The Rearing of *Phlebotomus* Sandflies (Diptera: Psychodidae) I. Technique[1]

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ABSTRACT

The methods and equipment described are used in rearing *Phlebotomus* sandflies, including: rearing vessels; larval food; control of molds; containers for capturing and maintaining adults; releasing cages; feeding cages; aspirator devices for capturing sandflies and loading feeding cages and other containers.

Since early in this century *Phlebotomus* sandflies[2] have been reared by a number of investigators concerned with these insects as annoying pests or in relation to sandfly fever, leishmaniasis or bartonellosis. A considerable variety of methods and types of apparatus have been devised to meet the basic requirements for sandfly rearing, which are: (i) providing the gravid females a moist surface for oviposition, (ii) a moist environment for development of the eggs and immature stages, (iii) suitable food for the larvae, (iv) containers or cages for the emerging adults and their subsequent confinement, feeding and experimental manipulation.

It is not the purpose of this paper to review all the various techniques but rather to give an account of those methods which have proved successful with the various species we have had occasion to rear. The experience of the senior author began in North China in 1924 (Young and Hertig 1926) in connection with studies on kala azar, with the rearing of *Phlebotomus chinensis, P. mongolensis* and *P. squamirostris,* followed by the rearing of *P. vexator* in the United States and of *P. verrucarum, P. noguchii* and *P. peruensis* in Peru, the last three in work on bartonellosis (Hertig 1942).

With the initiation in 1956 of the current study of the leishmaniasis transmission problem in Panama, it was found that our Panamanian species required a number of modifications of the methods which had worked well with several species in three continents. The adaptation of the technique to the peculiarities of our local species was continued by the junior author when she joined the staff of the project (6 months in 1957, January 1959 to the present) and assumed general charge of the rearing program. Part II of this series (Johnson and Hertig 1961) covers in detail the development and behavior in laboratory cultures of our principal man-biting species, together with notes on others of the 24 species, out of the 65 species known to occur in Panama, which we have reared. The taxonomy and distribution of *Phlebotomus* in Panama and elsewhere in the Americas have been the subjects of a series of papers by Fairchild and Hertig, most of which have appeared in the Annals of the Entomological Society of America, 1947 to 1959. These papers also contain incidental information about diurnal shelters of the adults and methods of collecting. It is not the intention, in the present paper, to go into the field aspects of *Phlebotomus.* It may be mentioned, however, that sandflies may be collected during the day, with the aid of tobacco smoke and a flashlight, in their resting places, such as tree buttresses, hollow trees, animal burrows, and leaf litter of the forest floor, low vegetation, rock crevices, holes in masonry, and to a very limited extent in Panama, in dwellings; at night, taken directly on human or animal baits or at lights, or in traps comparable to the well-known horse-baited stable trap, or in various types of light traps. In connection with certain items of equipment described in this report, their use in field collecting as well as in the laboratory is indicated.

METHODS AND EQUIPMENT USED IN REARING *Phlebotomus*

Breeding vessels; larval food

The maintenance of the larvae throughout their relatively long period of development is the phase of the rearing process which has usually presented the greatest difficulties. Therefore the most important

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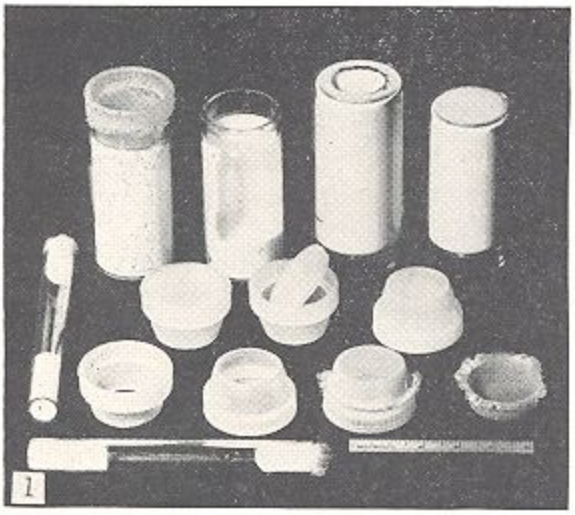
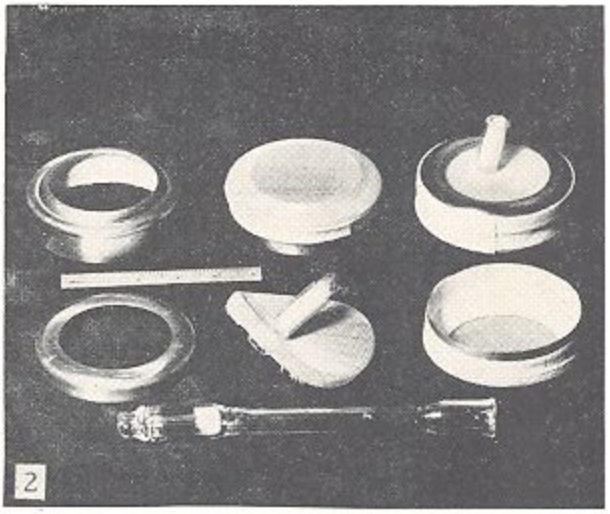
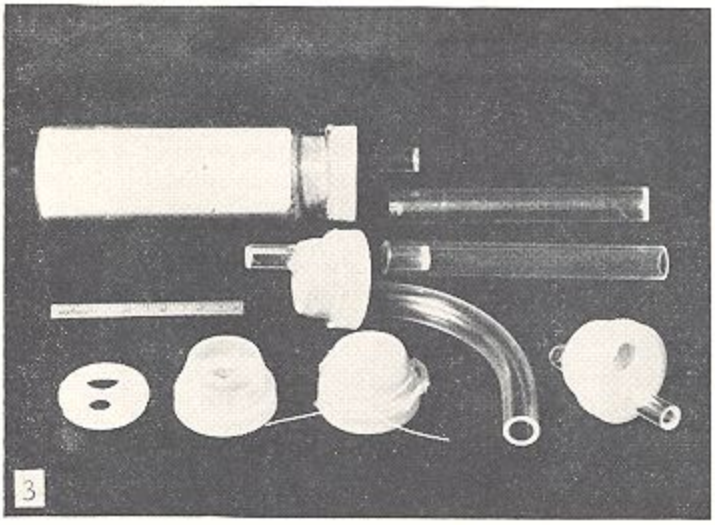
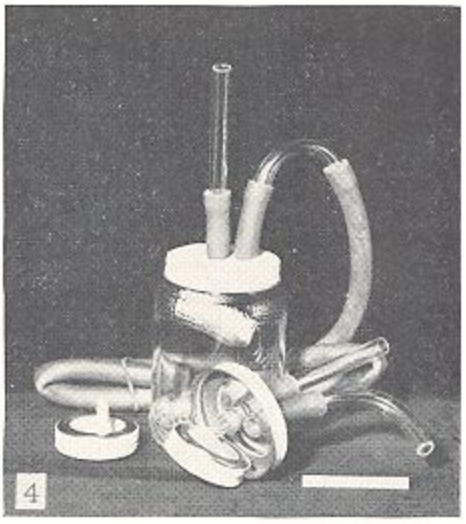
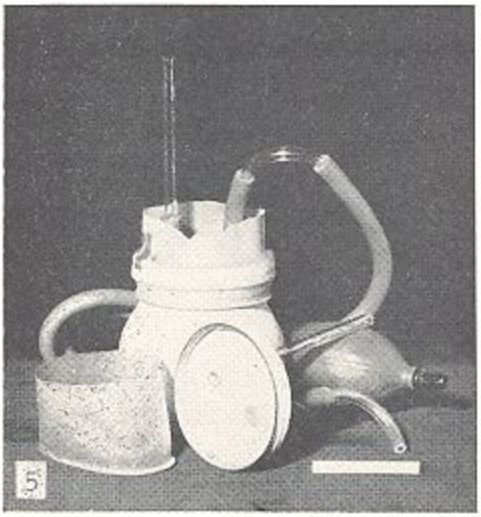
[2] Although not in accordance with the recommendations of the Committee on Common Names of Insects of this Society, this name was set solid in this paper at the request of the senior author.











