References and Notes

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25 August 1955

Quantitative Microinjection of Mosquitoes

A method for obtaining oocysts of the malaria parasite free from the mosquito stomach wall was sought as a further step toward the *in vitro* studies of the development of oocysts of *Plasmodium relictum* in *Culex tarsalis* (*1*). Following Weathersby's results (*2*) in obtaining infection of the mosquito (*Aedes aegypti*) with oocysts unattached to the stomach wall by injecting blood infected with the parasite (*Plasmodium gallinaceum*) into the hemocoele of the insect, an attempt was made to use this technique to obtain free oocysts (*3*).

The injection setup consisted of two parts, the injector and the insect holder (Fig. 1). Adapted from the "braking" pipette of Holter (*4*), the injector was made of two components: (i) a needle drawn from glass tubing forming a spindle with a capillary at each end and (ii) a glass tubing jacket, tapered at one end, into which the spindle was sealed and made airtight with Duco cement (Fig. 2, top). For injection, the injector was clamped firmly to a stand in a horizontal position and fastened to a rubber bulb that was provided with a check valve. Solutions to be injected were brought in touch with the point of the needle; the spindle then filled itself through capillary action. Known quantities of solution were obtained by using accurately calibrated spindles of different sizes.

The insect holder was a device similar to that of Wallis (*5*). It was made of glass tubing with a tapering end. The insect was held on the opening of this end by suction (Fig. 2, top). Successful attachment depended on a suitable size of the opening and the shape of the contact area of the insect. An opening with a curved surface could be ground to suit the particular need. The holder was mounted on a clamp that was attached to a mechanical stage by a spring-loaded lever. A screw in control of the lever provided vertical movement, as is shown in Fig. 1. Thus, within a sufficient range, the holder could be fitted to any position demanded.

The injection was carried out by setting the needle point of the injector in the center of the field of the dissecting microscope and then bringing the insect, impelled by the holder, to the needle. When the needle had pierced the body wall of the insect, pressure was maintained on the rubber bulb until the insect was drawn away from the needle in order to avoid backflow of the injected material.

The site selected for injection was the base of the metacoxa (Fig. 2, top); and the age of the mosquitoes used was not less than 3 days, for younger ones did not stand injection well. To immobilize the mosquitoes, a combination of carbon dioxide and cold was found satisfactory. The mosquitoes were first knocked down by CO_2, separated into small lots, and then kept inactive in an ice bath. A continuous CO_2 chamber in which the injection was carried out was found to be very convenient but was abandoned because of the possible effect on the solution to be injected.

Before injecting the parasitized blood of the canary, the technique of injection and the effect of the anticoagulants were tried on both sexes of the mosquitoes. The anticoagulants used were either sodium citrate or heparin in normal saline. At times, glucose was added to the saline-anticoagulant mixture as a possible essential food supplement for the activities of the parasites. Doses of 0.61 mg (comparable to the amount of blood in a fully engorged mosquito) each were injected into the females. Almost 100-percent survival was obtained regularly. Needles varying in diameter from 10 to 90 μ were equally safe, and in one experiment, a needle 300 μ in diameter was used on 13 females, nine of which survived for many days.

Repeated injections were also tried on a total of 21 females in two separate trials. These were fully injected three times on alternate days with a needle 40 μ in diameter, and 19 were still surviving on the third day following the last injection.

