BIOASSAY WITH *Drosophila melanogaster*, Meigen COMPARED WITH CHEMICAL ANALYSES OF TOXAPHENE AND DDT RESIDUES IN THE SOIL

By

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CHAPTER I

INTRODUCTION

Within the last decade the introduction of organic insecticides has made possible the control of many soil inhabiting insects by the application of these materials to the soil. This practice has fostered research on the effects of these materials on the plants that grow in treated soil and on the relative length of residual life of such compounds in the soil. The effects of these materials on the plants govern to some extent the amounts of insecticides that can be used. The length of residual life must be taken into account in the computation of the quantity of material needed each year to maintain a toxic level.

A cooperative project was initiated in 1951 by the Departments of Entomology and Agricultural Chemistry Research of the Oklahoma A. and M. College and the Hercules Powder Company to determine phytotoxicity and residual presistence of various levels of toxaphene incorporated into the soil. This was an insecticide weathering experiment set up under field conditions. Five levels of toxaphene; 5, 10, 20, 50 and 100 pounds per acre; and one level of DDT, 50 pounds per acre, were applied to the soil and mixed to a depth of eight inches. The first application was made in 1951, the second in 1952 and the third in 1953. Since 1953 no more insecticide has been applied to this soil.

Chemical analyses utilizing the method of Koblitsky and Chisholm (1949) for total organic chlorides were performed at one or two-month

intervals beginning early in 1951. Each year evaluations for phytotoxicity were made.

Since the total organic chlorides analysis had been used throughout this experiment to measure insecticide residues, it was deemed important to test this method by a biological assay. If values obtained from the biological assay were found to be lower than the chemical analysis results, one might assume that either the insecticide changed into non-toxic organic chlorides or that a masking agent had been introduced from the soil. However, if the values obtained from bioassay were higher, it would be assumed that the chemical analysis was not detecting all of the insecticide present.

If non-toxic organic chlorides were present the results of the total organic chlorides analysis would not be an accurate measurement of insecticide residues. However, if a cloaking agent from the soil reduced the toxic effects of the insecticide, the bioassay values would be in error.

In choosing a test animal it was desirable to use a readily available species which could easily be reared in the laboratory. Drosophila melanogaster Meig. was chosen because it fitted these requirements and offered the opportunity to test a species which apparently had not previously been used in soil bioassay experiments.

CHAPTER II

REVIEW OF THE LITERATURE

With the introduction of organic insecticides, such as DDT, that have long residual life and high toxicity there was immediate need for a means of chemically assaying residues of these materials. Schechter, et al. (1945) developed a method known as the Schechter-Haller method, which partially satisfied this need. This was a colorimetric analysis which would detect the respective quantities of all isomers of DDT and hexachlorocyclohexane in a sample. However, there was a need for a method of broader application because of the rapid development of the chlorinated terpenes following 1945. Koblitsky and Chisholm (1949) developed a method known as the total organic chlorides analysis which would detect all chlorinated insecticides. Since this analysis measures only the organic chlorides present in the sample it has the serious disadvantage of not being accurate where organic chlorides other than the insecticides are present. The quantity of insecticides is computed from the amount of chlorine found in the sample.

In view of this lack of specificity of the total organic chlorides method, biological experiments have been conducted to determine if the compounds under study decompose into non-toxic chlorine bearing compounds. Fleming, et al. (1951), working with 3rd. instar Japanese beetle larvae and Macrocentrus ancylivorus Rohwer, found that DDT and chlordane in the soil showed no signs of decomposing into non-toxic chlorine bearing compounds. In a later report, where chlordane

was used with 3rd. instar Japanese beetle larvae, Fleming, et al. (1954) arrived at the same conclusions, but the data presented did not appear to be in agreement with their conclusions. In the former report the bioassay and chemical analysis were very close for both DDT and chlordane. In the latter report the two analyses varied greatly. Terriere and Ingalsbe (1953) using mosquito larvae, Aedes vexans (Meig.), Aedes sticticus (Meig.), and Culex quinquifasciatus Say, analyzed seven chlorinated compounds which were applied to the soil at the rate of ten pounds per acre. Chemical and biological analyses were conducted the second and third years after application. They detected no difference between the chemical assay and the bioassay for aldrin, dieldrin, chlordane, heptachlor and DDT; however, the bioassay showed lower levels of toxaphene and BHC than the chemical method. It was assumed that both compounds exhibited a tendency to change into non-toxic chlorine bearing compounds.

Other than the test animals already mentioned, one additional arthropod has been used in the measurement of insecticide residues in the soil. Smith (1948) used woodlice or sowbugs (Oniscus ascellus Linn.) in direct contact with soil containing insecticide.

In the bioassays various methods of exposure to the insecticide residues have been developed. Fleming, et al. (1951) employed two methods of exposure, one involving direct contact of the test animal with the soil and the other requiring the extraction of the insecticide from the soil by the use of an organic solvent, usually benzene. Residues were deposited on filter paper by evaporation and the test animals placed in contact with these residues. Terriere and Ingalsbe (1953)

also used benzene extracts, but did not mention what method was used to disperse the extracts in the water with the mosquito larvae. Both papers reported difficulties arising from other soil constituents which were also dissolved in the benzene. Fleming, et al. (1951) stated that these materials could be removed with activated charcoal, but the process entailed substantial loss of insecticide. Terriere and Ingalsbe (1953) reported that 0.2 per cent sodium hdyroxide solution would perform the same function without removing any insecticide.

Many bioassay studies have been made using D. melanogaster Meig. as a test species. In the remainder of this section, twelve papers based on such studies using this species are cited. Stultz (1939) developed a method which consisted of spraying groups of flies caged in lantern globes. Morrison (1943) conducted toxicity tests with nicotine sulfate and nicotine alkaloids. This author found it necessary to standardize both the age of the flies and the quantity of flies per replicate because flies only one day old or over four days old were more susceptible than others, and an increase in the number of insects per replicate would increase the per cent kill. Lord (1944) used large mixed groups of flies caged in lantern globes to study the toxicity of nicotine and arsenic dusts which were applied to paper strips impregnated with honey. Glass shell vials lined with filter paper treated with DDT or related compounds and charged with fifteen adult flies were used by Morrison (1947) in biological assays of these compounds. Using both vials lined with filter paper and vials coated with insecticide, Proverbs and Morrison (1947) found that both types of tests gave reproducible results; however, an unexplained drop in mortality above a certain dosage level occurred with

the impregnated paper. Bartlett (1951) assayed insecticides with 50 to 100 flies caged in petri dishes lined with nine cm. filter papers impregnated with insecticide. Dresden and Oppenoorth (1953), King (1954), and Tattersfield, et al. (1953) used the mass spraying technique to develop strains of D. melanogaster Meig. which were resistant to chlorinated hydrocarbons. For bioassaying food residues, Sun and Pankaskie (1954) exposed flies to dry residues from benzene extracts or to osterized samples of contaminated food in four-ounce bottles.