FIGS. 1 to 6.—The small white scale is 5 cm. long.

items are the containers and the food for the larvae. The following are some of the combinations of breeding vessels and food which have been used by others:

Historical.—Earthen pot containing plaster block with earth and feces (lizard, rabbit or man); also tray in large cage (Waterston 1922); wooden box with garden soil plus lizard and other feces or insect bodies (Whittingham and Rook 1923); plaster block with central excavation containing pieces of stone and rabbit feces (Smith 1925); porous earthen vessel with earth and stable litter covered by a thick layer of dried mud, in which an opening gave access to the material beneath (McCombie Young et al. 1926); petri dish with filter paper, rabbit or goat feces, dried and ground (Christophers et al. 1926); porous earthen plate to which is transferred filter paper with eggs, rabbit or goat feces, at times enriched with dried blood (Shortt et al. 1926); plaster block or plaster-lined vessel, rabbit or goat feces with dried blood (Smith 1927). The above methods together with variations used later by several other workers were reviewed in some detail by Barretto (1942). He used chiefly porous earthen dishes. The larval food was soil mixed with material (mosses, lichens?) scraped from damp, shaded walls or the base of tree trunks, or soil rich in vegetable debris. He also found it desirable in the case of some species to "ripen" the food, a process used by Roubaud and Colas-Belcour (1927) and Ashner (1927), which consisted of holding the moistened mixture at 60° to 70° C. for a number of days.

In the above methods eggs were introduced either by transferring filter paper on which eggs had been laid or by confining the females in tubes, glass cylinders or by other means so that they laid their eggs directly in the rearing vessel or on the food material. During larval development the proper degree of moisture and humidity was achieved either by the direct addition of water, as in the case of filter paper in petri dishes, or, with porous vessels, by setting them in water or on moist cotton or sand. After pupation cloth covers, or tubes, glass cylinders and the like were arranged to receive the emerging adults, or the whole apparatus was placed within a cage.

Early in the work in China (Young and Hertig 1926) there were adopted as breeding vessels small unglazed earthen pots, lined with plaster of Paris, which served not only for the development of the young stages but also for holding live sandflies in the laboratory, securing oviposition and for the confinement of the emerging adults. These small pots, made for household use and obtained in the local markets, were "hard-fired" so that the degree of porosity was much less than that of ordinary flower pots, an advantage for our purposes. They were small enough (8 to 10 cm. in both diameter and depth) so that the interior could be inspected under the dissecting microscope. A lip permitted the easy fastening of a cloth cover with string or rubber bands. For the introduction of sandflies into the pots the silk bolting-cloth covers were provided with glass entry tubes. During larval development the pots were covered with earthen dishes, replaced after pupation by silk bolting cloth. The bolting cloth provided ventilation and permitted ready inspection of the adults. The plaster lining gave a white, porous surface, easily renewed. Also it was possible to judge the degree of moisture by the appearance of the plaster. Moisture was supplied by setting the thick base in water or on moist cotton. The larval food consisted of crushed feces of the Chinese striped hamster, which happened to be the most conveniently available source of rodent feces. A thin layer of food was sprinkled over the bottom of the pot shortly before hatching was expected, with more added from time to time as needed.

Essentially the same method was used in the case of *P. vexator*, a reptile-feeding species, collected at Plummer's Island, Maryland, and reared in Boston. The larval food consisted of dried guinea pig feces soaked in defibrinated horse or rabbit blood and then dried and ground. It was found that the size and shape of the small "individual-portion" size of the classic Boston bean pot made it ideal as a rearing vessel. They were made unglazed except for the lip, on special order, by the Dorchester Pottery Works, Dorchester, Mass. During the winter in the very dry air of the heated laboratory, it was desirable to enclose the pots and moist cotton in a more humid atmosphere, either under a bell jar or in cans with screw tops.

Porous Earthen Pots, Plaster-Lined.—These little

FIG. 1.—Plaster-lined vials, plastic stoppers modified to hold bolting cloth covers, for capturing and holding individual *Phlebotomus* sandflies, securing oviposition and rearing small lots. The four vials shown: 5-dram size ready for use; plaster lining partially removed; hole ground in bottom (7-dram size); bottom hole filled with plaster. Glass tubes, one end filled with plaster; useful for holding individuals of certain species.

FIG. 2.—Feeding cage; Monel metal frame, lined with plaster; bottom and cover of bolting cloth, the latter with glass entry tube; glass tube, bolting cloth screen in one end, for loading cage.

FIG. 3.—Plaster-lined vial (10-dram size) with plastic stopper modified to make an aspirating device for field collecting. The bottom of the stopper, partially cut away, is covered with bolting cloth, tied and cemented in place; the short entry tube has a removable extension; suction is applied through the removable curved tube fitted to a rubber tube.

FIG. 4.—Aspirating device for loading feeding cages; jar, metal cover with gasket; glass entry tube of feeding cage inserted in rubber entry tube of cover.

FIG. 5.—Aspirator top for rearing pot; metal cover fitted with entry and suction tubes; band cut from rubber glove holds cover in place with air-tight seal.

