METHODS FOR FOLLOWING THE FATE OF INFECTIOUS AGENTS FED TO SINGLE FLIES*

By LAWRENCE R. PENNER, Ph.D., and JOSEPH L. MELNICK, Ph.D.

(From the Section of Preventive Medicine, Yale University School of Medicine, New Haven, and Department of Zoology and Entomology, University of Connecticut, Storrs)

(Received for publication, May 17, 1952)

The use of precise methods to facilitate the handling, feeding, and collection of excreta from individual flies has been reported from this laboratory in preliminary fashion (1). These methods were developed in order to determine the fate of poliomyelitis virus in these insects. The purpose of the present paper is to report the methods in greater detail.

It would be sophistry to credit the origin of the methods we used to any one author, and certainly the precise methods which we have developed have been the result of many suggestions from methods originated and reported by other authors and from hints and ideas received from our laboratory assistants and other colleagues.

One outstanding contribution among the mass of literature available on methods of handling insects which has undoubtedly had a very great influence on the development of our techniques, is the work of Graham-Smith (11). He fastened chloroform-anesthetized blowflies, which had been fed syrup containing particles of India ink, on their sides with hot sealing wax at the bottom of a shallow tray and immediately filled the tray with water. The upper side of the fly was dissected away to expose the thoracic esophagus, crop duct, proventriculus, and ventriculus in order to watch the movements of the particles through the intestine of the fly. Plain and colored syrups were also fed to determine the fate of food which was fed to the flies. No doubt he had the fly in a workable position. Similarly, the techniques developed by Minnich (2, 3), Frings (4, 5), Chadwick (6), Williams (7), and the methods of many others have been helpful in the development of the equipment which we have found useful.

Numerous experiments with viruses have been conducted utilizing the methods reported here, and these are presented separately (8). The discussion which follows is in essence a report on the basic entomological procedures which we have followed.

Methods

Rearing.—All flies used in the experiments over a 4 year period have been laboratory reared. Most stock was originally obtained from wild flies, although *Phormia regina* was

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

established in the summer of 1948 with cultures received from Dr. Dietrich Bodenstein. *Musca domestica* were also supplied to us early in the summer of 1948 through the courtesy of Dr. George W. Barber of Rutgers University and Dr. Franklin C. Nelson, of Stanco Inc., New York. *Naidm* supplied by Dr. Neely Turner of the Connecticut Experiment Station at New Haven has been used in rearing larvae of *Musca domestica* and *Muscina assimilis*, and several varieties of dog biscuit based on the reports of Frings (9, 10) have been used in rearing *Phaenicia sericata*, *Phormia regina*, *Sarcophaga bullata*, (Fig. 1) and various members of the *Phaenicia* group. *Naidm* with a little brewer's yeast sprinkled on the moistened meal was considered to be superior to dog biscuit in rearing *Musca*. Marine fish have also been used in rearing procedures, particularly when eggs and larvae of wild flies were desired to

