FURTHER REFINEMENT OF A TECHNIQUE FOR TESTING CONTACT INSECTICIDES

by

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A THESIS

Submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Master of Science.

April 30th, 1943.

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Abstract

Drosophila melanogaster were treated with nicotine sulphate solution by both intermittent and continuous spraying methods. There was no clearly demonstrated superiority of one method over the other. An analysis of variance performed on observed mortalities expressed as angles of equal information indicated that increasing age of flies, increasing numbers of flies per cage, longer delays between filling of the cages and spraying, and increased proportions of males in the samples, raised observed mortalities significantly. The type of cloth used to cover the cages must be standardized. Data on fly ages and numbers of flies per cage were also put through the probit analysis of Bliss which indicated flies aged 5 days to be most susceptible and fly numbers to affect equally the mortalities due to all concentrations.

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I. INTRODUCTION

Although entomologists and biometricians have worked on the problem of comparative testing of contact insecticides for years, no perfectly satisfactory technique has as yet been evolved. Recent papers give valuable data on many of the factors which must be taken into consideration in this type of work. The present report is designed to confirm and enlarge certain of these findings, as well as to investigate several points as yet untouched. Though representing only a small contribution to a vast field these data will, it is hoped, add something to our knowledge of the many factors contributing to heterogeneity in the results from laboratory tests on contact insecticides. It may then be possible, by careful technique, to control the superficial variables with such success that only the inherent biological variation in the test animal will remain to prevent strict reproducibility of results. When that has been done the way will be clear for accurate comparisons of contact insecticides.

II. HISTORICAL REVIEW

In general, insecticides may be divided into three classes: (a) contact insecticides, (b) stomach poisons, and

(c) fumigants. Contact insecticides, which are the sole concern of this present paper, may be applied in laboratory tests in one of the following ways: (i) as a spray, (ii) by dipping, (iii) by use of a micropipette, and (iv) by dusting. The method of spraying may be further subdivided, as a matter of convenience, into two types: (1) in which the liquid is sprayed onto insects which are resting on a surface, and (2) in which the liquid is sprayed into a cabinet where the insects are flying around. The first of these two types was used exclusively in the tests recorded herein under "Experimental Results."

The historical aspect of testing insecticides has been presented in an excellent summary by Tattersfield (1939). It is proposed, therefore, to repeat here only the most important facts concerning researches on spraying, dipping and micropipette techniques together with brief notes on those papers which have appeared since the publication of Tattersfield's article.

A. Laboratory Methods

1. Spraying of Insects on a Surface

Tattersfield and Morris (1924) devised an apparatus for the testing of liquid insecticides. A dish of the test insects was placed under a glass tower while a measured quantity of liquid was sprayed over them by a blast of compressed air. Pressure of the air, quantity of insecticide and direction of the cone of spray were controlled but the usefulness of this

device was limited by the fact that the quantity of spray deposited was uniform only in a small area in the centre of the cone, tapering off rapidly toward the periphery. Later modifications in the atomizer (Tattersfield 1934) helped somewhat to remedy this defect though they did not eliminate it.

This type of apparatus has been widely used by other workers, including Stultz (1939), Potter (1941), Morrison (1943) and the writer. Stultz eliminated the glass tower and the dish of insects, using instead a glass lamp-chimney as a cage in which to hold about 300 Drosophila for each test. The spray cone was directed upward through a hole in the bottom of this container. Potter designed an entirely new nozzle which allowed an accurate centering of the liquid jet in the middle of the aperture of the air jet while also permitting a vertical adjustment of their relative positions. He retained, however, the spray reservoir and general method of operation of the Tattersfield apparatus, substituting a grounded metal tower for the original glass column. Morrison eliminated the tower and the wide dish for holding the test insects, using instead a glass tube 14 mm. in diameter and 45 mm. in length, covered at both ends with tulle, as a cage for his flies. This was centred in the spray cone so as to receive a maximum dose of the insecticide. Further modifications in the nozzle and method of spraying were introduced by the writer. These will be described fully in the next section, "Experimental Apparatus and Methods."

O'Kane et al (1930) used a commercial atomizer under controlled conditions of pressure, etc. The spray from this

was directed at an angle of 45° onto a revolving turn-table which exposed all sides of a cage of test insects to the stream of insecticide droplets. A further modification (O'Kane et al 1941) consisted of the introduction of a second jet of spray material. The atomizing devices in this apparatus were artist's air brushes mounted so that they focussed on the turn-table from opposite directions. In an effort to ensure equal individual dosages of insecticide, the insects, each held in a small metal clip, were suspended from a wire rack on the turn-table.

Jones et al (1935) also made use of a turn-table but under entirely different conditions. Their system was to place a glass column under the spray nozzle until it was filled with a mist of spray material. After a short interval during which the larger droplets settled to the table, this column was moved into position over a cage of insects and was allowed to remain there for a definite period of time. Later modifications, which included the substitution of aluminum for glass in the column and the addition of extra columns on the turn-table, resulted in improvement of the apparatus and an increase in the speed and ease with which data could be secured. The method is now commonly known as the Campbell turn-table method and has been used by Campbell et al (1934), Campbell and Sullivan (1934, 1938), Jones et al (1935), Zermuehlen and Allen (1936), Badertscher (1936), and others.

2. Spraying of Insects in a Cabinet

This method of testing insecticides has been used almost exclusively for household fly sprays with the house fly as a test animal. The original cabinet, described by Peet and Grady (1928) and later revised by Peet (1932), was essentially a small room measuring six feet in each dimension. Into this a definite amount of insecticide was sprayed through a number of apertures in the walls. After ten minutes the "knock-down" count was taken and the flies were put in cages in order to record the number dead in 24 hours. The Peet-Grady test has been adopted as the standard method for testing fly sprays in the United States and numerous workers have contributed to perfecting the technique now used.

H. H. Richardson (1931) described a similar though smaller cabinet. With this apparatus he counted the "knock-down" at half minute intervals until this value was over 50% of the total number of flies being tested. It was found that the time required to obtain a 50% paralysis was a very sensitive index of the strength of the spray, more sensitive in fact than the curve of mortalities after a 24 hour period.

3. Dipping Methods

Tattersfield (1939) lists half a dozen workers who have made use of the dipping method of testing insecticides. Of these, Craufurd-Benson (1938) had apparently the most accurate technique for rearing and dipping the test insects

and was able to secure fairly consistent results.

Morrison (1943) compared data secured by the spraying technique with those secured by immersion and found the former to be more consistent. Working with Drosophila, he found that varying amounts of air were retained as bubbles by the setae, especially when a saponin spreader was used. These were thought to affect the total absorption of nicotine through the body wall, making it difficult to secure high kills. Furthermore, although one worker could reproduce his own results with reasonable accuracy, it was more difficult to secure agreement between the data of different workers and the method was finally discarded in favour of the spraying technique.

The chief objections to the dipping method are:
(1) there is danger with most test animals of a stomach poison effect being present in addition to the contact effect, and
(2) the process is too different from common economic methods of applying insecticides to yield data of much real value.

4. Micropipette Methods

O'Kane et al (1933) used a platinum needle to apply a small drop of the pure insecticide to a selected area of the insects' integument. Studies were made on the more potent poisons available and of their effects after application to various parts of the body. It was suggested that this method might provide a suitable means for the investigation of the toxicity of new substances, preliminary to further tests of a more practical nature.

Nelson et al (1934) chilled their test insects for a few minutes in a refrigerator before making the test. When the insects had become motionless they were removed and placed on a cold marble slab while a calibrated, glass micropipette was used to place a measured droplet of poison on the mid-ventral thoracic region. By careful dilution of the various poisons it was possible to reduce their respective toxicities to a point where each produced approximately a 50% kill.

Once again it is obvious that this method is not at all comparable to economic methods. Furthermore, its authors state that it requires the development of a considerable degree of skill in manipulation.

B. Methods of Assessing Results

Once again reference must be made to the excellent summary of Tattersfield (1939). He states that toxic action of an insecticide may be quantitatively judged in three ways: (a) by the effect produced by different concentrations in a given time (plotted as a dosage-mortality curve), (b) by the effect produced at different intervals of time, concentrations being kept constant, (a time-mortality curve), and (c) by the effects produced at different intervals of time by different concentrations, (time-concentration curves). He lists a comprehensive series of references and emphasizes that it is necessary to review the original papers in order to appreciate the individual contributions of each. The first of these methods, i.e. the dosage-mortality curve, was used in the present work

and will be referred to chiefly in the following account.

More recently Bliss (1940) has described a mathematical treatment whereby dosage, time and mortality may be investigated simultaneously. Graphs may be drawn which give all combinations of two of these factors which will produce the desired effect at a fixed level of the third factor. The validity of such graphs may be tested by analysis of variance.

1. Classification of Results

Any review of the literature on toxicity studies will quickly reveal that various workers have adopted different methods of classifying their results. Some have divided the insects into living, slightly affected, seriously affected, moribund and dead. Others classify them merely as living or dead, including moribund in the latter classification. Still others prefer to place all insects capable of even the slightest movement in the category of the living. In each test, however, some time interval must be arbitrarily set up during which the poison may act before the results are counted. This interval should be chosen on a basis of a sound knowledge of the course of action of the insecticide and of the type of effect it is desired to secure by its use.

2. Probit Analysis

Early workers plotted concentration of the poison against percentage kill and secured in most cases a sigmoid

curve. This curve tails off at the upper and lower ends but usually approximates a straight line in the middle region. With such a line the best point for comparison of toxicity is that concentration producing a 50% kill, or the L.D. 50.

As has been pointed out by Bliss (1934a, 1935a) and others, this sigmoid curve may be considered to be an expression of the normal variation of susceptibility of individual insects in a random sample and is therefore only the cumulative form of the normal frequency distribution.

O'Kane et al (1930) postulated that, provided nicotine in concentrations which increase by equal increments acts as a straight-line force, the dosage-mortality curve should be a straight line when percentage kill is plotted on a probability scale against concentration on an ordinary scale. Using the data of Tattersfield and Gimingham (1927) he replotted the points but failed to secure a straight line. He concluded that his major premise was incorrect and that the effects of successive equal increases in concentration are not proportional. The actual lines which he secured in this work closely resembled absorption curves which are known to be logarithmic in nature. Accordingly he transposed percentage mortalities to what were called "re-valued datum points" by a procedure which was not fully explained until later (O'Kane et al 1934). It consisted of laying an arithmetical scale alongside the probability scale of mortalities with 0, 50 and 100 on the arithmetical scale being opposite 0.01, 50. and 99.99 respectively on the probability scale. The "re-valued datum points" could thereupon

be read directly from the graph on the arithmetical scale. Now, when the revalued datum points were plotted against the corresponding concentrations on logarithmic paper, a curve was secured which closely approached a straight line and could be so drawn.

Bliss (1934a, 1934b) followed this up with a method called the method of probits which permitted the plotting of dosage-mortality data as a straight line on ordinary graph paper instead of logarithmic paper. These probits were based on Pearson's Tables of the Probability Integral. Bliss's first table had 0.01% mortality equal to 0.0 on the probit scale, 50% mortality at the value of 5.0, and 99.99% mortality at 10. Intermediate values were calculated symmetrically. Substantially the same method was advocated at about the same time by Hemmingsen (1933), Gaddum (1933), and O'Kane et al (1934). Later in the same year Bliss (1934b) published a revised table in which the probit unit was redefined as equal to 5 plus (algebraically) the deviate of the normal curve expressed in terms of its standard deviation. By transforming the dosage to its logarithm and the percentage mortality to its probit value, dosage-mortality data could now be plotted on ordinary graph paper to give a straight line.