FIG. 6.—Rearing pots, unglazed earthenware, lined with plaster; moisture maintained by setting on cotton pads in petri dishes; newly established culture with larval food sprinkled on bottom and sides.

bean pots have been the standard rearing vessels throughout all subsequent work in Peru and Panama. The different batches of pots vary somewhat but the outside dimensions are approximately 80 mm. in height by 85 mm. in diameter at the widest part, 70 mm. at the lip, 64 mm. at the constriction below the lip, with the flat bottom 65 mm. in diameter (fig. 6). The walls and bottom are 7 to 8 mm. thick. The material is quite hard and only moderately porous, so that there is little danger of sudden changes in the moisture within the pot. The relatively large flat bottom provides not only considerable space for the larvae but assures good contact with the moist cotton on which the pot rests. The plaster lining is made by pouring into the pot, previously soaked in water and drained, a mixture of 50 cc. of dry commercial plaster of Paris in 25 ml. of water, rotating the pot to coat the walls and then allowing the rest of the plaster to flow to the bottom where it forms a layer about 8 mm. thick.

It should be noted that special types of plaster, e.g., that used by dentists, may contain substances (added for the purpose of modifying the setting characteristics) which are toxic to sandflies. In our experience, the plaster of Paris used by building contractors has been satisfactory.

The pots, with muslin covers, are autoclaved (20 minutes at 15 pounds) shortly before use. On discarding an exhausted culture the pots and bolting cloth or muslin covers are boiled and the pots washed with a brush. With repeated boiling and autoclaving and after several rounds of cultures the plaster lining becomes stained and the surface uneven. The old plaster when thoroughly wet is chipped and scraped out and replaced with a fresh lining. The porosity may in time become dangerously low. Such pots are either discarded or are marked and special attention given to make sure that the proper moisture is maintained, either by adding water directly to the plaster lining with a pipette or atomizer or by having the cotton pads frankly wet. The degree of moisture may be judged by the appearance of the plaster under the dissecting microscope, which must be learned by experience. The plaster should appear slightly "water-soaked" but a glistening film is to be avoided. The pads on which the pots rest are made by pressing cotton into petri dishes so as nearly to fill them (fig. 6). The petri dishes in turn are set in a stainless steel tray with water, which serves as a moat to protect against mites and ants. The moisture of the cotton is checked daily.

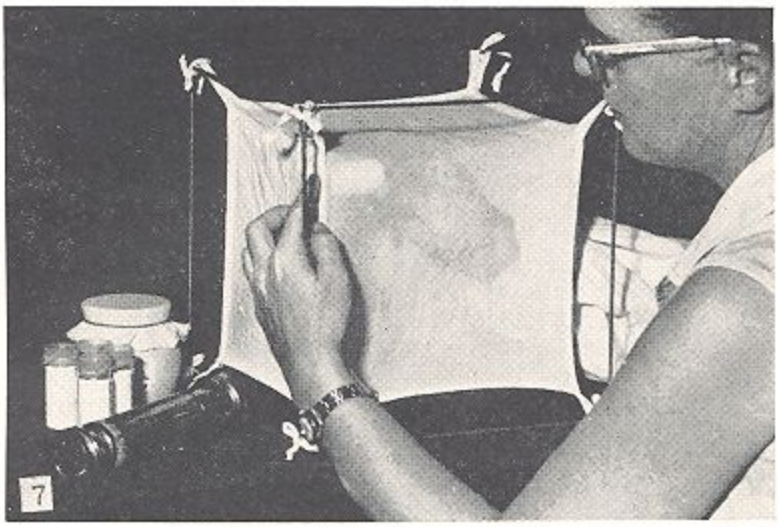
Larval Food.—A considerable number of substances in various combinations have been tried as larval food for our Panamanian species: rabbit, guinea pig, hamster, and sheep feces, alone or enriched by soaking in blood or adding powdered dried blood or commercial dried nutrient bacteriological media, dried yeast, decaying leaf litter and the underlying soil, green lichens on bark, ant-nest refuse, etc. While the larvae of various species developed well on the blood-enriched feces, the females thus reared were reluctant to take a blood meal. This may have been caused by the nutriment from the rich larval diet being carried over to the adult. Whatever the cause, the adults seemed to feed more readily without blood in the larval food.

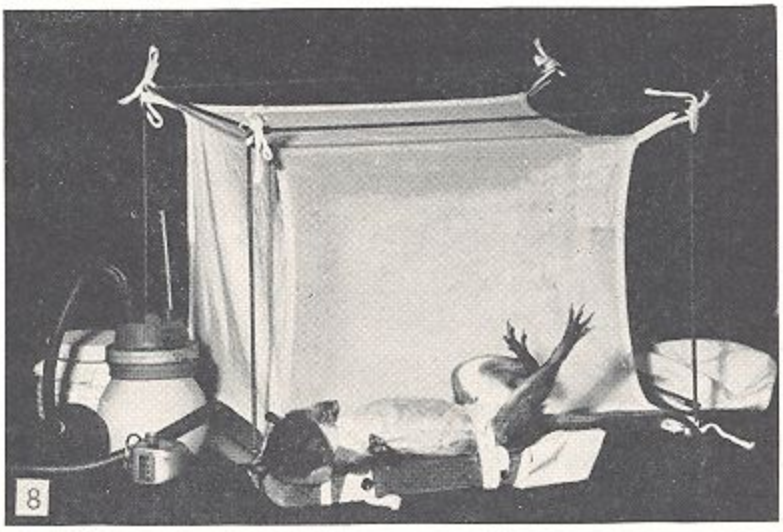
It was found in early trials that for some species it was desirable or even critical to have leaf litter present, whether the larvae fed on it or not. This was demonstrated by one dramatic instance. Newly hatched larvae of *P. nordestinus* were crawling away from the food (hamster feces plus dried sheep's blood) and climbing the bare walls of the pot. A small pile of well rotted detritus from under the leaves around a mango tree was placed on the bottom. Within 15 minutes some of the larvae had turned around and within 45 minutes a dozen or more were at the leaf debris. Thereafter they ate not the detritus but the food previously scorned. The 59 original eggs produced 56 adults. We have not had occasion to rear this species again nor have we observed any such striking behavior on the part of other species. However, this demonstration of the potential value of leaf litter led to its use as a standard ingredient of larval food.

