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LONG-TERM MAINTENANCE IN A NUTRIENT
MEDIUM OF ADULT ORGANS TAKEN FROM
ASEPTICALLY REARED MOSQUITOES,
ANOPHELES STEPHENSI

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Long-term maintenance in a nutrient medium of adult organs taken from
aseptically reared mosquitoes, Anopheles stephensi¹

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Efforts to cultivate the mosquito-borne phase of the malarial parasite in vitro, either in a defined medium or in a cell-cultured substrate maintained in a nutrient medium have met with little success. In both approaches, the addition of adult organs enhances the stepwise growth of sprongonic forms and development of oocytes into infective sporozoites. It appears that supplements of adult organs are necessary for the development of a system that will provide the environment required for a complete single step cultivation of the parasite.

This laboratory is presently maintaining primary cultures of larval cells and tissues of Anopheles stephensi for up to 60 days without change of nutrient medium (Ronquillo, et al., elsewhere in this colloquim). In order to supplement our cultures with healthy, germ-free adult organs, we attempted to rear aseptically, Anopheles stephensi mosquitoes. This paper reports these successful attempts and also the long-term maintainance of germ-free organs in a nutrient medium.

In the formulation of a rearing medium, we referred to studies done on the nutritional requirements of the developing larvae of several different species. Both a semi-defined diet of salts, lipid, protein, and vitamins, and a non-defined diet of liver extract and yeast were used. All procedures were done utilizing sterile techniques.

Newly oviposited eggs taken from a colony of mosquitoes maintained in our laboratory were surface sterilized, incubated for 48 hours, and hatched in sterile distilled water. In each 50 ml Bellco erlenmeyer flask were placed five of the newly hatched larvae along with five ml. of

the medium. Rearing was carried out in an incubator at 27°C with 70% relative humidity. Eight hours of light per day were provided with one 15 watt incandescent lamp. Records of growth were made daily. Contamination was tested by inoculation into thioglycolate (Bioquest). Pupae were removed from the rearing flasks to a specially constructed emergence chamber containing sterile distilled water. This chamber is essentially a stoppered test tube with a window in one side covered with nylon netting. The window provides a perch for the young adult and also a facility for later feedings. The chamber itself sits within another larger tube which is provided with a metal closure. The system thus remains sterile.

Twenty-four hour old adult females were allowed to take blood meals from surface-sterilized hamsters which either were free of malarial parasitemia^{or} were infected with Plasmodium berchei (HK65-A strain). All hamsters were sedated with 0.3 mgm (.05 grams) of sodium pentobarbital (Dialbutal) injected intraperitoneally.

The guts or other organs of the adults were dissected aseptically, using precision micro-instruments (Trident) and placed in the culture medium. Bellco culture dishes (12 x 30 mm) fitted with ground glass covers were used as containers.

Successful aseptic rearing of aedine mosquitoes has been reported. However, attempts by earlier workers to rear the anopheline mosquito failed.

Our attempts to rear Anopheles stephensi from newly hatched larvae to the adult form using either a semi-defined diet or non-defined diet have been successful. Adults emerging on the medium were almost always

unable to leave the surface. However, when pupae were transferred into sterile water, adults appeared healthy and active.

The difficulty in rearing anophelines on a prepared, sterile medium, may be purely a mechanical one. The anopheline feeds while resting just under the surface of the water that it inhabits. In both of our diets insoluble particulate matter was present which settles to the bottom of the rearing container. The particles are presumably unavailable to the surface-feeding larvae. When particulate matter was filtered from the liver extract and yeast diet, growth was not supported beyond 4th instar. It is interesting to note, however, that by replacing this particulate matter with inert carbon particles, growth and development approached normal experimental results. It may be then, that particles are necessary whether or not they are nutritionally important.

If test tubes rather than flasks were used, no growth beyond 4th instar occurred. Previous investigation, as far as we are aware, used only test tubes in which the depth of the media is greater than that of the same volume in a flask. The shallow medium in a flask appears to place the particulate material where it is available to the larvae. Any significant increase in the depth of the medium resulted in decreased survival.

Preliminary observations of the mid-gut of mosquitos dissected mosquitos provided with two blood meals from an infected hamster look very promising.

The availability of aseptically-reared insects can potentially serve many purposes and offers certain unique features and advantages to tissue culturists. Moreover, through the availability of aseptic organs, that can

be maintained in a nutrient medium, a necessary step towards the eventual cultivation of the mosquito-borne phase of the malarial parasite in one complete cycle has been accomplished.

As mentioned earlier, Ronquillo et al. reported the successful primary cultivation of tissues and cells of A. stephensi for prolonged periods of time. This was accomplished in a nutrient medium in which the pH may be varied (6.5 - 8.5) as well as the incubation temperature (20°C - 27°C). Therefore, in our aim to develop a receptive system for the cultivation of the parasites which consists of primary cell cultures with organ supplements, the latter should also remain viable in a similar latitude of pH and temperature range. Preliminary experiments regarding this aspect show promising results.

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ABSTRACT SUBMITTED TO III INTERNATIONAL COLLOQUIUM ON INVERTEBRATE
TISSUE CULTURE

Long-term maintenance in a nutrient medium, of adult organs, taken from
aseptically reared mosquitoes, Anopheles stephensi¹

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The need for aseptic organs of mosquitoes for use in the cultivation of parasites has been documented. Aseptic rearing of Aedes aegypti has been done and Trager (1935) and Akov (1961) succeeded in obtaining complete life cycles of this particular species. However, attempts of earlier workers to rear the anopheles mosquito to adult stage have met with failure. This paper describes the approach and improvements employed in the successful aseptic rearing of Anopheles stephensi in our laboratory. In the formulation of the nutrient media, we referred to the studies done on the nutritional requirements of the developing larvae of different species. Both a non-defined diet of liver extract and yeast (Trager, 1935) and a semi-defined diet (Akov, 1961) appear adequate for the successful aseptic rearing of A. stephensi. However, the difficulty arising is the availability of some of the nutrients to the larvae, which are definitely surface feeders. This problem has been overcome by rearing the larvae in flasks rather than in test tubes, thus keeping the media shallow so that the particulate materials become accessible to the larvae. Organs from aseptically-reared adults were dissected and placed in a nutrient medium (Ronquillo et al in press). They remain viable without loss of their integrity for as long as 30 days. The availability of aseptically-reared insects could serve many purposes and offers certain unique features and advantages to tissue culturists. Preliminary studies on the use of aseptically-reared A. stephensi and its dissected organs maintained in culture for the development of malarial parasites will be discussed.

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