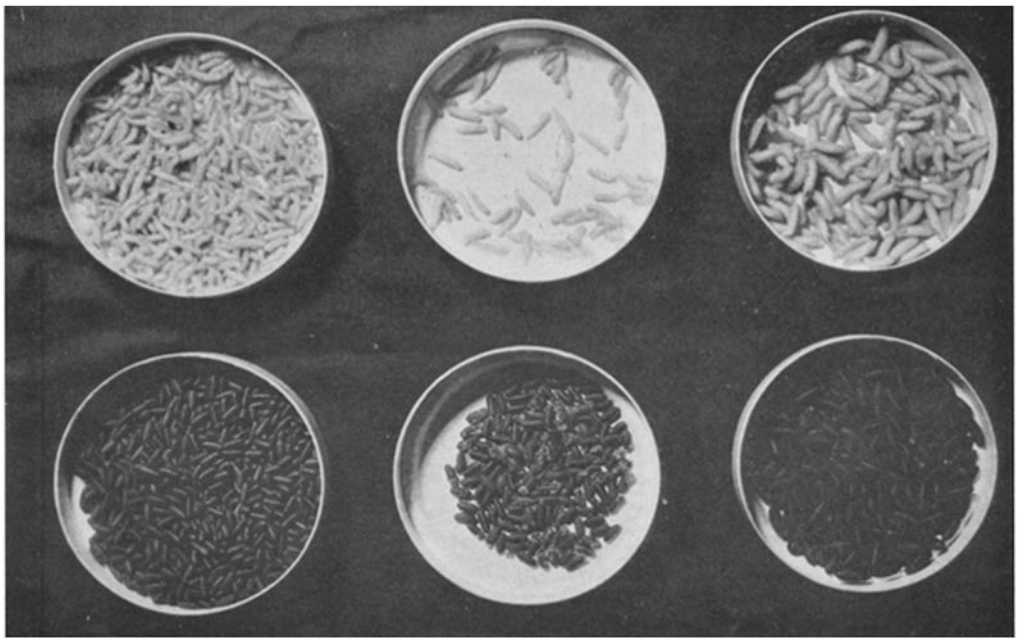

FIG. 1. Larvae and puparia of *Phaenicia sericata*, *Phormia regina*, and *Sarcophaga bullata* (left to right).

start cultures for the laboratory. For the sake of economy, empty fruit juice cans were utilized as rearing chambers for *Phormia regina*, other species of blowflies and sarcs. The eggs or the first stage larvae are placed in the medium and a double layer of cheese-cloth to be used as a cover is affixed with elastic bands. Two cans each are placed on 1 inch of sterile sea sand or other fine sand in rectangular metal boxes 10 by 6 by 6 inches. When prepupae are ready to migrate through the cheese-cloth, it is possible to collect them in the sand with appropriately sized sieves or large strainers or else the prepupae can be allowed to pupate in the sand and then be collected in the same manner. One must be careful to keep plenty of available food in the cans for the number of maggots present in order to prevent the disasters of overcrowding. Quart circular cardboard containers have been used in rearing *Musca domestica* larvae.

Emergence Cages.—Emergence cages for the flies (Fig. 2) were wooden boxes approxi-

mately 18 inches long by 10 inches wide by 12 ½ inches high with a middle partition making two inside chambers approximately 17 ½ inches long by 10 inches wide by 6 inches deep. One side was screened and two doors with hinges and eye hooks were placed on the other side to cover the two inside chambers. Into each chamber can be placed inverted beakers of molar sucrose and distilled water, respectively. We have used a 100 cc. or 150 cc. beaker inverted over two 9 cm. circles of filter paper in a Petri plate for the sugar solution and a 150 cc. or 200 cc. beaker for the water. The bottom of a quarter-pint container is filled with an adult fly food similar to the one used by Bang and Glaser (12) consisting of equal parts

FIG. 2. Cage for handling adult flies as they emerge.

of powdered whole milk, brewer's yeast, cane sugar, and whole wheat flour. Pablum has occasionally been added to this. The puparia of the fly species to be used in the next experiment are placed in the bottom of a quarter pint paper container and inserted in the chamber. Two or three hundred puparia will not overtax each chamber as they are to be kept there for such a short period of time. Usually the flies are mounted 1 or 2 days after emergence. This process involves darkening the screen side of the cage with dark cloth or newspaper, and allowing the flies to crawl into the lusteroid tubes at one end of the chamber. The lusteroid tubes are ⅞ inch in diameter at the open end and 4 ½ inches long. The tubes have been inserted into eight holes, four for each chamber, made with a ¾ inch bit and reamed out slightly to accommodate the ⅞ inch tube we use (Fig. 3). The four holes are placed near the top of each chamber for best passage by the flies into the tubes from the darkened chambers. This type of chamber facilitates the mounting procedure but is not

convenient for keeping flies or other insects for long periods of time. For our purposes, it was practical and economical. We did not build sleeves into the chambers although this may be desirable. The whole cage can be placed in a steam chamber to kill flies not used and the cage cleaned and reprepared accordingly for the next flies to be mounted.

Mounting the Flies.—Fly mounting expedites handling of the specimens, facilitates the keeping of individual records, allows a maximum of control and a minimum of equipment, and permits ready temperature regulation. It is obvious, however, that the conditions are unnatural in that the flies are not allowed to fly.