Soon after the appearance of these papers, Bliss (1935a) published detailed accounts of the method of plotting the dosage-mortality points, drawing the provisional regression line, determining weighting coefficients, computing the coordinates and slope of the regression line, and calculating the chi^2

test for goodness of fit of the observations about the computed line. Discussion of the variances of position and slope and the zone of error of the regression line were also included. This was followed by another paper (Bliss 1935b) which described methods for the comparison of the degree of agreement between different sets of dosage-mortality data.

Further work has been done on the statistical comparison of dosage-mortality data from different poisons but this does not directly concern us in a consideration of the present report.

This linear transformation has greatly improved the analysis of toxicity data. Not only does it permit the comparison of results at levels other than the L.D. 50 but it more readily reveals changes in the mode of toxic action which might be disguised by the sigmoid curve. It permits an approximate evaluation of the concentration likely to give 100% kill and gives statistical measures of the position and slope of the line. Furthermore, the chi^2 test measures the goodness of fit of the points about the line and allows the elimination of doubtful determinations.

3. Analysis of Variance

Owing to the fact that the analysis of variance was not originally designed for use with percentages, it is desirable that toxicity data on percentage mortalities should be put through some type of mathematical transformation before being analysed. Bliss (1938) stated that probits, which were devised for another purpose, would not meet the requirements. He

therefore proposed a table of angles of equal information as a basis for a satisfactory transformation. As the percentage varies in this table from 0% to 100%, the corresponding angular values vary from 0° to 90°. Fractions of an angle are given in decimals instead of minutes and seconds.

It is not proposed to refer at this point to any of the various textbooks of statistics which include in their contents a description of the method of analysis of variance but the author wishes to draw attention to a paper by Cochran (1938) in which various difficulties encountered in such work are discussed and illustrated.

III. EXPERIMENTAL APPARATUS AND METHODS

Work on a technique for testing contact insecticides has been carried on in Macdonald College for a number of years, culminating in the publication of a paper by Morrison (1943). The present research was undertaken for the purpose of examining certain sources of variation which had not been fully investigated or had not been investigated at all. Consequently the apparatus and technique already established were used almost without change. In certain specific details it was felt, however, that some modification might contribute to greater uniformity of results. In other cases the prevailing condition of war prevented the purchase of needed materials and a substitute material or method was utilized.

A. Breeding Technique

Adult Drosophila melanogaster were used as the test animal. They are very suitable for the work, being easily reared in large numbers throughout the year. The chief disadvantage lies in the fact that they must be fed during the course of the experiment, being unable to undergo a 24 hour period of starvation between spraying and counting.

Due to the uncertainty of the supply of bananas it was necessary to use the potato medium of Stultz (1939). This was unfortunate as the labor of feeding was much greater than when using bananas. It was soon decided that the recipe of Stultz contained more yeast than was actually required and this amount was cut to approximately 1 Royal yeast cake per 225 gms. of potato. During the greater part of the year this amount was not actually standardized by weighing, though it is thought that no great variations occurred. However, early in 1943 an examination of the paper by Lord (1942) revealed the importance of the yeast/potato ratio and from that time onward the standard of 1 cake of yeast per half pound of potatoes was adopted.

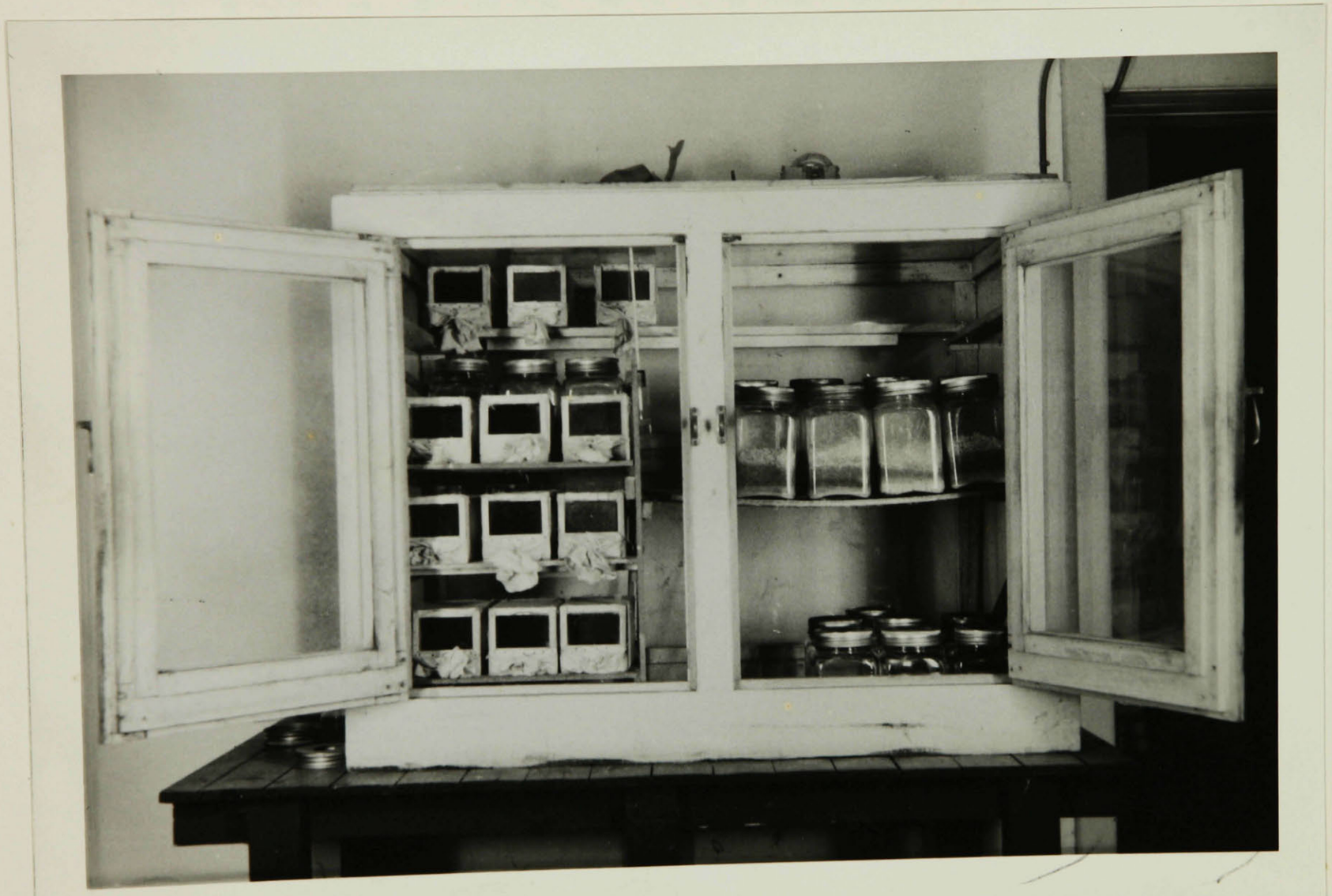
Excess moisture in the food medium is definitely detrimental to the larvae and it was early found necessary to use a minimum of potato water in moistening the yeast. Though Lord's method (l.c.) of using dry yeast was never adopted, it is thought that this would probably give a very satisfactory medium. The food was left in the breeding trays for only two days and was then removed to candy jars. No extra food was added at this time as it was found that the maggots utilized

only the top half inch of the medium and added food was only wasted. As soon as the earliest pupation was observed, clean sawdust was added (instead of bran) to provide a suitable medium for metamorphosis. This sawdust (preferably from a rip-saw) was thoroughly mixed in its container so as to ensure uniformity throughout each different experiment.

The boxes of flies and jars of culture medium were kept in a temperature cabinet at 23.5° C. (see Plate I, page 15). No control of humidity was attempted but it was found that the cultures gave off enough moisture to maintain a level of about 75% to 80% relative humidity at this temperature, in contrast to the humidity in the laboratory which ranged between 22% and 35% during the winter months. The humidity within the jars was probably closer to 100% though this was not tested. Under these conditions it was possible to bring through a generation of flies in about 10 to 12 days.

Newly emerged flies were taken off daily between 4 and 6 o'clock p.m. Any that did not readily fly or walk up into the collecting jar (see Morrison 1943) were allowed to escape into the laboratory, with the result that there was probably less than 1% of the population which did not come exactly within the age limits of any particular group. This small percentage consisted almost entirely of flies which were still in the process of spreading their wings after emergence. The total emergence for each day was transferred to a clean breeding box, complete with a tray of potato-yeast medium, and held until the population had attained the desired age. This

Plate I



Temperature cabinet containing breeding boxes and culture jars

was in contrast with the method of Stultz (l.c.) who fed his spraying cultures on honey solution. It was thought preferable to give the experimental flies full opportunity for oviposition in order to avoid any physiological variations which might be engendered by enforced restraint. In addition, the eggs thus secured were added to the general stock for production of further experimental populations. This latter factor had some influence on the choice of four days as an aging period for most of the work, since it was found that flies of this age had not yet begun to show any marked increase in susceptibility to nicotine sulphate. In any case the author is inclined to agree with the findings of Nelson et al (1934) that flies of any age are equally satisfactory, provided that the particular age chosen is used consistently throughout the experiment.

Considerable trouble was experienced with mites in the candy jars. Although no perfect solution to the problem was evolved it was found that certain methods helped to prevent serious infestations. Infested jars were discarded as soon as discovered. Every effort was made to provide a layer of dry sawdust for the newly emerged flies. This necessitated a rather dry medium together with the addition of sawdust only when the maggots were ready for pupation. Daily removal of adult flies was essential.

B. Cages and Covers

The cages used were 40 mm. in length by 14 mm. inside diameter. Although this may not have been the best size of cage

(Morrison, l.c.), at least uniformity was maintained. All old cages were carefully gauged between two converging bars before being accepted and new ones were cut from uniform stock. The cutting device consisted of a wooden block bored to a depth of about 30 mm. with a 3/4 inch bit. The stock tube was inserted to the full depth of this and was rotated against the edge of a sharp three-cornered file mounted at right angles to the direction of the tube and about one third of an inch from the surface of the wooden block. When a ring had been filed around each end of the long tube, the two cuts were completed by means of a hot resistance wire. The cut ends of the cages were ground on an emery wheel rotating at slow speed so as to assure smooth edges without the danger of reducing the true diameter, as often happens in annealing a tube.

The stock of 28-mesh tulle having been exhausted, an effort was made to secure a fresh supply. This proved to be impossible and it was found necessary to substitute cotton marquisette of 30 strands to the inch. This was not quite as satisfactory as the tulle, since the fibres had a tendency to soften up, thereby reducing the size of the apertures, when they were washed. Accordingly the covers were discarded when it was thought that they were getting too worn. A test later in the winter confirmed the importance of this factor.

C. Method of Filling Cages

The food tray was first removed from a box containing flies of the desired age. A candy jar equipped with a copper-

mesh bottom and a special lid designed to fit the window of the breeding box was used to collect the flies. Aside from these slight differences, the equipment and technique for filling cages were exactly the same as those used by Morrison.

D. Uniform Sampling

Every effort was made to secure a uniform sample of flies in each set of ten cages. Fifteen flies were placed in each cage and the total mortality from 10 cages (about 150 flies) was treated as a single replicate.

