THE IMPORTANCE OF THE LOCUS OF APPLICATION ON THE EFFECTIVENESS OF DDT AS A CONTACT INSECTICIDE FOR THE HOUSEFLY, MUSCA DOMESTICA L. (DIPTERA: MUSCIDAE)

by

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A THESIS

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I. INTRODUCTION.

DDT in its own right and as a typical representative of that class of insecticides now known as chlorinated hydrocarbons has proved one of our most important and interesting insect toxicants. Its mode of action though extensively studied still eludes us. The advent of phenomenally DDT-resistant housefly populations, in the short space of three years after its general use, has posed a challenging problem to be explained and has caused considerable anxiety as to what practical difficulties the development of widespread insect resistance to insecticides may bring about. Any study of the mode of entry and action of DDT on houseflies should, therefore, contribute worthwhile information.

The writer has limited himself to studying the importance of the locus of external application on the effectiveness of DDT as a contact toxicant. A survey of the literature failed to reveal what seemed adequate techniques for such a study. Much of the work reported here is, therefore, on newly designed and modified techniques for measuring very small amounts of insecticide, for breeding highly standardized and uniform test animals, for handling and immobilizing test animals and for applying treatments.

These new techniques have been applied in a logical series of thirteen experiments involving in all several thousand test flies handled individually. The results show that the fly integument is not simply a barrier to be traversed, as has been held in the past, but a tissue actively involved in the

phenomenon of DDT contact toxicity. The second feature that stands out is that haemolymph circulation beneath a locus of application governs the speed and possibly the intensity of action to be expected.

The new techniques and this revelation of the roles of integument and circulation in the action of DDT on houseflies are here claimed as original contributions to knowledge.

II. REVIEW OF THE LITERATURE.

Published information on techniques, rearing etc. are dealt with under appropriate headings to follow. The discussions of the results of the individual experiments contain references to most of the comparable experimental data found in the literature. It is therefore necessary here to mention only those papers which have stood out more or less as signposts to direct the lines of investigation. It is convenient to group these according to the locus of application studied.

A. REFERENCES TO THE EFFECT OF INSECTICIDAL APPLICATION.

a. On the legs.

Rajindar Pal (1950) reports that liquids spread more readily on the legs and antennae of Diptera than on the wings and other parts of the body, and thinks that this fact is important if the legs are the chief portal of entry of contact insecticides.

Potts and Vanderplank (1945) stated that the action of DDT can be as effective through contact with the feet as

through contact with the general integument on a large scale or via the spiracles. This appeared to hold true at least in species with well developed pulvilli.

Hickin (1945), on the other hand, claims that it is unlikely that the relative size of the pulvillus has any special significance other than that a larger pulvillus can hold a greater amount of insecticide and hence is more apt to retain a lethal dosage.

Hayes and Liu (1947) performed an histological examination of the tarsi of the housefly, the German cockroach and the Mexican bean beetle. Of these insects the housefly only was highly susceptible to DDT, and the housefly only possessed chemoreceptive sensilla on the tarsal segments.

Roeder and Weiant (1948) found the structures most sensitive to DDT in the cockroach to be the campaniform sensory cells of the trochanter, although all other sensory structures in the leg responded to the unstabilizing effect of DDT.

David (1946) stated that DDT collected by flies in insecticidal mists may be removed from the wings in the cleaning process and be absorbed through the legs.

b. On the wings.

David (1946) by adding Sudan III to the insecticide claimed to have traced DDT through the wings of the housefly. The dye either penetrated directly into the wings and later appeared in the Malpighian tubules from which it was excreted or, as already mentioned, may have been removed by the legs in the cleaning and have penetrated through the latter appendages. The

surface lethal dose was 6 µgm. per Kg. for males and 9 for females. He stated that the quantity collected on the wings was 3-4 times that collected on the body and contributed materially to the kill.

Lauger et al. (1946) applied a solution of DDT beneath the wings of fifty Musca domestica and at varying intervals killed the insects, dissected out the gut and Malpighian tubules, and assayed them for DDT. They obtained enough material from the carbon tetrachloride extracts to kill thirty flies. The DDT was said to pass through the epicuticula to the haemolymph, to be distributed by it to the nervous system and other organs and finally to be excreted through the Malpighian tubules into the hindgut.

Bodenstein (1946) claimed that the wings and legs of Drosophila virilis responded to DDT much more readily than the abdominal walls.

c. On the dorsal thorax.

Most insecticide screening techniques developed for use with small numbers of flies or roaches employ measured droplets of oil solution, emulsions or suspensions of insecticide dropped on the thorax from a calibrated loop or injection device. Menusan (1948) found that for Periplaneta americana the contact toxicity of DDT applied on the dorsal thorax was one-half that for blood-stream injection.

Yeager and Munson (1949) found that survival times for Musca domestica poisoned with DDT in corn oil applied to the dorsal thorax by means of a wire loop are hyperbolic, and

exhibit no region of inflection of the type reported for roaches injected with sodium metarsenite.

d. On the head.

Yeager and Munson (1945) injected a small amount of DDT in corn oil through a pinhole in the compound eye of a roach, Periplaneta americana, and found that it produced symptoms in the antennae but these did not appear posterior to the head. The heart had been cauterized to prevent cardiac circulation of the haemolymph.

Menusan (1948) reports that toxic doses for feeding are usually greater than those for contact.

e. On the central nervous system.

Bodenstein (1946) showed by isolation of the central nervous system from the portion of the body being treated that it was not necessary to the production of typical DDT responses.

Tobias et al. (1946) found that during the late prostrate phase of DDT poisoning, but not during the early hyperactive phase, the free acetylcholine content of the central nervous system rises about 200 per cent in both the housefly and the cockroach. In the latter nearly all the rise took place in the connectives.

Wett (1947) found that sections of the central nervous systems of larvae, pupae, and adults of Calliphora erythrocephala, Lucilia caesar and Phormia spp. killed by DDT showed no morphological changes.

Roeder and Weiant (1946) state that DDT has no

significant action on the central nervous system.

Lauger et al. (1946) reported the recovery of sufficient DDT from the thoracic ganglia of fifty poisoned flies to kill an additional five flies. They found as well that the symptoms of intoxication increased considerably to a peak at 30 minutes following treatment and then decreased until the death of the insect. This was said to indicate that the destruction of the nervous system had already progressed so far that all physiological functions had to come to an end. The authors claimed, too, that this was accompanied by a vacuolization of the cytoplasm and a dissolution of the cell nuclei of the nerve cells.

Sternburg and Kearns (1950) claimed that all attempts by them to duplicate the findings of Lauger et al. (1946) resulted in failure, but they admitted that since the manipulations involved were exceedingly difficult to master, the failure might have been due to faulty technique on their part.

According to Richards (1945), all histopathological and cytopathological pictures recorded for insect nerves with the possible exception of chromatin clumping are to be classed as "postmortem" and their further analysis is of doubtful value.

f. On the peripheral nerves.

Bodenstein (1946) working with Drosophila virilis found that one per cent DDT emulsion injected into the abdominal cavity caused muscular spasms and eventual death of adult larval flies. He ligatured various portions of larval and adult abdomens to separate them from the central nervous system and then

showed that these parts still responded to DDT, apparently through the peripheral nervous system alone. Again, by first blocking the nerves with phenobarbital, he showed that DDT no longer could cause muscular contractions and concluded that DDT acted on nerves alone and not on the muscle fibres.

Tobias and Kollros (1946) claimed that in the cockroach low dosages of DDT may excite motor nerves reflexly by impulses fired into the ganglion over afferent fibres, whereas high dosages may act on elements on the motor side of the ganglion and thus not require an intact reflex arc.

Roeder and Weiant (1946) reported that DDT emulsion perfused through the cockroach leg in dosages as low as 0.01 ppm. gives high frequency trains of axon spikes in afferent fibres and that tremors are due to intense afferent bombardment of motor neurons.

Dresden (1948) claimed that the effect of DDT on the cockroach was one of facilitation of the passage of impulses through the synapses followed by a general synaptic block.

Welsh (1947) suggested that the primary action of DDT on the nerves was physical interference at the lipoid surface of the axon.

Yeager and Munson (1945) by injecting ten per cent DDT in corn oil into cockroaches found that the DDT caused tremors only in motor neurons somewhere along their length but not at the ganglion or neuro-muscular junctions. They claim it acts more readily on motor than on sensory fibres and can cause repetitive discharges somewhere along the motor fibres.

III. APPARATUS, TECHNIQUES AND REARING THE TEST ANIMAL.

A. MEASUREMENT OF THE INSECTICIDE.

1. HISTORICAL REVIEW AND DISCUSSION.

The measurement of minute quantities of fluids has in the past been done by syringes or graduated capillaries with micrometer screw mechanisms to control the output. Trevan (1922), Storey (1933), McIndoo (1937) and Beard (1949) all used syringes fitted with micrometer heads which would deliver, for five divisions, 0.00124 c.c. For injection work these were fitted with 27 or 30 guage hypodermic needles. In 1948, Heal and Menusan developed an injection apparatus which would deliver 0.000015 c.c. per unit of graduated micropipette. The latter was mounted in a vertical position. The top of it was connected by a U-tube to a vertical glass tube in which a mercury column could be regulated in height by the manipulation of a screw controlled plunger. An air column above the mercury separated it from the fluid being measured.

Both of these types of devices had drawbacks: the micrometer head on the syringe type had to be turned accurately and so for proper injection two persons were required, one to watch the injection and the other to measure the fluid. Again the limit of drop size dispensed with any degree of accuracy apparently was that corresponding to five divisions or 0.00124 c.c. With the machine of Heal and Menusan there was the problem of an air cushion between the fluid and the mercury. As was found with the microdrop machine of the writer, an air bubble, no matter how minute, precluded the possibility of producing

droplets with any degree of accuracy. If air was present, the fluid would continue to be expelled after the forward motion of the plunger had been stopped and then would immediately draw back up the capillary tube. Production of uniform droplets under such circumstances was quite impossible. However, if all air was removed from the system, quantities dispensed with consecutive forward motions of the plunger were identical in volume.

2. MICRODROP MACHINE.

The microdrop machine used consisted of a regulated propelling device and a modified tuberculin syringe (fig. 1).

a. Propelling device.

A Cambridge rocking microtome was used as a base. A piece of three-quarter inch board was securely fastened to the back of the upward portion of the frame and to it was bolted a hardwood block which was grooved and divided, as shown in fig. 2, to accommodate the syringe.

The ratchet consisted of a brass wheel with a notched edge containing 478 notches. From the centre of this projected upwards a threaded bolt of 24 threads to the inch. The lever arm which contacted the plunger of the syringe rested on a nut on this bolt so that it was raised or lowered by turning the ratchet wheel. The wheel was turned by a ratchet device fastened beneath it as shown in fig. 2. The number of notches advanced with each forward motion of the ratchet was regulated by a movable bolt supported on the base of the machine.

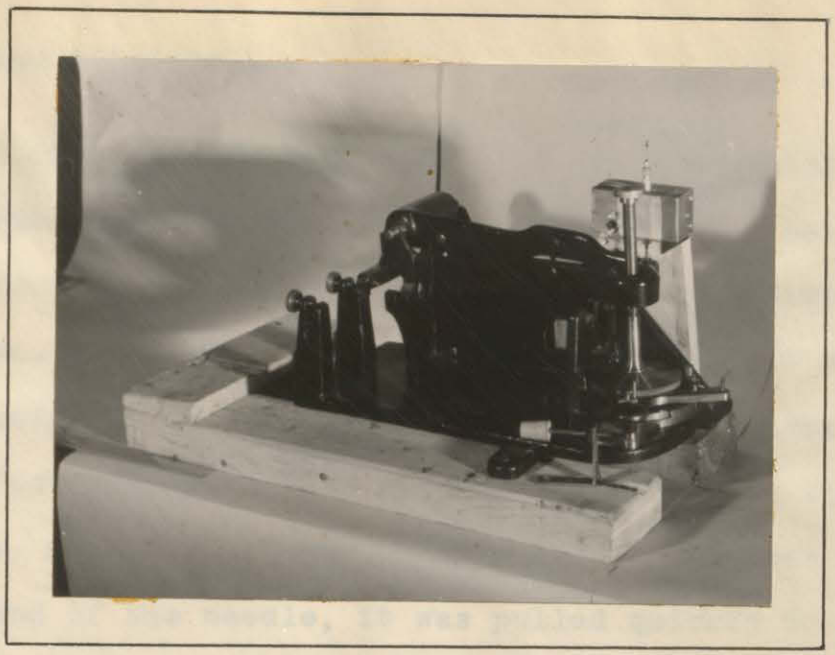


Fig. 1.- Cambridge rocking microtome frame converted into a microdrop machine.

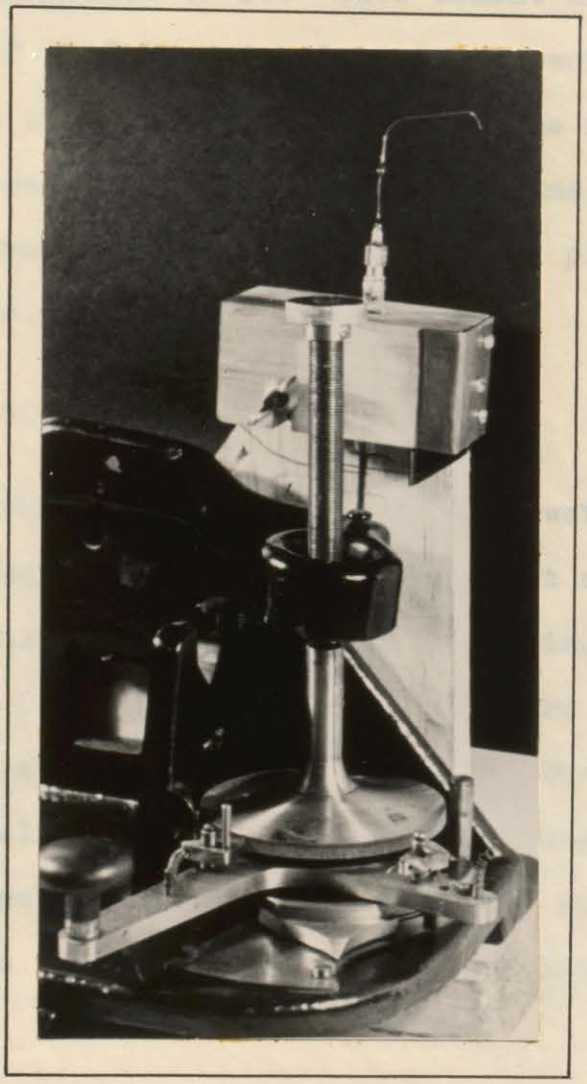


Fig. 2.- Ratchet propelling device and syringe holder of the microdrop machine.

b. Syringe and fittings.