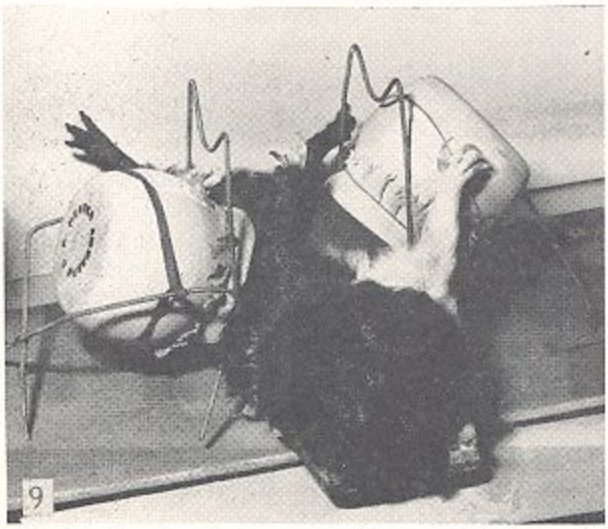
At a time when the regular food consisted of rabbit feces soaked in blood, a mixture satisfactory for a number of species, we were having difficulty in rearing other species, such as *panamensis* and *trapidoi*. It was found that fresh green lichens on a chip of bark from the forest were attractive, especially to *panamensis*. At times almost all the larvae in a pot were concentrated on the lichens, actively "grazing." On the other hand, two burrow-inhabiting species showed no interest in the lichens. We were reluctant to introduce fresh material, with mites and other organisms, into the cultures as a routine, but this difficulty was avoided by the development of the standard larval food containing decaying leaves.

We have finally settled on the following as the standard larval food: (i) Dried rabbit feces, autoclaved, dried, ground and sifted, retaining that which passes a screen with 20 meshes per inch but is held by the 40-mesh screen; (ii) decaying leaves from the forest floor, autoclaved, dried, crumbled by hand, rubbed through a 15-mesh screen onto the 40-mesh screen; (iii) dried bodies of various types of insects, mostly Diptera, Orthoptera, and Lepidoptera, finely cut up with a knife on a board and similarly sifted. It is important to discard the portion passing the finer screen since the dustlike particles adhere to the larvae and they become exhausted trying to free themselves. The bulk of the food consists of equal parts by volume of the rabbit feces and leaves, to each half-liter of which is added 20 cc. of the insect bodies. The mixture is autoclaved and stored. Small quantities, sufficient for a day's needs, are placed in muslin-covered vials and autoclaved within a day or so before use.

The food is applied with a small spatula which is dipped in 95% alcohol and burned off before being inserted in the stock vial. Food is first given a day or two before the eggs are due to hatch, sprinkling







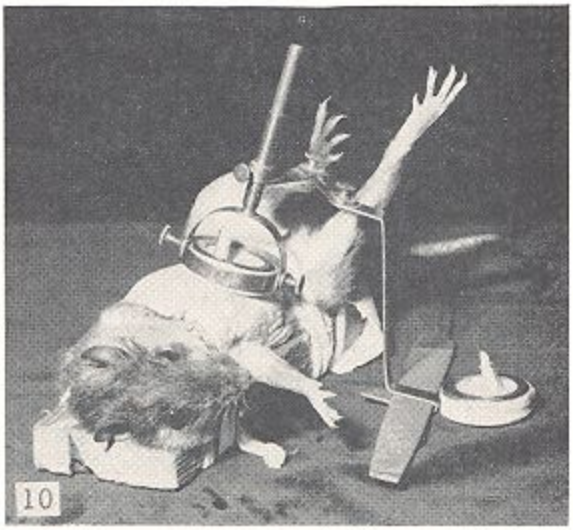


FIG. 7.—Small releasing cage (20 cm. cube) of muslin and nylon marquisette; the flexible material permits the free hand to aid in procedures within the cage.

FIG. 8.—Feeding sandflies on spiny rat, *Proechimys;* sandflies are released within the cage (medium size, 30 cm. long) and the immobilized animal, belly hairs clipped, is placed within; this in turn is placed under a frame covered with wet toweling.

FIG. 9.—Sandflies temporarily held in pots may feed directly through the bolting cloth covers; wire rack and rubber bands support two pots tilted against the clipped belly and sides of a guinea pig.

FIG. 10.—Apparatus for holding feeding cage in contact with animal's skin (spiny rat); cage is free to pivot on points of opposing set-screws.

a thin layer over the bottom of the pot and, for some species, part way up the walls, so that the larvae will not have to travel much more than their own length before encountering food, or if they have a tendency to climb, food will still be found nearby. Fresh food is added as needed. A culture with numerous older larvae may require fresh food every 2 or 3 days. We have considered it better to add food in successive layers sprinkled lightly over the older material, rather than give a large quantity all at once. In the case of certain species which are found on the surface of dead leaves in nature, such as *panamensis* and *pessoana*, small pieces of leaf, cut about 5 mm. square, are sprinkled over the regular food to give a more nearly authentic approximation of the natural environment.

Molds.—Molds have been found by most investigators to be troublesome at times. This is particularly true in a humid, tropical climate such as that of Panama. Nevertheless, a technique completely sterile throughout would probably not be desirable even were it feasible. Many of the fungi are in themselves palatable and they and other microorganisms probably form important parts of the larval diet. Actually it is not known whether any of the raw materials given as food would be satisfactory without the changes produced by the growth of microorganisms. The difficulties which molds cause are of two kinds:

a) The very young larvae may become entangled in the mycelia and never free themselves. This is especially the case with certain rapidly growing molds which produce a network of aerial mycelia. Usually by the end of the second instar the larvae can cope with this essentially mechanical problem. We have seen older larvae "graze" broad, clean areas in a thick, feltlike layer of mold. Heavy growths of mold may be removed with a needle which picks up a ball of mycelia as it sweeps through the growth. The aerial mycelia can also be pressed down into a mat, which presents fewer hazards for larvae, by the droplets of water from an atomizer.

b) Certain molds growing on the food are pathogenic to the larvae, notably a species of *Aspergillus* which appeared in our cultures for the first time about 2 years ago. This mold is dangerous chiefly when there is heavy growth. Apparently the larvae ingest spores which germinate in the intestine; the resulting mycelia invade the muscles of the thoracic region and kill the larvae. The persistence of this mold in our cultures necessitated a careful reexamination and revision of the procedures designed to prevent or reduce the introduction of microorganisms in general. Along with the measures outlined below, it was found that reasonable control could be secured with Mycostatin (Squibb Nystatin). A suspension in water, 2,500 units per cc., is sprayed into the cultures with a nasal atomizer. If the growth is heavy the pot must be sprayed every 2 or 3 days to prevent infection of the larvae. Since it seems that large concentrations of this antibiotic may be harmful to the larvae no more is used than absolutely necessary. Fortunately our strain of this *Aspergillus* seems not to have developed resistance to Mycostatin in the 18 months it has been used. There is no noticeable effect of this antibiotic on any other species of molds growing in our cultures. We have been able to confine this *Aspergillus* to cultures of certain sandfly strains which have been carried on for a number of successive laboratory generations and which we do not wish to lose. Autoclaving the affected cultures would probably eliminate the whole problem, but in the meantime another method gives promise of achieving the same result and saving these strains. Individual pupae are removed from an infested pot, washed several times in tap water and then placed in a clean, sterile pot.