Randomization of flies from different culture jars was assured by the method of taking the emergence from all jars and placing it in a single box for storage until the population was sprayed four days later.

The matter of uniform sex ratios and physiological condition presented rather a problem. Concluding, on the basis of results secured by Murray (1937) and Lord (1942), that the two sexes were attracted by light in different degrees and that physiological condition might also influence the speed with which the flies emerged from the box into the separatory jar, it was thought best to take off a portion of the flies, put them in cages, and then divide these cages equally between the different variates. Later the box was cleared again and the remaining quota of cages filled to make up the complete number needed for the test. Except in one small experiment on sexes, no check of sex ratios was made. However, due to this method of allotting the cages to the different groups and to the fact that often as many as 500 or 1000 flies were discarded after

all cages had been filled, it was thought that the sex ratio and physiological condition were fairly uniform between variates in any single experiment. This conclusion was borne out by the nonsignificant variance between replicates secured in Experiment I (Part IV, page 49) and Experiment J (Part IV, page 51) when all replicates were from the same population.

E. Transferring Technique

Thirty minutes after they had been sprayed the flies were transferred to homeopathic vials for storage until the following day. Every effort was made to complete the transfers exactly on schedule. This involved the necessity of having two workers. However, the writer and his assistant each confined himself to the same portion of the work each day in order that any differences due to different workers would be eliminated from the results.

F. Atmospheric Conditions

In the absence of air conditioning in the laboratory no control of temperature and humidity was possible during the tests. Records were kept, however, and it was found that temperatures in the room varied only between 22.5° and 24.0° C. throughout the entire winter. Relative humidities were low in the room but every effort was made to keep the flies in the breeding cabinet at about 80% relative humidity as much as possible except when they were actually being handled. It was hoped, in the light of Potter's work (l.c.) that these

variations would have no significant effect on the mortalities.

G. Preparation of Insecticide

In all tests nicotine in the form of nicotine sulphate was used as the insecticide. The stock solution of C.I.L. 40% nicotine was analysed by the standard A.O.A.C. method. Each day a 2% solution was prepared from the stock. Portions of this were immediately diluted further to give the desired concentrations. Throughout the work the concentrations were expressed in terms of percentage of nicotine alkaloid by weight (grams) present in each 100 c.c. of solution.

H. Spraying Apparatus

The experiments on flies of different ages and on different numbers of flies per cage were conducted with the modified Tattersfield apparatus used by Morrison (1943, Fig. 13). Pressure was 15 pounds per square inch and spraying rate was adjusted by the needle valve in the jet to approximately 1 c.c. in 20 seconds. The correct amount of insecticide was dropped from a burette into the spray cup as soon as each cage had been placed in the clip. It will be convenient in future to refer to this technique as intermittent spraying.

Difficulties in this method were soon apparent. Higher concentrations of nicotine sulphate tended to clog the jet, making frequent clearing necessary and rendering accurate timing of the spraying rate very difficult. In addition, the

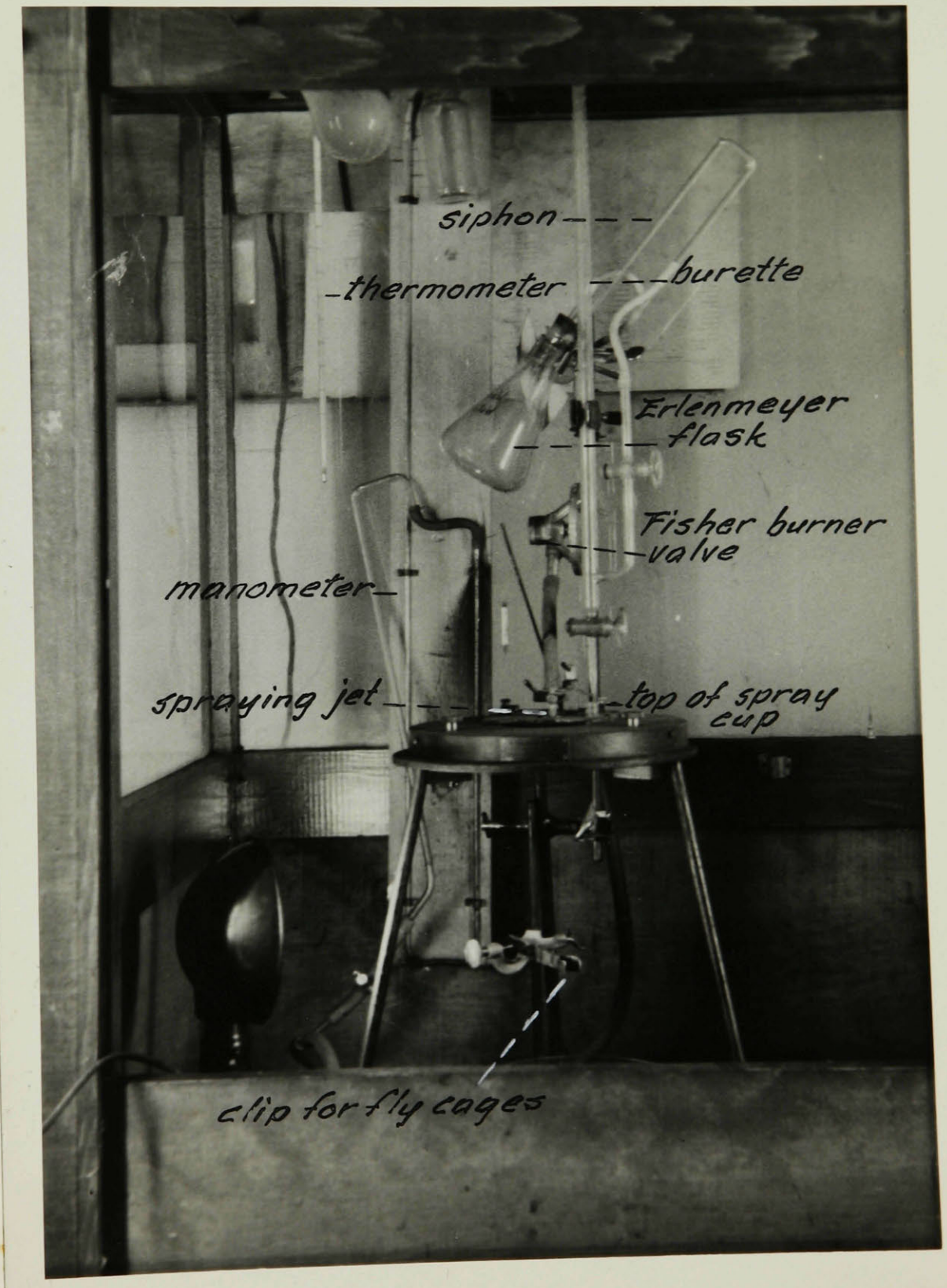
threads of the needle in the valve had become so badly worn that it would not maintain a constant adjustment for any length of time. In any case, the constant change of liquid level in the spray cup caused a corresponding variation in spraying rate during the application of each 1 c.c. portion while the last drop or two issued from the jet with a sudden spurt.

As a result of these observations it was decided to make use of some type of continuous spraying technique, using the time factor as a measure of the amount of material applied. Attempts to continue the use of the needle valve failed and it was found, moreover, that the clogging took place at the needle itself instead of in the tip of the jet, as had been previously supposed. Accordingly the needle (i.e. "nozzle adjustment", Fig. 13, Morrison 1943) was entirely removed and discarded. Since it was impossible to obtain replacement parts, the needle valve from a Fisher High Temperature Burner was connected by means of rubber tubing to the shaft which had previously held the needle (Plate II, page 22). Adjustment of this valve now controlled the rate at which liquid was aspirated from the spray cup and introduced into the air stream.

I. Calibration of the Jet for Continuous Spraying

The calibration of this device presented certain difficulties. It was found necessary to maintain the level of liquid in the spray cup above a certain mark or the spray material would issue from the jet in a series of very rapid surges instead of the steady and even stream desired. Several

Plate II



Spraying apparatus modified for continuous spraying

methods of measuring the rate of flow were tried but the most satisfactory proved to be as follows: as soon as the level of liquid in the spray cup had fallen to a certain mark, an accurately measured volume of spray liquid was dropped from the burette and the time required for the level to fall again to the original mark was recorded; then by adjusting the Fisher Burner valve, the spraying rate was modified until the apparatus was atomizing at the desired speed (in this work, 1 c.c. in 20 seconds).

It was recognized that rate of spraying varied as the level of liquid in the spray cup dropped during this calibration. However it was thought that if the level could be maintained during actual spraying at a point midway between the high and low marks reached during calibration, the spraying rate would be approximately the same as that secured in calibrating by means of the measured sample. All checks indicated this to be true within satisfactory limits.

Just as the level in the spray cup affected the rate of spraying, so it was found that the level in the burette affected the speed with which the liquid passed through the stop-cock into the spray cup. In an effort to minimize fluctuations in the latter, a 300 c.c. Erlenmeyer flask was connected by means of a siphon to the side-arm of the burette. By this means a relatively large volume of spray material could be applied with a negligible change in level in the burette.

J. Technique of Continuous Spraying

Although an effort was made to fix many of the

adjustments and to leave them unchanged thereafter, it was routine procedure at the beginning of each day's spraying to check the air pressure, position and direction of the jet, and centering of the cages in the cone of spray particles. As soon as the jet had been re-calibrated, the stop-cock on the burette was adjusted to maintain the level of liquid in the spray cup at the desired point (midway between the high and low points secured during calibration) and spraying was commenced.

A stop-watch was used to time all operations. The second-hand was started as soon as the first cage was inserted in the clip. When twenty seconds had elapsed the stop-watch was returned to zero while the cage was replaced by an unsprayed one (an operation which required about one second). As soon as the ten cages in that replicate had been sprayed a note was made of the time (thirty minutes hence) at which the flies should be transferred to vials. The box was placed in the breeding cabinet and a new one was taken out to be sprayed.

During the course of the experiment the operator kept constant watch on the air pressure, as read on the manometer, though this seldom varied significantly. Spraying rate was checked at least once for every 10 cages sprayed (i.e. once every 3 to $3\frac{1}{2}$ minutes). Only slight adjustments were required and these were not made unless an immediate recheck confirmed the necessity. The most difficult task was that of maintaining the liquid in the spray cup at the desired level since the nicotine solution, especially in the higher concentrations,

had a tendency to clog the stop-cock of the burette, thus slowing the rate of delivery and allowing the cup to become depleted.

Each cage was examined before being sprayed to see if it contained any flies which might have been damaged in filling and covering. When such flies were observed, their number was marked on the cage with a china-marking pencil and later on the vial during the transfer. This number was deducted from total dead when counting mortalities on the following day.

K. Duration of Each Experiment

The eight replicates for each experiment were always completed in the shortest possible period of days, it being hoped, in this way, to minimize any tendencies toward changing susceptibilities in successive generations of the test animal (Morrison 1943 page 44, Potter 1941 page 164). In the experiment on age of the test animal eight replicates were completed in only 9 days; on numbers of flies per cage, in 13 days; but the one on effect of delays in the daily schedule required a total of three weeks for completion.

L. Lack of Food During the Experiment

The flies were without food from the time the box was cleared in the early morning until the final transfer had been completed and the honey-soaked plug was inserted into the homeopathic vial. This was usually a period of less than four

hours at the most, except in the experiment on the effect of delays in the daily schedule. Since this period was always standardized within the experiment it was hoped to reduce the resulting errors to a minimum. Still the possibility that this factor might be a source of error was fully recognized (Potter 1941).

