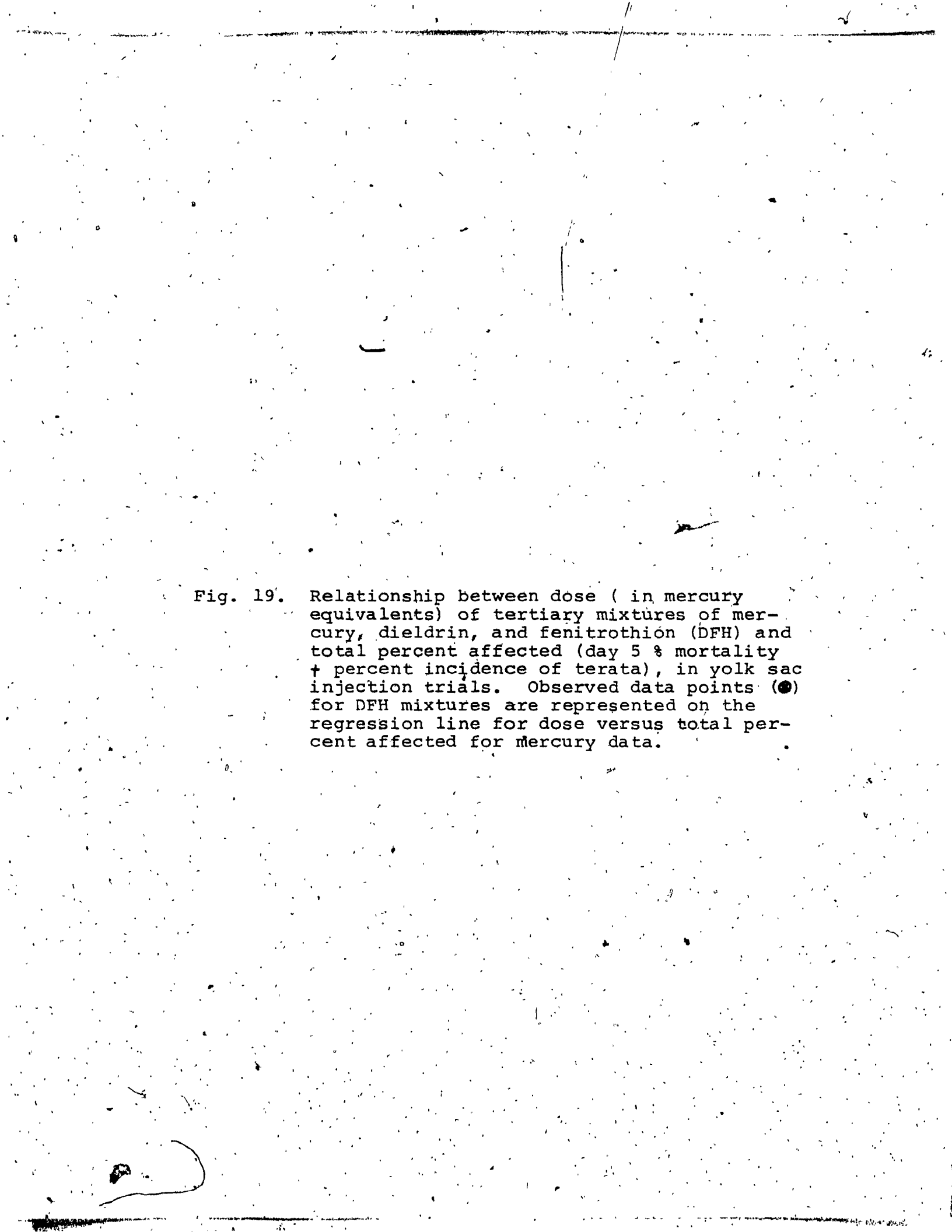
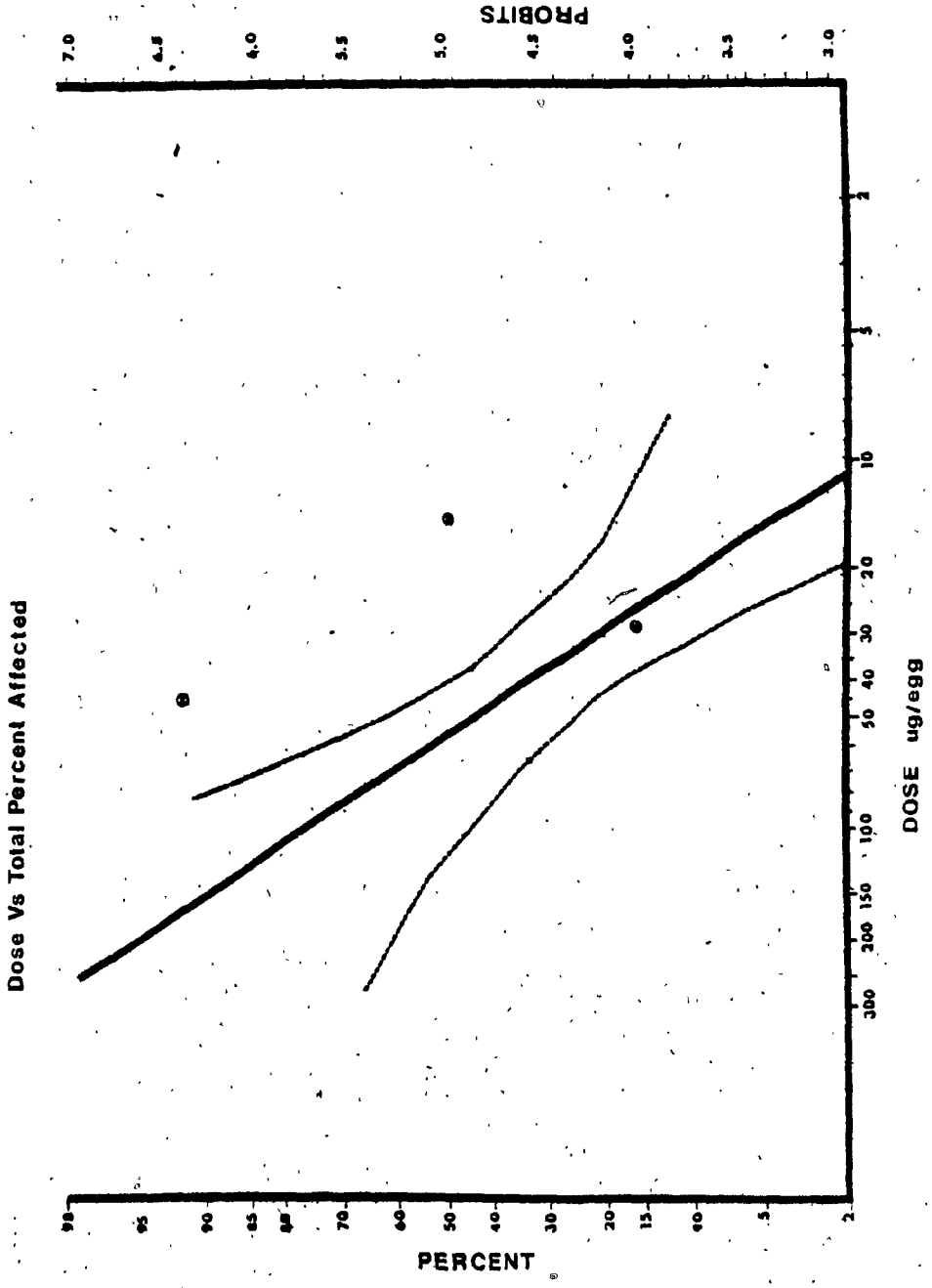