Crow (1954) found that the males and females of D. melanogaster Meig. exhibited different levels of susceptibility to DDT. The females were more resistant than the males to treatment with DDT. The LD_{50} for topical treatment of control males was .018 gamma and for control females it was .030 gamma. Ciferri and Scaramuzzi (1949) reported corresponding observations.

CHAPTER III

METHODS AND MATERIALS

General Procedure

In experiments where vials without filter paper were used, 0.5 ml. of 40 gammas per ml. extract was pipetted separately into each vial and was allowed to evaporate. Where filter paper was used, rectangles of filter paper 4 cm. x 4.5 cm. were either laid on a glass plate and 0.5 ml. of extract dropped on each one or they were dipped into the solution and laid on sheets of paper to dry. Hence, these vials contained only ten gammas instead of the usual twenty. In later tests the vials were discarded entirely. Petri dishes three and one-half inches in diameter became the new test receptacle. Equivalent amounts of extracts were pipetted into the dishes to give a concentration of 20 gammas in each dish. Filter papers nine cm. in diameter covered the bottom of each of the dishes. The extract was divided so that two-thirds of the volume was placed in the bottom and one-third in the top of the dish, in order to increase the treated surface.

The testing procedure for the vial experiments involved placing known numbers of anesthetized two-day old female flies into 15 x 50 mm. vials which have been treated with insecticide and completely randomized. Test samples, which consisted of female flies only, of ten or twenty flies per vial were used at various times. Ciferri and Scaramuzzi (1949) and Crow (1954) reported that the females were less susceptible than the males and gave slightly more

reproducible results. The vials were plugged with absorbent cotton. Food material, a five per cent honey solution, was placed on the plugs in most experiments. This practice was discontinued in later tests in an effort to reduce variation. Two drops of water on each plug replaced the honey solution to maintain the required humidity. Wooden racks seven inches long, three-fourths inch wide, and two inches high each held ten vials securely for handling purposes. The racks were placed in a constant temperature cabinet (76°F. $\pm$ 2°F.) until time for the mortality counts which were made successively at three or six-hour intervals. The criterion for death for all experiments was a motionless animal lying on its side with its wings folded vertically to the body (Plate 1).

The experiments using petri dishes as test containers were set up with two-day old flies which were anesthetized in the transfer cone, and a volume of approximately 0.4 ml. of them placed in each dish. This amount yielded a sample of approximately 200 individuals. The measured samples were then placed in randomized treated petri dishes. After a lapse of 15 hours the dishes were removed from the incubator and the dead flies were counted through the top of the dish with the aid of a large reading glass. Counting was facilitated by gridding the top of the dish. When all the flies were dead the total number of flies per dish was determined and the per cent mortality was calculated for each replicate.

Cultures (Plate 2)

A culture of "wild type" Drosophila melanogaster Meig. was obtained from the Carolina Biological Supply House. This nucleus culture was

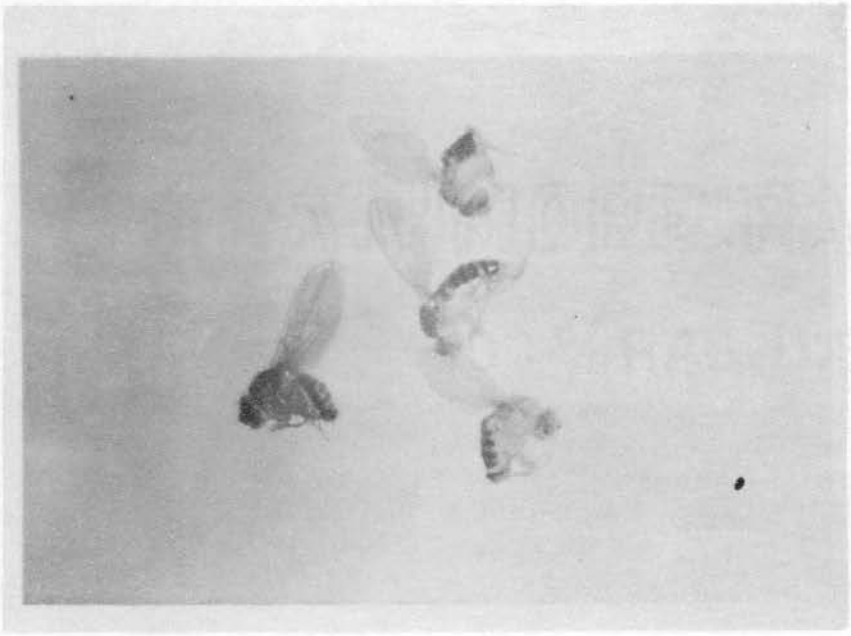

Plate 1. Dead D melanogaster.

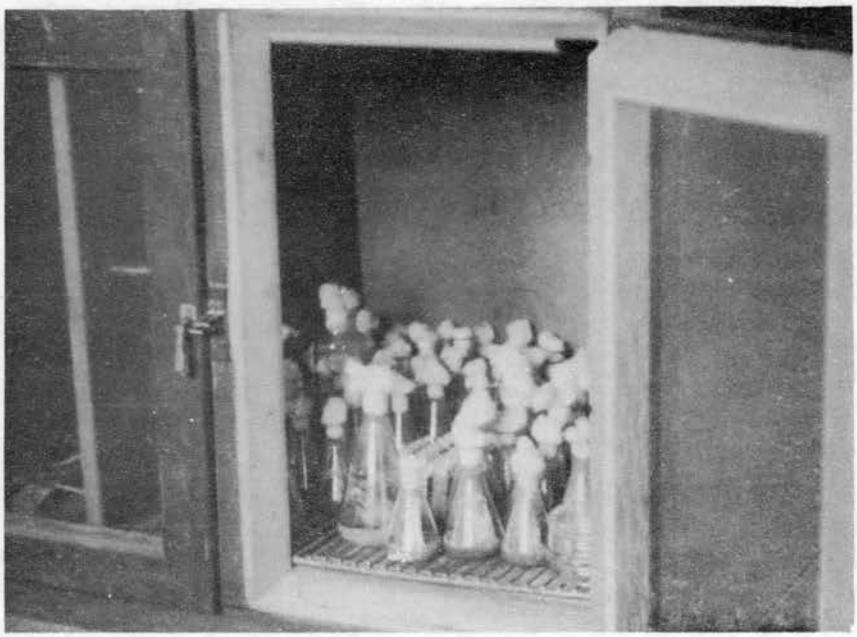

Plate 2. Close up of large incubator containing culture flasks.

expanded to provide the large number of flies necessary for the experimental work.