Our current technique, some features of which along with strict control of all procedures are the result of the *Aspergillus* invasion, is designed to limit as far as possible the introduction of mold spores to those which inevitably accompany the parent females, either wild or reared, and to reduce the sources of air-borne contamination in the laboratory. The sterilization of pots and covers has already been mentioned; other types of containers and accessories used for collecting and holding sandflies and at times for rearing, are boiled or otherwise sterilized; the cotton pads and petri dishes are boiled each week and the trays serving as moats are scrubbbed clean. Work surfaces are wiped off daily and dust in the air-conditioned laboratory is held to a minimum. The food is autoclaved and whatever is left over after a day's work is reautoclaved. Any instruments touching the inside of a rearing vessel are sterilized by dipping in alcohol and burning it off, before touching the stock of food or any other vessel. The cloth cages used in transferring and feeding sandflies contain nylon panels and cannot be boiled and mere washing does not kill fungus spores. We found that cross contamination of cultures with the pathogenic *Aspergillus* could be reduced by fumigating the cages. After being used two or three times the cages are placed in a closed jar with crystals of paraformaldehyde, left at least 12 hours and then aired for 24 hours. As a result of these various procedures the larvae in most of our cultures are well established before the molds pose any real threat.

In the attempt to develop methods of mass culture we have had some success in rearing large numbers of larvae in glass-sided aquaria. The bottom of the aquarium was filled to a depth of 25 to 50 mm. with alternate layers of autoclaved leaf litter and finely sifted soil from the forest, with a layer of leaves on top. The culture was moistened and eggs, washed into a small beaker of water, were sprinkled over the leaves. In some cases standard larval food was sprinkled over the surface from time to time. Aluminum foil covered three sides and the top to give subdued illumination and the aquarium was set in a moat of water. This method produced fair numbers of adults of the five species tried, but the adults

were reluctant to feed either inside the aquarium or when transferred to a releasing cage. This general method is being followed up with trials of other types of rearing boxes and arrangements for feeding the adults without removing them from the cultures.

Maintenance of individual adults; oviposition

Up to and including the early stages of the work in Peru, the pots were used for securing oviposition and holding the adults for experimental or other purposes. There was then devised the glass tube filled at one end with plaster of Paris (Hertig 1940) which, with Peruvian species, admirably served a number of purposes (fig. 1). These tubes were 80 mm. long with a bore of 8 to 9 mm., with the plaster plug extending about 15 mm. into the tube and with cotton at the other end. They were made by standing a bundle of tubes in freshly mixed plaster. The proper degree of moisture (just short of condensation on the glass) was maintained by standing the tubes in moist porous pots or on damp cotton, or whenever necessary, by standing in water for a few moments. They were used routinely for collecting sandflies in the field and transporting them to the laboratory, and for holding individuals for oviposition or any other purpose. The eggs were usually laid on the moist surface of the plaster and were transferred by washing into the porous pots. Individuals could be kept alive in these tubes at times as long as 3 weeks.

On undertaking the rearing of Panamanian sandflies it was hoped that we would have the benefit of the extreme simplicity, economy, convenience and versatility of the plaster-plug tubes. However, it was soon found that our local species rarely survived 24 hours in these tubes. The precise nature of the difficulty was never determined. The commonest hazard, condensation on the glass in which the sandflies would become trapped, was avoided. Modification of the tubes by increasing the diameter or by coating the walls with plaster for about 15 mm. above the surface of the plaster base, for better footing for the sandflies, was equally unsuccessful. It was therefore necessary to devise other containers.

Plaster-Lined Vials.—A satisfactory solution was reached by using a relatively large glass vial and endowing it with some of the characteristics of the porous pots, namely, a plaster base with moisture entering from below, considerable space, walls completely covered with plaster, and ventilation through a bolting-cloth top. Kimble "Opticlear" vials with plastic stoppers have been successfully modified for this purpose (fig. 1). We have used several sizes, namely, 10-dram, measuring 80 x 29 mm., 5-dram, 55 x 27 mm. (no longer manufactured), and 7-dram, 65 x 29 mm. All three have the same sized opening, 21 mm., so that the stoppers are interchangeable. In all cases the inside is coated with plaster in much the same way as the pots, so that the walls as high as the shoulder, i.e., all but the 7 to 8 mm. occupied by the stopper, are completely covered with plaster, with a layer in the bottom about 15 mm. thick. A hole is then ground in the bottom of the vial 13 to 15 mm. in diameter, using a small electric grinder fitted with an abrasive cylinder about 10 mm. in diameter. Since the grinding of glass should be done under water and to avoid immersing the hands when using an electric appliance, a band of adhesive tape is wrapped around the base of the vial with enough projecting to hold a pool of water several millimeters deep. The hole in the glass and any excavation in the underlying plaster is later filled with plaster to insure good contact with the cotton or other moist surface on which the vials stand. In filling the hole in the bottom of the vial the plaster should be poured, allowed to harden and then trimmed with a knife, since any attempt to spread plaster with a spatula decreases the porosity.

The plastic stopper is used to make a frame for holding the bolting cloth cover (fig. 1). The stopper is hollow, forming a little cup covered by a removable plastic disk. The bottom of this cup is then cut out and the edges smoothed with emery cloth. With the hollow plastic cylinder thus formed a piece of silk bolting cloth, 40 mm. in diameter, is pressed into the vial. The bolting cloth in effect forms the bottom of the stopper, with an area for ventilation 18 to 19 mm. in diameter, through which it is possible to inspect the interior with a low-power lens or with the dissecting microscope. In preparing a batch of vials the bolting cloth disks are wet before pressing them into the vials; after drying they retain their shape.

Individual fed or gravid females are kept in these "KM-vials" on damp cotton in petri dishes or, with large lots, set directly on very porous earthen dishes which are in turn set in a tray of water which serves also as a moat to protect against mites and ants. A boiled raisin is placed on the bolting cloth of each vial. In view of the demonstrated, but physiologically unexplained, importance of boiled raisins in the experimental transmission of Indian kala azar (Smith et al. 1941) all our adult sandflies have constant access to boiled raisins as a convenient source of both fluid and nourishment, plus whatever benefits may accrue in relation to leishmaniasis. We have determined that the raisins definitely increase the longevity of our sandflies. The vials are inspected daily for the presence of eggs, most of which are on the bottom. A flashlight held against the vial gives sufficient light for this purpose through the thin plaster lining. After oviposition the females are identified and the eggs are washed into the porous pots. If the eggs are not readily dislodged, which tends to be the case with certain species, they may be gently touched with the end of the fine pipette used in the washing process. Eggs of the same species may be pooled up to 200 or 300 eggs per pot.

Rearing in Vials.—The plaster-lined vials serve a number of other functions. Individuals or small lots of adults of either sex may be maintained in them for various experimental purposes. They may also be used for rearing small numbers, such as the progeny of a single female. When no adults are

present the bolting cloth is replaced by muslin.