Fig. 19. Relationship between dose (in mercury equivalents) of tertiary mixtures of mercury, dieldrin, and fenitrothion (DFH) and total percent affected (day 5 % mortality + percent incidence of terata), in yolk sac injection trials. Observed data points (●) for DFH mixtures are represented on the regression line for dose versus total percent affected for mercury data.


Dose Vs Total Percent Affected
PROBITS
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
DOSE ug/egg
1
2
3
10
20
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40
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100
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200
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PERCENT
99
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DISCUSSION

Term Studies: Control Mortality

The multiple toxicity model of Anderson & Weber (1975) has been successfully applied to the embryonic stage of a fish's life cycle (Gallimore, 1980). In applying the model to avian development, it was assumed that quantal data on the cumulative effects of the individual toxicants and their mixtures recorded at hatch would lend themselves to analyses. However, as evident in Table 2, a high and variable incidence of mortality occurred in propylene glycol controls for both air space- and yolk sac-injected test lots.

The high mortality in air-space injected propylene glycol controls suggests that either conditions for incubation were inadequate, or that the vehicle itself was highly toxic. The first consideration may be dismissed because of evidence provided by untreated controls. Deaths occurred in approximately one-fifth of untreated controls (Table 2) by day 21 (hatching day), a percentage which may appear high but which falls within the range projected for the species Gallus domesticus (Carter, 1964; Hamilton & Lillie, 1952; Romanoff, 1953). Furthermore, the recorded level of control mortality in this study was to be expected for the variety (White Rock). Arora (1969) noted that

hatching success differed significantly between Gallus domesticus varieties; in particular, those varieties selected for their rapid growth rate, such as White Rock, exhibited relatively low hatchability. In contrast, hatching success in standard varieties such as White Leghorn has been generally recorded in the vicinity of 90% (Verrett et al., 1964; McLaughlin et al., 1963). These differences in hatchability between different strains were demonstrated in an extensive study by Khera & Lyon (1968). It may be assumed, therefore, that the procedures followed for incubation were adequate, and that they were not a factor contributing to the high incidence of mortality in propylene glycol controls.

Although of no significance in air space injection trials, propylene glycol and the volume injected (saline) were significant lethal factors in yolk sac injection trials. Significantly higher ($p < 0.05$) mortality in propylene glycol-injected controls as compared to untreated controls was observed in all yolk sac injection trials (Table 2). The average incidence of propylene glycol mortality ($55.6 \pm 3.8\%$) is dramatically higher than that reported by Landauer & Salam (1971) and McLaughlin et al. (1963), but closely approximates that reported in studies where propylene glycol was injected on day 0 of incubation (Gebhardt & van Logten, 1968; Gebhardt, 1968; Khera & Lyon, 1968;

Walker, 1967). Both Gebhardt & van Logten (1968) and Khera & Lyon (1968) suggested that the simple violation of the yolk sac by a penetrating needle, and the mechanical disturbance of the yolk caused by the volume and solvency of the vehicle, were major factors in the mortality of propylene glycol controls. In our studies, needle penetration had no apparent adverse effects. However, physical displacement, as demonstrated in saline controls in these experiments, appeared to be a major factor contributing to the mortality recorded in propylene glycol-treated lots. Although the levels of mortality are higher in propylene glycol than in saline controls in yolk sac injection studies, the difference is not significant ($p < 0.05$). Therefore, propylene glycol may or may not have a specific chemo-toxicity. To compensate for the lethal effects of propylene glycol, the Abbott formula (Eq. 1) was applied to the data. Landauer & Salam (1972) have suggested that while propylene glycol may act as a selective lethal agent when injected into the yolk, it does not alter the course of normal morphogenesis.