Erlenmeyer flasks of capacities ranging from 125 ml. to 500 ml. were used for culturing the flies. Medium was placed in these flasks to an average depth of one inch. Folded filter papers were placed into each flask to provide pupation sites for the flies. To keep mold and bacterial growth to a minimum, mold inhibitor[1] was added to the medium and all flasks were autoclaved before any flies were placed in them. After stocking, the flasks were placed in an incubator at a constant temperature of 76°F. ± 2°F. After a two-day oviposition period the adult flies were removed from the flasks and destroyed to prevent any holdover of old flies when the new generation began emergence. On the first day of emergence the flies in the flasks were emptied into holding containers where they remained until they were two days old. Only two-day old adult flies were used in experimental work. Excess flies were transferred to other flasks for oviposition.

Medium

The medium employed for rearing purposes was a modification of a formula suggested by Spencer (1950). The modifications made by the author were changes in the amount of agar and brewer's yeast. The original formula called for 25 gm. of powdered agar and 60 ml. of brewer's yeast. The quantity of agar was increased to produce a more solid medium. Additional yeast was used to increase the index of nutrition of the medium.

---

1 Obtained from the Carolina Biological Supply House.

80 gm. of agar flakes in four liters of water

100 ml. of brewer's yeast

500 ml. of cornmeal in 500 cc. of water

500 ml. of dark corn syrup (Karo)

20 ml. of mold inhibitor dissolved in 10 ml. of ethyl alcohol

This medium, a general purpose type, was prepared in the following manner: 80 gm. of flaked agar were added to four liters of water and the mixture brought to a boil. One hundred ml. of brewer's yeast were stirred in, and the mixture boiled for fifteen minutes. The latter step was found to be unnecessary if the medium were autoclaved since the only reason for boiling the mixture was to kill the yeast. Five hundred ml. of cornmeal were mixed thoroughly with 500 ml. of water and then combined with 500 ml. of dark corn syrup. The cornmeal-syrup mixture was stirred into the hot agar-yeast mixture and the resulting medium was immediately poured into clean flasks. Filter papers were added and the flasks plugged with cotton and autoclaved.

Soil Extracts

Soil extracts, prepared by the Agricultural Chemistry Research Department, were made by extracting 500 gm. of soil with 500 ml. of benzene under agitation in a 1/2 gallon jar. The benzene was filtered and stored under refrigeration until used.

In order to standardize the procedure, the benzene extracts of soil in early experiments were either evaporated or diluted to a concentration of 40 gammas per ml. In later tests extracts were used in such quantities to produce equivalent amounts of insecticide instead

of dilution or concentration. These procedures reduced to a minimum the necessary number of controls and standards. Since equal amounts of insecticide were used under each of the two methods, mortality readings could be compared directly.

As indicated in the results, in certain of the tests the soil extracts were treated with 0.2 per cent sodium hydroxide at a rate of one part in two parts of extract. The mixture was placed in a separatory funnel, shaken briskly, and clipped onto a stand to allow the precipitate to settle out. The hydroxide solution appeared to saponify the foreign material and precipitated it as a curd-like substance which was neither soluble in benzene nor in water. After settling, which required about five minutes, the precipitate and sodium hydroxide were drawn off. Usually the entire process had to be repeated two or three times, depending on the quantity of foreign matter involved. When the process was completed, the cleared extract was drawn off and stored in a suitable container. In most cases the container was a 250 ml. flask with a ground-glass stopper. Extracts were kept under refrigeration to reduce evaporation to a minimum.

Standards

Standards, as defined in this paper, were solutions of technical toxaphene and benzene or technical DDT and benzene. The stock standard solutions were prepared by weighing by difference a small amount of insecticide on an analytical balance and adding enough c.p. benzene to make 100 ml. of solution. The concentration of insecticide in the stock solution did not make any difference as all reduction in gammas per ml. were made by dilution rather than by attempting to

weigh a specific amount of insecticide. The base level for standards was 20 or 40 gammas per ml. depending on the type of experiment.

Soil Standards

Soil standards were prepared by blending enough insecticide into 66 cubic inches of an insecticide-free soil to produce in the laboratory the equivalent of freshly treated soil for each application rate used in the weathering experiment. This insecticide was applied to the soil in the form of water suspension of an emulsifiable concentrate. This soil was extracted immediately by the staff of the Agricultural Chemistry Research Department. The insecticide content in gammas per ml. was computed from the amount of insecticide applied. The base level for all soil standards was 40 gammas per ml.

Incubators (Plates 3 and 4)

Two incubators were used during this work. A large one fitted with glass doors and heated by a 500 watt heating element was used for rearing flies. Insecticide was excluded from this incubator. For maintaining test vials or dishes at a constant temperature, a smaller incubator which was heated with a 100 watt bulb was used. Both cabinets were maintained at a constant 76°F. ± 2°F.

Special Equipment

In conducting this experimental work several pieces of special equipment were needed to facilitate handling of the flies. The author constructed the necessary equipment using sheet plastic,[1] acetone and scissors. This section is devoted to a description of the equipment which proved useful in this work.

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1 Plastacele, 0.02 inch thickness, E.I. du Pont de Nemours Co.

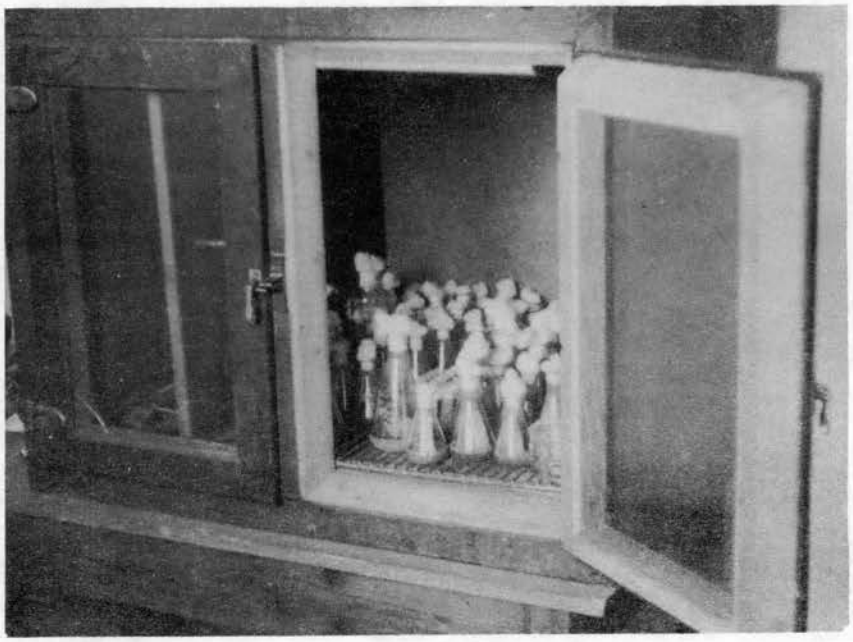


Plate 3. Large incubator containing culture flasks.