When ready to mount flies on the wax blocks, the emergence cage is darkened with paper or cloth to allow the flies to come in greater numbers to the lusteroid tubes. These tubes are pointed toward either artificial light or a window. Empty tubes are exchanged for those containing flies and these are lightly etherized in a lusteroid tube of similar size containing an ether-soaked plug of cotton in the bottom. Care should be taken that the cotton has enough ether in it but not enough to flow out of the wad onto the sides of the tube. The etherized flies are poured out on clean paper towels and picked up carefully by their feet with the clean forefinger and thumb of one hand while the other hand is used to heat to melting one

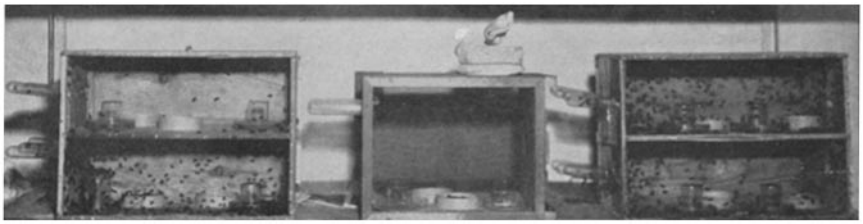

FIG. 3. Emergence cages showing emergence tubes.

side of the wax block on a small electric soldering iron. While the wax is still liquid on the side of the block, the fly is lightly pressed dorsal side down onto the surface of the block. A dissecting needle is flamed and heated in a gas burner, then applied a little distance from each side of the fly now on the wax block, and a small amount of subsequently melted paraffin is allowed to run under the thorax and over the wings to affix it more firmly. The flies revive shortly after this and there you have them (Fig. 4)! Flies are then sexed and the flies to be used placed in appropriate positions on the racks. One person can mount about one fly per minute. Care should be taken not to breathe the melted paraffin fumes or to get the paraffin too hot. Seven or eight flies can usually be etherized and mounted at a time before they return to consciousness. We have not found it necessary to feed them either sugar solution or water immediately after mounting and mortality is extremely low. They are left unfed until the next day when their virus feeding is to take place. Because they do not always feed as well then as they might, it is possible that a sucrose solution feeding should come at that time (24 hours) and then the virus feeding 24 hours later. In our experiments we have not usually delayed beyond the 1st day after mounting for virus feeding but would likely have obtained more ravenous feeding if we had. In most cases however, this was not considered necessary.

Flies are kept on the wax blocks until harvested or until they die when harvesting can be done, receiving daily feedings or otherwise as the individual experiment demands. They excrete on the wax paper below with a minimum of excreta loss. They rarely vomit, so the dejecta are almost entirely excreta spots and there is no difficulty in differentiating them.

The length of life of the fly species used during the summer experiments seems to be comparable to the length of life of unmounted flies kept in the laboratory. Some specimens of *Phormia regina* and *Phaenicia sericata* have been kept alive more than 2 months on the racks but for most of our experiments a shorter time has been used because of heavy mortalities due to various causes and particularly to unfavorable temperature conditions in the laboratory during part of the summer season. In general, for all species used, the survival of female flies is greater than of the males.

Description of the Fly Racks.—Several types of racks have been used in our experiments but we have found the following kind most satisfactory for this work. The essential part of

FIG. 4. *Phormia regina* mounted on paraffin block, and shown feeding with proboscis extended.

the rack is a 1 $\frac{1}{4}$ inch square piece of pine 12 inches long with ten $\frac{1}{8}$ inch holes drilled 1 inch deep into each of two opposite sides 1 inch apart and $\frac{3}{16}$ of an inch from the top. These alternate with each other so that no interference of opposing holes will result and consecutive numbers from 1 to 20 are marked above the holes on top of the rack piece. A piece of pine board 12 inches long by 5$\frac{1}{2}$ inches wide by $\frac{3}{4}$ of an inch thick is nailed lengthwise to the bottom of the rack pieces allowing the latter to rest in the center of the board. For each rack the heads of 8 common pins are removed and 2 of these headless pins are driven into the board $\frac{1}{4}$ inch from each end of the sides in order to facilitate fastening good grade wax paper on them for excreta collections. The glass rods bearing the flies on the wax blocks are inserted into these holes and their excreta can be collected daily or at will from the paper (Fig. 5). Each rack can be numbered at one end of the rack piece with tape or some other label. The racks can be arranged to stack on top of each other. They facilitate handling

when the flies are to be moved from one location to another; *e.g.*, from room to refrigerator shelf.