Khera & Lyon (1968) have argued that chick eggs, treated via the yolk sac injection procedure prior to incubation, are unsuitable toxicity models for term (21 day) studies. They based this conclusion on the fact that in such studies, there is a high incidence of

mortality in vehicle controls, a lack of dose-response relationships, and an unreliability of reproducible results. The results recorded at term in this study for the lethal effects of each of the three toxicants and their mixtures support this argument and concur with other studies (Clegg, 1964; Walker, 1967). For this reason, neither the yolk sac nor the air space-injected term studies were used to explore models of multiple toxicity. However, the method of subsampling used in these term studies permits a qualitative and quantitative examination of certain aspects of lethal, sublethal, and teratogenic effects. Firstly, these data showed that significant toxic effects may be demonstrated in yolk sac injected experiments during the early phases of development. Secondly, toxicant-treated chicks, upon hatch, were significantly different from controls in certain structural and behavioural characteristics.

Critical Periods of Development

Critical stages, as judged by the incidence of peak mortality, occur normally in development, and are related to the first day, the fourth and fifth days, and latter three days of incubation (Hamilton & Lillie, 1952; Romanoff, 1953). This time-related pattern was not significantly altered by any of the three toxicants or their mixtures, regardless of treatment. However,

in yolk sac-injected embryos, certain dieldrin-containing mixtures appeared to increase the mortality in the third critical phase. Furthermore, each of the individual toxic agents and certain mercury-containing mixtures significantly augmented the magnitude of mortality during the first critical phase in yolk sac injection trials (Fig. 9).

These latter results suggest that, when introduced beneath the blastodisc, toxicants reach critical target sites rapidly. At the concentrations used, the effects were often lethal. Unfortunately, it was not possible to evaluate the manner of death in toxicant-treated lots. Such investigations were prevented because of the rapid deterioration of tissues that follows death at an early stage of development. It was only possible to record that some development had occurred, as defined by the outline of the blastodisc.

The embryological events which normally occur during the first critical period are gastrulation and the initiation of organogenesis, particularly of the central nervous system and somatic musculature. In addition, transport mechanisms for the uptake of yolk and the initial development of the extraembryonic membranes are events associated with this period. One may assume that this or a combination of these events were adversely affected.

In air space-injected experiments, in which no significant ($p < 0.05$) deaths occurred during these stages, either the toxicants did not reach the embryos undergoing these events, or the levels encountered by the embryos were not effective. Although no attempts were made in this study to evaluate the diffusion rates of toxicants from the air space to the embryo, studies by Romanoff (1960) showed that a number of substances (eg. trypan blue) reached the embryo between four and seven days following air space injection prior to incubation. Romanoff explained the delayed toxic effects of these chemicals on the basis of their diffusion characteristics.

Mixtures, in this project, were studied only in yolk sac-injected trials. Their constituents were near or at their respective sub-threshold levels, yet mercury-containing mixtures had significantly ($p < 0.05$) greater lethal effects than controls at the first critical phase. Because, as a single agent it was highly lethal and was the common factor in these mixtures, mercury may have been the primary lethal agent. However, it would appear that mercury's effects were augmented in some way by the other toxic constituents in the mixture.

During the third critical phase, the normal stressors associated with mortality are respiratory, locomotory, and those related to yolk sac absorption

(Hamilton, 1952). Thus, the high incidence of lethality at this stage in embryos treated with binary mixtures of dieldrin may have been due to the augmentation of respiratory or yolk sac reabsorption stress. There is also the possibility that the lethal toxicity of dieldrin mixtures involved other targets which are not normally associated with control mortality at critical phase III. These events may arise during the third critical period, or they may occur prior to this phase. In the latter instance, the toxic effects may have occurred earlier and may have been manifested only during the third critical period.

In yolk sac-injected lots, there is a progressive decrease in the magnitude of mortality from the first through to the third critical phase for each of the three toxicants. In contrast, the incidence of mortality in air space injection trials would appear to increase for dieldrin, from the first to the third critical phases. For fenitrothion and mercury, the third critical phase is again the most sensitive, followed by the first critical phase. It would appear, therefore, that if the initial site of deposition was yolk sac, the first critical period was generally most affected, whereas if the site of deposition was the air space, the third critical phase was most susceptible.

Term Studies: Mortality in Toxicant-Treated Groups

While reasonably linear dose versus percent mortality curves were observed for 21-day incubated embryos treated with dieldrin or mercury according to the yolk sac injection procedure, this correlation was not observed with the fenitrothion-treated groups (Table 5a). The lack of a linear dose-response relationship in the fenitrothion-treated groups may be due, at least in part, to its rapid hydrolysis by the chick embryo. It is well-known that fenitrothion is rapidly hydrolyzed in many animals (NRCC, 1975); however, its metabolism in chick embryos is unknown. It is, nevertheless, quite possible that hydrolysis of fenitrothion occurs in the early chick embryo, since the yolk sac has formed extracellular enzymes by the end of the first day (Romanoff, 1960).

The lipophilic and persistent characteristics of both mercury and dieldrin have been well-established, and the tendency of birds to accumulate and concentrate these pollutants in eggs has also been documented (Birge & Roberts, 1976; Peakall & Lovett, 1971; Fowler et al., 1971; Koeman et al., 1967). In addition, the gradual uptake of the yolk throughout incubation would result in virtually continuous exposure of the embryo to dieldrin or mercury. These factors may therefore account, at least in part, for the dose-response correlations observed with these two contaminants.