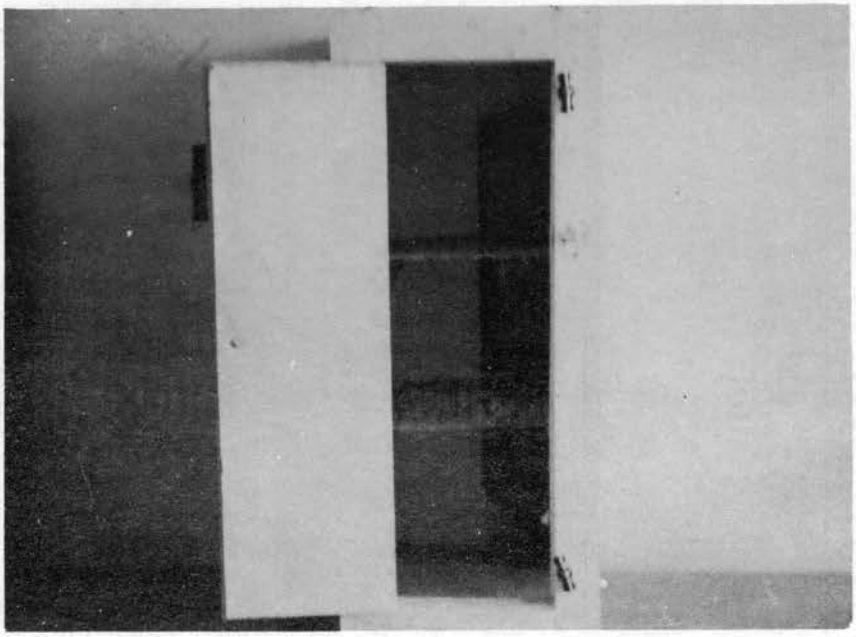


Plate 4. Small incubator used for holding tests at a constant temperature.

Transfer Cone (Plate 5, Figure 1)

A device was constructed to transfer flies from one container to another. It was a cone 13 inches long, four inches in diameter at the large end and three-eighths inch at the apex. The large end was covered with a sheet of plastic which was sealed in place and trimmed to fit. A plastic cylinder two inches in diameter and two and one-half inches long was attached to the plastic sheet which covered the end of the cone. Subsequently a hole was cut in the end of the cone to match the inside diameter of the cylinder. This formed an opening large enough to allow the necks of the culture flasks to be inserted. This device would have been just as efficient if it had been smaller.

Trapping Cylinder (Plate 6, Figure 2)

The trapping cylinder was a plastic cylinder 10 inches long and one and one-half inches in diameter. One end was fitted with a small plastic cone which had a vertex opening of three-eighths of an inch. The opposite end of the cylinder was also fitted with a plastic cone which was inserted so that the vertex was inside the cylinder. This cone had a vertex aperture of one-eighth inch. This device was used for the same purpose as the transfer cone. It was especially useful for transferring flies without the aid of carbon dioxide because the small aperture of the cone inside the cylinder permitted few if any of the flies to escape.

Counting Chamber (Plates 7 and 8, Figure 3)

The counting chamber was a plastic cylinder five inches long and three and one-half inches in diameter with a spout attached to the lower side of a sloping floor placed midway between the extremities of the cylinder. Carbon dioxide was introduced through a

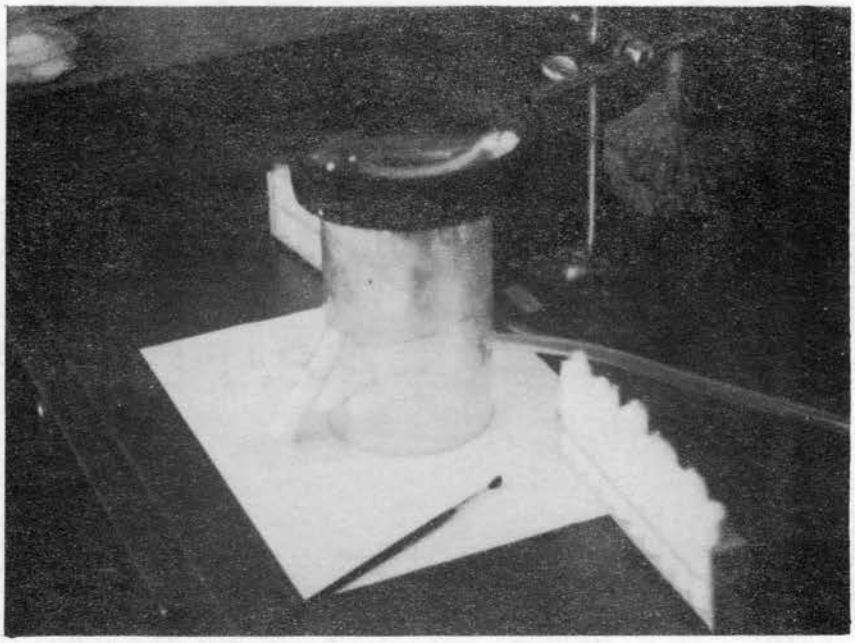


Plate 7. Counting chamber. (side view)

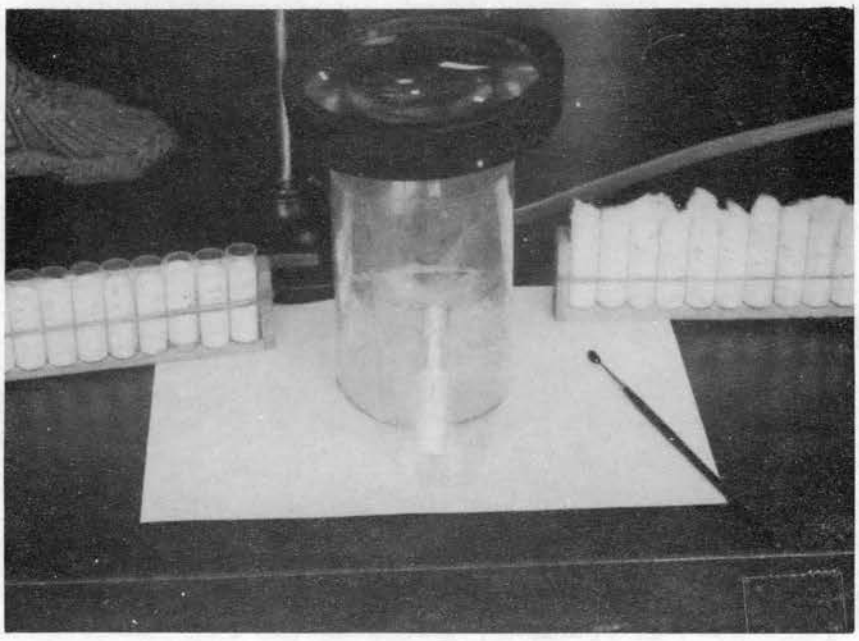
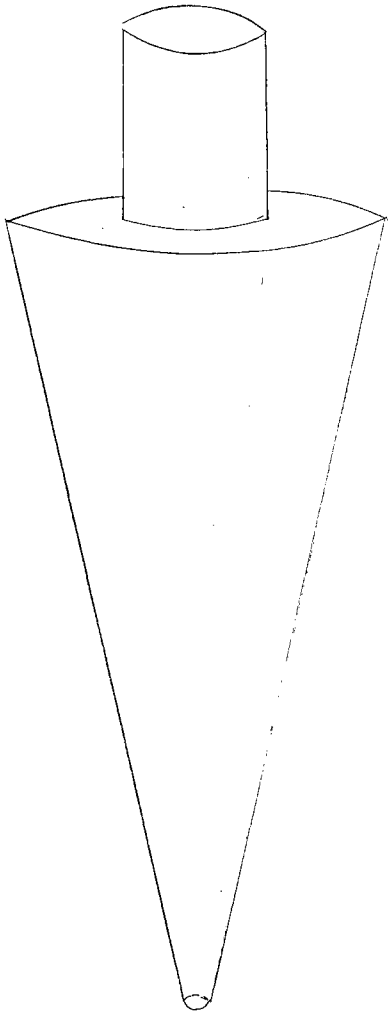
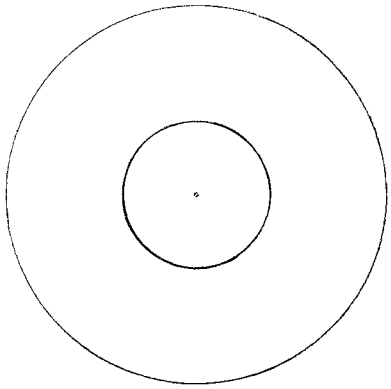