However, the males were less hardy,

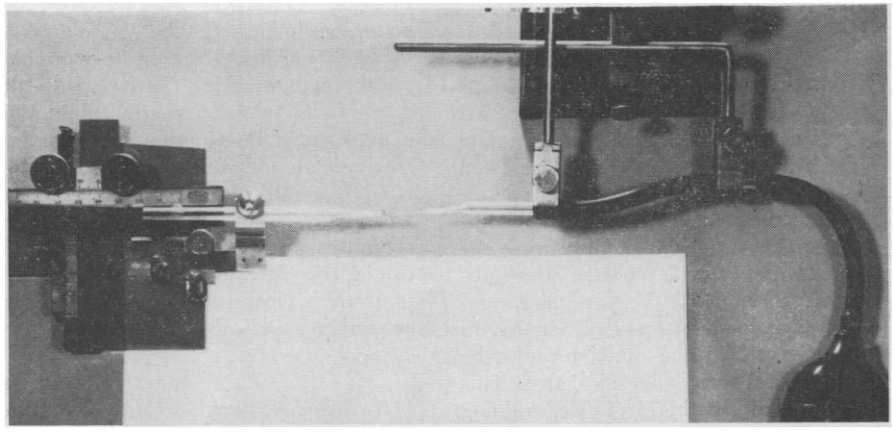

Fig. 1. Insect holder and injector.

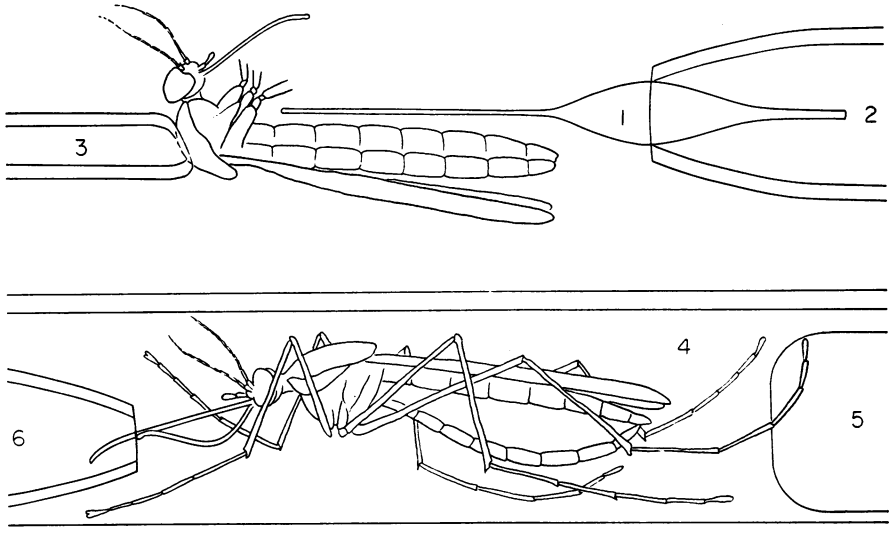

Fig. 2. (Top) Injection of mosquito; (bottom) forced feeding of mosquito. (1) Needle, (2) jacket, (3) insect holder, (4) glass tubing, (5) plastic rod, (6) micropipette.

and about 40 percent of them died on the third day after being injected with 0.41 mg of either heparinized or citrated saline solution. The needle diameter was about 40 μ.

For injecting parasitized blood, canary blood at the peak of infection was drawn. Either heparin, citrate, or additional glucose was added, or the blood was subjected to centrifugation and washed with normal saline; then it was injected into the mosquitoes. When saline-anticoagulant solution was used, the blood was diluted by one-fourth of its volume. However, when the blood cells were washed with normal saline without including an anticoagulant, the mixture was centrifuged, the supernatant was drawn away, and the original volume of the blood was restored with saline.

By the end of 2 weeks—the normal time for maturity of oocysts in the laboratory—the injected mosquitoes were dissected and examined for oocysts and sporozoites. The total number of the females surviving and examined was 73 out of 322 injected (23 percent), and of the males, five out of 118 (4 percent). In all cases the results were negative. Since the main purpose of this experiment was to obtain young oocysts free from the stomach wall of the mosquito for *in vitro* culture studies, the possible presence of oocysts and sporozoites was neither searched for histologically, nor was their presence tested by permitting the mosquitoes to bite a canary as Weathersby did in his unique experiments (*2, 6*).

To test the viability of the parasites, mosquitoes were fed artificially with a micropipette (*7*) that contained some of the sample of blood employed for injections (Fig. 2, bottom). Practically all the females that took the blood, heparinized or citrated, developed oocysts and sporozoites in a normal course. Viable eggs were laid and normal adults emerged in due time. A few males were also induced to feed, but none of them lived long enough to be dissected for the examination for any possible infection (*8*).