There are few dose-response studies in the literature regarding the effects of toxic agents upon developing avian embryos. This is undoubtedly a reflection of the difficulties encountered in obtaining a reasonable sample size of embryos at precisely the same stage of development. Large sample sizes may, in part, alleviate the considerable variation often observed, although inherent difficulties may persist. Rates of development of life-support systems vary between embryos, and such variance, however slight, may significantly influence the response of a large number of embryos exposed to a series of doses of a toxicant.

Teratogenesis

The incidence of post-hatch effects (terata) in chicks hatched from eggs treated with a single dose of either a binary or tertiary mixture of toxicants did not illustrate any sort of pattern. Of the binary mixtures, those containing dieldrin appeared to cause considerably higher proportions of chicks having discolored feathers, suggesting a greater potency of dieldrin upon those developmental processes whose disruption results in feather distortions. Despite the incidence of discolored feathers in mercury- and fenitrothion-treated chicks, none of the chicks hatched from eggs treated with binary mixtures of these substances exhibited feather discoloration. This may

be an indication of the threshold levels of mercury and fenitrothion required to induce this effect, since the concentrations of these toxicants in mixtures were substantially lower than those used in single agent tests. Similarly, no chicks hatched from eggs treated with a tertiary mixture exhibited feather discoloration. However, no definite conclusions can be drawn from the data for mixtures, since sample sizes were generally small (<10).

Although single doses of mercury or fenitrothion resulted in similar incidences of digit distortions (crooked feet and toes), all binary mixtures exhibited comparable incidences of this effect. These results must be interpreted with care, however, since sample sizes of chicks hatched from eggs treated with any of the mixtures were small (<10). None of the literature surveyed contained information regarding the incidence of this type of anomaly as a result of dieldrin or mercury exposure. Paul & Vadlamudi (1976) described similar effects in chicks exposed to 0.1 and 1.0% fenitrothion, injected into the air space of eggs at various times (days 4-12) during incubation.

The other parameters which were tested in hatched chicks were balance and righting ability. These behaviors were probably affected as a result of distorted limbs, or of absorption of the yolk sac containing pesticide residues on the final day of incuba-

tion, just prior to hatching. This latter observation is supported by the studies of Koeman et al. (1967), who reported results indicating the poisoning of young birds by insecticides following post-hatch absorption of the yolk sac. Chicks hatched from dieldrin-treated eggs exhibited a somewhat higher incidence of impaired righting ability, corroborating studies which indicate neuromotor dysfunction as a result of dieldrin treatment. However, this tendency was not observed in the data for chicks treated with binary or tertiary mixtures containing dieldrin; in fact, of all of the mixtures tested, binary mixtures containing dieldrin caused the lowest percentage of chicks affected with impaired righting ability. It is again probable that the levels of dieldrin in these mixtures were sub-threshold and hence, ineffective.

Single doses of fenitrothion or mercury resulted in significantly higher ($p < 0.05$) incidences of impaired balance, compared with controls. However, none of the binary mixtures used in these studies caused impaired balance. The concentrations of each of the toxicants in these mixtures were likely below the individual thresholds required to produce this effect.

The apparently high incidence of impaired balance caused by the tertiary mixture is not conclusive, since few chicks (<5) hatched from eggs treated with this mixture. As a result, no statistical comparisons were possible.

The apparent lack of a consistent relationship in potency is likely due to two factors: first, the small sample sizes of groups treated with mixtures, and second, the fact that those chicks which hatched were generally at least healthy enough to hatch, a process which demands a tremendous expenditure of energy and coordination.

Other post-hatch effects which were observed in the occasional chick were edematous cysts, wry neck, and unabsorbed yolk sacs. These occurred primarily in, but were not restricted to, chicks treated with either dieldrin-mercury or dieldrin-fenitrothion mixtures. However, at least one chick in each of the treated groups exhibited this phenomenon, which would appear to be a non-specific effect produced by several compounds. In addition, half of the chicks exhibitng edematous cysts were also afflicted with crooked toes, suggesting a possible syndrome. Further studies exploring these effects are required to form any conclusions regarding these observations. These effects were not seen in controls and are similar to those reported by Cecil et al. (1974). In this latter study, hens were fed various types of PCB's (polychlorinated biphenyls) at dietary concentrations of 20 ppm. for nine weeks. The similarity of effects produced by PCB's and by the toxicants tested in the present study suggest that a specific step in the

developmental sequence is being affected by all of these compounds. The present results also indicate that the effects observed are not caused by a common contaminant in the PCB formulations tested, as was postulated by Cecil et al. (1974), since the compounds used in the present study were all purified forms (> 93% purity).

Growth Rates

No conclusive statements can be made regarding growth, due to lack of statistically-supportive data. However, reduced growth was noted in most groups, particularly fenitrothion-treated lots, an observation concurrent with teratogenic studies by Paul and Vadlamudi (1976), who treated chick embryos with fenitrothion at various times during incubation and reported reduced growth in treated chicks. The apparent effects on growth do not, therefore, seem to be specific or restricted to any one toxicant.

Hatching Success

Hatching success was greatly reduced in most treated groups, although, as with growth, variable sample sizes did not permit statistical analysis. However, these observations of reduced hatching success tend to support those found in the literature. Hatchability was reportedly reduced by 30 and 45% in

chicken eggs exposed to 5 and 10 mg. of dieldrin, respectively, injected into the albumin (Smith et al., 1970). Mercury (as mercuric chloride) at levels of 0.5 mg./egg caused 100% mortality in yolk sac-injected eggs (McLaughlin et al., 1963), while both lower and higher levels have caused reduced hatchability in a variety of avian species (Birge & Roberts, 1976; Fimreite, 1971; EPA, 1971).