Plate 8. Counting chamber. (front view)



Side view



End view

Figure 1. Transfer Cone.

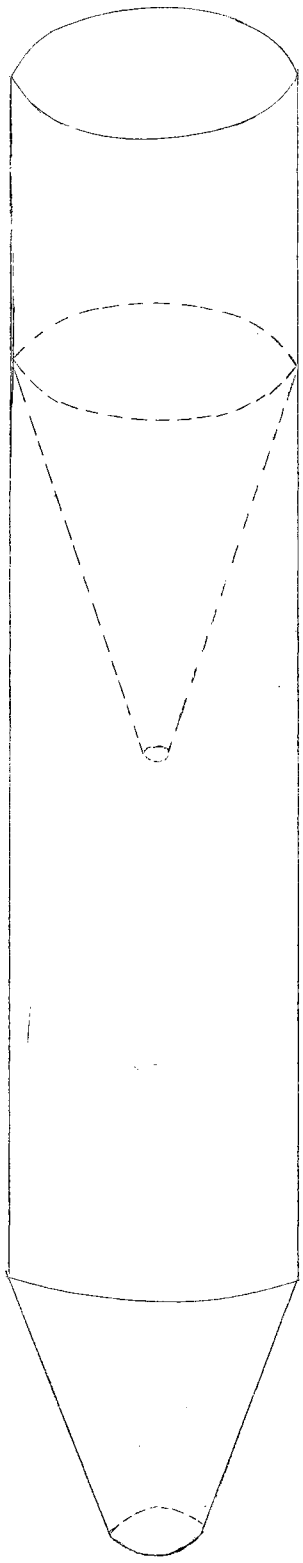


Figure 2. Trapping Cylinder.

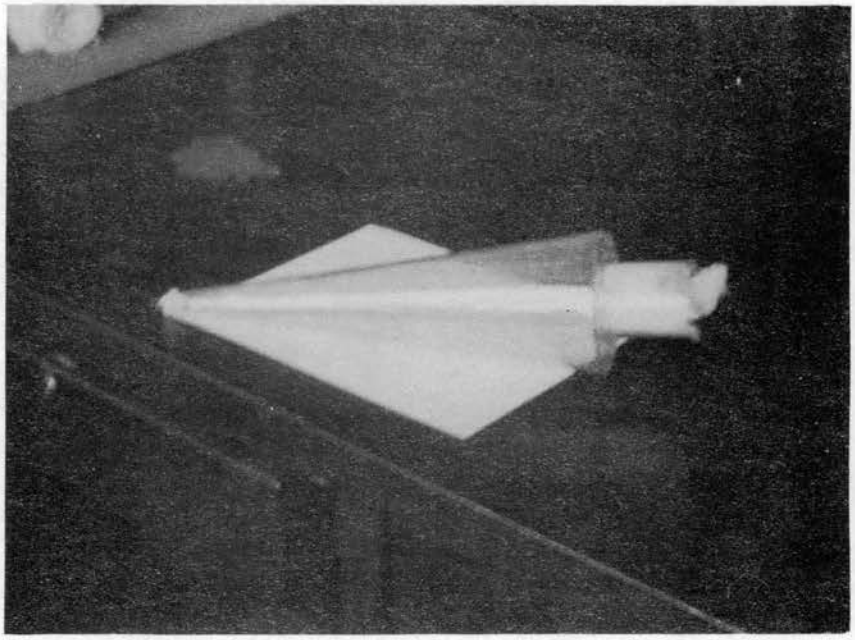


Plate 5. Transfer cone.