The glass rods or glass tubing $\frac{1}{8}$ inch or slightly less in diameter is cut into lengths of $2\frac{3}{4}$ inches and each rod has a rectangular piece of low melting point (47–49°C.) paraffin molded over one end. The block of wax is approximately $\frac{3}{4}$ by $\frac{1}{4}$ inch by $\frac{1}{2}$ inch and allows about 2 inches of the glass rod to be free of wax for convenient handling. A quick dip in melted paraffin after it is molded into shape will make it acceptable for affixing live flies when needed. The waxless ends of these rods fit into the inch-deep $\frac{1}{8}$ inch diameter holes on the rack piece

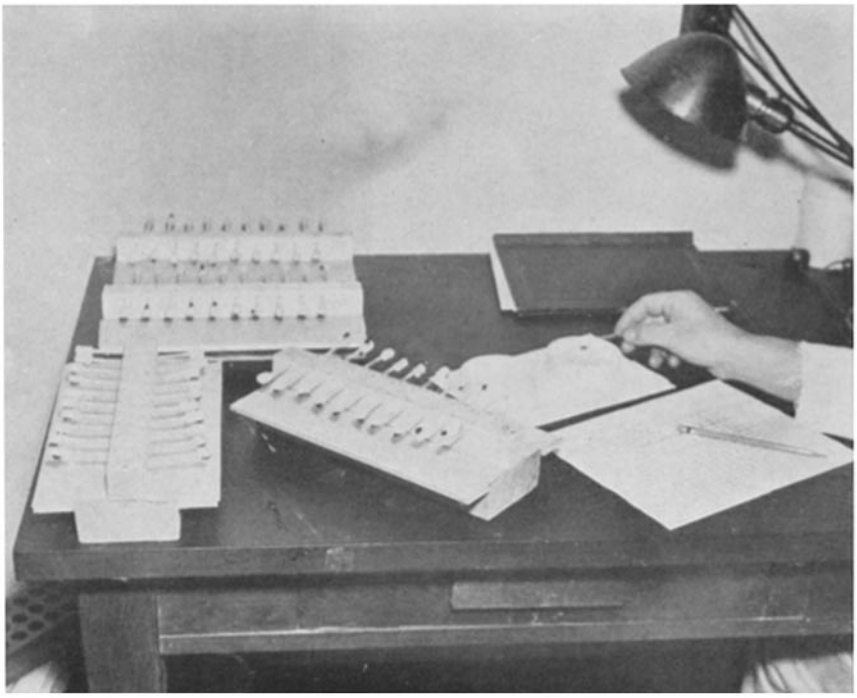

FIG. 5. Experimental arrangement, showing fly racks, a fly being fed, and record sheet.

previously described. Before mounting the flies it is wise to have as many of the racks made up as necessary for the experiment.

Cage for Racks.—The newspaper-covered floor of a cheese-cloth cage 70 by 25 by 40 inches with a door 28 by 36 inches in the center of one side has been used to keep the racks of flies which had been fed virus material. The floor of the cage holds 15 racks of 20 flies each or a total of 300 flies at one time. If necessary, additional shelves can be added for more racks but due to the amount of time required to tend the flies adequately, not many individual experiments will involve more than 300 flies. The cage has been kept on a mobile table which is 37 inches high with a top slightly larger than the floor of the cage. This has been a convenient level for opening and closing the cage and facilitates manipulation of the fly racks. The cage is an additional precaution in event a fly frees itself from the wax block. This never happens when correct mounting procedures have been followed. The cage also prevents possible entry of a wild fly or other wild insects from outside which might occur in spite of

the well screened laboratories. The cloth-covered cage serves as further protection for the flies from possible disturbing factors such as people walking by. A thermometer has been kept on the inside wall of the cage so that temperatures could be recorded.