Chemical Analyses

The lack of detectable liver residues of fenitrothion is not surprising, since, as previously mentioned, fenitrothion is rapidly hydrolyzed by many organisms (NRCC, 1975) and probably by the early chick embryo as well. Furthermore, even if the chick embryo is not capable of metabolizing fenitrothion, which is unlikely due to the presence of yolk-metabolizing enzymes early in development, the hatched chick almost certainly would be capable of doing so. Since many of the liver samples were taken from 6- or 7-day old hatched chicks, there would have been ample time for the metabolism of fenitrothion during the post-hatch period, prior to sacrifice.

It is probable that this latter factor is responsible for the low ($<$ 1 ppm.) residue levels of both dieldrin and mercury. Residues of mercury have been reported in various tissues of a number of avian

species, at levels as high as 200 ppm. for liver-kidney composites (Nelson et al., 1971); hence, mercury can accumulate in tissues when there are sufficiently high levels of exposure.

Skeletal Analysis

Cleared skeletons were examined for morphological defects, and although no apparent correlation between dose and incidence or severity of effect was established, affected chicks from most groups treated with either individual toxicants or a mixture of toxicants were observed. The lack of any notable dose-response relationship is again likely due to the low sample size in some groups, particularly mixtures, and also to the fact that over half of the skeletons were of chicks at least sufficiently healthy to hatch.

T.A. and T.I. Data

The T.A. formula developed for these studies appears to be a useful tool for distinguishing between the toxic and teratogenic effects of contaminants. For example, using this approach on dieldrin data, it may be concluded that dieldrin is not highly teratogenic, since response is positively correlated with dose in both day 5 percent mortality and percent affected with terata. However, the combined points representing day 5 percent mortality plus percent terata are somewhat

scattered, and do not conform well to a linear relationship. It was concluded that this variance is the consequence of the separate actions of dieldrin, one embryotoxic and lethal, the other teratogenic and possibily lethal as well, and that these two events are not well-correlated. Results for both dieldrin and fenitrothion indicate that the toxic and teratogenic actions of each of these substances are independent; ie., their lethal effects are specifically results of their toxicity, rather than coincidental manifestations of severe teratogenicity. Mercury, on the other hand, exhibits an inverse relationship between dose and T.A. response, suggesting a correlation between lethality and teratogenicity. In the case of mercury-treated eggs, it would appear that some embryos were so profoundly affected that mortality resulted; this is not to be confused with mortality simply due to mercury's toxicity. These findings seem to be consistent with those of Birge & Roberts (1976), who reported a high sensitivity of chick embryos to metallic toxicants, such as mercury, when injected into the yolk sac prior to incubation.

The patterns of response observed with T.A. data are similar to those observed with the application of the TI index to the data. This lends further support to the hypothesis that the toxic and teratogenic effects of mercury are related, while those of

fenitrothion, and to a lesser extent, dieldrin, are independent.

Five-Day Studies: Quantal Response

The dose versus day 5 percent mortality for both dieldrin and mercury treatments were well-correlated ($r = 0.72$ and 0.95, respectively), although a weaker correlation with respect to fenitrothion data was obtained ($r = 0.52$). However, as with the 21-day mortality, the combination of day 5 percent mortality and incidence of sublethal effects by day 5 resulted in linear dose-response relationships for all three toxicants (Tables 15-17).

When no reasonably linear dose-response relationships for dose versus percent incidence of terata by day 5 could be established for either dieldrin or mercury ($r = 0.21$ and -0.09, respectively), it became apparent that the mortality observed in treated eggs was in fact, to a certain extent, related to the incidence of terata. Although a number of studies have attempted to evaluate the teratogenic potential of mercury and dieldrin, and a few of fenitrothion, only rarely has there been any indication of an effort to correlate dose with either mortality or incidence of terata in developing avian embryos. Furthermore, the evaluation of the severity of teratogenic effects has been largely qualitative, with the exception of a study

by Schom & Abbott (1978). Application of a TI (teratogenic index) devised specifically for these studies, and consideration of mortality (at least early mortality within the first 24 hours) as a teratogenic effect, resulted in a reasonably linear dose-resoponse relationship for each toxicant (Table 13). In addition, the combination of day 5 percent mortality and incidence of terata (sublethal effects by day 5 of incubation) yielded good linear correlations between dose and total percent response for each contaminant tested (Figs. 13-15).

The significance of these findings is that they demonstrate a reasonably successful method of quantifying teratogenic data. Other studies, such as Paul & Valamudi's (1976) teratogenic study of fenitrothion, have described but have failed to demonstrate any linear correlation between the dose of the toxicant tested and the observed response (lethal or sublethal).

Terata and Sublethal Effects

Since significant ($p < 0.05$) differences in % mortality were observed for toxicants and their mixtures primarily during the first and second critical periods (up to day 5, or stages 24-27 of development), quantal evaluation of toxicants was based upon mortality and/or teratogenic effects incurred by day 5 of development. No significant ($p < 0.05$) incidences of any teratogenic or lethal effects were observed in embryos

incubated for five days in air space injection experiments.

Sublethal effects were evident in embryos in yolk sac injection experiments from all three toxicant groups and their mixtures, and at nearly all doses. The doses at which no terata were observed were generally low and were considered to be at sub-threshold levels. This incidence of observed terata (sublethal effects) is similar for both mercury- and fenitrothion-treated groups (Table 6). This suggests that the potencies of these compounds with respect to induction of these effects are nearly equal, since dosages of both compounds were within the same range (10-160 ug./egg). Dieldrin treatments induced considerably lower frequencies of response for all effects except left rather than right hand (inverse) torsion. However, none of the treatments resulted in incidences of terata that were significantly ($p < 0.05$) different from control groups.