During ecological studies carried out by Mr. Wilford J. Hanson as a member of our group, 1957 to 1960, he recovered from natural breeding places a large number of larvae, of which several hundred were reared to the adult stage (Hanson 1961; see also Part II, table 1, Johnson and Hertig 1961). These larvae, unharmed by the screen-washing and sugar flotation technique, were reared in KM-vials with the standard larval food.

Field Capture with Vials.—One of the most important uses to which we have put these vials is the direct capture of fed females in the field, either with horse or human bait. Small carrying cases (aluminum lunch boxes, perforated for ventilation) are loaded in the laboratory with 25 to 50 vials, each complete with bolting cloth stopper. The vials are moistened in the field. The fed females are easily caught by placing the open vial over them as they rest on the horse, person or some nearby object. On being disturbed they fly into the vial and usually stay within so that the stopper is easily replaced. If the return to the laboratory involves a prolonged period in the heat of the following day, the vials rest on a wire rack with plastic bags of ice beneath. The sandflies are then maintained in the laboratory in their original vials, thus avoiding the labor and trauma to the sandflies involved in sorting and transferring the catch.

After use the vials and bolting cloth are boiled, while the plastic stoppers, which will not stand boiling, are dropped into 1:500 "Roccal" disinfectant, rinsed and then put into 70 percent alcohol. The vials are cooled gradually, and always under water, by allowing a small stream from the tap to flow into the hot water. Cooling in air increases greatly the danger of cracking. A certain amount of cracking occurs in any case, radiating from the hole in the bottom, the result of stresses set up in grinding. Cracked vials may be bound with adhesive tape and often survive repeated use and boiling.

Our first vials were of the 10-dram size but it was soon found that the smaller 5-dram vials (fig. 1) were equally satisfactory or even better for most purposes, particularly when examination was to be made with the dissecting microscope. With the discontinuance of their manufacture, however, we have been forced to use the 7-dram vials as the smallest size with stoppers which fit our general stock.

Aspirator Vial.—The large 10-dram vial, however, with the versatile plastic stopper transformed to make an aspirating device, is extremely useful in the rapid "bulk" collection of sandflies in the field and in some laboratory transfer procedures (fig. 3). We are indebted to our colleague, Dr. G. B. Fairchild, for the basic design of this apparatus. With a cork borer a hole is made in both the bottom and the removable top of the stopper to receive a short length (45 to 50 mm.) of ¼-inch methacrylate tubing, which serves as the entry tube; another hole is made only in the top to receive the ⅜-inch plastic tip of the suction tube. These holes are made slightly smaller than the respective tubes, so that the latter are firmly held by the elastic material of the stopper. Leaving support for the hole already made, as much of the rest of the bottom as possible is cut away. Bolting cloth, previously shaped to fit the stopper by being pressed wet into the vial and allowed to dry, is placed over the cut-out bottom, fastened with a clove hitch of stout thread, and coated with plastic cement wherever it is in contact with the plastic stopper. When the cement has hardened the entry-tube hole is cut through the bolting cloth with a cork borer, the cover is slipped back into its circular groove, the entry tube inserted to project equally above and below, and cemented in place. An 80-mm. length of ⅜-inch plastic tube serves as a removable extension when slipped over the ¼-inch entry tube.[3]

The aspirator tube consists of a convenient length (about 70 cm.) of rubber tubing fitted with a 100-mm. length of ⅜-inch methacrylate tubing with a right-angle bend and beveled for easy insertion into the appropriate hole in the top of the stopper. The short plastic tube can be smoothly bent by inserting each end in rubber tubing and suspending it in boiling water by the two rubber tubes held together. The only force necessary is supplied by the elasticity of the rubber; the ends of the plastic are protected against deformation. Suction can be applied directly by mouth or by using a rubber suction-pressure bulb. The latter is desirable particularly in dealing with large numbers of sandflies in the laboratory, to avoid inhaling scales, dust, etc. In the field each collector is provided with a number of aspirator vials plus a suction tube and an extension entry tube. The vials are moistened in the field before use. It is well to put no more than 15 sandflies in one vial if they are to be kept alive. The suction should be as gentle as possible since fed or gravid females are easily injured by too brusque an entry into the vial and, furthermore, there is inevitably some trauma from the agitation of the entire catch with each fresh entry.

Feeding adults

Virtually all species of *Phlebotomus* in nature suck blood only between dusk and dawn, although in dense forests some species occasionally feed also during the day. In the laboratory, however, the daytime feeding behavior varies greatly as between different species. In the early work on kala azar in India, *argentipes* in a releasing cage would feed on the arm of a patient only at night, although daytime feeding was later induced if the sandflies were kept in darkness. In our own experience certain species, e.g., *chinensis* and *mongolensis* in China, *verrucarum* in Peru, very rarely bite during the day either in nature or when liberated in a cage

[3] The series of sizes of methacrylate tubing commercially available, with outside diameters differing by ⅛ inch, have walls 1/16 inch thick and fit accurately over the next smaller size—a great convenience in various laboratory applications. It can be cut with a hack saw and the ends smoothed and beveled with an ordinary flat file, or for the inner edges, with a countersink. Methacrylate tubing is not yet generally listed in scientific supply catalogues; ours was obtained from Crystal-X Corporation, Lenni Mills, Pennsylvania.

with an animal nor do they attack the hand of the operator within the cage. Nevertheless, the same sandflies a few moments later when confined close to animal or human skin in a small feeding cage may feed at once.

In Panama the half-dozen principal forest species which attack man, horses, and other mammals, rarely bite in the daytime in nature and usually do not become active until after the brief twilight. However, in the laboratory two of these species, *sanguinarius* and *gomezi*, may feed readily in full daylight if liberated in a cage with an immobilized animal (fig. 8). They may also attack the operator's hand with such promptness and eagerness, even though the hand is in the cage for only a few moments at a time and is usually in motion, that it is necessary at times to wear a rubber glove as protection. The other species at best feed reluctantly and erratically. Night trials with some yielded no better results. Although there are unresolved difficulties in the feeding of these species, for the present at least, all our laboratory procedures are carried out during the day.

We have as yet made no attempt to determine the critical combination of factors which lead sandflies to leave their shelters and seek a blood meal, but it is obvious that this activity is usually preceded by a decrease in both light and temperature and a corresponding rise in humidity. In early trials we found that feeding results were improved by transferring the operation to another air-conditioned room with a slightly lower temperature. We then adopted the routine of manipulating the air-conditioning in the sandfly room so that the night temperature, 28° to 29° C., was somewhat higher than during the day, 26° to 27° C., thus reversing the conditions found in nature. As will be seen in the following descriptions of feeding operations, whenever there is prolonged exposure in a cloth cage, high humidity, necessary for sheer survival, is maintained in semi-darkness.