These techniques may well serve advantageously for research on mosquitoes carrying viruses, such as the recent work of McLean (*9*).

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8 August 1955

Ethylene Oxide for Sterilizing Diets

Hawk and Mickelsen (*1*) showed that treatment of diets for rats with ethylene oxide produced deleterious effects. Thiamine was almost completely destroyed, but this was not the sole effect, for supplementation with either thiamine or a complete vitamin mixture did not significantly stimulate growth.

At our laboratory, ethylene oxide in both the liquid and the gaseous (*2*) forms has been successfully used to sterilize liver brei, a chemically defined diet (*3*), and various mixtures of these two foods that were used to rear the larvae of the dipterous parasite *Pseudosarcophaga affinis* (Fall.) (*4*).

Liquid ethylene oxide was used to sterilize the liver brei. Working in a cold room (1°C) with thoroughly chilled materials, we pipetted 1 ml of ethylene oxide into 250-ml florence flasks containing 100 g of liver brei. After 2 hr in the cold room, the flasks were allowed to reach room temperature in a well-ventilated area. They were then placed in an incubator at 37°C for 24 hours to remove toxic traces of ethylene oxide. Fifty larvae were reared individually on aliquots of the sterile brei. They thrived.

Gaseous ethylene oxide was used to sterilize 66 chemically defined diets containing various amounts of liver. Forty milliliters of each diet, without added vitamins, were placed in 250-ml florence flasks and sterilized. A low-pressure supply of gas was obtained by partially inflating a valveless inner tube. The gas in the inner tube was used immediately to reduce the time of contact with the rubber. The flasks containing the material to be sterilized were put in a 250-mm vacuum desiccator, and the pressure was reduced 25 to 26 in.-Hg below ambient. The gas was admitted from the inner tube until the pressure in the desiccator was within 5 in.-Hg of the ambient pressure. The cycle of evacuation and admission of carboxide was repeated once.

The material was allowed to remain in the atmosphere of ethylene oxide for at least 24 hours, and then the desiccator was flushed with air three or four times. The diets were allowed to stand at room temperature for approximately 24 hours before use. The vitamins, in solution, were then filtered through a bacteriological filter and added to the medium under aseptic conditions. In all, 3250 larvae were reared individually on diets that had been sterilized in this manner, and only four were contaminated. On media that contained no liver, whether sterilized by autoclaving or by ethylene oxide, 50 percent of the larvae reached the third instar in about 5 days. Ethylene oxide, therefore, did not have a deleterious effect on the diet.

The difference between these results and those of Hawk and Mickelsen (*1*) may be a result of differences between parasitic larvae and rats, but several additional reasons can be suggested. The vitamins of the chemically defined diets were not exposed to ethylene oxide. The diets were treated in a buffered solution, not in the dry state (as the rat diet was treated), and carbon dioxide was present as well as ethylene oxide. These differences in technique may well have combined to prevent the development of the high *p*H to which Hawk and Mickelsen attributed some of the injurious effects of ethylene oxide. In the liver brei that was treated with liquid ethylene oxide, there probably was a considerable buffering action, as well as some protective combinations of nutrilites not present in the rat diet.

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2. Carboxide, Carbide and Carbon Chemical Company, 30 E. 42 St., New York 17; a mixture of 10 percent ethylene oxide and 90 percent carbon dioxide supplied in cylinders under pressure of 800 lb/in.^{2}
3. This diet was similar to that described by H. L. House [*Can. J. Zool.* **32**, 331 (1954)] but with glucose reduced to 0.5 percent, salt mixture reduced to 0.33 percent, and KOH, rather than NaOH, used to adjust acidity.
4. This report is contribution No. 290, Chemistry Division, and No. 3346, Entomology Division, Science Service, Canada Department of Agriculture, Ottawa.

24 August 1955

Correction

The filter paper strips used in V. L. Johnson and J. S. Dunlap "Electrophoretic separation of hemoglobins from the chicken" [*Science* **122**, 1186 (1955)] should be listed as follows: S. and S. No. 2043 B.

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