The pattern of enhanced incidence of effects by mixtures of the compounds tested was also observed for these sublethal effects. For example, in single agent trials (Table 6), all agents induced an incidence of embryos exhibiting abnormal eye development. The highest incidence was seen in mercury-treated groups, while the lowest was in dieldrin-treated groups. However, the percent incidence of this effect even in

dieldrin-treated groups was not as high as in any groups treated with either binary or tertiary mixtures containing dieldrin. Averaging the data for the percent incidence of abnormal eye development in groups treated with binary mixtures, the order of potency was DF > DH > FH. There was, however, considerable variation about the mean for each group. The incidence of these sublethal effects in nature has not been reported, although it is probably very low, since the occurrence of these anomalies in controls was nearly zero.

With respect to circulatory disturbances and abnormal eye development, the order of potency was DFH > DF > DH > FH, while curiously enough, this order was exactly reversed with respect to left rather than right hand (inverse) torsion. It is also interesting to note that most embryos exhibiting this inverse torsion were generally otherwise morphologically normal. It would appear, then, that development, at least to day 5 of incubation, is not affected by this inverse torsion. Whether or not more profound effects upon subsequent development are exerted by this unusual torsion is not known, since most eggs were opened and examined on day 5 of incubation. No known published reports on the natural occurrence or consequence of inverse torsion were found, although the incidence in controls in the present experiments was extremely low (average $\pm$ std. deviation = 1.4 ± 1.6%).

It is also interesting to note that many of the embryos having circulatory disorders often exhibited reduced or abnormal eye development as well. Again, there is a dearth of literature on this subject; however, it is possible that a common temporal or morphological step in developmental pathways may be affected by these compounds. Certainly, the data do not suggest specific effects by any of the agents tested.

While there is some degree of correlation ($r = 0.69$) between the incidence of terata and dose of fenitrothion in yolk sac injection experiments, there is no correlation between the incidence of terata and dose of either dieldrin or mercury. As with mortality, the most likely factors responsible for this lack of clear dose-response correlation are variation in availability to, and uptake of the toxicant by, the embryo. This supports findings previously reported in the literature for pesticide evaluations using the chick embryo (Khera & Lyon, 1968; Walker, 1968; Clegg, 1964), although not all of the pesticides tested in the present study have been evaluated with respect to embryotoxicity using this model system.

Another point of interest is that the lack of a dose-response relationship for dieldrin- and mercury-treated embryos with respect to the incidence of sublethal effects in 5-day embryos is precisely the

opposite situation observed for dose versus percent mortality (5 days) relationships. To test whether or not this phenomenon was coincidental, the incidence of sublethal effects (Table 6) and percent mortality (5 days) were combined, and correlation between dose and total response was tested for each toxicant. Very good correlation between dose and total percent affected (incidence of sublethal effects + percent mortality at 5 days) was observed for both fenitrothion and mercury data ($r = 0.85$ and 0.95, respectively), although the data for dieldrin was not well-correlated ($r = 0.41$). This suggests that mortality and incidence of sublethal effects are, to a certain extent, somehow related in mercury- and fenitrothion-treated groups.

Mixtures

Using quantal response curves for individual agents as a basis for predicting responses due to multiple toxicant exposure, it was found that the observed interactions between the contaminants tested in this study were either additive or supra-additive. The results observed for mercury-fenitrothion combinations with respect to total percent affected data (day 5 mortality + percent affected with terata) tend to support those found in the literature. In a study by Dieter & Ludke (1975), male Coturnix quail (Coturnix coturnix japonica) were given morsodren (4 ppm. as

methyl mercury) in their diet for 18 weeks, and subsequently orally dosed with various levels of parathion (2,4,6,8, and 10 mg./kg.). Their results indicated that mercury potentiated the toxicity and biochemical effects of a popular organophosphate insecticide, parathion, which is similar in action and structure to fenitrothion (Pesticide Manual, 1977). On the other hand, responses observed for dieldrin-mercury and dieldrin-fenitrothion mixtures are not compatible with those described in published works. Triolo and Coon (1966) examined interactions between a variety of chlorinated hydrocarbon and organophosphate insecticides. They reported observing protective effects by the chlorinated hydrocarbon compounds with respect to reducing the effects of the organophosphates. Such protection was apparently the result of increased detoxification capacity by hepatic microsomal enzymes which had been induced by the chlorinated hydrocarbon insecticides. It is possible that these results differ from those in the present study due to the difference in specific compounds tested, despite the same general nature of the substances. For instance, while fenitrothion and parathion, both organophosphate insecticides, are structurally and chemically similar, parathion's toxicity to mammals is primarily the result of its metabolism to a more toxic compound, paraoxon.

Conversely, it is believed that fenitrothion does not require metabolism to exert its primary toxic effects (NRCC, 1975; Melnikov, 1971). The metabolism of both parathion and fenitrothion probably differs between mammalian and avian species, and these differences may, in part, explain the discrepancies between the results of the present studies and those of previously reported work.

In addition to the variation in test species and in compounds used, different parameters were examined in the present study as compared to that of Triolo and Coon (1966). In the latter work, toxicity rather than teratogenicity was evaluated.