A 0.25 c.c. tuberculin syringe was fitted with a 22 guage hypodermic needle. The ground glass joint was made benzene-tight with liquid solder applied in three layers, allowing each to dry in turn. The hypodermic needle was clipped off with a pair of tin snips and the end filed so that the bore was open. To this was fastened in a flame a piece of soft glass tubing of diameter 3 mm. and a bore of 1 mm. While the glass was still soft at the end of the needle, it was pulled quickly to form a fine capillary tube. The glass-metal joint was cooled slowly above the flame and while still quite hot a stick of "Hi-pyseal" cement was touched to it. This cement hardens at a high temperature of 300° F. and helped to prevent any cracking due to strains in the glass as it cooled. The fine capillary tubing was then bent in two places in the flame so that the tube pointed downwards when the syringe was placed in position on the machine.

c. Calibration of the machine.

The syringe was filled with water, secured in position, and the plunger advanced by the ratchet until the water came from the end of the capillary tube. Since the lever arm was pivoted at its base and described an arc at its distal end, the latter was travelling parallel to the syringe through only approximately 2 cm. Therefore, the plunger of the syringe had to be advanced until the lever arm was pushing in a direction parallel to the plunger. A clean weighing bottle was weighed

accurately and then used to catch the water forced from the syringe by twenty revolutions of the ratchet wheel. Calculation of the amount of water delivered by one notch of the ratchet wheel was as follows:

Weight of weighing bottle plus water - 12.76845 gms.
Weight of weighing bottle - 12.61935 gms.
Weight of water - 0.14910 gms.

The circumference of the ratchet wheel had 478 notches. Twenty revolutions represented 20 x 478 = 9,560 notches.

Advancing 9,560 notches delivered 0.14910 gms. of water.

Advancing 1 notch delivered 0.0000156 c.c. of water at 24°C.

d. Reproducability of droplet size.

To determine whether the successive droplets produced by the machine were of uniform size, two methods of measuring were used: (a) measurement in capillary tubes; and (b) measurement by photomicrograph.

In the former a piece of soft glass tubing was drawn out rapidly from a heated portion 1 cm. in length into a tube about 2-3 feet in length. One end was left at the original size to be held in the mouth to provide suction. The other end was broken off where its bore diameter was just slightly larger than the outside diameter of the end of the capillary tube of the delivery syringe. Using distilled water, drops were racked from the microdrop machine and drawn into the small end of the long capillary tube. When the tube was filled with droplets, it was fastened to a ruler or a piece of fine grid paper and the droplets drawn forward to a point marked on the scale. The droplet

could be kept stationary by pressure in the tube until its length was recorded from the scale. Successive droplets were drawn to the same mark and measured. Any irregularities in the diameter of the tube were not of any consequence since all droplets were measured in the same portion of it. From one such trial, using six notches on the ratchet wheel per droplet, the readings were as follows:

13.0 mm.	13.0 mm.	*12.5 mm.
13.0 "	13.0 "	13.0 "
*12.9 "	13.0 "	13.0 "
*13.5 "	13.0 "	13.0 "
*13.6 "	13.0 "	13.0 "
*12.0 "	*13.5 "	13.0 "

The readings marked with an asterisk were due to the formation of the droplets too close together with consequent leakage from one to the other; however they averaged out to equal the other uniform readings.

Measurement of single droplets representing one notch on the ratchet wheel was difficult owing to evaporation and to the problem of getting the water into the capillary tube cleanly for measurement. Larger drops drew away cleanly and evenly.

The second method was as follows: About 100 droplets of supersaturated DDT in benzene were formed on the surface of water in a receiving dish and placed on the stage of a dissecting microscope. A 100 watt lamp was placed at one side of the stage and a cylinder of white card stood upright over the dish so that air currents were removed; at the same time the light was reflected from the card providing an even illumination of the droplets. A 35 mm. Mercury model 1 camera was used to photograph the droplets. A one-quarter inch black card tube fitted over the lens placed it at the eye point so that it could be focussed for infinity. The uniformity of the droplets can be

seen in fig. 4 b.

3. PRODUCTION OF DROPLETS OF SUPERSATURATED DDT IN BENZENE.

In actual practice the water in the syringe was replaced by benzene which dissolved the DDT and permitted the production of supersaturated droplets.

Since solid DDT (1-trichloro-2, 2-bis (p-chlorophenyl) ethane) could not be placed and held accurately on the insect cuticle and since the benzene solution as it came from the microdrop machine spread over a large area, it was necessary to reduce the amount of benzene to a minimum without causing crystallization of the DDT. The surface used to catch the droplets from the machine had to have such a low affinity for the DDT solution that the drops could be removed to be placed on the insect. For this purpose water containing a minute quantity of "Aerosol", a surface active agent, proved satisfactory. As the surface of the water was brought to the end of the capillary tube, the DDT droplet formed there left the tube cleanly and spread out on the surface of the water. The benzene evaporated rapidly and the droplet shrank in size until the solution had a consistency about that of glycerine. Since the droplet was very difficult to see on the surface of the water through the binocular microscope, it was found that a mirror cemented to the bottom of the receptacle containing the water reflected the light of the lamp back through the droplet making it clearly visible. While in the process of formation the droplets were strongly attracted to each other, but when the benzene was evaporated,

the droplets appeared to take on similar electrical charges and were repelled; this permitted a large number of droplets to be formed without coalescence on the same water surface.

4. DETERMINATION OF THE ACTUAL AMOUNTS OF DDT AND BENZENE IN THE SUPERSATURATED DROPLETS.

Preliminary measurements of the output of the micro-drop machine were made at the beginning of the project and the concentration of the benzene solution of p, p'-DDT fixed at approximately 1.00%. Later on the exact concentration was calculated as 1.070%.

The amount of DDT and the percentage of benzene per droplet of supersaturated solution were determined as follows:

The syringe was filled with the DDT solution and a quantity forced out upon the collecting water surface by turning the propelling wheel through forty revolutions. This volume represented 40 x 478 or 19,120 notches on the wheel and equalled 19,120 x 0.0000156 c.c. or 0.2983 c.c. When the solution had shrunken by evaporation of the benzene to a small supersaturated droplet, it was taken up on a glass rod, seeded out to a crystalline mass and then weighed. Seeding was done by rubbing a very fine glass filament over the surface of crystalline DDT and then touching it to the supersaturated droplet. The seed crystal picked up in this procedure was too minute to see under high power magnification. The DDT was then dissolved away in benzene and the glass rod weighed again. Two weighings gave the following:

	No. 1	No. 2	Average
DDT plus rod -	0.0245 gm.	0.0247 gm.	0.0246 gm.
Rod -	0.0214 gm.	0.0214 gm.	0.0214 gm.
DDT -	0.0031 gm.	0.0033 gm.	0.0032 gm.

Weight of DDT in one droplet representing one notch on the ratchet wheel = 0.0031 ÷ 19120 = 0.000000167 gms. = 0.167 γgm.

A third quantity of 1.07% solution of DDT in benzene was evaporated on water to the supersaturated state and then taken up on the glass rod for weighing. The amount of benzene in the supersaturated solution was determined thus:

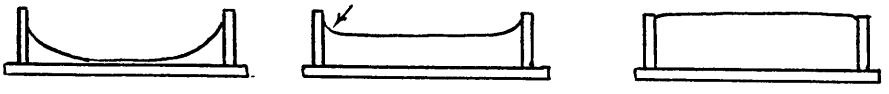
Weight of rod plus supersaturate -	0.0254 gm.
Weight of rod plus DDT -	0.0250 gm.
Weight of rod -	0.0215 gm.
Weight of benzene -	0.0004 gm.
Weight of supersaturate -	0.0039 gm.

Per cent benzene = $\frac{4}{39}$ x 100 = 10.26

Actual amount of benzene per unit droplet = 0.0191 γgm.

5. EFFECT OF THE QUANTITY OF SURFACE ACTIVE AGENT AND THE SHAPE OF THE MENISCUS ON THE FORMATION OF DROPLETS ON WATER.

In preparing the water surface to receive the droplets of benzene solution of DDT, one drop of a 1.0% solution of "Aerosol" was shaken up with 100 c.c. of distilled water and poured into the glass vessel until the meniscus tended to flatten out, as shown in figs. 3 a, b and c.



a b c

Fig. 3.- Cross-section diagrams of a droplet receiving vessel showing three depths of water and the meniscus shape for each.

A delicate balance existed between the concentration of the surface active agent and the meniscus shape. When the concentration of "Aerosol" was too great the droplet remained piled up as on a waxy surface and while evaporating very slowly tended to be attracted to and to coalesce with those already formed. If too low, the droplet spread instantaneously over the whole water surface and disintegrated into a film of minute particles. Using the ideal concentration, a droplet placed on the sloping portion of the meniscus (fig. 3 b, arrow) spread quickly into an irregularly shaped triangular film with its apex toward the centre of the vessel. This film then gradually decreased in area to a tiny circular, viscous bead which was repelled by similar ones as it was pulled by gravity towards the centre. While the benzene was evaporating, the flat film exhibited a clean smooth edge and brilliant diffraction colours. If the meniscus was as in fig. 3 a, the surface forces were so strong that it was almost impossible to form a supersaturated drop even with the ideal concentration of "Aerosol". If the meniscus was as in fig. 3 c, spilling of the water usually occurred or the drops wandered to the side of the vessel and adhered to the glass. In the latter case crystallization was apt to ensue and this in turn would disrupt the surface forces

and prevent proper droplet formation.

Under ideal conditions droplets could be placed on the edge of the meniscus at the rate of one every second and a half and these travelled toward the centre to lie in a tight group as shown in fig. 4. Any draft of air prevented the formation of droplets regardless of the amount of care taken with all other factors.

B. APPLICATORS FOR SUPERSATURATED MICRODROPS OF DDT IN BENZENE.

The most convenient applicator was made from soft glass rod or tubing 3 mm. in diameter and 10 cm. in length. One end was rounded off and the other drawn out to half the original diameter. The latter was cut off 0.5 cm. from the base and with the aid of a binocular microscope and a micro-flame was drawn out into a fine flexible filament 1 mm. in length and only a few microns in diameter. The micro-flame used for drawing the final filament was made by attaching a No. 27 hypodermic needle to the gas line by a piece of rubber tubing without any air intake. The flame could be reduced to 1-2 mm. in diameter for making very tiny filaments.

1. USE OF THE APPLICATORS.

Generally when a wire loop or glass loop is used with oil solutions or pure liquid materials a small amount adheres to the applicator and serves as an extra source of error. However, by using the supersaturated solution of DDT in benzene the droplet became spherical when taken from the water and came

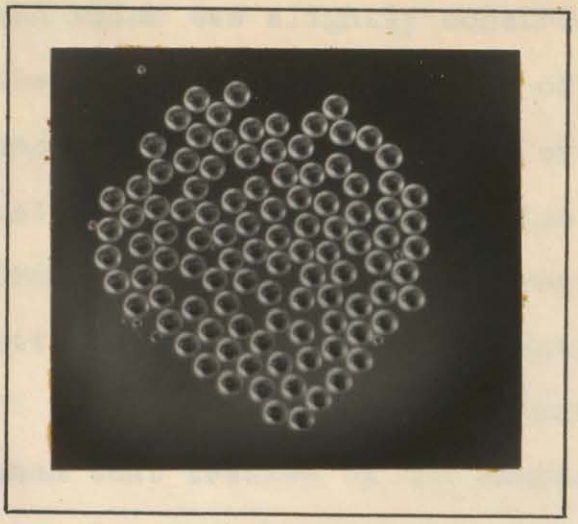
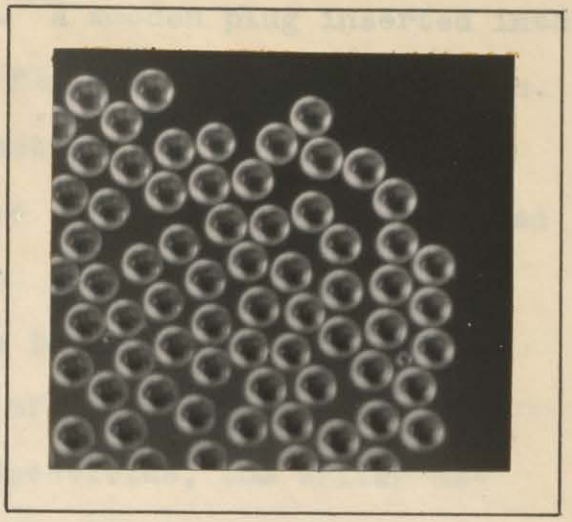


Fig. 4 a.- Photomicrograph of a large group of supersaturated droplets on a water surface.



b.- Same as fig. 4 a enlarged to show uniformity of droplet size.

away cleanly from the applicator when touched to any surface. The problem of adhesion of the droplet to the water surface was overcome by tugging it suddenly upward so that it did not have time to slide back off the applicator. If the applicator filament were too long and too flexible it would whip about and deposit the droplet some distance up from the tip from where it could not be removed. On the other hand, if too stiff and thick it would often break off. In either case, it would not release the entire droplet to the insect being treated.

C. HOLDERS FOR THE TEST ANIMAL, MUSCA DOMESTICA L.

The only holder for individual flies yet described in the literature was that devised by McIndoo (1937) for insecticide injection tests on Calliphora spp., Phormia spp., and Lucilia spp. Each fly was made to pass backwards into a small glass tube until its abdomen projected to the exterior from the

end which was slightly constricted. A wooden plug inserted into the tube contacted the head of the fly and held it in position. About an hour was required for injection of twenty flies by using this immobilizing device since each fly had to be removed from the holder following treatment. Such a technique could not be used for contact insecticide tests.

To prevent contamination of areas of the body other than that treated by the contact insecticide, the writer designed simple holders in which all the flies treated could be left until mortalities were to be recorded.

The basic material for all holders used in testing individual flies was "Scotch brand" cellulose tape one-half inch in width fitted into the regular metal dispenser.

A piece of tape 18-20 mm. in length was torn off, reversed, and then stuck to the free end of the tape still in the dispenser. The amount of overlap was 7 mm. to suit the size of fly being used. The composite piece was then torn off and stuck to the slide as shown in fig. 5.

Three different types of holders were developed from this basic plan by using various tools as follows:

Type 1. For tests where the leg or a portion of the leg of the housefly was to be treated a hole was made in the centre of the overlap portion by a horizontal push with the tool shown in fig. 8. By keeping the bevelled face of the tool upwards a flap was cut and pushed through as shown in fig. 9 a, b. The flap then was turned back firmly by being pressed against the tool while still inserted; otherwise it sometimes returned

to its original position and occluded the opening.

When the coxal segment or region of the body adjacent to it was to be treated, the hole was made considerably larger (1.5 - 2 mm. in diameter) to allow for the insertion of the applicator with the insecticide.

Type 2. For treatment on the wings, ventral or dorsal abdomen and head, a tool like that in fig. 8 but slightly larger was used but with the bevelled face downwards. It was pushed horizontally through the overlap region of the tape at about one-third up from the lower margin. This cut out a pear-shaped piece which remained stuck to the slide as the free end was being pulled upwards (fig. 6, 11).

Type 3. For treatment on the dorsal thorax, the tool shown in fig. 10 was used as in type 2 to cut a flap which stuck to the slide while the free end was pulled upwards. This tool was notched slightly at the end and had parallel sides so that the flap was not pear-shaped but rectangular and from 1.0 mm. in width at the apex to 1.5 mm. at the base (fig. 7).