Feeding.—Feeding with potometers has enabled us to measure conveniently the amount of virus solution ingested and has allowed us to set up some semiquantitative experiments. Early experiments without them were conducted for qualitative knowledge but it soon appeared necessary to know whether quantitative studies could be accomplished. After experimenting with several home-made types of potometers, or "swigmeters," as they are commonly called, and attempting to use a type reported by Frings and Frings (13) and also one which the latter sent to us to try, we finally decided on one made from pipettes of 0.1 or 0.2 cc. capacity graduated in thousandths. The tip of the pipette is sealed off by using a

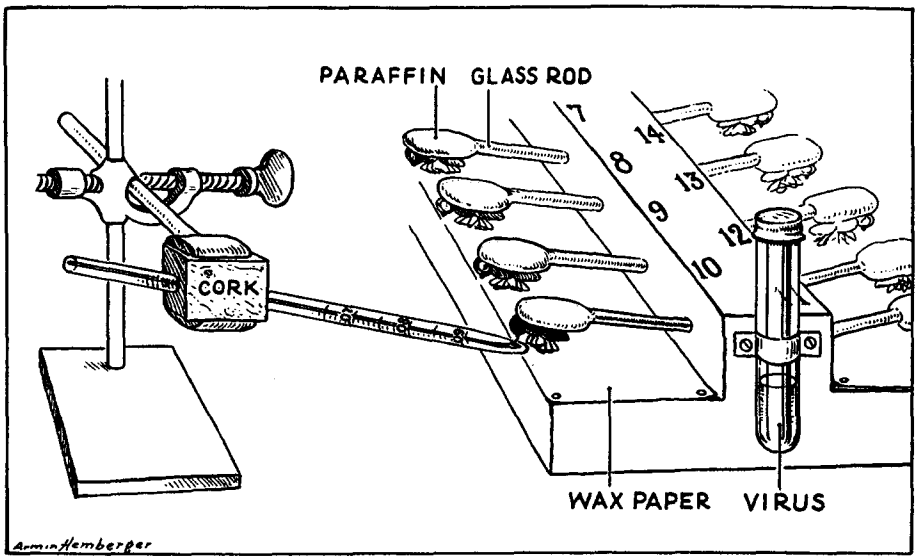

FIG. 6. Drawing of arrangement for quantitative feeding of virus to flies.

small air torch flame, then a hole is blown into the pipette at the end of the graduation to fit the labellum of the fly species to be fed (Fig. 6). Measuring can then be made with greater accuracy than by any other method we have used. Species of flies (discussed elsewhere (8)) will vary but it is a rare fly that will take more than 0.04 cc. of solution and we usually limit them to a given amount, much less, on V day (virus feeding day). Each fly's intake, of course, is recorded on special sheets devised for that purpose. Not only can each fly be kept satisfactorily but a complete record of its excreta, vomitus, and other habits can be ascertained and records kept on the same sheet.

Filling the Potometers.—A syringe or rubber bulb affixed to a rubber tube which is of such size as to fit over the basal end of the potometer can be used effectively to suck virus into the potometer (Fig. 6). One must be careful not to bring the virus preparation above the graduation in the tube.

Harvesting the Excreta.—We carefully add to each excreta spot 0.01 cc. of a phosphate solution (M/100 dibasic sodium phosphate) with a 1/4 cc. graduated syringe and a 27 size needle. We have occasionally used buffers made up in 1 per cent inactivated monkey serum.

The syringe needle is used to mix and then suck up the mixture. It is then frozen in a tube until time for animal inoculation for virus assay. Sometimes the excreta spot is quite sticky and may stick in the needle if mixing is not done properly. One must also be careful not to scratch the wax from the paper, thereby puncturing the paper and losing valuable material. Papers are changed daily after collections. We have had little trouble with lost excreta spots but have attempted to keep a complete record of any possible loss.

Harvesting the Flies.—Mounted flies are collected when dead, or whenever harvesting is desired, by clipping off the legs, head, and wings with a sterile razor blade. Hence the body including the crop, ventriculus, and other internal parts is retained for testing with a minimum of accessory material. The waste material is placed with the wax block and rod into a container of 70 per cent ethyl alcohol. The wax block and rod can be cleaned and used again if needed.

SUMMARY

Methods have been devised which facilitate the handling, feeding, and collection of excreta from individual flies bred in the laboratory. Using these methods, quantitative studies can be carried out in which known amounts of infectious material are fed to flies and the fate of these agents determined. It has been possible to determine the persistence of excretion of viruses, and the amount of the ingested virus recovered in the excreta and in the bodies of the flies as well.

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