The only other studies of chlorinated hydrocarbon and organophosphate interactions found in the literature surveyed was a report on pesticide synergism of Kreitzer and Spann (1973). In their study, young quail (Coturnix coturnix japonica) and pheasant (Phasianus colchicus) were exposed via the feed for five consecutive days to one of several binary mixtures of various pesticides, including dieldrin (HEOD) and diazinon, an organophosphate compound, at assorted concentrations. The action between these two substances was reported to be strictly additive, and no synergism was observed. As was the case with the dieldrin-fenitrothion mixtures previously discussed, the dissimilarity of results between the present work

and these published studies may be largely attributed to differences in route of administration and age of the test subjects (7-16 days old in the Kreitzer and Spann study).

The results for the tertiary mixtures indicate at least additive and probably greater than additive effects. Furthermore, a nearly linear relationship is observed when the data points for these tertiary mixtures are plotted on the graph of dose versus total percent affected common slope line for mercury (Fig. 22). This linear function exhibits a similar slope to the plotted common slope regression line for mercury, thus providing further support for greater than additive action of tertiary combinations.

No published reports of tertiary mixtures of these or similar pesticides were found in the literature surveyed. The results of the present study appear to be of some significance, since the dosage levels of all three contaminants in these mixtures were generally near or at subthreshold concentrations, as might be the case in nature. This is at odds with a statement expressed by Guthrie and Donaldson (1973), who examined the toxicity and reproductive effects of dieldrin upon several avian species. Their conclusion, based on one agent, was that the levels found in nature were not sufficient to provoke a hazard to the reproductive success of avian wildlife. However, it may be

concluded from the present research that exposure of birds to sublethal levels of environmental contaminants, either concurrently or sequentially, may be hazardous to the individual organism, and possibly to the population as a whole.

Summary

The primary objective of these studies was to examine both the toxicity and teratogenicity of three common environmental contaminants (dieldrin, fenitrothion, and mercuric chloride) to birds. Each of the three agents tested exhibited a significant level of toxicity, but the route of administration influenced the nature and extent of this toxicity.

Term (21-day) studies were found to be unsuitable for the establishment of dose-response relationships, due to a number of uncontrollable external variables which come into play during the final three days of development. This confirmed previous findings (Khera & Lyon, 1968; Walker, 1968; Clegg, 1964) regarding the use of the chick embryo as a toxicity model. Therefore, the initial five days of development was selected as a more appropriate period for quantal evaluation of toxicity. All three toxicants were found to exhibit positive correlation between dosage and lethality, with dieldrin and mercury being nearly equipotent, and fenitrothion being the least potent.

Furthermore, teratogenic effects were observed in embryos treated with any of the three toxicants. These effects included circulatory abnormalities, reduced eye development, and inverse torsion in the embryonic period, and feather discoloration, limb and digit distortion, and neuromotor dysfunction in the

post-hatch period. There was little correlation between the amount of toxicant injected and the incidence of terata, a phenomenon frequently observed in teratogenic studies.

In an effort to overcome the difficulties encountered in quantifying teratogenic effects, three approaches were employed to treat the data. The first was to include mortality as a profound teratogenic effect. When this was done, a strong correlation between total response (day 5% mortality + % incidence of terata) and dose was observed for each of the three contaminants.

The second approach involved an indexation of terata on a temporal basis with respect to development. This approach was less successful in establishing dose-response relationships.

Finally, it was suspected that teratogenic effects were being masked by coincidental mortality. Hence, an attempt was made to separate the toxicity and teratogenicity of each contaminant using a formula (T.A. formula) devised for this purpose. This approach allowed determination of the selective action of the compounds tested. Dieldrin was found to be a potent teratogen, although it caused considerable embryotoxicity as well. Both mortality and terata resulted from exposure to fenitrothion, but its action did not appear to be selectively embryotoxic or

teratogenic. Mercuric chloride was highly teratogenic; however, its embryotoxicity sometimes masked its teratogenicity, especially at the higher doses tested.

Exposure, either concurrently or sequentially, to more than one contaminant may frequently occur in the environment. To date, a limited number of studies have examined potential interactions of such pollutants, and these interactions were investigated in the present work. All binary and tertiary mixtures demonstrated additive or supra-additive responses, suggesting that interactions between the various toxicants occurred. This augmentation of response was particularly evident in embryos treated with mixtures composed of apparent sub-threshold levels of toxicants. Since the concentrations of each toxicant tested were comparable to residue levels detected in many avian species in nature, the potential interactions of these contaminants, even at apparently harmless levels, constitute significant hazards to both individuals and to the reproductive success of certain avian populations.

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APPENDIX I

Photographs of control and treated embryos from both air space and yolk sac injection trials.

Fig. 20. Control (untreated) chick, 7 days post-hatch.

Fig. 21. Seven-day old chick hatched from egg treated with 1 ug. dieldrin, injected into the yolk sac after 4 hours' incubation. Note the curved toes and abnormal (darkened) feathering.

Fig. 22. Seven-day old chick hatched from egg treated with 1 ug. dieldrin, injected into the yolk sac after 4 hours' incubation. Note the mildly affected (curved) toes, particularly on the left foot of the chick.

Fig. 23. Seven-day old chick hatched from an egg treated with propylene glycol, injected into the yolk sac after 4 hours' incubation. Curved toes were occasionally observed in propylene glycol-treated groups.