Releasing Cages.—Beginning with the work in China the releasing cages have in all cases been made of cloth, with at least two panels of some sort of netting, with no internal seams to obstruct visibility, and suspended by the corners in a frame. The first models were made entirely of silk bolting cloth, except for the muslin sleeve, and measured about 20 x 20 x 30 cm. Later a cubical cage with net internal dimensions of 20 cm. on a side, was found more suitable for routine releasing and sorting as well as for certain feeding operations. Later it was found desirable to have only two panels of bolting cloth (top and front) with the other panels of muslin giving a white, opaque background. A suitable grade of bolting cloth has about 20 meshes per linear centimeter. In Panama the 20-cm. cube with the same arrangement of netting and muslin panels is our standard releasing cage (fig. 7). In our current models, as a substitute for the silk bolting cloth, we have used nylon "marquisette," a fabric with the same weave as bolting cloth, i.e., the warp threads are double and pass on either side of the weft threads with a twist between each two, resulting in firmly locked square or rectangular meshes. Our nylon has about 16 x 22 meshes per linear centimeter. The finer nylon threads afford somewhat better visibility than the bolting cloth.

The cloth cages have various advantages over those with metal screen or other rigid material, quite apart from their extreme lightness and portability. The hand outside the cage enters actively into many operations. The finger tip on the outside of the cage aids in the capture of a sandfly when a vial or tube is placed over it (fig. 7). Containers may be firmly held by the free hand while corks, stoppers and covers are removed or replaced by the hand in the cage.

After considerable experience with different seamstresses and tailors, we have found it desirable to prepare the fabric ourselves. It is washed to preshrink it and remove sizing, and then with drawing board and T-square each seam, fold and cut is carefully marked; after cutting the folds are pressed with an iron. The muslin sleeve, 28 to 30 cm. long, is set into an opening 11 to 13 cm. square, the size depending on the objects, such as animal cages, which are to be inserted. The 6-mm. seams are double sewed with all cut edges "buried" within them and project flangelike at all edges of the cube, except that no seam is necessary between the two netting panels which are made of one piece. Even though it is possible to form the four muslin panels as one piece it is well to have the extra strength of the seams. The cage is suspended in its frame by two 20-cm. strands of tape sewed to each corner. With all seams on the outside there are no hiding places for sandflies except in the sleeve and relatively few seem to enter the sleeve. A rubber band holds the sleeve snug, but not too tight, around the arm. After withdrawing the arm the sleeve is brushed downward so that the surface presented to the inside of the cage is as nearly vertical as possible.

The cubical frame is made of ⅛-inch (3.2 mm.) brass rod, 25 cm. on a side, i.e., 5 cm. greater in each dimension than the suspended cage. To make the entire apparatus conveniently portable, the four vertical rods fit easily but not too loosely into 15-mm. lengths of brass tubing welded to the corners of the top and bottom parts of the frame. The tension of cage and tapes holds the frame together. The four loose rods are easily removed with the cage still tied to the corners of top and bottom; the whole apparatus can be collapsed for transportation and quickly set up again. (Like the cages themselves, the frames have passed through several developmental stages. At one time the removable vertical rods and the corresponding tubes were screwed together with right- and left-hand threads. The current arrangement is simpler and just as effective.)

Feeding Adults in Releasing Cages.—For the purpose of feeding sandflies liberated with an immobilized animal, two larger sizes of cage are in use. They are designed to accommodate an animal such

as a spiny rat, *Proechimys* (fig. 8), or a guinea pig. These cages are longer and broader but only slightly higher than the small releasing cages. The materials and general method of construction are the same. The inside dimensions are 30 x 25 cm. with a height of 21.5 cm. (fig. 8) and 40 x 35 x 21 cm. The sleeve is inserted at one end with an opening of 14 x 14 cm. In the larger cage three of the long panels, top, front and back, are of nylon marquisette while in the smaller one only the top and front are of netting (with the sleeve at the right). (In making a cage with the latter arrangement the needs of a left-handed operator should be considered; the small cubical cages with two netting panels and the larger one with three are suitable for either hand.)

The frames for the larger cages, made of the same brass rod, with four of the rods removable, have all dimensions 5 cm. greater than those of the corresponding cage. To support the weight of the animal if the cage is to be moved, a broad sheet of aluminum bent at the ends rests on the bottom end rods of the frame. For the purpose of maintaining a high degree of humidity during feeding operations, and also to provide semi-darkness, a large frame capable of accommodating any of the cages with their frames, is draped with wet toweling. The frame measures 56 x 51 x 32 cm. The cloth, blue Turkish toweling, is cut and sewed to form a boxlike hood over the frame, with one end hanging free. The top panel is supported by a rack made of coarse metal screening resting on the top of the frame.

In practice the animal is tied with wide gauze bandage, belly uppermost, on a narrow board (fig. 8). The belly hairs are removed with electric animal clippers. The animal is placed in the cage and the sandflies liberated from the breeding pots. The animal is free to move its head and legs but the cages are of such size that the cloth is rarely engaged by the animal's claws. After about an hour the animal is removed and the fed and unfed sandflies are transferred to separate clean, sterile pots, moistened and fitted with an aspirating device described below (fig. 5). About 20 fed females may be put into one pot, accompanied by an equal number of males, since copulation may not take place until after the blood meal. Depending on the numbers involved and the requirements for future operations, the unfed females and the rest of the males are either discarded or aspirated into other pots, with as many as 100 sandflies, females and/or males, to a pot. The aspirator tops must now be removed and replaced with bolting cloth without liberating sandflies in the process. The sandflies are immobilized by chilling the pots in a refrigerator at 4.5° C. for about 20 minutes. The exchange of covers is made, a raisin is placed on the bolting cloth, a muslin cover secured over all and the pots placed on cotton pads as in the routine handling of the cultures.

Aspirator Top for Pots.—The aspirator tops are made from the metal lids of glass jars, of such a size that they just fit over the top of the pots (fig. 5). (Lids of 6-ounce coffee jars happen to be about right.) Two holes are drilled somewhat smaller than the rubber tubing, about 10 mm. outside diameter, which will be squeezed into them to form the entry and suction tubes and which project about 25 mm. and 50 mm. respectively on the outside and about 5 mm. inside. The inner end of the suction tube is covered with bolting cloth; the entry tube is left open. The lid is held in place by a cylindrical band 50 mm. wide cut from the wrist of a rubber glove, which also makes an air-tight seal between lid and pot. When ready for use an 8-cm. glass extension is inserted in the entry tube, and the same aspirating tube with suction-pressure bulb, previously described, is fitted into the other rubber tube.