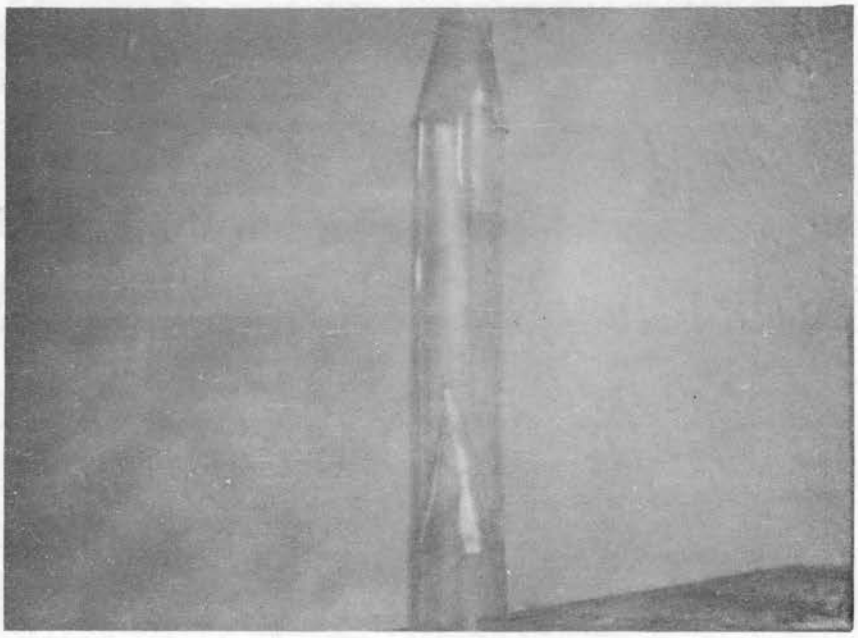
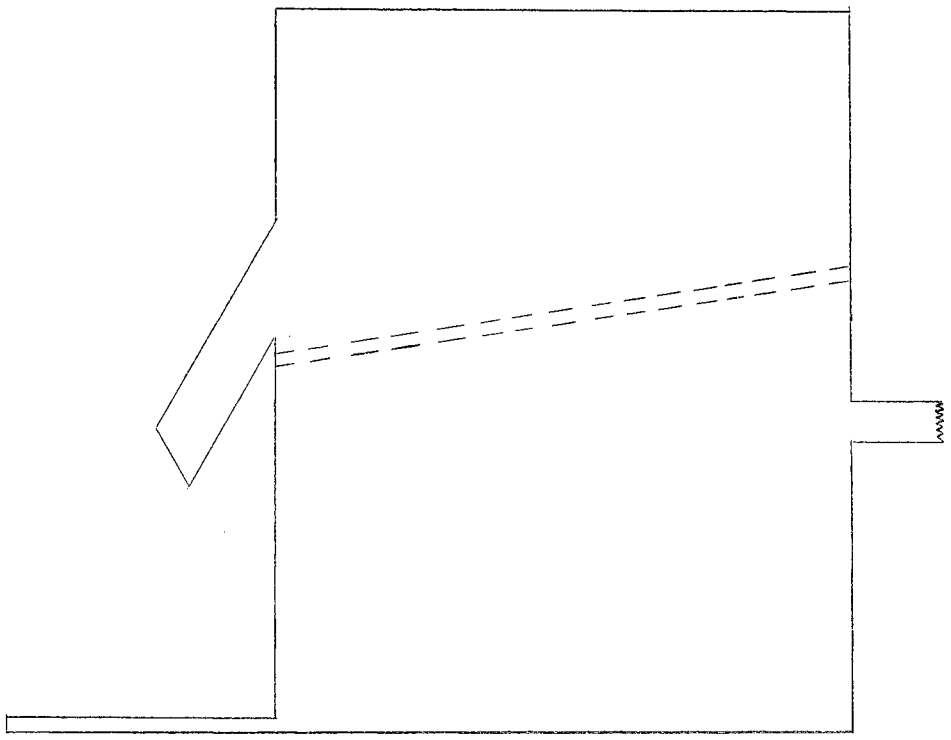
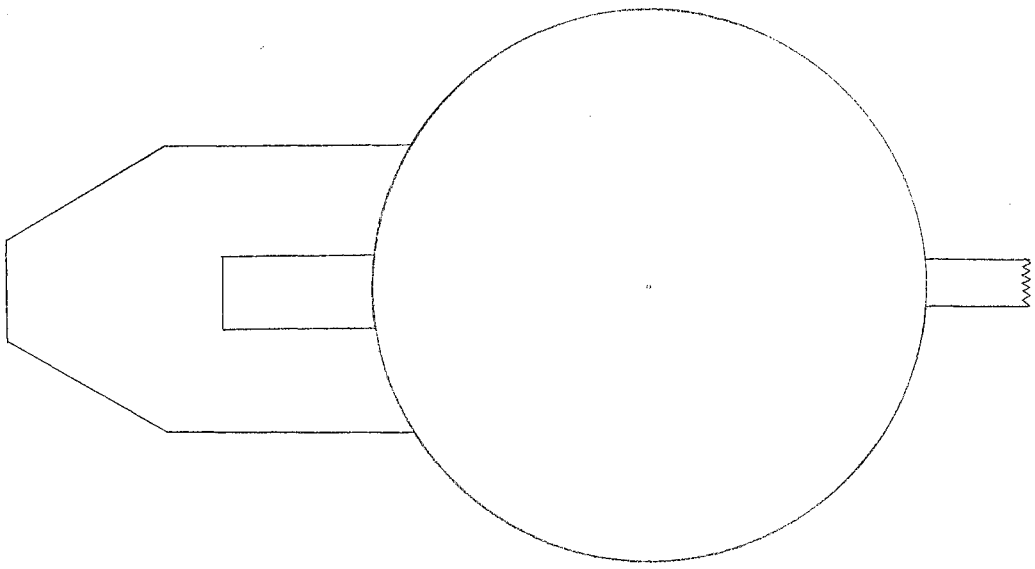


Plate 6. Trapping cylinder.



Side view



Top view

Figure 3. Counting Chamber.

hole in the side of the cylinder under the floor. The gas flowed upward through two holes in the high side of the sloping floor and downward over the anesthetized flies.

CHAPTER IV

RESULTS

Experiments with Vials

In early tests soil extracts as furnished by the chemist and not modified in any way were tested in vials. The results of a total of 12 tests and 4,200 flies are given in Tables I and II.

It is notable that with treated vials a wide range of toxicity appears among the different extracts and standard solutions (Table I). The toxicity range was from 1.6 per cent mortality at twelve hours with the 10 lb. extract to 77 per cent mortality at 12 hours with the 100 lb. extract. The 50 lb. DDT rate showed a 34.3 per cent mortality at twelve hours. Both the toxaphene and DDT standard solutions approached 100 per cent mortality by or before twelve hours. These figures show a marked trend of increasing toxicity with increasing application rates despite considerable variation.

The tests using treated filter paper in vials did not show the sharp increase in toxicity with an increase in application rate that was apparent in the treated vials, but a moderate trend of increasing toxicity was exhibited except at the 20 lb. level (Table II). This extract was a replacement and not of the original series and an error in chemical analysis is indicated because toxicity of this magnitude was not encountered with 20 lb. extract at any other time. The treated filter paper in vials showed a closer relationship between the standard solution and the extracts of the higher application rates, which was a better indication of the truth than the

TABLE I

Percentage mortality[1] of D. melanogaster in vials[2] treated with soil extracts and standard solutions, January-February, 1955.

	Toxaphene					DDT		
Test No.	Annual Application Rates 10 lbs.	20 lbs.	50 lbs.	100 lbs.	Standard solution	Ann. Ap. Rate 50 lbs.	Standard solution	No treatment
1	0	0	50	82	100	70	100	0
2	2	2	54	72	100	18	100	0
3	0	0	4	50	100	28	100	4
4	0	0	14	80	100	22	94	0
5	8	2	48	90	100	36	100	0
6	0	0	16	88	100	32	98	0
Average	1.6	0.6	31.0	77.0	100	34.3	98.6	0.6

1 An average of five replicate vials at 12 hours after treatment.

2 Treated with 10 gammas of insecticide and containing 10 flies each.

TABLE II

Percentage mortality[1] of D. melanogaster in vials containing filter paper[2] treated with soil extracts and standard solutions, January-February, 1955.

Test No.	Toxaphene: Annual Application Rates 10 lbs.	20 lbs.	50 lbs.	100 lbs.	Standard solution	No Treatment
1	27	93	35	82	93	8
2	34	100	76	88	78	22
3	30	82	60	53	79	11
4	14	40	33	72	29	7
5	20	60	17	42	66	7
6	55	90	79	78	88	5
Average	30.0	70.5	50.1	69.1	72.1	10.0

1 Based on an average of five replicate vials containing 20 flies each at 12 hours after treatment.

2 Treated with 10 gammas of insecticide.

relationship between the standard solutions and the extracts of the higher application rates in treated vials. For example, in tests with filter paper (Table II) the average per cent mortality for the standard was 72.1 and that for 100 lbs. was 69.1 while the corresponding figures in tests with vials only (Table I) were 100.0 and 77.0, respectively.