D. PRODUCTION OF THE TEST ANIMAL MUSCA DOMESTICA L.

1. CULTURE OF LARVAE.

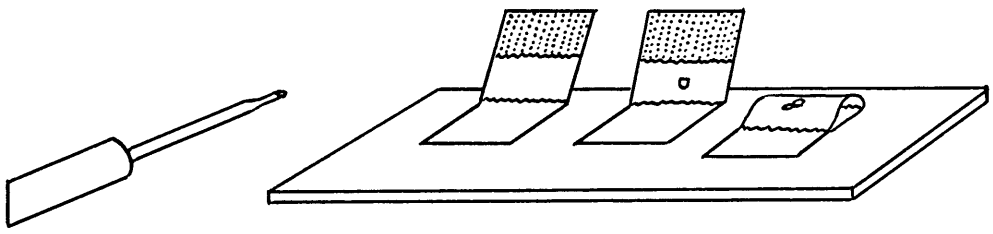
a. Larval medium.

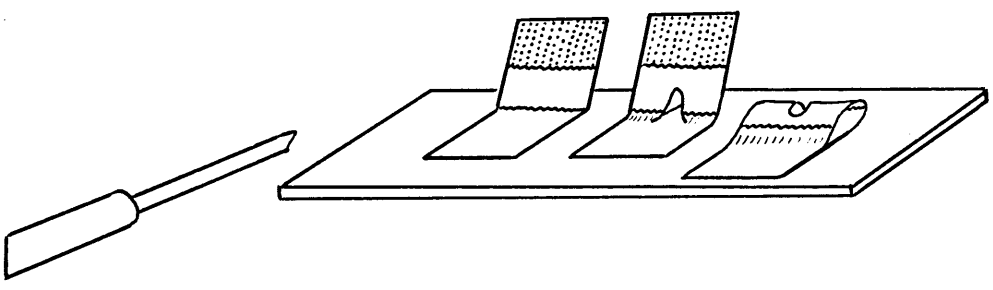
(i) Historical. Glaser (1923) reared larvae of the housefly in fresh horse manure, fermenting urine-soaked straw, or cow dung packed loosely into one gallon battery jars and moistened with water. Noting that most investigators lost their fly cultures in December when the larvae died after attaining

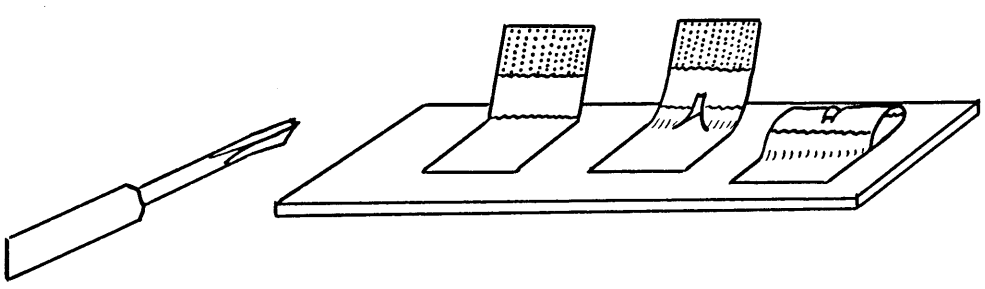
Fig. 5.- Punching tool and the three steps in the productio of holder Type 1 which exposes one leg of the fly treatment. Actual size.

Fig. 6.- Punching tool and three steps followed in making holder Type 2 which exposes the head or abdomen of the fly for treatment. Actual size.

Fig. 7.- Punching tool and three steps followed in making holder Type 3 which exposes the thorax and holds it securely during the cutting of "islands" in the integument. Actual size.







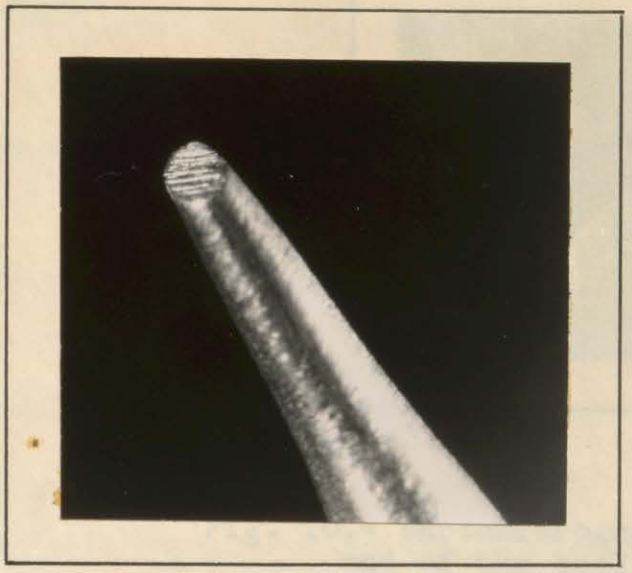


Fig. 8.- Photomicrograph of tapered end of tool for punching holes in tape for holder, Type 1,

Scale: 1 cm. = 1 mm.

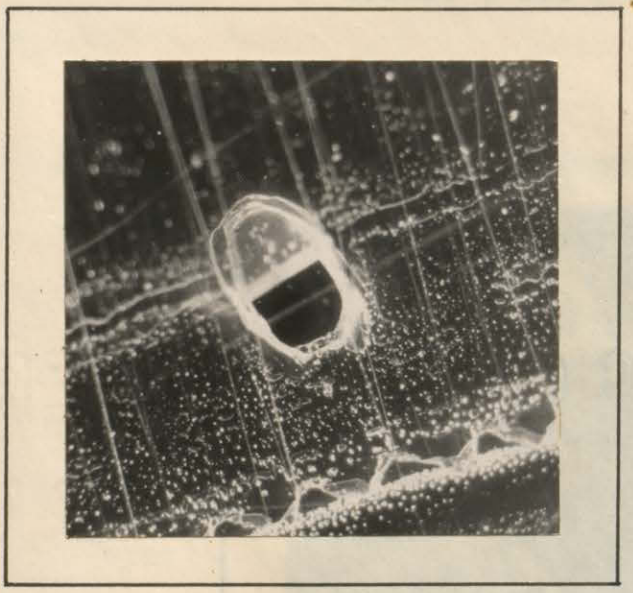


Fig. 9 a.- Photomicrograph of flap pushed through tape of holder Type 1, anterior view; flap is plainly visible through the tape.

Scale: 1 cm. = 1 mm.

Fig. 9 b.- Photomicrograph of flap cut as in 9 a except that the tape has been folded forward and stuck down to the position seen in fig. 5 c.

Scale: 1 cm. = 1 mm.

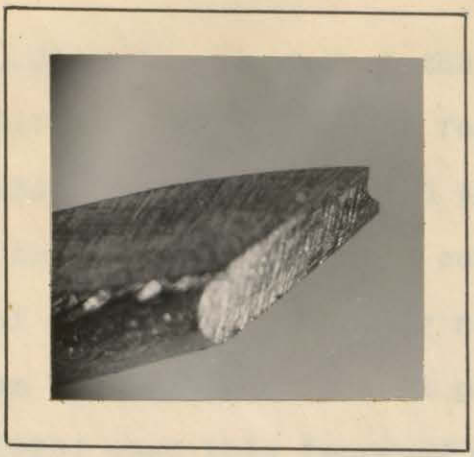


Fig. 10.- Photomicrograph of the end of the tool for punching holes for tape holder Type 3. Scale: 1 cm. = 1 mm.

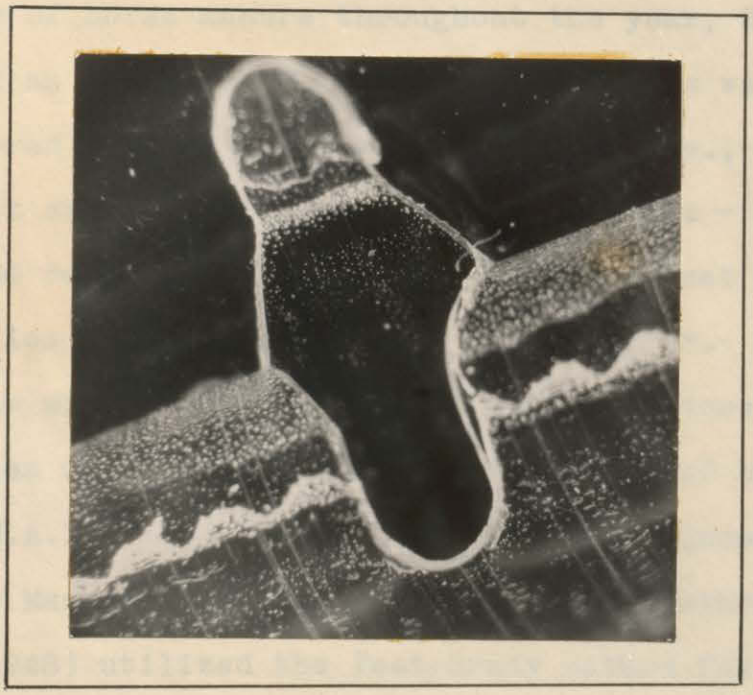


Fig. 11.- Photomicrograph of the hole in the tape holder Type 2; top view as seen also in fig. 6 c. Scale 1 cm. = 1 mm.

only half their growth, Glaser assumed that the horse dung at that time of year was deficient in necessary food factors or that the necessary yeasts and bacteria failed to grow in the medium. He found that the addition of a few cubic cemtimeters of a heavy suspension of yeast cells in water restored the nutritive value of the medium and permitted larval production throughout the year. Grady (1928) used this improved medium with success. In 1931, Hockenyos found that the larvae preferred a mixture of hog manure and horse manure to pure horse manure and also that the cultures were more productive if kept in three gallon crocks.

Since it was impossible to obtain a uniform fresh, mite-free supply of horse manure throughout the year, Richardson (1932) developed an "artificial horse manure". This was composed of wheat bran - $3\frac{1}{4}$ lbs., alfalfa meal - $1\frac{3}{4}$ lbs., water - 5,000 c.c., yeast suspension - 300 c.c., and Diamalt - 25 c.c. Though he gave no record of results, he did state that the medium reared flies successfully throughout the year. This medium as such or with slight variations in proportions of constituents has been used since 1932 by all workers and has been adopted by the N.A.I.D.M. (National Association of Insecticide and Disinfectant Manufacturers) as the Peet-Grady Method. Wilkes et al. (1948) utilized the Peet-Grady method for studying the biology and large scale production of laboratory populations. The culture jars were six inches in diameter and nine inches high and were filled with medium to a depth of seven inches. Although the battery jars were kept in a room at 26.7°C., the temperature in the centre of the medium ranged as high as

45°C. by the third day and then gradually declined to 29°C. by the ninth when pupation was almost complete. The larvae were found to prefer a temperature of 40-42°C. and so congregated at the layer of medium within that range.

These methods are quite satisfactory in productivity and suitable for rearing enormous quantities of flies at a time; however, for smaller laboratory tests where the numbers of individuals used are restricted to small samples by tedious experimental technique, a more convenient and precise culture practice is essential. Geberich (1948) studied thirty-five bacteriological media as possible habitats for rearing houseflies for experimental purposes and found ten of them to be satisfactory. No one, it seems, has attempted to utilize any of these media in continuous rearing for experimental work. In 1949 Hafez found a simple breeding method which required only milk, absorbent cotton, and two-pound jam jars, and which produced conveniently about two hundred flies per jar. The life cycle of these flies was shorter than that recorded in all previously recognized standard methods.

b. Preliminary rearing trials.

A culture of Musca domestica L. was established with pupae received from a Peet-Grady susceptible strain being used for insecticide screening tests by H.A.U. Monro at the Dominion Fumigation Laboratory in Montreal, P.Q.

The method of Hafez appeared in preliminary trials to give the most promise and so some time was spent in studying the effects of varying the type of absorbent substrate, the quantity

of milk, the temperature, the humidity and the quantity of light falling on the bottles. Sawdust (16-mesh screenings) and milk (homogenized to provide uniformity) gave the poorest results. The larvae grew slowly and pupated while still quite small. Fungi and moulds grew rapidly and quite likely competed with the larvae for food.

A bran and milk mixture was considerably more suitable and the larvae attained a much larger size before pupating. They worked vigorously through the medium and appeared quite healthy, but, as will be seen from table I, they could not compare in size or life cycle with those of the new standard.

Larvae started well in sterilized cellucotton and milk but invariably declined by the second day of rearing. Fermentation was very slow, the larvae worked sluggishly, and soon became thin and elongated; the resulting crop of pupae were extremely variable in size, shape and age. None were fit to use in experimental work.

Non-sterilized cellucotton and milk (Fisher and Morrison, 1949) was superior to all media so far reported both in this preliminary work or by other workers. The milk underwent gradual fermentation and appeared to support the growth of bacteria essential to the health and vigour of the larvae. As the latter worked through the cellucotton it swelled to about twice its previous size and became perforated like a sponge giving good aeration to the culture. Table I indicates the superior quality of this medium by the number, size and uniformity of pupae produced. A few pitfalls ironed out in the preliminary work led to the following conclusions:

High temperatures force larvae to congregate at the cooler upper layers of the medium and prevent their proper nutrition. Low ones increase the length of larval life and cause the medium to go foul.

Excess humidity in the culture disrupts the natural course of fermentation and growth of bacteria and also forces the larvae to the surface so that they crawl upwards and out of the culture jars.

Since housefly larvae are negatively phototrophic and quickly retreat into the medium when exposed to light, a darkened cabinet is most suitable to hold the culture bottles.

Homogenized whole milk has not been compared with skim milk, but obviously should be more uniform than non-homogenized milk for use in the medium.

c. Rearing procedure adopted.

Eggs laid during the night were washed from the absorbent cotton pads (fig. 12) into a 50 c.c. beaker the following morning. Any large clusters that floated were decanted into a test tube and broken up by shaking. The eggs were then washed several times with distilled water and 0.4 c.c. measured out by allowing them to settle in a graduated vial (a two-inch portion of a 10 c.c. pipette with a copper bottom fastened on with cement).

The culture jars found to be most convenient were pint milk bottles. Cellucotton (non-sterilized) squares measuring $2\frac{3}{4}$" to the side were cut from the large five pound rolls with a paper cutter and the corners trimmed off with a scissors to make round pads; these fitted snugly into the bottoms of the

TABLE I

Number of cultures	Medium	Number of pupae left on screens of various sizes				Total number of pupae	Length of life cycle
		7/64"	6½/64"	6/64"	5½/64"		days
8	non-sterilized cellucotton plus homogenized milk	(average of 8 days rearing on regular culture medium) 17	1,003	555	155	1,730	8-9
8	sterilized cellucotton plus homogenized milk	(1 representative day of 10 days) 0	24	91	268	692	10-13
8	bran and homogenized milk	(1 representative day of 2 weeks rearing) 0	326	832	1,261	2,491	10-13
8	non-sterilized cellucotton plus homogenized milk	(1st day on return to regular culture medium) 29	1,473	402	51	1,980	8-9

pint bottles (fig. 13 a). Each pad had seventy-two plies of tissue. Sixty cubic centimeters of homogenized milk were then poured on the pad in one motion so as to cover it quickly and give uniform wetting from the top down (fig. 13 b). Now the 0.4 c.c. of eggs were tipped into 16 c.c. of distilled water and thoroughly dispersed in it. With the aid of a 1 c.c. dropper, 2 c.c. of the egg suspension were added to each of eight culture bottles. The test tube was inverted before every dropperful was removed to insure uniformity. Each culture bottle received 0.05 c.c. of eggs containing from 300 to 350. The bottles were then loosely stoppered with absorbent cotton and placed in a darkened cabinet (fig. 14) at 28-29°C. At no time did the internal temperature of the medium exceed the cabinet temperature by more than one degree centigrade.