It was further recognized that the honey-plug itself was possibly a source of error but no practical substitute could be devised which would serve in the case of Drosophila. It should be noted here, however, that fermentation rarely took place and that most of the difficulties were connected with moisture (and possibly some honey) causing the flies to stick to the sides of the vials. This was reduced by binding each group of ten vials together with an elastic band and standing them upright so that the honey-plug was at the bottom of the vial.

M. Statistical Analysis

Twenty-four hours after spraying, the vials were examined and the number of living and dead flies recorded. All flies capable of movement were recorded as living. When, on rare occasions, the flies were found to have been affected by fermentation, the honey-plug was removed and the vial left open to the air. Within a few hours most of them would recover and escape. The dead were then counted, the living determined by subtraction from the total, and the values recorded.

Totals of living and dead in each replicate of ten cages were then taken and percentage mortality was determined

to the first decimal place. Mortalities in the check cages were uniformly satisfactory and it was never necessary to correct for this value. Percentage mortalities were transposed to angles of equal information for use in the analysis of variance. The Table of Angles given in Bliss (1937) gives values to two significant decimals and is to be preferred to the abbreviated table found in the English translation of this paper (Bliss 1938). In each experiment the sums of squares for various interactions were calculated but, when they proved to be not significant, were not removed. Except in the experiment on effect of delays in the daily schedule the data were then plotted graphically as a dosage-mortality curve, using the log. concentration and the probit values of the total mortalities for eight replicates according to the method of Bliss (1935a). An electric calculator was necessarily used in all these computations, values being carried to a number of decimal places.

IV. EXPERIMENTAL RESULTS

A. Analysis of Nicotine Supply

An analysis of the nicotine sulphate supply, (C.I.L. 40%), by the standard A.O.A.C. method revealed a true value of 47.5% nicotine (expressed as the alkaloid) in this sample. This value was accordingly used in calculating the volumes necessary to mix the 2% stock solution and the various concentrations desired for the particular experiment under consideration.

B. Experiment on Effect of Age of Flies

In the experiment on effect of age of flies, culture jars were cleared every afternoon between 4 and 6 p.m. Flies which had emerged during the preceding 24 hours were taken off and placed in a breeding box in the temperature cabinet. On the morning of the following day these were used in the experiment as "flies aged 1 day." Their actual ages, therefore, ranged between 18 and 42 hours. Flies aged 3 days and 5 days were handled according to a corresponding plan, their actual ages being 66 to 90 hours and 114 to 138 hours respectively. Probably less than 1% of the flies were outside these limits, and most of them only by an insignificant amount.

Each age group was sprayed with a water check and concentrations of 0.5%, 0.75%, 1.1%, 1.25% and 1.5% nicotine at the rate of 1 c.c. in 20 seconds. Eight replicates of the experiment were secured in a total of 13 consecutive days. Results of the analysis of variance for this experiment are given in TABLE I on page 29 and the parameters of the dosage-mortality curves for flies of different ages are to be found in TABLE II on page 30. Figure 1, page 31, shows the calculated regression lines secured from these data.

C. Experiment on Effect of Numbers of Flies per Cage

This experiment was designed to supplement the work of Morrison (1943) by confirming the evidence of parallelism in his tests of 15 and 150 flies per container.

TABLE I: Analysis of Variance for Experiment on Flies (Drosophila melanogaster) of Different Ages Sprayed with Nicotine Sulphate (8 replicates)

	Sum of Squares	Degrees of Freedom	Variance	F	F for 5% P	F for 1% P
For ages of flies	1,631.67	2	815.84	23.09	3.09	4.82
For replicates	4,407.34	7	629.62	17.82	2.19	2.99
For concentrations	13,681.94	4	3,420.49	96.82	2.46	3.51
For error	3,745.34	106	35.33			
For total	23,466.29	119				

Standard deviation for error: 5.944°

Interaction of ages and concentrations was not significant.

TABLE II: Calculation of the Dosage-mortality Curves for Flies (Drosophila melanogaster) of Different Ages Sprayed with Nicotine Sulphate (8 replicates)

	n'	n	Range of Mortality in Probits*	$\bar{x}$	$\bar{y}$	b	Chi^2
Aged 1 day	5	3	3.49 to 5.03	2.0275	4.5564	3.2273	26.4003
Aged 3 days	5	3	3.61 to 5.09	2.0239	4.6082	3.0940	33.6230
Aged 5 days	5	3	4.06 to 5.27	1.9967	4.8164	2.5552	12.2592

Chi^2 for 3 degrees of freedom at 5% P : 7.815

Chi^2 for 3 degrees of freedom at 1% P : 11.341

* These probit values were read from the calculated regression line at the lowest and highest concentrations used. They are not necessarily the probits of the mortalities obtained experimentally with these concentrations.

Figure 1:- Probit-log. dosage regression line for flies (Drosophila melanogaster) of different ages sprayed with nicotine sulphate (eight replicates)

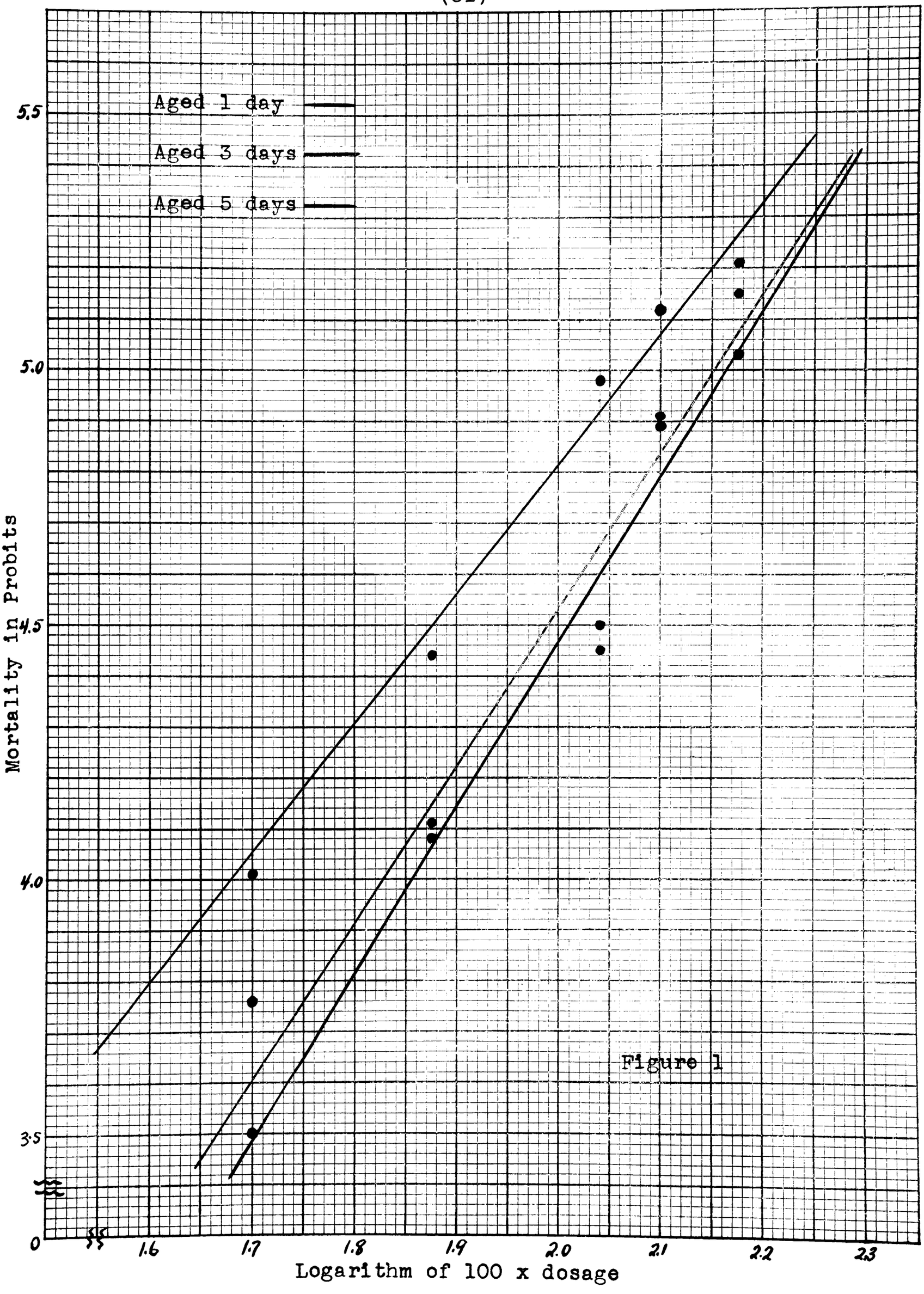

Aged 1 day
Aged 3 days
Aged 5 days
Mortality in Probits
5.5
5.0
4.5
4.0
3.5
0
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
Logarithm of 100 x dosage

Figure 1

Standard cages 40 mm. in length by 14 mm. inside diameter were used throughout the experiment. These were divided into three lots which contained cages of 16, 32 and 64 flies each, respectively. Since it was impossible to count with perfect accuracy, limits of permissible variation within the lots were set at 12 to 24, 25 to 48, and 49 to 120 flies. Cages which failed to qualify for one of these were discarded from the results. A total of 128 flies was considered to be a variate; thus 8 cages of 16 flies each, or 4 cages of 32 flies each, or 2 cages of 64 flies each, were totalled to give a single percentage mortality value.

Each lot was sprayed with a water check and concentrations of 0.5%, 0.75%, 1.1%, 1.25%, 1.5% and 1.75% nicotine at the rate of 1 c.c. per 20 seconds. The flies were aged four days following the clearing of the culture jars. Eight replicates of the experiment were completed in a total of 9 consecutive days. Results of the analysis of variance for this experiment are given in TABLE III on page 33 and parameters of the dosage-mortality curves for different numbers of flies per cage are to be found in TABLE IV on page 34. Figure 2, page 35, shows the calculated regression lines secured from these data.

As was explained in the section on "Statistical Analysis," (page 26), it was customary in the calculation of the regression lines to take the total number of flies for 8 replicates of each treatment, secure the aggregate percentage mortality based on this sum, and transpose this mortality to a probit value. This gave one point on the regression line to correspond with each treatment (usually 5 or 6 points) and the

TABLE III: Analysis of Variance for Experiment on Numbers of Flies (*Drosophila melanogaster*) per Cage, Using Nicotine Sulphate Spray (8 replicates)

	Sum of Squares	Degrees of Freedom	Variance	F	F for 5% P	F for 1% P
For numbers of flies per cage	5,814.43	2	2,907.22	63.04	3.07	4.78
For replicates	11,108.67	7	1,586.95	34.41	2.17	2.95
For concentrations	19,732.68	5	3,946.54	85.57	2.29	3.17
For error	5,949.41	129	46.12			
For total	42,605.19	143				

Standard deviation for error: 6.791°

Interaction of numbers and concentrations was not significant.