Neither the tests with treated vials nor the tests with treated filter paper in vials were satisfactory because both were subject to extreme variation. Tests were conducted to determine if the variation encountered in previous experiments was due to a difference in the quantity of residue in vials or to a variable population. On three consecutive days, vials which had been treated only once were charged with three different samples of flies. Tests 1, 2, and 3 (Table II) were made in the vials on the first, second, and third days, respectively. Similar results are shown in tests 4, 5 and 6. It is evident that even the averages of five replicates show variation up to 50 per cent. These data show that the variation is not due to differences in residue deposits.

Since the holding incubator was heated with a light bulb, tests were conducted to determine if the phototropic response of the flies was a factor of variation. The results obtained when the flies were kept in darkness are given in tests 4-6, Table II. It can be noted that this made no difference. The factors of variation were not specifically known, but were thought to be in the population.

The last resort was a change in the entire procedure which involved the use of large samples of unsexed flies. It was observed in previous

counting operations that the distribution of males and females was reasonably constant with the females slightly more numerous than the males. This normal distribution apparently nullified the difference in susceptibility between the males and females. It has been reported that the males are more variable than the females (Crow, 1953); however, any variation due to this factor would be more than balanced by the time factor involved in the counting and sexing technique. The variation with this procedure was less than 15 per cent.

Experiments with Petri Dishes

The results of four experiments, utilizing petri dishes as test container in which 27,570 flies were used are shown in Tables III and IV.

These experiments were set up with extracts treated with 0.2 per cent sodium hydroxide and extracts not treated and were tested simultaneously. This procedure was calculated to show the value of sodium hydroxide treatment. These data were analyzed by computing an F[1] value for the mortalities obtained for both the treated and untreated extracts (Tables VII and VIII). The F value for the fly mortalities where treated extracts were used showed that none of the annual application rates were significantly different from the standard solutions except at the 5 lb. level. This difference was obviously significant without a statistical analysis. On the other hand a significant F value was computed for the fly mortalities where untreated extracts were used (Table IV). This indicated that masking was apparent at the lower

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1 Computed as outlined in Statistical Methods by Snedecor (1946).

TABLE III

Percentage mortality[1] of D. melanogaster in petri dishes containing filter paper[2] treated with soil extracts and standard solutions which were treated with 0.2 per cent sodium hydroxide, April, 1955

Replicate No.[5]	Toxaphene Annual Application Rate 5 lbs.	10 lbs.	20 lbs.	50 lbs.	100 lbs.	Standard solution	DDT Ann. Ap. rate 50 lbs.	DDT Standard solution	No treatment
1	100	43	63	57	56	49	26	54	6
2	100	62	60	44	60	53	26	49	8
3	100	45	56	62	57	59[4]	38	50	7
4	100	45	55	47	54	51[4]	38	54	7
5	100	53	55	53	54	53	37	47	9
6	100 [86][3]	55	55	60	58	61 [5][3]	35	63	9
7	100 [80]	54	57	60	55	61 [7]	40	61	8
8	100 [91]	59	55	61	60	59 [5]	47	58	9
9	100 [94]	60	54	53	60	57 [6]	45	54	6
10	100 [87]	56	53	57	59	49 [6]	39	53	5
11	100 [91]	58	56	54	56	56 [6]	36	52	2
12	100 [88]	56	56	54	52	54 [8]	36	56	5
13	100 [94]					54 [11]			
Average	100 [89]	53.8	55.4	55.1	56.7	55.0[6.8]	36.9	59.2	6.7

1 Based on reading at 15 hours.

2 20 gammas of insecticide divided two-thirds to the filter paper and one-third to the top of the dish.

3 Figures in parentheses based on readings at five hours.

4 Average of two replicates.

5 Approximately 200 flies per replicate [dish].

TABLE IV

Percentage mortality[1] of D. melanogaster in petri dishes containing filter paper[2] treated with soil extracts and standard solutions, April, 1955.

Replicate No.[5]	Toxaphene Annual Application Rate 5 lbs.	10 lbs.	20 lbs.	50 lbs.	100 lbs.	Standard solution	DDT Ann. Ap. Rate 50 lbs.	Standard solution	No treatment
1	28	39	50	60	60	51	24	54	7
2	36	39	57	48	64	53	18	47	9
3							25	56	
4	40	54	53	59	58	61[3]	34	63	9
5	40	54	51	60	60	58[3]	41	61	6
6	44	50	52	51	49	52[4]	31	53	5
7	37	48	48	54	52	54[4]	28	52	3
Average	37.5	47.3	51.8	55.3	57.0	54.8	32.8	55.1	6.5

1 Based on reading at 15 hours.

2 20 gammas of insecticide divided two-thirds to the filter paper and one-third to the top of the dish.

3 Average of three replicates.

4 Average of two replicates.

5 Approximately 200 flies per replicate [dish].

annual application rates. The mean mortality of the 5 lb. level was 37.5 per cent as compared with 54.8 per cent for the standard solution, a reduction of 41 per cent because of masking. An indication of this phenomenon was shown by the mean value increasing with the application rate.

The DDT extracts were compared with their standards as a paired experiment by computing a t[1] value. Both the mortalities for the treated and untreated extracts were significantly lower than the standard solutions at the 5 per cent level. However, that of the untreated extract was significant at the 2 per cent level which indicates that a slight masking effect may be incurred with the untreated extract.

An F was computed for the data on the soil standards (Tables V & IX) which showed that differences among annual application rates were not present. This infers that there was no initial breakdown or tie up of toxaphene or DDT in the soil. Masking did not occur with these extracts even though they were not treated with 0.2 per cent sodium hydroxide because the soil used to make up the standards was oven dried. It was found that the process of drying in an oven removed masking effects. A subsequent experiment was conducted to prove that soil from the same vicinity would contain materials which would mask the presence of fresh insecticide (Table VI). The 5 lb. level was chosen because masking effect was most marked when the insecticide concentration was low in an extract. This extract proved to be about 50 per cent more toxic when treated with 0.2 per cent sodium hydroxide.

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1 Computed as outlined in Statistical Methods by Snedecor (1946).

TABLE V

Percentage mortality[1] of D. melanogaster in petri dishes containing filter paper[2] treated with soil standards[3] and standard solutions, March, 1955.