On the third day, when the larvae approached maturity, fine sawdust was poured on the culture pad to a depth of two inches. The larvae crawled up into the sawdust and pupated within the next two days (fig. 13 d).

2. HANDLING OF PUPAE.

a. Separation of pupae from medium.

(i) Apparatus. Separation of pupae from the medium has been done in a number of ways by various workers. For the Peet-Grady method David and Havrey in 1941 devised a type of fanning mill which separated the pupae from the dried upper layer of medium as it was poured over a sloping screen in a wind tunnel. Wilkes et al. (1948) and Fisher and Morrison (1949) used a fan over a shallow tray to blow away the medium

Fig. 12.- Oviposition split pad of absorbent cotton wet with water and milk. Eggs laid in the slot represent a good production for one night.

a b c d

Fig. 13. - Larval culture bottles.

a. Cellucotton pads dry.
b. Cellucotton pads wet with homogenized milk.
c. Cellucotton pads worked by larvae into spongy mass.
d. Pupae formed in sawdust on top of the cellucotton.

Fig. 14.- Rearing cabinet opened to show the larval cultures. Note the glass windows have been painted black to darken the interior.

Fig. 15.- Modified vacuum cleaner for separating pupae from the sawdust taken from the larval culture bottle.

or removed it by repeated washing in water.

All of these methods were dirty and blew the dry medium about in the air of the room or, in the case of washing, required subsequent drying of the pupae. To eliminate the trouble, Goodhue and Linnard (1950) made a separator in which a fan set up a rapid circulation of air downwards through a metal cone into a dust-tight jar. The whirling air created a suction through a pipe attached to the side of the cone. This aspirated the dry medium from the pupae in a can with a screen bottom held at the end of the suction pipe.

A simpler and more easily constructed modification of the above aspirator type separator was made by the writer from a discarded vacuum cleaner fan. A large bag of unbleached canton flannel (fig. 15) was made and fitted with a zipper in the large end for easy removal of sawdust. To the intake opening of the fan was fixed with rubber bands a cardboard tube, double-elbowed so that the distal end opened downwards. Into this end was glued a tapered cork ring. A lantern globe with a circular piece of very fine copper screen fixed to the lower end with de Khotinsky cement held the pupae and sawdust for separation.

(ii) Procedure. When the pupae had become medium brown in colour the sawdust and pupae were poured out into the separating jar and the latter turned slowly under the end of the aspirator pipe. Usually from 2-4 cultures could be cleaned at once depending on the quantity of sawdust in each bottle. The ovoid shape of the lantern globe created turbulence and a decrease in wind velocity near the middle, and so prevented the

pupae from being drawn up into the suction pipe.

b. Standardization of pupae.

The procedure in insecticide screening followed by the Peet-Grady and other methods using houseflies has been to produce flies of as uniform quality as possible and then to use numbers large enough to approximate the normal curve. Where techniques are more difficult and only a hundred or two flies can be handled at the time allotted to the test, a small sample of very uniform individuals must be used. Since weighing of flies and adjusting the dosage per gram of body weight for each was impossible in the testing procedure used here, another and simpler method was employed: After separation from the sawdust the pupae were placed on a stack of barley grain screens of the following sizes (fig. 16): top screen 7/64", second screen 6½/64", third screen 6/64". The majority of the pupae went through the top screen; it took out only the very largest. All the pupae which did not pass through or stick in the second screen were poured off into a dish and the screen cleared of those stuck in the slots. This batch was put over the same screen about five times until only 5-10 remained lodged in it, and was then placed in a petri dish one inch deep labelled "A" (for experimental use). Those which stuck in the second screen were combined with the very large pupae and those which would not pass through the third screen and were put into a petri dish labelled "B" (for rearing). This procedure of screening provided daily about 700-1,000 pupae with an average weight of 22 mg. containing about 40% males and 60% females with an emergence of

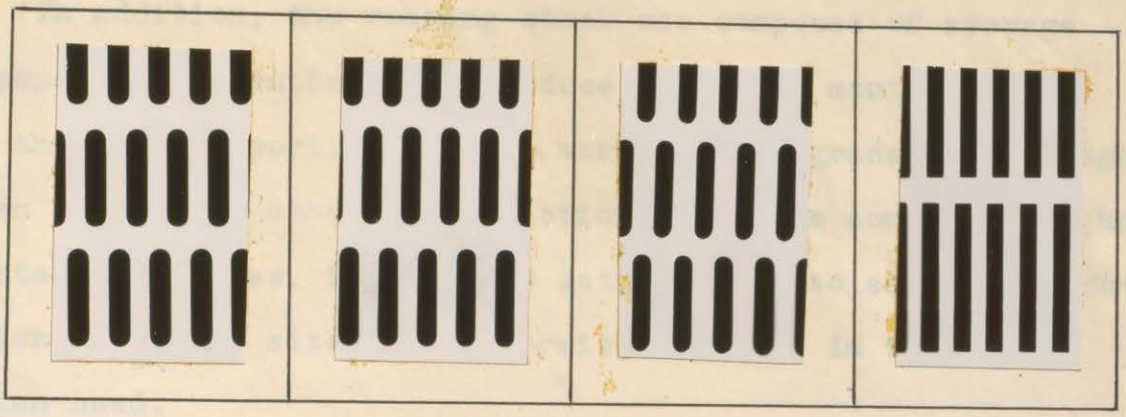


Fig. 16.- Barley screens used for segregating pupae according to size. a = 7/64", b = 6½/64", c = 6/64", d = 5½/64".

Fig. 17.- Cage for adult breeding stock of Musca domestica.

98+%. In addition, the rearing stock was composed of average size pupae which continued to produce regularly month after month the same proportions in the various size grades. Although, as seen in table 2 other size fractions of pupae contained higher percentages of males, it was more satisfactory to sacrifice numbers for the large size and uniformity of those in the narrow fraction used.

3. CARE OF THE ADULTS.

a. Rearing cages.

The adults of Musca domestica L. used for rearing were kept in cages 12" x 12" x 12" (fig. 17). Two sides and the top were covered with copper screening; the wooden back and front were removable and were held in place with metal fasteners; the bottom was made of galvanized iron. The front panel had a circular opening of 7 inch diameter fitted with a 1 foot cotton sleeve to allow for restocking, feeding of the flies and removal of the oviposition dishes. Both front and back panels had cleats to make the cage tight to prevent the escape of flies through cracks at the sides. The presence of detachable ends provided for easy cleaning of the cages with soap and water after egg collection had been suspended, and the rigid metal floor prevented any spilled liquids, pupal cases and dust from falling through to the cages below.

From a study of the oviposition habits of the flies it was found that six such cages constituted the most convenient battery for continuous rearing.

The cages were kept in a rearing room at a temperature

TABLE 2.

Screen size	Number of flies checked	Number emerged	Number of males	Number of females
7/64"	97	94	22	72
	54	54	16	38
	91	89	19	70
Total	242	237	57	180
Percentage		98	24	76
$6\frac{1}{2}$/64"	100	100	43	57
(Top of screen)	100	99	34	66
Total	200	199	77	123
Percentage		99.9	38	62
$6\frac{1}{2}$/64" (In the meshes of the screen)	100	97	57	40
	100	89	44	45
Total	200	186	101	85
Percentage		93	54	46
6/64"	100	92	53	39
	100	97	62	35
Total	200	189	115	74
Percentage		94.5	60	40

of 28-30°C. and a relative humidity of 40-70 per cent.

b. Handling the breeding stock.

The procedure for handling the breeding population of flies is most easily understood by following the eleven day plan as outlined below:

Sun.	Mon.	Tues.	Wed.	Thurs.	Fri.	Sat.	Sun.	Mon.	Tues.	Wed.	
A	A	A-	A-	A-	A						
	B	B	B-	B-	B-	B					
		C	C	C-	C-	C-	C				
			D	D	D-	D-	D-	D			
				E	E	E-	E-	E-	E		
					F	F	F-	F-	F-	F	
						A	A	A-	A-	A-	A

The letters A, B, ... F designate breeding cages and the dashes refer to the presence of an oviposition dish on a specific day.

From the chart we see that cage A was stocked with newly emerged male and female flies on Sunday. A feeding pad of absorbent cotton in a petri dish wet with fresh milk diluted 1:1 with water and with 2 c.c. of granulated sugar added on top was placed in cage A on Sunday, and renewed on Monday and removed on Tuesday evening. On Tuesday evening an oviposition dish of absorbent cotton prepared as shown in fig. 12 and using milk diluted 1:5 with water replaced the feeding dish. This was a "scouting dish" to ascertain the onset of egg laying. Usually the oviposition started in earnest on the third day and so eggs were collected on Wednesday and Thursday.

On Friday the flies were cleared from cage A, eggs

were collected from cages B, C and D, and feeding pads were provided for cages E and F. On Saturday cage A was restocked and the cycle begun again.

Old flies were killed by flushing the cage with hot water when it was removed for washing.

4. DISCUSSION ON HANDLING OF BREEDING STOCK.

The greatest deviation from the customary method of handling adult flies was in the maintenance of the breeding stock for only five days; this provided a preoviposition period of three days and an oviposition period of two days. This short preoviposition period appeared to be due to the diet of milk and sugar given to the flies. Glaser (1923) fed adult flies on several diets consisting of starch, bouillon, sucrose, blood serum, and glucose in varying combinations and found that with the most suitable foods they required a preoviposition period of 11-24 days. Grady (1928) fed the flies milk, lump sugar, sweetened bread and yeast suspension. Extra animal proteins were found unnecessary. The females laid eggs in a little over three days after emergence in some cases. Wilkes et al. (1948) reported that female flies began laying about three days after emergence and that the rate of oviposition decreased rapidly after the first five days of egg-laying. With the above facts in mind, the writer developed the scheme described in the previous section and found it to give excellent results in number of eggs produced per day, and in number of pupae per culture from those eggs. Killing of the stock after five days and

thorough cleaning of the cages removed all problems associated with the growth and spread of organisms pathogenic to the fly, with reduced vitality (high mortality of males, and decreasing fecundity of females with increasing age) and with the effect of cyclic variations in egg production, as noted by Wilkes et al. (1948).

5. SEXING OF FLIES.

The fact that female flies are considerably more resistant to insecticides than the males precluded the use of a mixed population in tests using small samples. Also, since females tended to vary in size and vitality depending on the development of their ovaries and, in some cases, laid considerable quantities of eggs by the fifth day, it was found preferable to use only males. According to Grady (1928) flies reach their greatest activity and resistance to insecticides between the fourth and fifth days after emergence, and so the male flies were kept for five days in cloth covered cages as illustrated in fig. 18 and fed diluted milk and dry granulated sugar in a waxed removable tray (fig. 19).

Since the writer found sexing with the use of ether both tedious and disagreeable, a machine was devised which permitted the separation of the flies by visible morphological characters. The machine, a modification of that reported previously (Fisher and Morrison, 1949), is illustrated in plate I and is operated as follows:

Pupae that had turned black in colour were placed in the metal container A which was stoppered with a No. 3 cork and

Fig. 18.- Cotton covered cage 9" x 5" x 5" for storing the adult houseflies to be used for experiments.

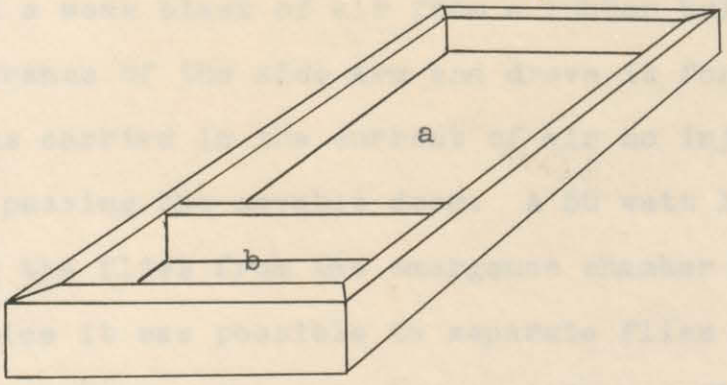


Fig. 19.- Removable tray for feeding the adult flies in the cage shown in fig. 18.

a. Chamber for dry granulated sugar.
b. Waxed chamber for absorbent cotton wetted with milk diluted 1:5 with water.

left in the rearing cabinet at 28°C. for 6-8 hours after the first flies emerged. The can **A** was then taken to the sexing machine and the screened end covered with an opaque plastic bag. The rubber covered end of the glass section B was then fitted into the end of the emergence can. A pipe cleaner in the observation chamber B kept the flies in until the apparatus was set up. Two cloth covered cages were fitted to the arms of the Y-tube C by tight elastic bands over the cloth sleeves. The pipe cleaner was removed and the observation tube inserted into the stem of the Y-tube. As the flies emerged from the chamber A and crawled along the observation tube B, the males could easily be distinguished by their dark sub-genital plates plainly visible from above or in the mirrors below. By means of the finger-operated key N the door d could be turned to direct the females into one cage and the males into the other. To assist in the separation a weak blast of air from a rubber bulb hit each fly at the entrance of the side arm and drove it forward. Since the fly was carried in the current of air no injury to it resulted in passing the movable door. A 50 watt lamp assisted in attracting the flies from the emergence chamber to the cages. With practice it was possible to separate flies at the rate of 1600-1800 per hour. Any crippled or deformed flies could be directed into the cage with the females. Flies from pupae of sizes any smaller than those described about for experimental use would have crowded in the 5 mm. observation tube and made separation more difficult.

PLATE I - SEXING MACHINE

Legend

A - Darkened emergence chamber.

B - Observation tube.

C - Y-tube separator.

D - Cotton covered cage for males.

E - cotton covered cage for females.

K - Rubber bulb.

M - Mirrors.

d - Copper door for directing flies to right or left arm of the Y-tube.

g - Cylindrical metal tube soldered to the emergence chamber to support the rubber tube i.