TABLE IV: Calculation of the Dosage-mortality Curves for Different Numbers of Flies (Drosophila melanogaster) per Cage, Using Nicotine Sulphate Spray (8 replicates)

	n'	n	Range of Mortality in Probits*	$\bar{x}$	$\bar{y}$	b	Chi^2
64 flies per cage	6	4	4.87 to 6.22	2.0258	5.6825	2.4655	15.2334
32 flies per cage	6	4	4.48 to 5.99	2.0194	5.3102	2.6079	42.2619
16 flies per cage	6	4	4.21 to 5.63	1.9882	4.9682	2.5643	20.3273

Chi^2 for 4 degrees of freedom at 5% P : 9.488

Chi^2 for 4 degrees of freedom at 1% P : 13.277

* These probit values were read from the calculated regression line at the lowest and highest concentrations used. They are not necessarily the probits of the mortalities obtained experimentally with these concentrations.

Figure 2:- Probit-log. dosage regression lines for different numbers of flies (Drosophila melanogaster) per cage, using nicotine sulphate spray (8 replicates)

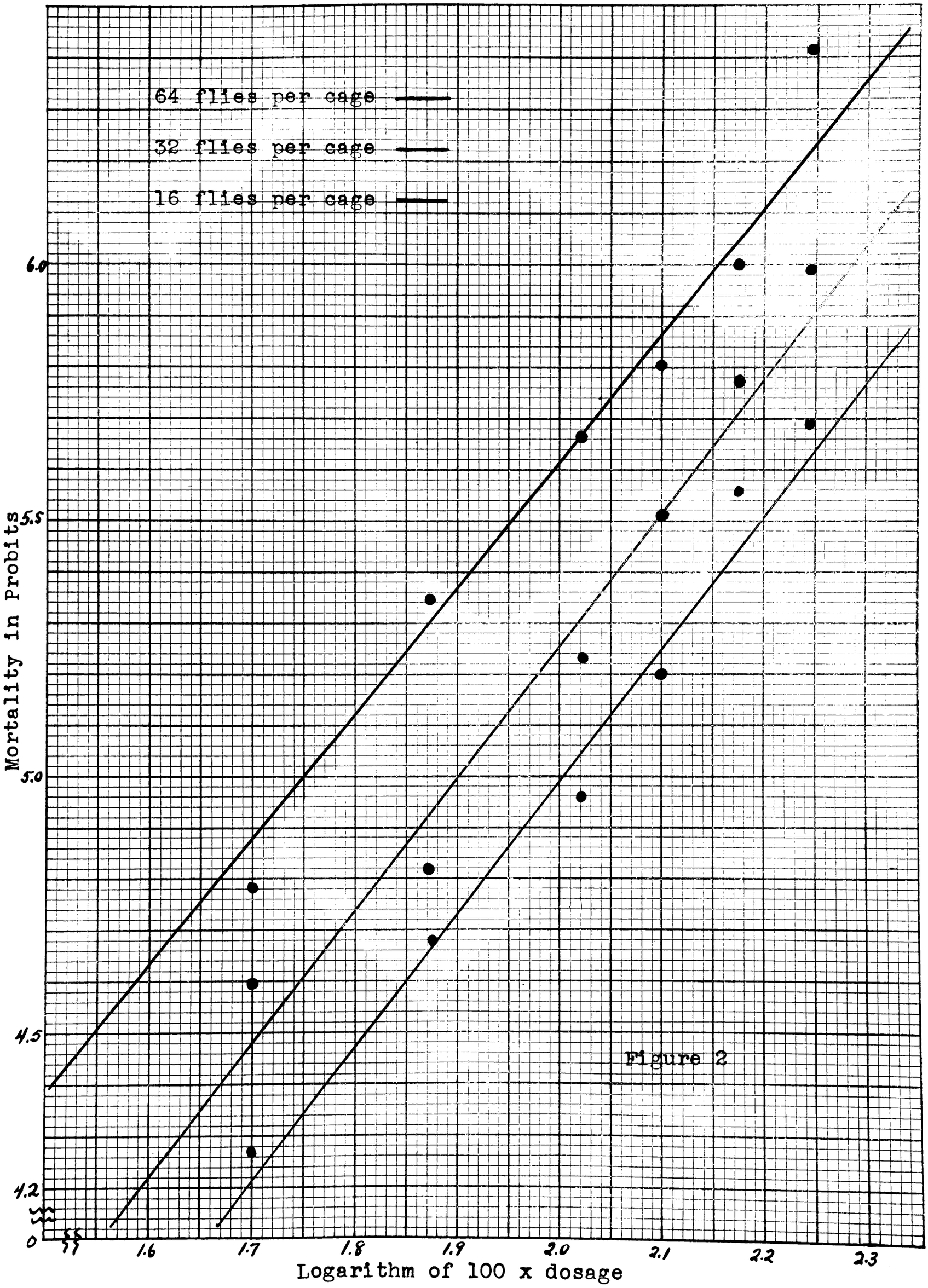

64 flies per cage
32 flies per cage
16 flies per cage
6.0
5.5
5.0
4.5
4.2
0
Mortality in Probits
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
Logarithm of 100 x dosage

Figure 2

degrees of freedom were correspondingly less than the number of points (3 and 4 degrees of freedom respectively) (Bliss 1935a).

Noting that Bliss (1935a) in his example used each value in each series (a series corresponding to a replicate in the present work) as a point in plotting and calculating his regression line, and thereby increased the number of degrees of freedom, the author determined to try a similar analysis with some of the present data. It was realized that the chi^2 value would be much larger but it was hoped that the great increase in the number of degrees of freedom would counterbalance this and give a less unsatisfactory test of homogeneity. The raw data on 32 flies per cage were chosen for the proposed analysis. Each separate variate was plotted in the graph and used in the computation. The following values, which may be compared with values for 32 flies per cage in TABLE IV, page 34, were secured:

n'	n	$\bar{x}$	$\bar{y}$	b	Chi^2	$\sqrt{2X^2} - \sqrt{2n-1}$
48	46	2.0194	5.3440	2.8631	878.0007	32.34417*

* See Table of Chi^2 in Fisher (1938) or Paterson (1939)

D. Tests on Rate of Spraying and Amount of Deposit

On November 18 and 19, 1942, cages were sprayed by the intermittent method in order to discover what correlation existed

between spraying time and amount of spray deposited. One cubic centimeter of 1.5% nicotine solution was atomized on each cage and the time required for the application was carefully recorded with a stop-watch. No effort was made to obtain uniformity of spraying time, as had previously been done, but instead, the jet was deliberately allowed to become obstructed by spray materials. During these two experiments the spraying time varied between 8 and 100 seconds, though most of the values lay between 8 and 60 seconds, and the spray deposits ranged from 10 to 80 milligrams.

Cages were first filled with a plug of absorbent cotton and covered on both ends with marquisette. No flies were used on these two days. Each cage was placed in a shell vial closed with a rubber stopper and was weighed. After weighing, the cage was removed from the vial, sprayed and immediately returned to the vial. The latter was promptly stoppered in order to prevent loss of weight due to evaporation in the dry atmosphere of the laboratory (relative humidity about 40%). During the actual spraying the top of the cage was encircled by a metal shield which prevented any nicotine solution from adhering to the outside of the glass tube or to the marquisette except across the end of the tube. The combination of vial and cage was then reweighed and the amount of spray deposit determined by subtraction.

On November 20 and 21 the experiment was repeated but with about 15 flies in each cage and without the absorbent cotton.

When the data were calculated the following values of the coefficient of correlation, r, were secured:

Date	Number of cages	Percent nicotine	r	Coefficient of correlation for
Nov.18,19, 20,21.	91	1.5	-0.924	spraying time X amount of deposit
Nov. 20	39	1.5	-0.151	amount of deposit X percent mortality
Nov. 21	11	0.75	-0.045	"
Nov. 21	29	1.5	-0.075	"

Noticing the high negative correlation between time of spraying and amount of deposit, the writer concluded that for further work of a preliminary nature it would be sufficient to correlate spraying time and percentage mortality. Data on these factors could be accumulated much more rapidly than if each tube were required to be weighed but if this data indicated any significant correlation it might be possible at a later date to repeat the work in such a manner as to actually correlate weight of deposit and percentage mortality. Moreover, during the process of weighing the cages sprayed on November 21st, the flies had of necessity been left in the tightly stoppered vials for several hours and the resulting mortalities had been unduly high.

Accordingly, further data were secured on November 30th and December 4th respectively. On November 30th 100 cages, each containing about 15 flies aged three days, were sprayed with

1 c.c. of 0.9% nicotine. Spraying times ranged from 10 to 70 seconds and were recorded with a stop-watch. The flies were transferred to homeopathic vials after 30 minutes and mortalities were counted after 24 hours according to the usual method. Mortalities from these cages were still rather high, most of them being over 80%, so the work was repeated on December 4th when 97 cages were sprayed with 0.5% nicotine in exactly the same manner. This time the spraying times ranged once again from about 7 to 60 seconds while mortalities were chiefly below 50%. Naturally, since no weighing was done, it was unnecessary to place the individual cages inside shell vials at any time during these two experiments. The following results were secured:

Date	Number of cages	Percent nicotine	r	Coefficient of correlation for
Nov. 30	100	0.9	0.258	spraying time X percent mortality
Dec. 4	97	0.5	-0.208	spraying time X percent mortality

E. Tests on a Continuous Spraying Technique

The author was ready at this time to accept the evidence that amount of deposit could vary within certain limits without having a significant effect on mortality and was prepared to continue with the so-called intermittent type of spraying. It was proposed to conduct one more experiment, however, in order to determine within what limits it was possible by careful

attention to the performance of the jet to maintain the variations in spray deposit on a number of different cages. Before this experiment could be performed the needle in the valve of the jet became so worn as to be entirely unsatisfactory.

Accordingly, the "continuous spraying" technique was devised and its performance was calibrated (see "Spraying Apparatus", page 20, and "Calibration of the Jet for Continuous Spraying", page 21). During the final test the jet was adjusted to deliver 1 c.c. of 1.5% nicotine sulphate in 20 seconds and was allowed to run without further correction for 60 minutes. During this time the spraying rate was checked approximately every five minutes. Recorded spraying times varied between 18.6 and 21.8 seconds but this range was considered satisfactory in view of the fact that the 1 c.c. portions of nicotine solution were measured in haste with a 50 c.c. burette. In later work it was found that spraying rate, once properly adjusted, would sometimes do over 100 cages with concentrations ranging up to 1.5% nicotine without any necessity of readjustment. Higher concentrations usually required a slight correction of the needle valve.

F. Experiments on Intermittent versus Continuous Spraying

The next step seemed to be an experiment to compare the two types of spraying, intermittent and continuous, in order to justify the adoption of the latter. Such experiments were attempted on December 7th, 14th and 18th.

On December 7th about 200 cages were prepared in the usual manner, each having about 15 flies aged 4 days in it. During the morning 90 of these were sprayed by the intermittent method using 0.8% nicotine and a few were sprayed with water as a check. After dinner another 90 were sprayed by the continuous method and again a few were sprayed with water as a check. The cages were not grouped in any particular arrangement and mortalities, when counted 24 hours later, were recorded in random order. When the standard deviation of the individual cages and the standard deviation of the mean obtained by averaging the individual cages had been determined, it was thought that similar statistics for groups of 10 cages might be of interest. Mortalities were therefore placed in groups of 10 in the order of counting and the analysis was performed.

The experiment was repeated on December 14th with the same concentration of nicotine but on this occasion 100 cages were sprayed in groups of 10's. On the following day mortalities were counted, the various groups being recorded in the same order as that in which they had been sprayed. The chief difference in procedure was that the continuous type of spraying was used in the morning and the intermittent in the afternoon. When the data had been analysed the results were about as expected with one exception. In both tests, and for no apparent reason, mortalities were higher in the morning than in the afternoon.