Replicate No.[4]	Toxaphene Annual Application Rate 5 lbs.	10 lbs.	20 lbs.	50 lbs.	100 lbs.	Standard solution	Ann. Ap. Rate 50 lbs.	Standard solution	No treatment
1	49	56	59	52	47	54	49	56	3
2	53	48	44	46	53	52	53	49	1
3	46	51	48	50	51	55	52	55	0
4	52	44	49	47	55	51	50	49	4
5	53	54	53	55	43	55	51	54	2
6	51	52	51	52	54	48	46	52	5
7	47	46	47	45	54	54	47	51	3
8	53	50	52	53	49	44	48	45	0
9	52	44	51	55	46	51	52	53	1
10	46	50	52	51	58	52	49	50	5
11	51	51	46	50	49	51	52	49	3
12	55	53	49	47	48	46	51	52	2
Average	50.6	49.9	50.0	50.2	50.5	51.0	50.0	51.2	2.3

1 Based on reading at 12 hours.

2 20 gammas of insecticide divided two-thirds to the filter paper and one-third to the top of the dish.

3 Extracts of freshly treated soil.

4 Approximately 200 flies per replicate [dish].

TABLE VI

Percentage mortality[1] of D. melanogaster in petri dishes containing filter paper[2] treated with 5 lb. level of soil standards[3], May, 1955.

Replicate No.	5 lb. extract not treated with sodium hydroxide	5 lb. extract treated with sodium hydroxide	Standard solution	No treatment
1	17	57	44	4
2	26	44	59	2
3	24	53	55	3
4	30	49	49	5
5	26	52	52	1
6	21	50	47	6
7	19	46	56	3
8	22	56	51	4
9	25	53	53	5
10	20	51	48	2
Average	23.0	51.1	51.4	3.5

1 Based on a reading at 15 hours.

2 20 gammas of insecticide divided two-thirds to the filter paper and one-third to the top of the dish.

3 Extracts of freshly treated soil.

4 Approximately 200 flies per replicate [dish].

TABLE VII

Analysis of variance for data presented in Table III.

Source of Variation	D.F.	S.S.	M.S.
Individuals	59	1212	
Replication	11	61	5.5
Error	48	1151	23.9

F = 0.230

TABLE VIII

Analysis of variance for data presented in Table IV.

Source of Variation	D.F.	S.S.	M.S.
Individuals	35	2387	
Replication	5	1603	320.6
Error	30	784	26.1

F = 5.255

TABLE IX

Analysis of variance for data presented in Table V.

Source of Variation	D.F.	S.S.	M.S.
Individuals	71	1288	
Replication	11	11	1
Error	60	1277	21.2

$F = 0.047$

DISCUSSION

The data presented in Tables I and II cannot be used in an evaluation of toxaphene residues as later tests with extracts treated with sodium hydroxide have proved that the deviations among the extracts of different application rates were caused by masking entities. In these vial tests it can be noted that the standard solution of toxaphene showed more toxicity than the higher application rate extracts (Table I). This condition does not appear in the later experimental work. In these tests the residue deposit was limited to the bottom of the vial. It is thought that the great concentration of the residue magnified the effect of the masking factors.

Masking of the insecticidal properties of toxaphene in benzene extracts of the soil have been shown to occur (Tables I, II, IV, and VI). Terriere and Ingalsbe (1953) have reported the masking agents in their tests to be oils, fats, and waxes from the soil which could be removed by 0.2 per cent sodium hydroxide. This treatment was effective in removing masking effects in this work. No masking effect was found in the first tests on soil standards (Table V); however, the soil in these tests was given the special treatment of oven drying. A later experiment prepared from soils that were not oven dried exhibited masking qualities (Table VI). Since masking effects occurred in tests with extracts of freshly treated soil samples, it appears that time is not a factor in the production of these entities. These factors seem to be oils, fats, and waxes as identified by Terriere and Ingalsbe (1953) that may occur normally in soils. This is supported by evidence that

these factors may be eliminated by heat or by sodium hydroxide treatment. The degree of masking was inversely correlated with the rates of application. It would seem that this correlation occurred because the extracts were used in amounts to produce equivalent quantities of insecticides; larger volumes of extract and therefore, larger amounts of masking entities were added to the test containers in the lower application rate treatments. Effective masking was incurred when the extract contained less than 15 gammas per ml. or 15 p.p.m.

The p.p.m. of insecticide were computed according to the biological assay for all sodium hydroxide treated extracts. The p.p.m. of insecticide were calculated on a ratio between the mortalities of the standard solutions and that of the extracts (Table X). Significant differences were found only at the 5 lb. toxaphene and 50 lb. DDT levels.

The reason for the extreme toxicity of 5 lb. toxaphene level has not been determined. The calculated toxicant content for this extract was 10 p.p.m., which was equal to one-third of the total insecticide added to the soil in three applications. This high toxicity does not appear to be an accurate measurement of the insecticide remaining at the 5 lb. level because higher application rates show lower insecticide concentrations. This can be noted in Tables III and IV, which present the results of tests all made simultaneously. It has been shown with a 5 lb. soil standard (Table VI) that masking accounted for a reduction in toxicity of approximately 50 per cent. In view of these records, little weight should be given to the abnormally high toxicity figures for the treated 5 lb. extract (Table III). It is not

TABLE X

P.p.m. of insecticide in the soil as determined by chemical and biological assays of soil extracts.

Insecticide residue in soil of Lake Carl Blackwell		
Annual application rate pounds per acre	Chemical analysis toxicant p.p.m.	Biological analysis toxicant p.p.m.
5	0.9	10
10	2.9	2
20	3.8	3
50	15.0	15
100	23.9	24
DDT	14.7	9

thought that masking or an error in chemical analysis could account for this situation.

Except for these levels all of the levels treated with sodium hydroxide were not different from each other or from the standard, which indicates that toxaphene does not change into non-toxic chlorine bearing compounds and that an analysis for total organic chlorides would be an effective measure of the toxaphene present in soil except at very low levels of insecticide concentration.

It was found in these experiments that the soil extract of DDT was significantly less toxic than the DDT standards. This trend suggests that DDT may change into non-toxic organic chlorides or into less toxic isomers.

SUMMARY

Since the total organic chlorides analysis had been used to measure the residues of toxaphene and DDT present in insecticide weathering plots, it was desired to test by a biological assay the accuracy of this method. This was accomplished by testing the toxicity of residue deposits from benzene extracts of the soil with Drosophila melanogaster Meig. A total of 21 experiments, utilizing 55,078 flies were made.

Shell vials 15 x 50 mm. were found to be unsatisfactory because the sample size was limited by the small containers. This was a disadvantage since the population of flies was very variable. Petri dishes combined with samples of approximately 200 flies produced reasonably good results.

A masking effect on insecticide toxicity was detected in extracts of insecticide weathered in soil or extracts of freshly treated soil where the insecticide contained in the extracts was less than 15 p.p.m. These masking entities gave a yellow color to the benzene extract and were thought to be oils, fats, and waxes which normally occur in the soil. This was substantiated by the fact that masking was removed by washing the extracts with 0.2 per cent sodium hydroxide and by oven drying the soil.

The bioassay agreed with the chemical analysis of toxaphene at all application rates with one exception. The bioassay data for the 5 lb. application were contradictory, but it is thought that the chemical analysis was also reliable for this level. The DDT soil extract showed significantly less insecticide than was indicated by chemical analysis.

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