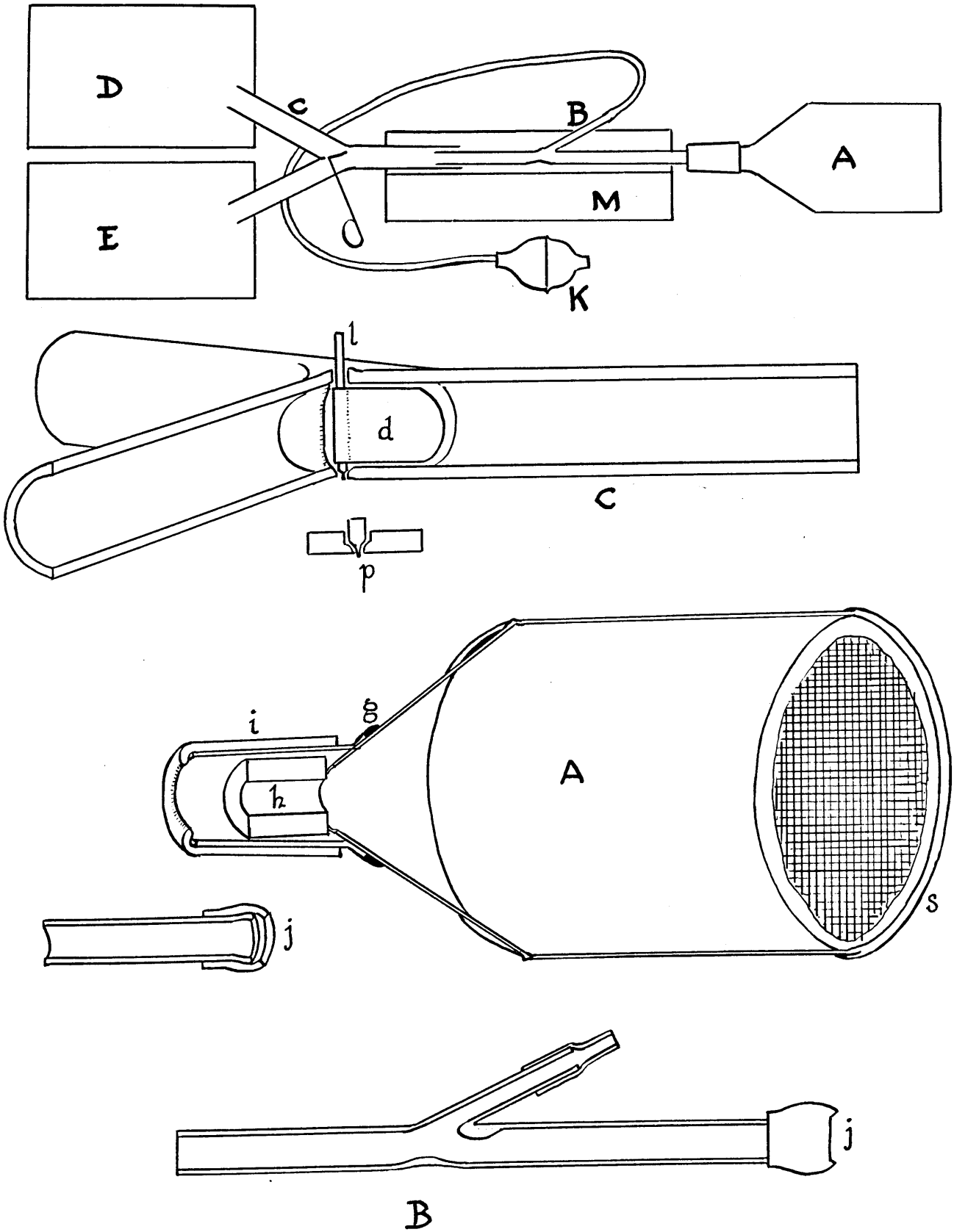
h - Short piece of rubber tubing to guide the flies into the observation tube and to hold the latter firmly in position against the outer rubber lip of i.

i - Piece of rubber tubing to form the flexible lip which holds the observation tube in place.

j - Rubber tubing cemented to the end of the observation tube to provide an expanded end which will not slip from the exit of the emergence chamber.

p - Pivot point for the copper door d.

PLATE I.



6. RESUME OF STANDARDIZING TECHNIQUES FOR THE TEST ANIMAL.

1. Larvae were standardized by using:
   a. Identical small culture media units with internal temperature variation of not more than 1°C.
   b. Eggs from batches laid within a period of 10-12 hours.
   c. 300-350 eggs per culture so that sufficient food was available to all larvae.
2. Pupae were standardized as to size by screening several times over barley seed screens. Pupae from a narrow size fraction emerged within a narrow time interval. (The method of DeBach (1942) for separating larvae every hour from those that had pupated was not regarded as necessary in addition to the method used here for pupae.)
3. a. Adults were sexed and only the males used (see page 40). Males were taken from those flies which emerged within the first eight hours.
   b. Males were fed milk and sugar and kept at 27.5 - 28.0°C.
   c. All flies were five days old when tested.

E. IMMOBILIZING AND TREATING THE TEST ANIMAL.

1. REMOVAL OF FLIES FROM STORAGE CAGES.

The food tray was first removed from the storage cage. While a pint bottle was held firmly in the mouth of the cotton sleeve with the left hand, the whole cage was knocked

sharply against the leg with the right. This dislodged the flies which then dropped through the sleeve into the bottle. Since they dropped quickly, they were not injured by repeated jarring. This method was resorted to because the phototactic response in the flies was so variable that many vigorous healthy flies could not be removed by attraction to light. When the flies had all been knocked into the bottle it was fitted with a cork having a half inch hole in its centre and a metal slide door as shown in fig. 20. From this bottle the flies were shaken by centrifugal force into 7 c.c. vials (5-6 per vial) by opening the slide door and swinging the pint bottle quickly through a short arc. The vials were stoppered with No. 3 corks or absorbent cotton plugs and arranged on the table. Any possible variation in size or vitality from vial to vial could then be taken care of by random selection of the vials for treatment.

2. ANAESTHETIZATION.

A standard carbon dioxide Kipp was used as a source of anaesthetic. To the outlet pipe was fitted, with one and one-half feet of rubber tubing, a glass tube drawn to a small aperture and fixed into a No. 3 cork stopper (fig. 21). The flow of CO_2 was regulated to a slow stream which kept the flies anaesthetized for only about thirty seconds until they could be placed in their holders.

3. PLACING FLIES IN HOLDERS.

Type 1. A piece of electrician's friction tape about three inches long was fastened to the table top with cellulose

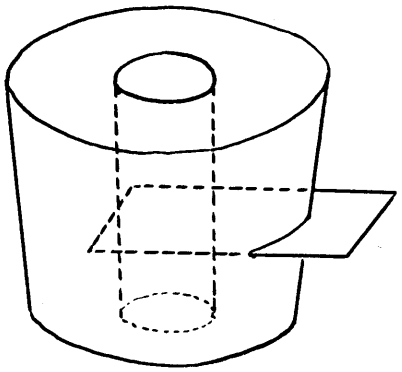


Fig. 20. - Cork stopper with metal slide gate for removal of flies to vials from the pint milk bottle.

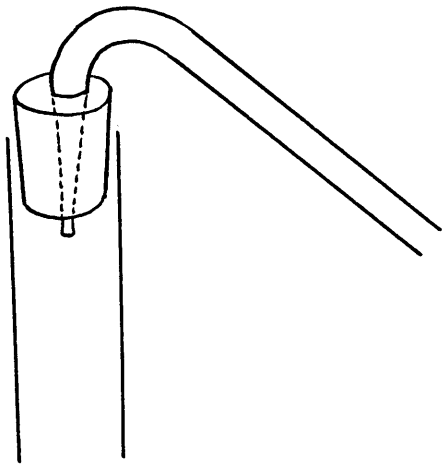
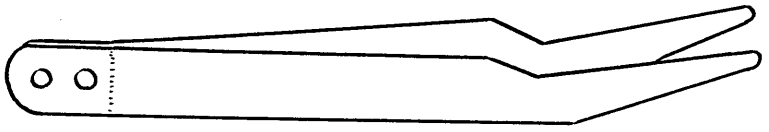
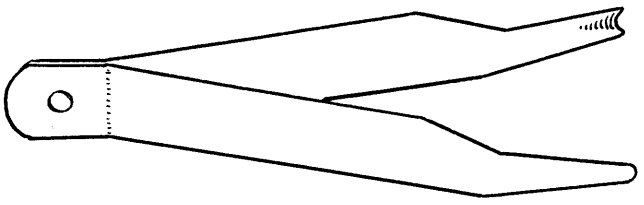


Fig. 21.- Cork stopper attachment for the Kipp generator outlet tube to fit into the 7 c.c. vials for anaesthetizing the flies.



a



b

Fig. 22.-

a. Featherweight forceps for the right hand.

b. Featherweight forceps for the left hand; note the concave inner surface on the tip of one arm to aid in holding the fly being placed in the holder.

tape. This held the slides and prevented them from slipping while the flies were being placed in the holders.

Two types of forceps were used for this procedure (fig. 21 a, b). Both were made of light weight metal and would not injure the fly regardless of the pressure applied with the fingers. The fly was held facing left by the forceps in the left hand; the leg to be treated was extended and pushed through the opening in the tape. The leg was then grasped lightly with the forceps in the right hand and pulled forward until the body of the fly was in firm contact with the tape. The free end of the tape was then turned forward and stuck down so that the fly became enclosed in a transparent half cylinder which left no sticky portion in contact with it. Five flies were fastened thus to each slide in a matter of 2-3 minutes. As the procedure was quite rapid, 2-3 flies could be fixed down before they had revived too much for easy handling. Having the flies held close to one edge of the slide permitted the slides to be stacked quite close together in a small space as shown in fig. 23. This allowed for the use of the relatively small post-treatment chamber shown in fig. 24.

Type 2. The fly was held as above with the head to the left, and its left wing worked through the hole gently so as to avoid tearing it. The free end of the tape was then secured as before. The wing was grasped very gently by the second pair of forceps to avoid injury by tearing. To insert the head the fly was grasped by the right wing in the right hand forceps and slid forward so that the legs were dragged backwards; the free end of the tape was secured by the left hand. For exposing the

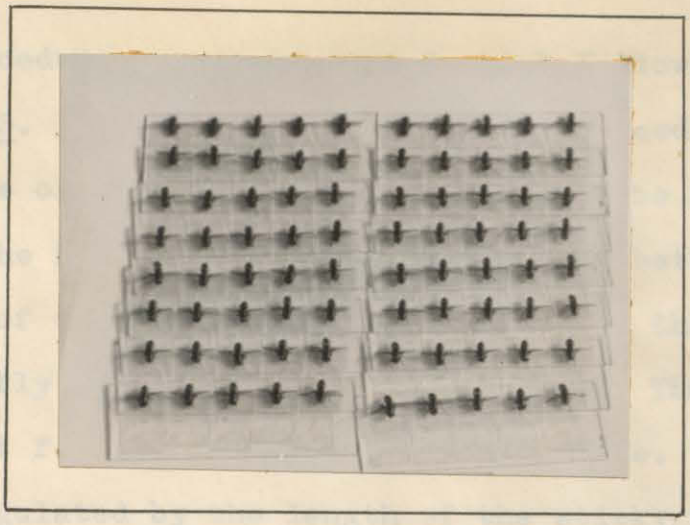


Fig. 23.- Slides of flies in holders as arranged in the post-treatment chamber. Placing the tape holders near the edge of the slide permits such convenient stacking.

Fig. 24.- Post-treatment chamber which has a water jacket to permit the cooling of the air to the required temperature.

abdomen the procedure was as in type 3 which follows.

Type 3. Here the friction tape was placed parallel to the forward line of vision of the operator and the slide stuck to it. While the free end of the tape was held between the thumb and forefinger of the left hand, the abdomen of the fly was manoeuvered gently through the rectangular gap. The free end was then brought forward and stuck down as before. The size of the hole was regulated by the length of the sticky portion of the tape that was pulled from the slide in making it. If too large the fly wriggled free after several hours, and, if too small, it suffered from cramped quarters. As can be seen from fig. 25, the whole dorsal surface of the thorax and abdomen could be left exposed while the fly was held firmly and comfortably. The wings spread out laterally preventing the fly from backing out.

4. APPLICATION OF THE DROPS.

Two binocular microscopes were used in the treatment procedure. The vessel containing the insecticide droplets was placed on the stage of the one to the right of the operator. An elastic band placed in a horizontal position around the stage of the left hand microscope fitted between the tape holders on the slide and held it stationary during treatment. In this way the left hand was free to handle any tool when necessary to manipulate the portion of the fly being treated, as well as to guide and steady the right hand while the droplet was being applied to the insect. After treatment of one fly the slide could be advanced to the next tape holder and held there by the rubber

band so that additional centering and focussing of the fly was not necessary. The droplet of DDT was picked up from the water surface as described on page 19 carried to the fly and deposited on the exact spot to be treated by touching the tip of the applicator to it. The droplet settled on the cuticle of the fly, spread slowly to about twice its former diameter and disappeared beneath the waxy surface plates; this left a spot only slightly darker in colour than the surrounding integument to indicate the locus of application.

IV. EXPERIMENTS CONDUCTED, RESULTS AND DISCUSSION.

A. EXPERIMENT I.

a. Object.

To test the hypotheses:

(1) That pure crystalline DDT can be absorbed in lethal amounts by the tarsal integument.

(2) That crystalline DDT on the pretarsal integument causes irritation and fluid secretion which in turn dissolves the DDT and facilitates its absorption in lethal amounts.

b. Procedure.

Test No. 1. Fifteen male Musca domestica L. were placed in holders with the left hind leg of each fly exposed. The leg outside of the hole in the tape was supported on a glass block. This prevented breakage or injury from bending. A mass of 0.67 ugm. of solid DDT was applied firmly to the exposed tarsus of each leg by means of a fine strip of cellulose tape.

Applicators and dosages for this test were prepared as follows:

A 3" x $\frac{3}{4}$" strip of cellulose tape with the sticky side out was fixed longitudinally on a glass microscope slide. A second narrow strip of tape 1.5 mm. in width was placed sticky side down also lengthwise upon the median line of the former. With a razor blade the whole length of the tape was cut crosswise into tiny strips 1-1.5 mm. in width. Each strip now consisted of a tiny square of smooth tape with a sticky tab at either end for application to the insect. A droplet of supersaturated benzene containing 0.67 ugm. of DDT was placed on each tiny smooth square and seeded so that the DDT crystallized and adhered tightly to it (fig. 26).

c. Results.

No mortality obtained in 24 hours.

Test No. 2. Thirty flies treated as above, but with fifteen of these the DDT was pressed firmly in among the setae of the tarsus.

Results.

No mortality in 24 hours.

Test No. 3. Fourteen flies were treated as before, the DDT being applied and pressed firmly into the pulvilli of the tarsi.

Results.

A copious secretion was produced between the pulvilli

by a few individuals, but this in no case appeared to increase the dissolution of the DDT. One fly died, but in view of the vigour of the remainder, this was probably a weak specimen that would have died without the presence of DDT.

Test No. 4. Finely divided DDT was sprinkled on the sticky side of strips of cellulose tape attached to slides and all the loose crystals removed by tapping. Forty flies each with one hind leg free to move were then placed in holders. The slides were inverted over these tapes so that only the tip of the tarsus contacted the DDT. The flies scratched at the DDT-covered surfaces until the masses of setae on the ventral surface of the distal segments became matted with tiny crystals.

Results.

In all cases 100% mortality occurred in 24 hours.

d. Discussion.