The experiment was therefore performed again on December 18th, using 100 cages for each type of spraying as before, arranging the cages in groups of 10's and recording

the mortalities in the order of spraying, but using 0.9% nicotine and spraying by the intermittent method in the morning and the continuous method in the afternoon. The arithmetic mean of the individual mortalities (and also the total mortality) was once again higher for the morning spray than for the afternoon. TABLE V, page 43, gives a summary of the data secured from these three experiments.

G. Experiment on Effects Secured When Both Nicotine and Test Animals Were Allowed to Stand for a Period of Time

The fact that in the three preceding experiments the mortalities secured in the morning were higher than those secured in the afternoon, regardless of the method of spraying, suggested that there might be some definite cause of the variation. This was especially so since such results were the opposite of those which would naturally be expected.

Accordingly a rough, preliminary experiment was designed to indicate whether nicotine produced a different effect after it had been allowed to remain standing for a period of some hours and whether the flies themselves were more or less susceptible after a similar interval of time. A 0.9% nicotine solution was prepared at 7 a.m. During the 2 hour period between 8 and 10 a.m. 180 cages were filled with about 15 flies each and at 10 a.m. another solution of 0.9% nicotine was prepared. Between that time and 12 o'clock noon, 40 cages in 4 groups of 10 cages each, were sprayed with one solution,

TABLE V: Statistics from Data Secured in Comparing Intermittent and Continuous Methods of Spraying

Variate	Type of Spraying	Total Mortality	Standard Deviation	S.D. of the mean	Type of Spraying	Total Mortality	Standard Deviation	S.D. of the mean
	Morning Spraying				Afternoon Spraying			
December 7th, using 0.8% nicotine, mortalities counted at random and later grouped in order of counting.								
Individual cages	Intermittent	37.64%	24.11%	2.50%	Continuous	17.14%	18.38%	1.92%
Groups of 10 cages			5.23%	1.74%			7.47%	2.49%
December 14th, using 0.8% nicotine, cages sprayed in groups of 10 and groups counted in order of spraying.								
Individual cages	Continuous	29.83%	32.24%	3.22%	Intermittent	16.5%	15.73%	1.57%
Groups of 10 cages			6.86%	2.17%			6.50%	2.05%
December 18th, using 0.9% nicotine, cages sprayed in groups of 10 and groups counted in order of spraying.								
Individual cages	Intermittent	54.68%	17.94%	1.80%	Continuous	48.47%	17.86%	1.79%
Groups of 10 cages			3.10%	0.98%			3.69%	1.17%

40 cages similarly grouped were sprayed with the second solution, and 10 were sprayed with water as a check. During the afternoon the remaining cages were similarly treated with the same two solutions (which were now 9 and 6 hours old, respectively,) at 4 p.m. Mortalities were counted after the usual 24 hour period, percentage mortalities were transposed to angles of equal information (Bliss 1937) and analysed by the analysis of variance.

The following statistics were secured:

	Sum of Squares	Degrees of Freedom	Variance	F	F for 5% P	F for 1% P
For aging of flies	94.92	1	94.92	12.28	4.67	9.07
For aging of nicotine	2.12	1	2.12	Not significant (less than 7.73)		
For error	100.53	13	7.73			
For total	197.57	15				

Standard deviation for error: 2.78°

Average mortality for flies sprayed in the morning was 57.7%.
Average mortality for flies sprayed in the afternoon was 65.8%.

These results were taken to indicate that in all probability nicotine does not change in toxicity upon standing for a few hours. The value of F for "aging of flies" seemed high enough to warrant further investigation, however, and an adequate experiment was consequently designed.

H. Experiment on Effect of Delays in the Daily Schedule

No attempt was made in designing this experiment to provide for the separate analysis of effects of delays on the nicotine and on the flies. Instead it seemed more profitable merely to secure the cumulative effect of all factors concerned in such a delay.

Each day 180 cages were filled with approximately 15 flies each, the flies having been previously aged four days. These were divided into three lots of six groups of 10 cages per group. The three lots were sprayed at 10.30 a.m., 1 p.m. and 3.30 p.m. respectively with a water check and concentrations of 0.7%, 0.8%, 0.9%, 1.0% and 1.1% nicotine at the rate of 1 c.c. per 20 seconds (using the continuous method). Eight replicates of the experiment were completed over a period of three weeks.

Results of the analysis of variance for this experiment are given in TABLE VI on page 46. The provisional regression lines are shown in Figure 3, page 47, but, due to the fact that mortalities were too low to be considered satisfactory, the calculated regression lines were not obtained nor was the chi^2 test computed. TABLE VII, page 48, gives the percentage mortality for each treatment based on the total number of flies tested in eight replicates. Should the experiment be repeated, higher mortalities would give more reliable data.

TABLE VI: Analysis of Variance for Experiment on Effect of Delays in the Spraying Schedule When Flies (Drosophila melanogaster) were Sprayed with Nicotine Sulphate

	Sum of Squares	Degrees of Freedom	Variance	F	F for 5% P	F for 1% P
For times of day	2,563.14	2	1,281.57	76.30	3.07	4.82
For replications	1,747.30	7	249.61	14.86	2.19	2.99
For concentrations	703.21	4	175.80	10.47	2.46	3.51
For error	1,780.47	106	16.80			
For total	6,794.13	119				

Standard deviation for error: 4.098°

Figure 3:- Provisional probit-log. dosage regression lines for flies (Drosophila melanogaster) sprayed with nicotine sulphate after different periods of delay in the spraying schedule.

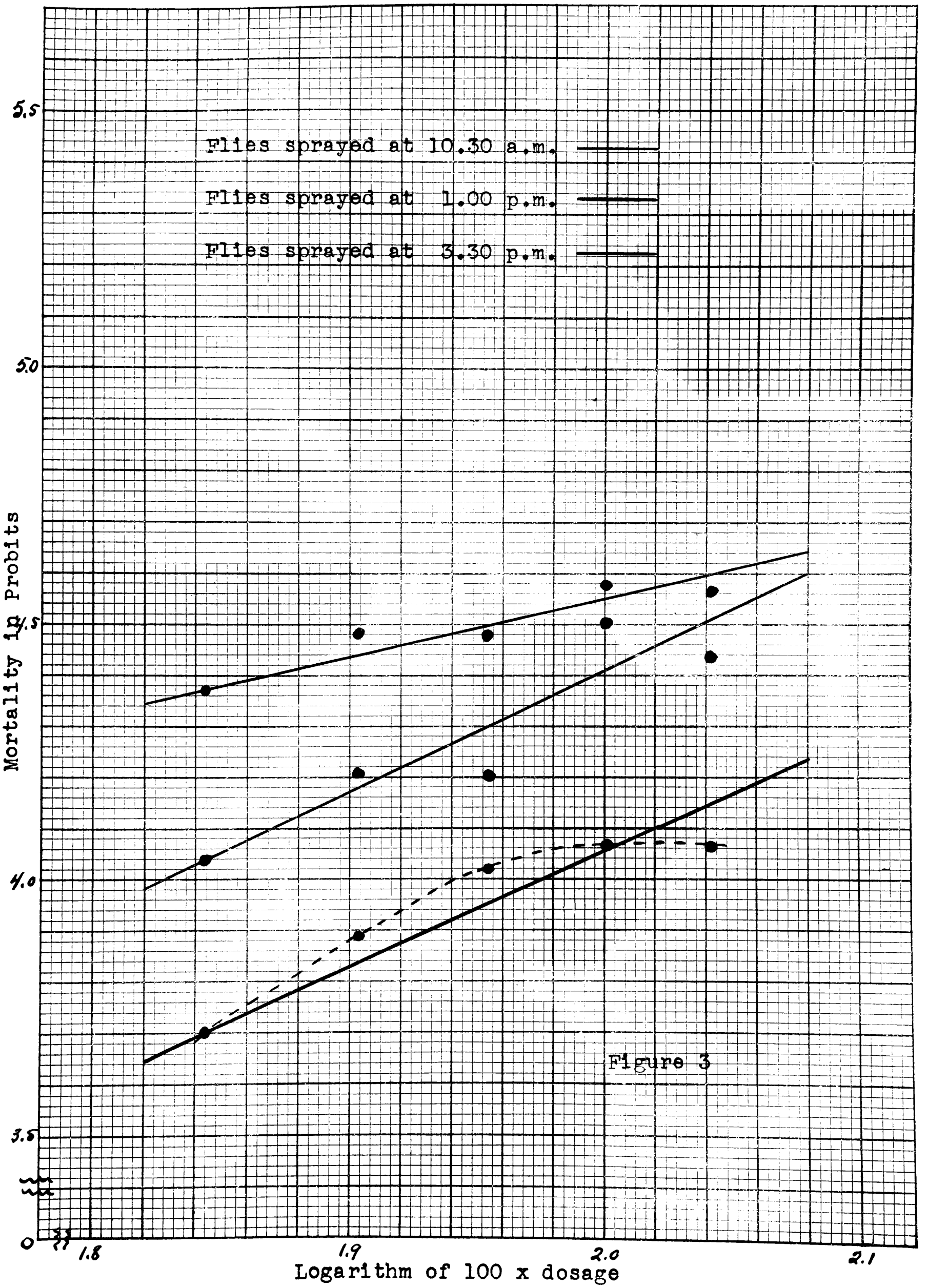

Flies sprayed at 10.30 a.m.
Flies sprayed at 1.00 p.m.
Flies sprayed at 3.30 p.m.
Mortality in Probits
5.5
5.0
4.5
4.0
3.5
0
1.8
1.9
2.0
2.1
Logarithm of 100 x dosage

Figure 3

TABLE VII: Percentage mortality for each treatment based on total number of flies (Drosophila melanogaster) sprayed with nicotine sulphate in 8 replicates of experiment on effect of delays in the spraying schedule.

Dosage of nicotine		0.7%	0.8%	0.9%	1.0%	1.1%
Time of Spraying						
10.30 a.m.	Total flies	1208	1135	1190	1205	1228
	% mortality	9.69	13.39	16.39	17.43	17.43
1.00 p.m.	Total flies	1132	1205	1173	1161	1176
	% mortality	16.77	21.33	21.14	30.92	28.57
3.30 p.m.	Total flies	1209	1193	1237	1200	1167
	% mortality	26.39	30.18	29.99	33.50	33.08

I. Experiment on the Effect of Different Types of Cloth Covers

It had been noticed for some time that the marquisette covers on the cages had a tendency to soften with washing, thus causing the openings between the threads to become somewhat reduced in size. The difficulty had been overcome by replacing the old covers with new ones as soon as it seemed necessary but it was finally decided to do a brief experiment in order to see whether the type of cloth had any effect on the mortality produced.