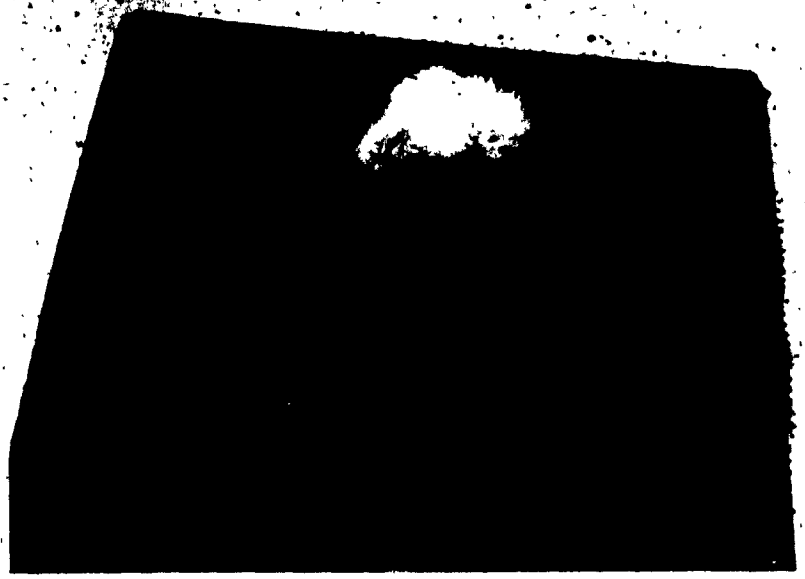

Fig. 24. Seven-day old chick hatched from egg treated with 2 ug. dieldrin, injected into the yolk sac after 4 hours' incubation. Mild monolateral (right leg) locomotory dysfunction is evident.

Fig. 25. Seven-day old chick hatched from egg treated with 20 ug. fenitrothion, injected into the yolk sac after 4 hours' incubation. Moderate single limb dysfunction and digital abnormalities (crooked toes) are apparent.

Fig. 26. Seven-day old chick hatched from an egg treated with 5 ug. dieldrin, injected into the yolk sac after 4 hours' incubation. Note the crooked toes and severe loss of limb control, as demonstrated by the chick's splayed posture.

Fig. 27. Four-day old chick hatched from an egg treated with 160 ug. of fenitrothion, injected into the yolk sac after 4 hours' incubation. Severe digital distortion (crooked and curved toes) and locomotory dysfunction are evident.

Fig. 28. Skeleton of a chick treated with 10 ug. fenitrothion, injected into the yolk sac after 4 hours' incubation. Skeleton is stained with Alizarin Red S. Note the distorted limb (crooked foot) and general retardation of growth. The ppm. notation refers to the concentration of the toxicant in the test solution, rather than in the egg.

Fig. 29. Skeleton of a chick hatched from egg treated with 10 ug. mercury ($HgCl_2$), injected into the yolk sac after 4 hours' incubation. Skeleton is stained with Alizarin Red S. Digital distortions (curved and crooked toes) are evident. The ppm. notation refers to the concentration of the toxicant in test solution, not in the egg.

Fig. 30. Photomicrograph (6X) of five-day old chick embryo; untreated control.

Fig. 31. Photomicrograph (6X) of five-day old chick embryo treated with a tertiary mixture of fenitrothion, dieldrin, and mercuric chloride, injected into the yolk sac after 4 hours' incubation. Note the partial torsion distortion (twisted spine) and reduced eye development.

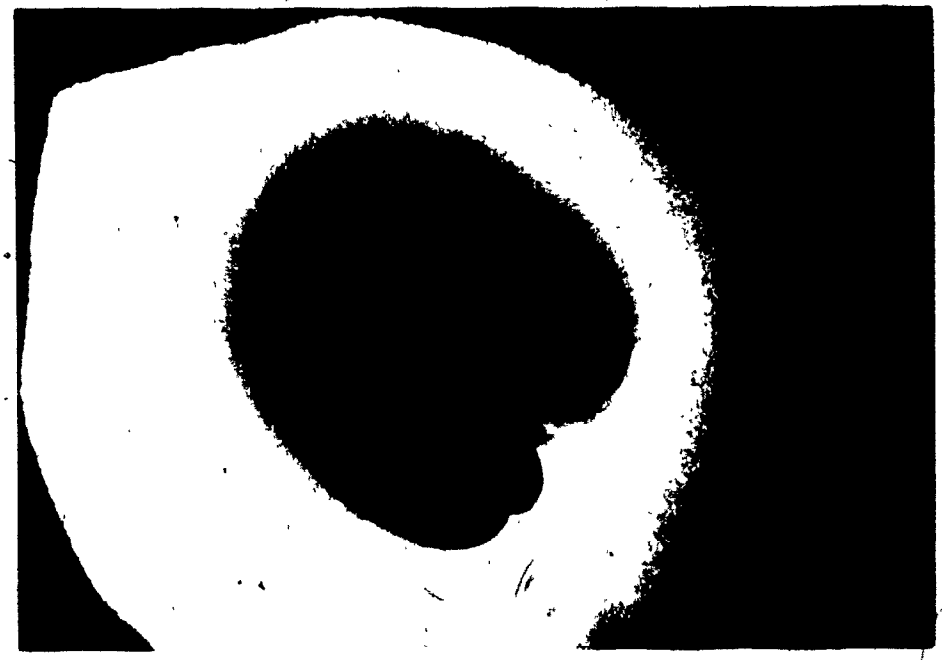

Fig. 32. Photomicrograph (6X) of a five-day old chick embryo, treated with 18.4 ug. mercury (as $HgCl_2$), injected into the yolk sac after 4 hours' incubation. This embryo is one member of a pair of "Siamese twins" (see below). Note the reduced eye development, distorted limb buds, and abnormal torsion (twisted spine).

Fig. 33. Photomicrograph (6X) of a pair of "Siamese twins" ; five-day chick embryos were treated with 18.4 ug. mercury (as $HgCl_2$) injected into the yolk sac after 4 hours' incubation. Note that the embryo on the left appears to be a normal 5-day embryo, while the embryo on the right is severely affected with terata.