Feeding Through Bolting Cloth Pot Covers.—Females held in pots after having failed to feed when liberated may be fed directly through the bolting cloth covers. The arm of a volunteer (choosing one for whom the reaction to the bites is not severe) merely rests on one or two pots for about 15 minutes. For feeding on a guinea pig, which is the more usual practice, the animal is tied on its back with gauze strips and the hair clipped from the belly and both sides (fig. 9). A wire rack, with rubber bands, supports a pot on either side of the immobilized animal in such a way that the weight of the tilted pots holds the bolting cloth in contact with the skin.

Small Feeding Cages.—In feeding small numbers of sandflies some investigators have simply put them into test tubes and held the open end in contact with the skin. Throughout all our work we have used small feeding cages in which the sandflies, always within about 15 mm. of the skin, can feed through bolting (fig. 2). These cages are the lineal descendants of Wolbach's louse-feeding cage (Wolbach et al. 1922). In the early work in China the senior author used cages which had been presented to him by Dr. Wolbach. They were made from tinned metal ointment boxes with the top and bottom cut out and replaced by bolting cloth. To adapt these cages to flying insects the only modification necessary was to insert a glass entry tube in the bolting cloth cover. Later, to avoid corrosion, the sheet-metal stock was of Monel metal. The metal parts were made in commercial shops, the shaping being done not by stamping but by a process known as spinning, in which a revolving sheet of metal is gradually pressed over a form. The current models are 40 mm. in diameter by 14 mm. in depth, with openings in both top and bottom 29 mm. in diameter. Around the lower part of the bottom is a projecting "head," over which the bolting cloth is fastened, made either by soldering in place a circle of wire or by having such projection "spun" during manufacture. The narrow rim of the bottom is pressed outward so that the bolting cloth can be drawn taut over its inner edge. (Otherwise sandflies would wedge themselves into loose places between metal and cloth.) The metal cover is made just large enough to slip snugly over the bolting cloth cover as the latter is pressed

into place. Wet bolting cloth is pressed over the bottom with a loose wire ring, bound over the projecting head with a clove hitch of stout thread and allowed to dry. It is then trimmed and bound with a narrow strip of adhesive tape. The bolting cloth cover is shaped by being pressed on when wet and allowed to dry. To fix the entry tube (22 mm. long, of glass or plastic) in the bolting cloth cover, six star-shaped cuts are made, the tube is inserted to project about 8 mm. into the cage, the six points are bound to the tube with thread and then coated with plastic cement. The inner metal surface of the cage is coated with plaster of Paris to provide both moisture and proper footing for the sandflies. With the cover pressed on, a strip of adhesive tape around the cage holds it safely in place. It seems that the volatile substance which gives fresh adhesive tape its characteristic odor is toxic to sandflies, but the tape is safe to use after exposure in the air for several days. A stock of such "seasoned" strips is maintained on glass plates or other exposed surfaces.

Sandflies are transferred to the feeding cages by methods described below. The cages, usually with no more than 15 sandflies, are routinely held in contact with the skin of the host animal with gauze strips or by a device described below. Those sandflies which are ready to take a blood meal will usually have completed feeding within 15 or 20 minutes. The cages are opened in a releasing cage and the specimens transferred to appropriate vials or pots.

When feeding sandflies on human leishmanial lesions it is necessary to mask the lesion with aluminum foil, so that the sandflies have access only to the narrow border of indurated, unulcerated skin surrounding the central ulceration, this being the area where the parasites are concentrated.

Loading Feeding Cages.—The feeding cages are loaded by means of a transfer tube or an aspirating device. The transfer tube (fig. 2), made by a glassblower of two sizes of tubing, is 9 cm. long, the middle 5 cm. with a bore of about 4 mm., the 2 cm. at either end of a somewhat larger size, about 6 mm., which can slip easily over the entry tube of the cage. A bolting cloth screen is fitted in one end. The sandflies are caught one at a time by putting the open end over them as they rest on the sides of the releasing cage. Once inside the tube the finger is held over the open end and the fly makes its way or is shaken into the narrow portion, where its movement is restricted, since it cannot fly, and where it may be inspected with a handlens. Meanwhile the feeding cage, set in a slot cut in a pasteboard box, is held firmly with the entry tube horizontal. The cotton stopper is removed from the latter. When the sandfly is seen to be at a safe distance from the open end, the transfer tube is shifted quickly from the finger tip to the entry tube and the sandfly blown gently into the cage with a rubber bulb. (The first model of the transfer tube, devised in China, had a syringelike plunger which "nudged" the sandfly and caused it to run into the cage.)

Aspirator for Feeding Cages.—The aspirating device for loading feeding cages is made out of a glass jar with screw top of metal and an air-tight gasket (fig. 4). We have used the smallest available jar which will easily receive the feeding cage. It measures 68 mm. in diameter by 98 mm. in height, the inside diameter of the mouth being 47 mm. Two holes, about 9 mm. in diameter are drilled in the metal cover, one located so as to correspond with the somewhat eccentrically placed entry tube of the cage. Short lengths of rubber tubing, outside diameter 12 mm., are squeezed into the holes so as to project 30 mm. outside and 10 to 12 mm. inside. The inner segment of the rubber entry tube is wrapped with wire to insure a firm grip on the feeding cage's glass entry tube as the latter is pushed into place with the fingers. In use, the feeding cage hangs by its entry tube inside the jar. Outside, the rubber entry tube is provided with a plastic 8-cm. extension while the other rubber tube receives the suction tube previously described, which is used interchangeably for all our aspirator devices. After the sandflies have been aspirated into the feeding cage, the latter is removed by grasping the entry tube with rubber-tipped, curved haemostatic forceps and pulling it free.

Holder for Feeding Cage.—For feeding sandflies on Chinese striped hamsters, an apparatus was constructed in which the feeding cage was held by the sharp points of two screws set opposite one another in a metal ring, which in turn was fixed to a shaft free to move vertically in a tubular metal sleeve (fig. 10). The latter was held by an adjustable arm clamped to the side of the screen rack on which the animal was tied. This arrangement permitted the cage, resting on the animal's belly, to pivot on the two points and move vertically with the respiratory movements. The weight of the cage plus the movable metal parts was counterbalanced by rubber bands so that the pressure was just enough to maintain contact of cage with skin. A metal guard prevented the hamster's reaching the cage with its teeth. This apparatus is quite useful in the case of small animals which can be satisfactorily immobilized. We have used the original Chinese model with guinea pigs and spiny rats. These animals can easily bear the weight of the apparatus. Instead of being clamped to a fixed object it merely rests on the table, prevented from tipping over by a metal bar, such as a large file, inserted in the clamp (fig. 10).

Artificial Feeding.—For the purpose of infecting sandflies with *Leishmania* they were fed artificially a mixture of culture and defibrinated blood. The use of membranes gave only indifferent results, while the pipette method of Hertig and Hertig (1927) was very successful in an extended series of such feedings. These techniques and the related experimental work, in which a large proportion of the sandflies became infected, will be the subject of a separate report.

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