Four types of cloth were used: tulle (remnants discarded from previous work in this department), fine Brussels netting,

unused marquisette, and used marquisette which had been frequently washed. Fifty cages, each containing 15 flies aged 4 days, were covered with each type of cloth. The cages, arranged in groups of 10's, were sprayed with 0.9% nicotine but during the course of the experiment it was observed that the Brussels netting was of too large a mesh and was allowing most of the Drosophila to escape. It was accordingly discarded and the experiment was continued with the remaining three types of cloth. Mortalities were counted after 24 hours, the groups being recorded in the order of spraying. The percentages were transposed to angles of equal information and analysed by the analysis of variance with the following results:

	Sum of Squares	Degrees of Freedom	Variance	F	F for 5% P	F for 1% P
For different types of cloth	355.45	2	177.73	6.205	4.46	8.65
For replicates	102.78	4	25.70	Not significant		
For error	229.12	8	28.64			
For total	687.35	14				

Standard deviation for error: 5.35°

J. Experiment on Physiological Condition of the Flies

As has been proven by many workers, the physiological state of the flies has a pronounced effect on the mortalities

secured in toxicity tests. The highly significant F tests for replicates in the experiments which involved 8 replications on different days in the present work are sufficient proof of that fact. It was thought, however, that possibly phototropistic responses might be of some use in the selection of a sample of flies which would give more uniform and predictable results under test conditions.

Several devices designed to collect flies from the regular spraying culture on the basis of their responses to light were tried but none proved satisfactory. It was finally decided to clear the culture in the regular manner (see "Method of Filling Cages", page 17) but without undue disturbance of the flies, which had previously been dark-adapted for a period of 18 hours. Those which rose readily into the collecting jar were classified as "early" flies. Those which did not rise voluntarily to the light but required to be shaken out of the box were classified as "late" flies and were kept in a separate container. These flies were used to fill 100 cages (10 groups) from each category. Two groups, one of early and one of late flies, were used in the checks. The remaining nine groups of each type were sprayed with 0.9% nicotine, thus giving a total of 18 variates for the experiment. Flies aged four days were used. The mortalities were counted after 24 hours and, after being transposed to angles of equal information, were used in the analysis of variance. The resulting statistics are to be found in the table at the top of page 51.

	Sum of Squares	Degrees of Freedom	Variance	F	F for 5% P	F for 1% P
For different types of flies	0.0235	1	0.0235	Not significant		
For replicates	24.44	8	3.06	Not significant		
For error	117.77	8	14.72			
For total	142.24	17				

Standard deviation for error: 3.836

K. Test of Homogeneity of Data

It having been proved in the foregoing section that the "early" and "late" flies were definitely of the same population, it was decided to use the 18 variates in this experiment as a means of determining the homogeneity of results secured by the technique of spraying which had been developed. The chi^2 test of Snedecor and Irwin (1933) was used and gave a value of chi^2 equal to 28.477. (The chi^2 for 17 degrees of freedom at 5% P is 27.587 and at 2% P is 30.995.)

L. Experiment on Sex Ratios and the Relative Susceptibility of Male and Female Flies

As soon as the mortalities had been recorded in the normal way for the experiment on the physiological condition of the flies, the same cages were counted once again. Both

living and dead were sexed and recorded separately in order to give the percentage mortality of male and female Drosophila. The work was slow and very hard on the eyes with the result that it was possible to sex only about 1265 flies during the remainder of the day. Since there had not yet been any chance to examine the data from the experiment on physiological condition of the flies, it was thought best to select cages from both "early" and "late" flies in case there should prove to be a significant difference between these two categories. This was done, the first four groups of each category being sexed and recorded. The data secured are given in TABLE VIII, page 53.

V. DISCUSSION OF RESULTS

A. Effect of Age of Flies

It is generally agreed by those interested in toxicity tests that age of the test animal is one of the most important factors to be considered. Morrison (1943) records the results of experiments on this factor by Maxwell and by Cameron and Prebble. He himself did an experiment on ages of flies in which he found 1 day old flies to be most susceptible and 4 day old flies least susceptible. Except for this one test, Morrison used a randomized mixture of flies varying in age from a few hours up to the age to be tested (usually 4 days). In spite of the great increase in work involved, the author decided to use flies within a 24 hour range of age in all tests, hoping thereby to secure

TABLE VIII: Data on Relative Susceptibility of Male and Female *Drosophila melanogaster* when sprayed with nicotine sulphate

Group	Total Number of Flies	Total Mortality	Number of Males	Mortality of Males	Number of Females	Mortality of Females	Sex Ratio Males / Females
Early Flies							
1	168	30.7%	77	39.0%	91	22.0%	46/54
2	167	20.0%	63	41.3%	104	7.7%	38/62
3	169	22.9%	64	36.0%	105	12.4%	38/62
4	184	25.9%	95	45.3%	89	25.3%	52/48
Total	688		299		389	Average	$43\frac{1}{2}/56\frac{1}{2}$
Late Flies							
1	142	22.7%	78	30.8%	64	12.5%	55/45
2	140	27.9%	71	39.5%	69	17.4%	51/49
3	157	35.7%	84	52.4%	73	15.1%	53/47
4	138	28.7%	66	33.3%	72	15.3%	48/52
Total	577		299		278	Average	52/48
Average (8 replic.)		26.8%		39.7%		16.0%	

greater uniformity of results. As previously stated, great care was exercised in clearing the cultures and the number of flies which exceeded the stated age was negligible.

Since Morrison and others using the banana medium cleared their culture jars early in the morning of the day of the experiment, their 1 day old flies ranged from 0 to 24 hours while, as previously explained, those flies listed herein as "aged 1 day" were actually from 18 to 42 hours old. This will account for the fact that the writer did not secure indications of such high susceptibility in the lowest age-group as did some of the other workers.

The significance of the age factor is unquestionable, since the F value is over seven times as great as the F for 5% P (TABLE I, page 29). An increase in susceptibility was noted as the data on flies aged 1, 3 and 5 days were compared, being especially prominent in the last group, (TABLE II, page 30, and Figure 1, page 31) but the chi^2 test indicated greater uniformity of results in flies aged 5 days. It might be worth noting also that Morrison (1943, Fig. 9, page 50) secured a smaller chi^2 value for 4 and 5 day than for 3 day old flies. These factors were taken into consideration in the choice of 4 days as an aging period. In any case, the author agrees with Nelson et al (1934) that flies of any age are equally satisfactory, provided that the particular age chosen is used consistently throughout the experiment.

Further comparisons with the work of Morrison (l.c.) were not possible since he used nicotine sulphate in a 1% saponin

solution and determined only 3 points, while the author used nicotine sulphate alone and determined 5 points on the regression line.

It will be noted in Figure 1, page 31, that the slope of the regression line for flies aged 5 days is considerably less than corresponding values for either 3 day or 1 day flies, being 2.55 as compared with 3.09 and 3.22 respectively. Should these values be confirmed in a repetition of this experiment it would mean that the comparative susceptibility of flies aged 5 days is increased by a greater amount in the case of the lower concentrations than of the higher ones. This will indicate that, in the absence of parallelism of the regression lines, results of experiments with the same toxicant on different ages of Drosophila may not be truly compared at any one level of mortality. It will thus be possible to make such comparisons only by means of the parameters of slope and position of the regression line.

B. Effect of Numbers of Flies per Cage

Morrison (1943) found that mortality steadily increased with increases in the number of flies per cage. He suspected but did not demonstrate a parallelism between the regression lines for different numbers per container, using the spraying technique. In addition, his chi^2 values indicated less heterogeneity in the data when the smaller numbers were used.

Stultz (1939), who used lantern globes as cages, stated, "Spray tests with nicotine sulphate.....do not indicate

any significant correlation between number of flies per globe and percentage kill."

The experiment herein recorded (IV, C, page 28)*

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* (IV, C, page 28) This reference is to Section IV (EXPERIMENTAL RESULTS), Part C (Experiment on Effect of Numbers of Flies per Cage) which is found on page 28 of this report.

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definitely supports the findings of Morrison (l.c.). The F value for numbers of flies per cage in the analysis of variance (TABLE III, page 33) is over 20 times the value of F for 5% P. This degree of significance cannot be doubted. Furthermore, the parallelism between the regression lines is also demonstrated within close limits (see TABLE IV, page 34, and Figure 2, page 35) since the values of b, the slope, for 16, 32 and 64 flies per cage are respectively 2.56, 2.61 and 2.47, when determined on the basis of 6 points. The chi^2 values (TABLE IV) are also of interest since that for 16 flies per cage is comparatively satisfactory though it should be noted that the value for 64 flies per cage is even lower.

Such evidence makes it necessary that Stultz's statement be qualified in some manner. It may be that a significant correlation exists only when some factor such as absolute size of the cage, or average volume of cage per fly, is below a certain threshold value. What this factor may be the author did not try to discover but it is evident that one must exist and that Stultz was working outside the limits of its effect. The parallelism of slopes in the regression lines allows us to conclude that this undiscovered factor acts with equal intensity

regardless of the concentration of chemical being used.

When the author decided to investigate the effect of using the mortality from each concentration in each replicate in the calculation of the regression line and the value of chi^2, Morrison's paper had not yet been published. Upon reviewing the latter it was found that he had suggested the same procedure. The results secured (page 36) do not make this analysis one to be advocated for experiments involving many replicates. As Morrison anticipated, the calculation was considerably more laborious. Moreover, the increase of chi^2 due to the use of many points about the regression line was not in any way compensated for by the increase in the number of degrees of freedom. Consequently the value of 32.344 secured with 46 degrees of freedom (page 36) was 16 times the value of 2 which is considered to be satisfactory, while the chi^2 value of 42.262 secured from the same data using only 4 degrees of freedom (TABLE IV, page 34) was less than $4\frac{1}{2}$ times the value of 9.488 obtained from the chi^2 table at 5% P. This demonstrated that, when drawing a regression line in toxicity tests, the method of plotting the mortality secured from the totals of many replicates is easier and gives a lower value of chi^2 than does the method of plotting the individual values secured in each replicate. This is natural enough since the averaging of the results from a number of replicates tends primarily to remove the effects of extreme variation in some of them.

C. Effect of Variations in Spraying Technique

Callaway and Musgrave (1940) kept concentration of the insecticide constant but secured variations in effect by varying the amount of deposit. They found that percentage mortality increased with increased deposit. Potter (1941) also found that percentage mortality increased with increased deposit, provided the concentration being applied was above the lower threshold of toxicity.

Morrison (1943) found that the rate of application (1 c.c. in 10 seconds, or 1 c.c. in 25 seconds) was not an important factor within the limits tested, though he adopted the slower rate on the basis of a lower chi^2 value and lower variance in the data. He did not actually measure the deposit. The writer, in similar experiments, found that 1 c.c. of 1.5% nicotine sprayed in 10 seconds gave an average deposit of 70 milligrams while the same quantity sprayed in 25 seconds gave an average deposit of 48 milligrams. It is therefore logical to assume that Morrison actually varied the amount of deposit without securing a significant effect.

The writer found results similar to Morrison's when using the intermittent spraying technique. The problem was not fully investigated nor was it investigated at all after the adoption of the constant spraying method. This latter would, it is thought, prove to be an excellent method for such an investigation.

In this experiment the actual amount of spray material placed in the reservoir was always constant at 1 c.c. per cage.

The amount reaching the cage and, it is presumed, the test insects was measured at first by weighing (IV, D, page 36). This amount was found to depend directly on the condition of the jet. When the latter was clean, the liquid was quickly applied in relatively large droplets with a consequently heavy deposit. As the jet became somewhat plugged with nicotine the spray required much longer for atomization, the droplets became much finer, and less of the resulting mist became deposited on the cage and its contents. Although the application was relatively much heavier than that secured by Potter (as much as 80 milligrams on a cage 14 mm. in diameter as compared with Potter's deposits of up to 140 mg. on a Petri dish 9 cm. in diameter) and the concentration of 1.5% nicotine was definitely high in the toxic range, the coefficients secured failed to show any significant correlation between amount of deposit and percentage mortality. Further work correlating spraying time (which varied inversely as the weight of deposit, having a coefficient of correlation of -0.924) with percentage mortality resulted in correlation coefficients of greater numerical size but still not large enough to be considered significant (IV, D, page 39).