Test No. 4 indicated that DDT in a fine form and sufficient dosage will kill flies simply by contact with the tarsus.

DDT is not rapidly soluble in the cuticle of the fly. This can be seen from the fact that there was no perceptible decrease in size of the 0.67/ugm. crystalline mass pressed on the tarsus, and that the fly itself was apparently unaffected by its presence after 24 hours.

Contact of solid dry DDT with the pulvilli of one leg alone has no lethal effect on the housefly since an amount of

crystalline material less than half lethal dosage was not absorbed by them. The findings of Potts and Vanderplank (1945), therefore, that tsetse flies and other species with well developed pulvilli receive a lethal dosage through the feet alone apparently has nothing whatsoever to do with the presence of pulvilli as absorbing surfaces. It is much more likely due to the fact that insects having well developed pulvilli are usually able to walk upon vertical walls and on ceilings and thereby accummulate on the tarsi sufficient insecticidal residue to cause death.

Hickin (1945) states, in answer to Potts' and Vanderplanks' statement, that the size of the pulvillus is not a deciding factor in the toxicity of insecticides, and that it is unlikely that the relative size of the pulvillus has any special significance other than that a larger one can hold a greater amount of insecticide and hence be more apt to have a lethal dosage.

It might be argued that possibly the pulvilli of all six tarsi together could absorb a toxic dosage of crystalline DDT. However, since no decrease could be seen in the 0.67/ugm. applied to the pulvilli of one tarsus, and also since 1/ugm. applied as a benzene supersaturated droplet disappeared completely into the cuticle and caused only 30% mortality (see experiment III), it is very unlikely that the above could obtain. The pulvilli do not appear to present an absorbing surface large enough or permeable enough to take in a lethal dosage of DDT from the solid surface of crystals and contact with one or several of the tarsal segments as well may be necessary.

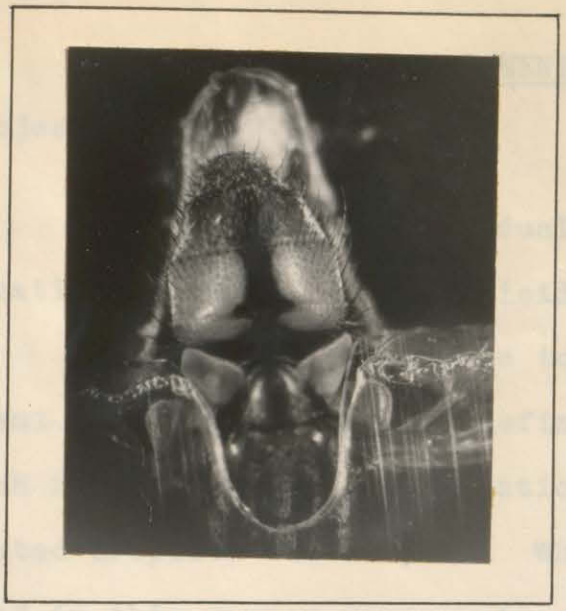


Fig. 25.- Photomicrograph of cellulose tape holder Type 2 showing the abdomen and posterior portion of the thorax of the fly protruding through the hole while the wings are extended laterally within.

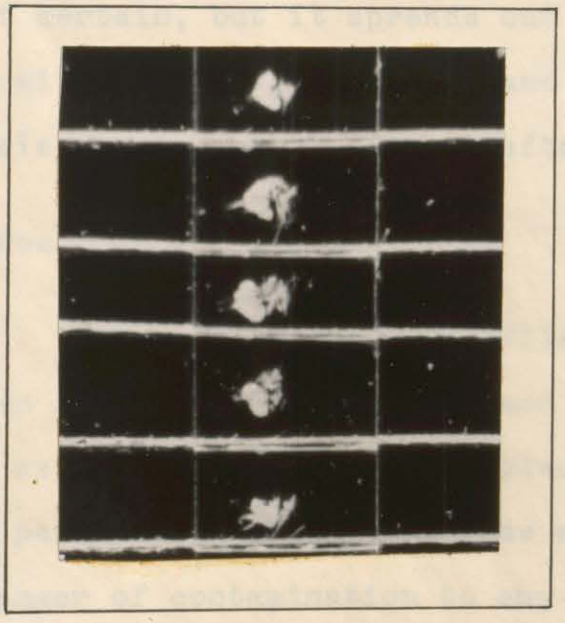


Fig. 26.- Photomicrograph of cellulose tape bands for applying crystalline DDT to the tarsus of the housefly. The DDT is crystallized on the central non-sticky portion. Sticky tabs at either end secure the band to the slide holding the fly.

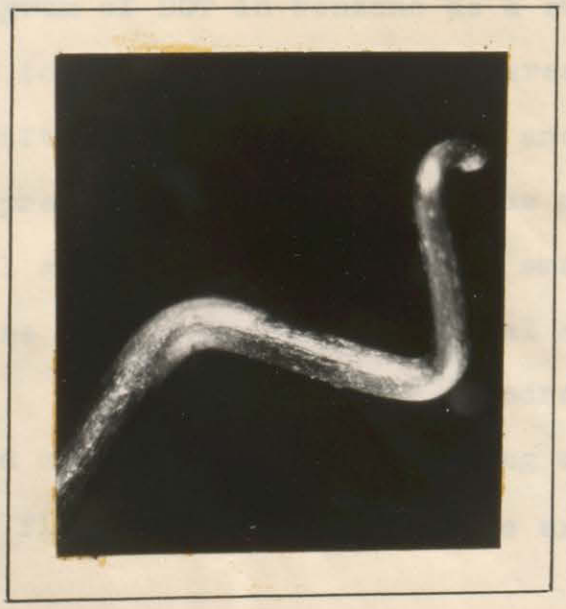


Fig. 27.- Photomicrograph of the end of a tool made from a No. 2 insect pin. This particular shape was found most convenient for holding the legs still during the application of droplets of supersaturated DDT in benzene.

B. EXPERIMENT II.

a. Object.

To compare the individual tarsal segments as loci of application of DDT as an insecticide.

Since it is impossible to apply solid DDT to the integument in such a way that a definite quantity will be absorbed by each fly at a designated location, the method of using supersaturated droplets was adopted. Whether or not the total dosage applied in this manner manages to penetrate the cuticle entirely is not certain, but it spreads out over a small area and disappears within a very short time and any attempt to seed out crystals of DDT on the surface after that time is unsuccessful.

b. Procedure.

One hundred and five flies comprising six groups of fifteen for six treatment loci and one for a check were anaesthetized as already described and placed in holders of type I; these permitted each fly the free movement of one hind leg without danger of contamination to any other area of the body. One microgram of DDT in benzene as a supersaturated droplet was applied to each of six different areas of the tarsus: pulvilli, and fifth, fourth, third, second and first tarsomeres. The droplet spread slowly among the setae on the ventral side of the tarsal segments and only in the small fifth segment did it extend as far as the intersegmental conjunctiva.

The test using one hundred and fifteen flies was repeated on each of ten days making a total of one hundred and fifty flies per treatment in the experiment.

c. Results.

The mortality totals for the ten replicates in order of size were as follows:

Locus of application	Dead out of 150	Differences between successive pairs	Differences from maximum mortality
First tarsomere	66		
Second tarsomere	51	15	
Third tarsomere	44	7	22
Fourth tarsomere	44	0	22
Fifth tarsomere	41	3	25
Pulvilli	36	5	30
Check	0		

The following is an analysis of variance on the data:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$ (n_2=44)	$P_{.01}$ (n_2=44)
Total	59	302				
Replicates	5	135	27	10.89	2.43	3.46
Treatments	9	55.6	6.18	2.49	2.10	2.84
Error	45	111.4	2.48			

Necessary difference between any pair of observed mortality totals ($P = .05$) = $\sqrt{2.48 \times 10 \times 2} \times 1.95996 = 13.79$

Necessary difference ($P = .01$) = $\sqrt{2.48 \times 10 \times 2} \times 2.57582 = 18.13$

From this analysis it is seen that:

1.) The first tarsomere is significantly better than the second and very significantly better than the third, fourth, fifth tarsomeres and the pulvilli as a locus of application.

2.) The second tarsomere is significantly better than the pretarsus,

but there is no significant difference between the third, fourth and fifth segments and the pulvilli.

d. Discussion.

Hayes and Liu (1947) made an histological study on the tarsus of the housefly and proposed that the presence of chemoreceptive sensilla found on the ventral sides of the tarsal segments might be especially concerned with the effectiveness of DDT as a contact insecticide. They thought the lack of chemoreceptive organs on the tarsi of Blatella germanica L. and the adult and larva of Epilachna varivestis Huls. to be correlated with a lack of susceptibility to DDT. However, in view of the fact that the first tarsomere of the housefly has no chemoreceptive sensilla on it and at the same time is very significantly more susceptible to DDT than the third, fourth and fifth which have large numbers of them, it would seem quite obvious that the effectiveness of DDT in this insect does not depend on the chemoreceptive sensilla. The cuticle overlying a chemoreceptive sensillum is usually quite thin but there is nothing to indicate that cuticle thickness has any relation with penetration of DDT. Weismann (1947) claimed that resistant flies from Sweden had thicker cuticulae than those of a susceptible strain in Switzerland but this could very easily have been due to natural variation in flies from different regions. Also March and Lewallen (1950) could find no significant difference in cuticle thickness between resistant and non-resistant flies.

The presence of glands in the pulvilli does not enhance to any perceptible extent the penetration and effectiveness of DDT.

C. EXPERIMENT III.

Since experiment II indicated that the structures such as chemoreceptive sensilla were not responsible for the increasing effectiveness of DDT as the point of application was moved up the leg from the pulvilli, it was thought that perhaps proximity to the central nervous system might be the determining factor.

Therefore it was decided to compare other portions of the leg with the pulvilli as the standard. These areas were: the tibio-tarsal membrane, tibia, tibio-femoral membrane, femur, and coxo-trochanteral membrane.

a. Procedure.

Seventy flies composing six groups of ten for areas treated and one group of ten as a check were placed in cellulose tape holders with the hind leg free as before. A droplet containing 1.0/ugm. of DDT was placed on the area of the leg designated above while it was held stationary by means of the wire tool of fig. 27. When treated the whole lot was removed to a chamber at a temperature of 21-22°C. and R.H. 40-70%. This test was repeated on each of ten days to make a total of one hundred flies per treatment.

b. Results.

The mortalities at the 24 hour check time in order of size were as follows:

Locus of application	Dead out of 100	Differences between successive pairs	Differences from maximum mortality
Tibio-femoral membrane	61		
Tibia	58	3	
Coxo-trochanteral membrane	51	7	10
Tibio-tarsal membrane	48	3	13
Femur	48	0	13
Pulvilli	31	17	30
Check	0		

Analysis of Variance:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$	$P_{.01}$
Total	59	169				
Replicates	9	29.1	3.23	1.73	2.09	2.83
Treatments	5	55.5	11.10	5.94	2.43 (n_2=44)	3.46
Error	45	84.4	1.87			

Necessary difference between any pair of observed mortality totals (P = .05) = $\sqrt{1.87 \times 10 \times 2} \times 2.042 = 12.48$

Necessary difference (P = .01) = $\sqrt{1.87 \times 10 \times 2} \times 2.750 = 16.802$

From this analysis it is seen that:

1.) All areas are very significantly better than the pretarsus as a locus of application for DDT.

2.) The tibio-femoral membrane is significantly better than both the tibio-tarsal membrane and the femur but not the tibia or coxo-trochanteral membrane.

3.) There is no significant difference between the tibia, tibio-tarsal membrane, femur and coxo-trochanteral membrane.

c. Discussion.

The results of this experiment substantiate the suggestion made at the beginning that the DDT becomes increasingly effective as the locus of application approaches the body of the fly. The presence of the minute quantity of benzene apparently makes the DDT penetrate well through both sclerotized hard cuticle and non-sclerotized membranous areas, but the lack of a significant difference between the sclerotized tibia or femur and the tibio-tarsal membrane may be due to reduced penetration of the DDT through the sclerotized exocuticle. The superiority of the tibio-femoral membrane over the femur may not be due altogether to the lack of exocuticle in it, but more likely to the presence of the pulsatile organ or "accessory heart" which lies directly beneath it. The beating of this organ causes the membrane to move up and down at a considerable rate so that the DDT might be assisted in penetration by its rapid removal from the inner surface of the integument; it may also be rapidly dispersed in the haemolymph flowing past the undulating heart membrane. Other possibilities will be discussed following descriptions of experiments on other parts of the body.

D. EXPERIMENT IV.

The previous experiment showed that there was no difference in mortality between flies treated on the tibio-femoral and coxo-trochanteral membranes of the hind leg. Although the coxa is a very short segment it was considered necessary to determine whether the coxal membrane in being closer to the central nervous system was a more effective locus of application than the

coxo-trochanteral membrane. The comparison was made directly between the above two membranes and indirectly between the coxal and tibio-femoral membranes:

1. In the direct comparison eight replicates of thirty flies each gave a total of eighty flies per area treated and eighty as a check; 1.0/ugm. of DDT in supersaturated benzene was used for each fly treated. The mortalities at 24 hours were 56 for the coxal membrane and 61 for the coxo-trochanteral membrane. This difference of 5 was not significant and could be attributed to normal variation in the flies treated.

2. The indirect comparison afforded a further check on the tibio-femoral membrane as well as indicating the relative effectiveness of coxal and coxo-trochanteral membranes by comparison with the data from experiment III.

Ten flies were treated with 1.0/ugm. DDT on the tibio-femoral membrane of the hind leg and ten on the coxal membrane; ten were left as checks with no treatment. This was repeated ten times giving a total of one hundred flies per area and one hundred for checks.

The mortalities after 24 hours were:

Locus of application	Dead out of 100	Differences between successive pairs	Differences from maximum mortality
Tibio-femoral membrane	71		
Coxal membrane	67	4	4
Check	0		

The following is an analysis of variance on these data:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$	$P_{.01}$
Total	19	101.8				
Replicates	9	92.8	10.3	11.4	3.18	5.35
Treatments	1	0.8	0.8	0.9	5.12	10.56
Error	9	8.2	0.9			

There is evidently no significance in the treatment difference between the coxal membrane and the tibio-femoral membrane of the hind leg.

a. Discussion.

From experiment III and the second part of experiment IV we can see that the coxal membrane differs from the coxo-trochanteral membrane by 6; in the first part of experiment IV the difference is 5. The coxal membrane mortality total is the larger in the former case and the smaller in the latter; therefore there very definitely is no difference in effectiveness between the two membranes of the hindleg as loci for DDT application.

E. EXPERIMENT V.

The question now arose as to whether or not the foreleg of the fly would react in a manner similar to that found for the hindleg. Therefore an experiment was performed in which the pretarsus, the tibio-femoral membrane, and the coxal membrane were compared by treatment with the supersaturated droplet containing 1.0/ugm. DDT in benzene. The holder used for experiments III and IV was used here again except that the aperture in the tape was slightly larger. The front leg of the fly is considerably

shorter than the hindleg so the hole had to be large enough to contain the whole of the coxa in order to prevent the fly from pulling the leg free and escaping. Mortalities were recorded after 24 hours.