It is probable that Potter's statement is true as far as it goes. He says that "Where the concentration of the poison is below a certain threshold value, variation of the deposit within wide limits has little or no effect." He goes on to state that above this threshold the mortality is affected by the weight of deposit. It seems, then, that weight of deposit as well as concentration of poison may have different effects

in different ranges. The amounts Potter used (ranging up to 2.4 mg. per sq. cm. when calculated on the basis of 155 mg. on a 9 cm. Petri dish) were within the range where variations in deposit have an effect on percentage mortality while the amounts used by the author (ranging up to about 52 mg. per sq. cm. when calculated on the basis of 80 mg. on a cage 1.4 cm. in diameter) were so far above this range that variations in amount of deposit had little or no effect.

No comments are necessary in regard to the tests on continuous spraying (IV, E, page 39). In an experiment which is still in progress at the time of writing, careful checks have indicated that the method of calibration described is indeed accurate. The present work requires that 20 cages be sprayed with each concentration of nicotine. This makes it unnecessary to use the Erlenmeyer flask and siphon. Instead, the insecticide is placed directly in the burette. The jet is checked for spraying rate (1 c.c. in 20 seconds) at the beginning of each new set of 20 cages. Then the level of liquid in the burette is read and spraying is commenced. The cages are sprayed in a total of about 7 minutes and the level of liquid is once again noted. If the operator has watched the liquid level in the spray cup carefully the amount of spray material used is never less than 20 c.c. and seldom more than 22 c.c. Such accuracy is somewhat more difficult to achieve with the more concentrated solutions.

In the comparison of intermittent and continuous methods of spraying a review of the data in TABLE V, page 43,

leads to a conclusion that there is no clear evidence for the superiority of either method. The standard deviations of the experiments and standard deviations of the means are comparable in magnitude. In any case, evidence had already been secured that the spraying rate was more constant for each 1 c.c. portion when using the continuous method but further evidence indicated that amount of deposit, within the range of applications recorded in this work, had little effect on mortality. The author felt justified in concluding, therefore, that the substitution of continuous for intermittent spraying would certainly not introduce any greater heterogeneity in the results of experiments and would provide some possibility of improvement in the consistency of the data.

The most interesting observation was that mortalities were consistently higher in the morning than in the afternoon, regardless of the type of spraying being used. This was the opposite of what would naturally be expected and further experiments failed to discover any reason or explanation for the phenomenon.

D. Effect of Delays

The variance for aging of nicotine in the preliminary experiment (IV, G, page 42) was so low that it was taken to indicate a definite lack of significance in this factor. The F test for aging of flies, on the other hand, was significant and led to a decision to perform a more comprehensive experiment. It was interesting to note that mortalities were somewhat higher

in the afternoon than in the morning, in contrast with results secured in experiments on intermittent versus continuous spraying.

In the later experiment (IV, H, page 45) the author was led to the choice of these concentrations by the results secured on December 18th (TABLE V, page 43) when 200 cages sprayed with 0.9% nicotine produced an average mortality close to 50%. The only explanation for the low mortalities secured in January is that the breeding cabinet was fumigated with nicofume during the Christmas week. Though subsequently well ventilated, the cabinet may have retained enough nicotine to kill the weaker flies with the result that these tests were actually run on what was for all practical purposes a stock of flies selected on the basis of resistance to nicotine.

In any case, the F test for delays in the daily schedule ("times of day" in TABLE VI, page 46) was highly significant and there can be no doubt that such delays have a marked influence on results secured in laboratory tests with insecticides, particularly when the test animal is unable to get along without food and water for a period of 24 hours. The obvious correction for this factor is to make all haste with the experiment so as to finish it within the shortest possible period of time. All parts of the work should be done on a strict time-schedule and deviations from this schedule should be eliminated as far as is possible. Should any unforeseen difficulties cause a serious loss of time during the experiment the results should not be accepted until a careful scrutiny proves that the mortalities are comparable to those secured in other replicates.

The regression lines in Figure 3, page 47, and the percentage mortalities in TABLE VII, page 48, indicate that a progressive increase in susceptibility took place as the period of delay increased in length. This is the result one would naturally expect and the weight of evidence from 8 replicates in this experiment should be sufficient together with a similar result secured in the preliminary experiment (IV, G, page 42) to overbalance the unexplained results secured in the 3 tests on intermittent versus continuous spraying (TABLE V, page 43). It will be noticed that the points for flies sprayed at 10.30 a.m. follow a definite curve but no conclusions may safely be drawn from any of the provisional regression lines because of the low mortalities secured.

E. Effect of Different Types of Cloth Covers

Although this was not a comprehensive test, the fact that the F value secured was greater than F for 5% P (IV, I, page 48) indicates that the use of different types of cloth in covering the cages may have a significant effect on the mortalities secured. If this is so, it is logical to conclude that a cloth such as marquisette, which softens with continued use, may bring about a progressive change in the level of mortalities secured. In such a case the only safe procedure is to discard the covers after a certain number of tests, before the softening has proceeded far enough to influence the results.

F. Homogeneity of the Data

Throughout this work and also in a review of the literature, it was found that smaller samples tended to produce smaller values of chi^2. Callaway and Musgrave (1940) secured a measure of homogeneity in 5 tests which consisted of 14, 7, 8, 11, and 8 determinations respectively while heterogeneity was demonstrated in the 3 tests which consisted of 40, 20 and 19 determinations. Each point was determined by a test of less than 100 eggs. Potter (1941), using about 50 or 100 insects in the determination of each point on his regression lines, secured values of chi^2 which were in general very satisfactory, indicating definite homogeneity in all except one of his tests. Morrison (1943), on the other hand, using 8 or 10 replicates of about 150 flies each, a total of 1200 to 1500 flies for each point about the regression line, secured indications of heterogeneity in all tests except one. The author, using 8 replicates of 150 flies each, found similar indications of heterogeneity.

Morrison (l.c.) stated: "The very large chi^2 suggests that the theory involved in the application of the method of probits probably does not describe the data and some curve other than a straight line would fit the converted data better." This may not necessarily be so. Moore and Bliss (1942) plotted the regression lines of 7 different chemicals each replicated 3 times in tests on aphids. On the basis of this work they stated: "Inspection of the diagrams for the individual series indicated no systematic departure from linearity. The plotted points either adhered closely to a straight line or followed a

concave as often as a convex trend." Upon combining all of these lines into one composite line, they said, "It is apparent from the figure that there was no consistent non-linear trend which would warrant shifting to some transformations other than the log-concentration and the probit."

Although they maintained the suitability of the straight line as a measure of the probit-log. dosage relationship, Moore and Bliss (l.c.) found values of chi^2 indicative of heterogeneity in 13 of the 21 series and "a highly significant heterogeneity for the experiment as a whole." In explanation of this fact, they stated: "The number of aphids per pot averaged more than 560. So large a number reduced the sampling error in estimating the percentage of dead aphids on the plants of each pot to a relatively small value and exposed the heterogeneity of the four points about their computed curve." This, then, would also seem to be the explanation of the high values of chi^2 found by Morrison in his work and by the author in the experiments on age of flies (IV, B, page 28) and numbers of flies per cage (IV, C, page 28). Again in their discussion of heterogeneity, Moore and Bliss (l.c.) say, "Conclusions depend primarily, therefore, upon differences between curves and the consistency of these differences rather than upon inferences drawn from the degree of their internal homogeneity."

In contrast to the high values of chi^2 in the two experiments where so many flies were tested, we may look at the value secured when 18 variates, each consisting of only 150 flies, were put through the chi^2 test of Snedecor and Irwin (1933) (IV, K, page 51). In this test the chi^2 of 28.477

is about at the level of 4% P which is well above the 1% P accepted as being satisfactory by such workers as Murray (1937) and Callaway and Musgrave (1940).

It seems a valid conclusion, therefore, that the use of large numbers of flies in establishing a point on the regression line is not unjustified although the chi^2 values secured will be quite high. At least such a procedure will have the effect of reducing the sampling error and the resulting line will be more satisfactory for work in comparative testing of contact insecticides than if it were based on smaller samples.

G. Sex Ratios and Relative Susceptibility of Male and Female Flies

Murray (1937) working on houseflies and Lord (1942) working on Drosophila both found males predominating in the first cages filled from any single population of flies. The author, on the basis of only one day's work, was unable to confirm this fact but it is probable that the reversal of sex ratios (TABLE VIII, page 53) was due to chance. Murray and Lord make no mention of the condition under which the flies were kept previous to the experiment and the ratios recorded in TABLE VIII may have been secured as a direct result of the fact that the flies had been kept in absolute darkness for a period of 18 hours just before the experiment.

With regard to the relative susceptibilities of the two sexes there is no disagreement. All three experiments demonstrate the greater susceptibility of the male.

It is evident, therefore, that the matter of sex ratio of the test animals is one of considerable importance and probably contributes in a marked degree to variations in the mortalities secured. It will be necessary for this reason to devise some method of reporting the susceptibilities separately according to sex (as suggested by Murray) or of securing constant sex ratios between variates in the experiment.

VI. SUMMARY

1. The potato-yeast medium of Stultz was found to be satisfactory for rearing Drosophila though it involved more work than did the banana medium.

2. Age of the flies had a very definite effect on their susceptibility to nicotine sulphate. Flies aged 5 days were the most susceptible of those tested. It is essential to use flies of the same age in all replicates of the experiment.

3. Increased numbers of flies per cage increased the observed mortalities with the technique described herein.

4. Parallelism of the regression lines for 16, 32 and 64 flies per cage was clearly demonstrated.

5. Provided equal amounts of spray material were placed in the spray cup when using the intermittent method of spraying, increase in length of spraying time produced a decrease in amount of deposit on each cage.

6. There was no significant correlation between

amount of deposit (within the limits tested) and percentage mortality when using the intermittent spraying technique.

7. The continuous spraying technique eliminated many apparent irregularities in application of the spray material but failed to produce any statistical improvement in homogeneity of the data.

8. A delay in the daily spraying schedule resulted in an increase in observed mortalities which appeared due to the flies themselves becoming progressively more susceptible rather than to any inherent change in the nicotine sulphate solutions on standing.

9. The type of cloth used to cover the cages had a significant effect on the mortalities secured.

10. Experiments designed to select from the spraying population by means of phototropistic responses a sample of flies of uniform physiological condition were not successful in demonstrating any significant differences.

11. The relatively greater susceptibility of male flies over females when sprayed with nicotine sulphate was confirmed.

12. A brief experiment on sexes of Drosophila indicated a preponderance of females in the early cages when the culture was cleared under a light. The data were not conclusive but indicated the necessity of standardizing this factor in establishing any satisfactory technique.

13. The use of each variate in each replicate in calculating the regression line by the method of probits was tried and found to have no advantage over the use of the mean

of all replicates of that variate in plotting points about the line.

VII. ACKNOWLEDGMENTS

The author wishes to express his thanks to Dr. W. H. Brittain for the supervision of this research and to Dr. F. O. Morrison for his invaluable guidance in the study of the statistics involved and his assistance with the spraying and transferring of the test insects.

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