Sixteen replicate tests each of five flies per area treated plus five for a check made up a total of three hundred and twenty flies for the experiment.

Results were as follows:

Locus of application	Dead out of 80	Differences between successive pairs	Differences from maximum mortality
Tibio-femoral membrane	53		
Coxal membrane	50	3	
Pretarsus	39	11	14
Check	0		

Analysis of variance:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$	$P_{.01}$
Total	47	79				
Replicates	15	36	2.40	2.40	2.04 (n_1=14)	2.74
Treatments	2	13	6.50	6.50	3.32	5.39
Error	30	30	1.00			

Necessary difference between any pair of observed mortality totals (P = .05) = $\sqrt{1.00 \times 16 \times 2} \times 2.0420 = 11.53$

Necessary difference (P = .01) = $\sqrt{1.00 \times 16 \times 2} \times 2.750 = 15.53$

There is no difference between the tibio-femoral membrane and the coxal membrane of the foreleg as a locus of application of DDT but both are superior to the pretarsus.

a. Conclusion.

The variability in effectiveness of the locus of application of DDT on the foreleg is similar to that of the hindleg.

F. EXPERIMENT VI.

Having established the trend of effectiveness along the foreleg and hindleg, it appeared logical that it would not have to be repeated for the middle leg.

A comparison of similar areas of the three legs of the housefly was therefore made.

In experiment VI, the tibio-femoral membranes of pro-, meso- and metathoracic legs were treated with 1.0/ugm. of DDT supersaturated benzene. One hundred flies were treated for each leg and one hundred left not treated as checks. After 24 hours the mortalities for the different areas were recorded thus:

Locus of application	Dead out of 100	Differences between successive pairs	Differences from maximum mortality
Tibio-femoral membrane of foreleg	74		
Tibio-femoral membrane of midleg	58	16	
Tibio-femoral membrane of hindleg	54	4	20
Check	0		

The following is an analysis of variance on these data:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$	$P_{.01}$
Total	29	100.8				
Replicates	9	39.1	4.34	1.99	2.46	3.60
Treatments	2	22.4	11.2	5.14	3.55	6.01
Error	18	39.3	2.18			

Necessary difference between any pair of observed mortality totals ($P = .05$) = $\sqrt{2.18 \times 10 \times 2} \times 2.101 = 13.87$

Necessary difference ($P = .01$) = $\sqrt{2.18 \times 10 \times 2} \times 2.878 = 18.99$

a. Conclusions.

The tibio-femoral membrane of the foreleg is significantly better than that of the midleg and very significantly better than that of the hindleg as a locus of application for DDT.

It seems that there is a gradient of effectiveness of DDT increasing from posterior to anterior in the legs of the housefly.

G. EXPERIMENT VII.

Since observed mortality increased as the locus of DDT application on the legs moved anteriorly, it might be that the circulation of the blood could vary sufficiently in the locomotor appendages to bring this about.

Experiment VII was therefore designed to determine whether the same differences would be observed when application was made on the bases of the legs of the insect. Here the constricted passages within the legs could have no serious effect

on the flow of blood. Since the coxal membrane of the middle leg was quite difficult to contact with the holder being used, the coxo-trochanteral membrane was treated instead. The diameter of the middle leg at this point is, however, very small so that we might expect it to differ in susceptibility from the coxal membrane which forms part of the venter of the mesothorax.

As in experiment VI, one hundred flies were treated for each area and one hundred were used as checks. Using 1.0/ugm. DDT again, the mortalities at 24 hours were as follows:

Locus of application	Dead out of 100	Differences between successive pairs	Differences from maximum mortality
Coxal membrane of foreleg	82		
Coxal membrane of hindleg	65	17	
Coxo-trochanteral membrane of middle leg	47	18	35
Check	0		

The analysis of variance was done thus:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$	$P_{.01}$
Total	29	107.5				
Replicates	9	22.2	2.47	1.86	2.46	3.60
Treatments	2	61.3	30.65	23.05	3.55	6.01
Error	18	24.0	1.33			

Necessary difference between any pair of observed mortality totals (P = .05) = $\sqrt{1.33 \times 10 \times 2} \times 2.101 = 10.84$

Necessary difference (P = .01) = $\sqrt{1.33 \times 10 \times 2} \times 2.878 = 14.85$

a. Conclusion.

The coxal membrane of the foreleg is very significantly

better than either that of the middle leg or that of the hind leg; this further proves that there is a higher level of susceptibility toward the anterior end of the animal.

b. Discussion.

The low mortality total for the coxo-trochanteral membrane of the middle leg, as predicted at the beginning, does not fit into the gradient picture shown for the tibio-femoral membranes; the constriction at the coxa coupled with the fact that the middle leg is not used as actively in cleaning movements, etc. as are the foreleg and the hind leg could together cause a considerably slower circulation of haemolymph than obtains at the coxal membranes; this would cause, therefore, a relative reduction in susceptibility at the coxo-trochanteral membrane and in no way cancels out the possibility that the coxal membrane might have conformed with the gradient if it could have been investigated.

Hockenyos and Lilly (1932) found that for nicotine injected into Celerio lineata larvae the toxicity increased as the point of injection approached the head where the largest ganglia occur. However, nicotine causes ganglion synaptic block in the central nervous system and it would be expected that the effect would increase as the point of application approached the brain.

Tobias and Kollros (1946) claim that symptoms of DDT poisoning in the cockroach, Periplaneta americana, can occur in amputated legs, in legs whose nerves have been cut, and in legs whose segmental ganglia have been destroyed, and that it is

possible for DDT to produce its motor effects by action on some structure or structures peripheral to the segmental ganglion.

Whether or not the same is true entirely for houseflies is not certain. The gradient of nervous potential of the central nervous system could still hold and peripheral nervous reactions carry on at different potentials increasing towards the head.

H. EXPERIMENT VIII.

The tibio-femoral membrane of the leg which covered a pulsatile organ proved superior to membranes distal to it and equal to the coxal membrane as a locus for application of DDT and so it was thought that integumental areas over the pulsatile organs of the wings might also be effective portals of entry.

Thomsen (1938) claims four pulsatile organs in the vein bases of each wing of a number of Diptera. The writer found two of these easily but treatment was restricted to the largest and most easily reached at the junction of the First Anal and the Cubito-Anal cross-vein.

Ten flies were treated with 1.0/ugm. DDT on the tibio-femoral membrane of the foreleg, ten on the pulsating membrane of the wing and ten kept as checks. The test was replicated eleven times and at 24 hours mortalities were recorded:

Locus of application	Dead out of 110	Difference
Tibio-femoral membrane	71	
Junction of 1st Anal and Cubito-Anal cross-vein	64	7
Check	0	

The following is an analysis of variance:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$	$P_{.01}$
Total	21	62.6				
Replicates	10	20.1	2.01	0.50	2.97	4.85
Treatments	1	2.2	2.2	0.55	4.96	10.04
Error	10	40.3	4.03			

Necessary difference between any pair of observed mortality totals ($P = .05$) = $\sqrt{4.03 \times 11 \times 2} \times 2.228 = 20.9$

Necessary difference ($P = .01$) = $\sqrt{4.03 \times 11 \times 2} \times 3.169 = 29.8$

a. Conclusions.

There is no significant difference between the tibio-femoral membrane and the pulsatile membrane at the junction of the Cubito-Anal cross-vein and the 1st Anal vein as a locus of application for DDT.

Since there is no significant difference between the coxal membrane and the tibio-femoral membrane of the foreleg, then it is safe to assume that there is no significant difference between the coxal membrane and the wing membrane just mentioned as loci for the application of DDT. However, since the coxal membranes of the meso- and metathorax are very significantly less effective than that of the prothorax, then the wing membrane should be considerably better than the coxal membranes of the same antero-postero region of the body. The blood is aspirated from the wing veins by the accessory hearts of the basal portions of the wing, so any insecticide or insecticidal product would be carried directly to the dorsal vessel. On the other hand, blood from the coxal region of the same thoracic segment

must pass upwards through the perivisceral sinus to the pericardial sinus before being admitted to the heart for subsequent dispersal to all parts of the body. The difference in distance might account for the difference between ventral and dorsal regions of the body.

I. EXPERIMENT IX.

Experiments up to Number VIII showed that the more anterior portions of the body of the fly tended to be more effective loci for the application of DDT. As the most anterior locus treated so far was the coxal membrane of the prothorax, it was decided that the head region should be the next area for investigation.

One microgram of DDT in a supersaturated benzene droplet was applied to each of four areas: the pseudotracheae of the labellae, the basal membrane of the antennae, the dorsal surface of the compound eye, and the dorsal side of the cervical membrane. Holders of type 2 (fig. 28) were employed to immobilize the flies. Ten flies were used for each treatment and the test was replicated ten times. One hundred flies were also used as checks. Preliminary tests showed that mortality was complete at 24 hours for those treated on the labellae so the counts were made after 16 hours instead. The dosage could have been reduced but, as the results showed later on, 1.0 µgm. was not too much for the other areas treated and the results could more easily be compared with those of previous experiments.

Mortalities were recorded at 16 hours:

Locus of application	Dead out of 100	Differences between successive pairs	Differences from maximum mortality
Labellae	78		
Basal membrane of antennae	62	16	
Dorsum of compound eye	52	10	26
Dorsal side of cervical membrane	47	5	31
Check	0		

An analysis of variance showed the following:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$	$P_{.01}$
Total	39	225				
Replicates	9	56.1	6.23	1.99	2.25	3.14
Treatments	3	84.5	28.17	9.00	2.96	4.60
Error	27	84.4	3.13			

Necessary difference between any pair of observed mortality totals ($P = .05$) = $\sqrt{3.13 \times 10 \times 2} \times 2.052 = 16.23$

Necessary difference ($P = .01$) = $\sqrt{3.13 \times 10 \times 2} \times 2.771 = 21.92$

a. Conclusions.

The labellae as a locus of application for DDT were nearly significantly better than the antennal membrane and were very significantly better than both the compound eye and the cervical membrane.

There was no significant difference between the antennal basal membrane, the dorsal side of the compound eye and the dorsal cervical membrane.

Presumably the DDT was dispersed rapidly via the mouth parts to the digestive tract or was spread over a larger area of

integument by capillary action in the pseudotracheae or by transport to the cuticular lining of the foregut by the fluids. This suggested the possibility that the same dosage of DDT dispersed over a larger area would be more effective, and that if the spreading, visible or invisible within the cuticular lipoid wax complex, could be limited to a definite area, the mortality might be reduced accordingly. Hence experiment X.

J. EXPERIMENT X.

The problem of limiting the spread of a fat soluble insecticide in a lipoid wax complex appeared at first to be impossible. No lipophobic material placed on the surface could prevent the diffusion of DDT beneath the surface and so the only alternative appeared to be the interruption of the cuticular structure completely by incision. Cutting of the integument of the housefly could not be done with any degree of accuracy on any portion of the body that tended to move when touched with the cutting instrument. Several types of holder were used to keep the fly stationary but the only one which proved at all satisfactory was that of type 2, fig. 6. The hole in this tape holder was broader toward the abdomen and the fly still had too much freedom of movement. A type of tool was then developed which had a notched end and parallel sides (fig. 11) and cut a rectangular hole of type 3, fig. 7. This proved very successful in maintaining the fly in position while at the same time allowing it sufficient freedom to prevent injury from cramped quarters.

The tool for cutting the integument was made from a piece of soft glass rod 8 cm. in length and 3 mm. in diameter.

The end was drawn out to a very fine, short, conical point with a microflame and then broken off at an angle to leave an extremely sharp cutting edge. A large number of these had to be made before one was found that was satisfactory for the operation.

A special lighting device had to be used here to eliminate the heat and drying action of the microscope lamp. A rod of leucite 1 inch in diameter and 12 inches long was placed in front of a 100 watt lamp so that the operating field was flooded with a cool light from the end of the rod. Wet cotton was placed about the field so that the humidity was raised sufficiently to prevent undue evaporation from the incision in the fly.

All flies for a test were placed in the tape holders before the actual operation and treatment began. Under the high power of the dissecting microscope squares were cut in the mesonota of the flies as shown in figs. 29 and 30. The large square was bounded in front by the transverse suture, laterally by the dorso-central rows of setae and posteriorly by the last setae in the acrostichal rows. The small square comprised approximately one-quarter of the area of the large square. With care it was possible to cut these squares of integument free on all sides without shifting them in any way from this original position; haemolymph welling up slightly through the incision coagulated somewhat and served as a barrier to the diffusion of the DDT-benzene droplet which was placed in the exact centre of each square.

Five flies were operated upon, treated with 1.0/ugm. of supersaturated DDT in benzene within about 2.5 to 3 minutes

Fig. 28.- Photomicrograph of a fly held in tape holder type 2 exposing the head and neck region for treatment as in experiment IX.

Fig. 29.- Photomicrograph of mesonotum of housefly showing the smaller square cut in the integument as per experiment X.

Fig. 30.- Photomicrograph of mesonotum of housefly showing the large square cut in the integument as described in experiment X.

and then were removed immediately to the post-treatment chamber. This chamber was kept at a temperature of 20-21°C. at all times and a relative humidity maintained at 90-100% by a layer of wet absorbent cotton on the top shelf and another on the floor beneath the screen carrying the treated flies.

Ten flies with large squares cut, ten with small squares cut, and ten with intact integument were treated for each test. Ten checks each with a large square cut in the mesonotum were kept without further treatment. The test was replicated ten times and mortality counts recorded at 20 hours:

Locus of application	Dead out of 100	Differences between successive pairs	Differences from maximum mortality
Intact integument	67		
Large square	49	18	
Small square	29	20	38
Check	0		

The following is the analysis of variance on these data:

Source of variation	Degrees of freedom	Sums of squares	Variance	F.	$P_{.05}$	$P_{.01}$
Total	29	154.2				
Replicates	9	65.5	7.28	7.99	2.46	3.60
Treatments	2	72.3	36.15	39.68	3.55	6.01
Error	18	16.4	0.911			

Necessary difference between any pair of observed mortality totals ($P = .05$) = $\sqrt{0.911 \times 10 \times 2} \times 2.101 = 8.97$

Necessary difference ($P = .01$) = $\sqrt{0.911 \times 10 \times 2} \times 2.878 = 10.29$

a. Conclusions.

There are very significant differences between areas treated.

The intact integument is more effective than the large square, and the latter is superior to the small square in promoting the toxic effect of DDT.

b. Discussion.

Three factors may be concerned in this variation in effectiveness of DDT due to the surface area affected:

1. The area of intact lipoid-wax layers surrounding the point of application may determine the range of diffusion of the insecticide.
2. The area of intact integument surrounding the point of application may determine the number of sensory endings that will be stimulated by the DDT.
3. The area of intact integument surrounding the point of application may determine the number of hypodermal cells that can be affected and made to respond by producing toxic cell metabolites due to DDT stimulation or to produce toxic degradation products from the DDT that gains entry.

First of all, it is logical that the distance that the insecticide can diffuse in the cuticle of the integument will greatly influence the degree of penetration and subsequent effect of that material and so a larger area should produce a higher mortality, as has been demonstrated.

Now, depending on this diffusion are the factors

described in numbers 2 and 3.

Roeder and Weiant (1948) found that all sensory organs such as tactile setae etc. in the cockroach leg responded to the unstabilizing effects of DDT. It is thus possible that the DDT in the housefly might diffuse to a large number of sensory setae in the intact integument and that the difference in number of these sensilla affected in the large and small squares might have been sufficient to account for the very significant mortality difference obtained between them.

Some morphologists claim to have demonstrated the presence of a nerve net beneath the integument of insects which they describe as the anastomozing of the nerve end fibres to form a reticulated system. Some even show connections between nerves, but do not state whether the fibres themselves remain separate or are joined within the nerve sheaths.

If a nerve net is present in a housefly mesonotum, the area of integument attacked by the insecticide would cover a corresponding area of nerve net and so the greater the area treated, the greater would be the toxic effect. Whether the nerve net depends upon the complete reflex arc or can cause contractions in muscles without the mediation of a ganglion is not known. Yeager and Munson (1945) claimed that DDT caused impulse trains in the motor nerves between the ganglion and the myo-neural junctions, so it is not impossible that portions of motor fibres in a nerve net could be so affected and cause the characteristic contractions. In this way we may account for the lack of effect of DDT on the central nervous system reported by Roeder and Weiant (1946) and Wett (1947).

The third suggestion stems from the recent work of Kearns and Sternburg (1950) on the metabolism of DDT in resistant and susceptible houseflies. They have found that resistant flies can degrade DDT to DDE (1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene) and DDA (bis-(p-chlorophenyl) acetic acid) in the cuticle-hypoderm cells and in the digestive tract and that this degradation is sufficiently rapid to account for the resistance of the houseflies. Winteringham et al. (1951) found that this degradation was not rapid enough to account for the resistance of flies used by them. However, they both agreed that in susceptible flies where no degradation of DDT to DDE and DDA takes place, a small portion of the initial dose applied was degraded to some other material that could not be recognized by any chemical test then being applied. They considered that it was this small part which was active at the site of action.

If the portion of the DDT which kills the insect is changed by the cuticle-hypoderm cells to another and possibly more toxic principle, then the relative effects of the applied dosages in the different flies will be correlated with the number of cuticle-hypoderm cells available for the breakdown of DDT. Hence the intact integument in the tests reported here was better than the large square and the latter was superior to the small square as a locus for the application of DDT.

Winteringham et al. (1951) further state that the metabolism is enzymic in nature and the enzymic system is either destroyed or made inaccessible by the destruction of the tissue. From this it may be suggested that possibly the DDT would kill the cuticle-hypoderm cells if it were to be restricted to a small

area and thereby keep a high concentration localized. In the larger square, where the DDT would have a chance to diffuse farther from the point of application a few cells might be killed in the immediate vicinity of the point of application but a number would remain in a sufficiently healthy condition to sustain the enzymatic processes necessary to produce a small amount of toxic breakdown product of DDT.

Obviously, then, if no restriction is imposed on the diffusion of DDT, a large number of viable hypoderm cells would produce a substantial amount of toxin from it.

Although several workers already mentioned have induced typical DDT symptoms by perfusion of emulsions and suspensions of DDT directly onto the isolated nerves of cockroaches, this in no way obviates the possibility of a more toxic material being produced by the cells themselves or by degradation of DDT and which likewise could produce typical impulse trains, convulsive twitches, tetanus and finally the death of the insect. It would also, in view of the small amounts needed, be very difficult to inject or perfuse tissue without integumental contamination.

Bodenstein (1946) removed small portions of integument, with muscles, nerves and trachea attached,from the abdomens of Drosophila virilis and kept them alive in Ringer's solution for several hours. He showed that a DDT emulsion added to the Ringer's solution could illicit the typical DDT symptoms in the muscles attached to the integument. These symptoms began 30 seconds after the introduction of the DDT. Although he shows that the effect of the toxin is on the peripheral nerves only,

there is still left the possibility that the DDT can diffuse into the hypoderm cells and be degraded or cause the production of a toxic secretion within an extremely short time; enzymatic hydrolyses such as the breakdown of acetylcholine by cholinesterase can take place in a matter of milliseconds (Loewi, 1945).

Two questions arose at this time:

1. Would pure crystalline DDT be toxic if placed directly in the haemolymph among the tissues or would its passage through the integument be a necessity?
2. Would the presence of an excess of cuticular waxes at the point of application enhance or hinder the penetration and effect of DDT?

Two experiments were done on various numbers of flies to answer these questions:

K. EXPERIMENT XI.

It was expected that injection of pure DDT would effect some mortality and so it was compared with external application on intact integument. The first test used fifteen flies per area treated; 1.0/ugm. of supersaturated DDT in benzene was applied to the areas noted as external, and 6.0/ugm. of DDT crystallized on a fine glass filament was placed into the muscle tissue of the thorax. This was done by cutting a flap of integument and carefully lifting one edge with the cutting tool. The large crystalline mass was then inserted and held in place while the glass filament was withdrawn from it. The flap was replaced and the would appeared to be sealed by coagulating haemolymph in a very short time. To guard against possible

dessication, however, the treated flies were placed in the moist post-treatment chamber. After 20 hours mortalities were as follows:

Intact integument - 5

Crystallized DDT injected - 2

Check - 0

The very low kill with the crystallized material was thought then to be due to the small surface area of the mass in contact with the haemolymph.

A second test was therefore designed to increase the effective surface of the crystalline mass enormously in relation to its volume. The 6.0/ugm. quantities of DDT as supersaturated benzene were crystallized by seeding and then placed in a benzene-air atmosphere until a bloom of microscopic sharp needle-like crystals projected from the surface in all directions. These bloomed crystalline masses were then used as intramuscular injections as in the previous test. The DDT supersaturated benzene droplets for the external treatments were doubled to 2.0/ugm. of actual DDT. Twenty flies were treated for each locus and the mortalities at 24 hours were as follows:

2.0/ugm. on intact integument - 16

6.0/ugm. internally - 1

Check - 0

This very definitely shows that the haemolymph does not effectively dissolve DDT and transport a lethal dosage to a site of action.

L. EXPERIMENT XII.

The fat-soluble waxes and lipoids of the cuticles of one hundred male houseflies were removed by shaking with 10 c.c. of benzene and the resulting extract used to make up a 1.07% solution of DDT. Supersaturated droplets of this solution were compared with those from the regular benzene solution by treating the tibio-femoral membrane of the hind leg. One hundred flies were used for each treatment and the check and mortalities at 20 hours were as follows:

Benzene plus DDT	-	54
Benzene plus DDT plus wax	-	56
Check	-	0

An analysis of variance shows that there is absolutely no difference between the treatments, e.g.:

Total sum of squares	-	85.00
Replicate sum of squares	-	55.00
Treatment sum of squares	-	0.02
Error sum of squares	-	29.98

The incorporation of extra cuticular waxes has no effect at all on the effectiveness of DDT.

M. EXPERIMENT XIII.

a. Object.

To study speed of knockdown and mortality in relation to locus of application and the relation between mortality and availability of food to the fly.

b. Procedure.

Sixty flies were treated on the labellae with 1.0/ugm. DDT and released in a pint milk bottle stoppered with moistened absorbent cotton; sixty were treated on the tibio-femoral membrane of the hindleg and similarly released; sixty were kept as a check without treatment.

c. Results:

Time in hours	Labellae		Tibio-femoral membrane		Check	
	Knockdown	Death	Knockdown	Death	Knockdown	Death
0.5	53	0	0	0	0	0
1.0	51	0	23	0	0	0
2.0	48	1	41	0	0	0
3.0	48	2	41	4	0	0

Crops of all dead flies were exposed by dissection and drawings made to check on any correlation between crop contents and rapidity of death.

Plate 2 shows the relative sizes of the crops from flies killed by DDT, from flies treated with DDT but dissected before death, and from untreated living flies. All crops appear about the same size within each time zone. However, a dead fly with a full crop may still have died of starvation if paralysis had prevented evacuation of the crop. The midgut in some dead individuals appeared to be still quite well filled with food and so the DDT must have so upset the metabolic activity of the fly that digestion and absorption no longer functioned normally to supply energy to the tissues.

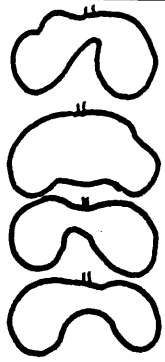
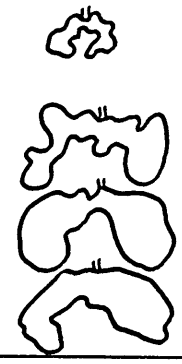
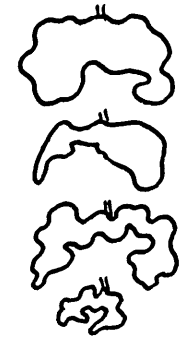
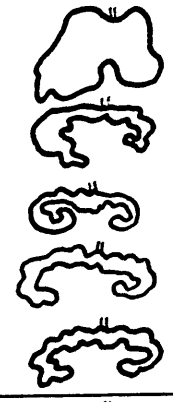
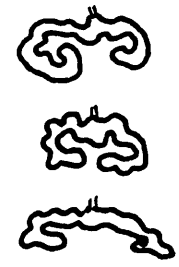
Dissection time	Treated and still alive	Treated and dead	Check no treatment
4 hrs.			
5 hrs.			
20 hrs.			
fed at 20 hrs. dissected at 23 hrs.			

PLATE 2.- Drawings of the relative sizes of crops of flies treated on the labellae.

The knockdown rate of the flies treated on different areas as shown in section c may have several explanations:

The phenomenon of nervous coordination depends on two nerve centres, the suboesophageal ganglion and the brain. The former is responsible for the activation of the insect in locomotion, while the latter acts as an inhibitor to it. The two together then keep the fly on an even keel. Any blocking of the activity of the brain or excessive increase in the activity of the suboesophageal ganglion would unbalance the equilibrium and the fly could no longer make coordinated walking movements; hence knockdown.

The hind leg of a fly treated on the tibio-femoral membrane became paralysed first and extended to its full length; this was followed in a short time by partial paralysis of the other hind leg so that the fly could no longer rub its hind legs together in the cleaning movements. General lack of coordination did not occur till about an hour had passed.

Since knockdown occurred in 30 minutes with flies treated on the labellae and only in an hour or more with those treated on the hind leg, it seems that either the poison was distributed to all locomotor appendages much more quickly via the digestive tract or it acted much sooner on the centres responsible for the coordination of the fly when applied there.

It is possible that poison would require considerable time to travel from one hind leg throughout the body in order to paralyse the peripheral nervous systems of all the legs, but the knockdown in those treated on the mouth parts occurred without the stiffening paralysis of the legs typical of that from treatment

on the legs.

Yeager and Munson (1949) claim that time of knockdown is correlated with the concentration of the applied toxin but that once knockdown has occurred the paralysis carries on at the same rate regardless of the concentration.

If this is true, the differences in time of knockdown can be correlated with the time necessary for the threshold knockdown concentration to accumulate at the side of action.

V. SUMMARY AND CONCLUSIONS.

A finely drawn out glass tube fused to a truncated steel hypodermic needle was cemented in turn to a 0.25 c.c. tuberculin syringe fixed vertically over the lever arm of a Cambridge rocking microtome. Movements of the syringe plunger were governed by the microtome lever arm. Drops as small as 1.5/uml. were produced with precision.

Microdrops of a benzene solution of DDT were collected and evaporated to a supersaturated state on the surface of water containing a minute proportion of a surface active agent. These microdrops, containing as little as 0.167/ugm. of DDT, could be applied to the insect as such by tiny glass applicators or seeded out and applied as crystalline masses.

Cellulose tape strips cut into suitable sizes, overlapped and punched with special tools in the non-adhesive overlap region were fixed to microscope slides to form "straight jackets" for the harmless immobilization of Musca domestica adults. These could be made to expose any desired portion of the body for a spot treatment while at the same time preventing

contamination elsewhere on the body.

Musca domestica L. were reared on a medium of homogenized milk and non-sterilized cellucotton in pint milk bottles. This method gave very uniform temperature and nutritional conditions and combined with screen grading of pupae and selection of five day old male adults only for tests provided a highly standardized test animal. A machine designed to separate the sexes by visible morphological characters without aid of anaesthetics was used throughout.

Microdrops containing 1.0/ugm. DDT in benzene both in the supersaturated and crystallized states were used as spotting treatments to compare the relative effectiveness of sixteen different areas of integument on the legs, wings, thorax and head of adult flies as loci of application for DDT. In general ten replicates of ten flies each were used for each locus, two to six areas being compared in each experiment.

Similar and much larger dosages were inserted as crystalline masses into the muscles of the thorax through flaps cut in the mesonota. Other spot treatments of the supersaturated DDT solution were made on "islands" cut in the mesonotal integument to correlate effectiveness of DDT with size of surface affected. One test was conducted spotting DDT dissolved in a benzene extract of cuticular waxes and a final one to associate locus of application with speed of knockdown.

In most instances the criterion of DDT activity was mortality at 24 hours though actual rate of action was measured in one instance. An analysis of variance was conducted on total observed dead in each experiment to establish significant

differences.

Conclusions drawn from the observed results are as follows:

1. DDT is not extremely soluble in the cuticle and a very large dosage of fine crystals must be pushed in among the tarsal setae to cause death.
2. The pulvilli alone will not permit a toxic dosage of DDT to enter the body of the fly if the DDT is in the dry crystalline form.
3. Chemoreceptive sensory organs of the tarsi are not responsible for increased toxicity as segments having none at all are superior as loci of application of DDT.
4. The effectiveness of DDT increases along the tarsus from the distal pulvilli towards the tibio-tarsal joint.
5. The sclerotization of the femur and tibia may be the cause of an observed reduction in penetration of DDT and consequently make the unsclerotized membranes of the leg more effective ports of entry.
6. The pulsatile organ of the tibio-femoral joint appears to enhance the effectiveness of DDT applied to that region when compared to other areas closer to the body.
7. There is a gradient of effectiveness for DDT as its locus of application approaches the body along the locomotor appendages, and also as it approaches the anterior end of the animal.
8. Integument over the pulsatile organ of the wing is a better locus for application of DDT than is the ventral coxal region of the same thoracic segment.
9. The labellae are very effective in permitting the DDT to enter

and take effect either by penetration through the proboscis itself or by diffusion throughout the digestive tract or over the inner surface of the foregut.

10. DDT as a solid crystalline mass is not an effective toxin for the housefly when injected into the thoracic muscles but is very toxic when applied to the exterior of the overlying integument as a supersaturated solution in benzene.
11. The effectiveness of DDT applied to the mesonotum is correlated with the area of integument over which it can diffuse in the lipoid-wax layer of the epicuticle.
12. An increase in the amount of cuticular waxes at the locus of application neither enhances nor decreases the toxicity of DDT in the housefly.
13. Knockdown occurs much sooner with flies treated on the labellae than with those treated on the tibio-femoral membrane.
14. Dead flies often still show well filled crops and midguts.

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