weight of the liver decreased significantly with the growth of tumor (Fig. 1 and 2—legends). Finally, careful examination of the carcass of the tumor mouse revealed the loss of stored fat so characteristic of tumor growth.

The growth of tumor in mice and rats differed in at least one respect which might account for the species difference in the effect on cytochrome oxidase activity. The tumor rats of Table I were sacrified at about 2½ months after receiving transplants, when tumors comprised about 40% of total B.W. On the other hand, mice that were given transplants had grown barely palpable tumors in the same length of time. Spontaneous tumors, once they appeared, grew at approximately the same rate as the transplanted. Conceivably, the stress of rapid tumor growth in the rat with Walker carcinoma 256, a tumor which has been shown to increase the caloric requirements of the host(6,7), may account for the increased activity of the terminal oxidative enzyme, cytochrome oxidase; whereas, in the mouse, in which tumor grew slowly, the degree of stress may not have been sufficient to bring about increased enzymatic activity. Further work is necessary in order to explain the decrease in cytochrome oxidase activity in the liver of the tumor mouse.

Summary. In C3H female mice growing either spontaneous or transplanted breast carcinoma the cytochrome oxidase activity of liver homogenates decreased as the tumor attained an increasingly larger percentage of total body weight. On the other hand, confirming previous results with Rochester rats, an increase in the activity of this enzyme was found in liver homogenates from Sprague-Dawley rats bearing Walker carcinoma 256. In tumor-free animals, mice had significantly higher cytochrome oxidase activity than rats of either strain.

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Transmission of Sindbis Virus by *Aedes aegypti* to a Chick Embryo Cell Culture System.* (29955)

JAMES O. MASON†, THOMAS E. FROTHINGHAM, ANDREW SPIELMAN AND THOMAS H. WELLER
Department of Tropical Public Health, Harvard School of Public Health, Boston, Mass.

Sentinel animals commonly are used for surveillance of the arthropod transmission of viral agents. Viruses have been demonstrated in endemic and epizootic environments by the use of non-immune monkeys(1), mice(2), and chickens(3). There are, however, certain limitations which restrict the usefulness of this approach. Difficulties in transportation, maintenance, and protection are inherent in the use of animals. Furthermore, except where virus transmission is indicated by obvious disease, the sentinel animal must be tested for presence of virus or for antibody conversion. To detect a low order of virus transmission, the investigator is faced with the task of processing numerous specimens. The results of such tests often reflect past activity and fail to stimulate expanded investigative or control efforts at a time when

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they would be most productive.

Ideally, a sentinel system should simplify the management of animals, and indicate within a few days whether transmission has occurred. A cell culture in which arboviruses form plaques would possess these properties, provided that problems of vector attraction and stimulus to feeding could be solved and that feeding could be achieved through a membrane that would exclude contamination.

A possible means of inducing mosquitoes to feed on a cell culture was indicated when adenine nucleotides were identified as components of blood responsible for gorging(4,5). The work of Collins *et al*(6) suggested trial of an animal-derived protective membrane. This report describes the development of a membrane-protected chick cell culture system which stimulates feeding by female *Aedes aegypti*. Sindbis virus is transmissible to such cultures by infected mosquitoes and its presence is indicated by the formation of plaques.

Materials and methods. Cell cultures. Cells were derived from trypsinized 10-day-old chick embryos. For virus titrations, cultures were prepared by adding 20×10^6 cells in 5 ml growth medium to Petri dishes of 5 cm diameter. Growth medium was made up of 9 parts Earle's salt solution(7) containing 0.5 g lactalbumin hydrolysate per 100 ml and one part heated (56°C, 30 min) horse serum.‡ Agar overlay medium consisted of Earle's solution containing Difco§ Bacto Agar 1.0 g, gelatin 0.5 g, Difco yeast extract 0.1 g, and neutral red 0.003 g per 100 ml. Penicillin and streptomycin were included in all solutions at 100 units and 100 μg per ml respectively. Cultures were incubated at 36°C in humidified air containing 5% CO_2.

As a result of experiments described below, a cell culture system was developed which possessed the capacity to attract and induce female *A. aegypti* to feed. Materials employed in these cultures were prepared as follows. Growth medium was modified by reducing the $NaHCO_3$ concentration to 0.021 g per 100 ml and by adding 4 ml of a 0.05 N tris‖ buffer (pH 7.5) in Earle's solution. This was designated tris-growth medium. Agar overlay medium was modified by replacing the $NaHCO_3$ with 5 ml of the tris buffer per 100 ml; this was designated tris-agar medium. Chick embryo cell suspension (8) was prepared by mixing tris-growth medium containing 2.5×10^8 cells per ml with an equal volume of tris-agar medium in a 40°C water bath.

Adenosine triphosphate¶ (ATP) solution was prepared by adding 0.31 g of the disodium salt to 5 ml tris-growth medium. The ATP was dissolved and pH adjusted to 7.5 by addition of 0.85 to 0.90 ml of 1 N NaOH. This solution was sterilized by filtration.

Animal derived membranes (untreated Baudruche**) were cut into discs 5.5 cm in diameter and sterilized by exposing both surfaces to ultraviolet light (2537Å at 5.5 cm, 10 min each side).††

Virus. Sindbis virus (strain MP 684), in its 7th mouse brain passage since isolation from a pool of *Mansonia fuscopennata* collected near Entebbe, Uganda, was furnished by the Rockefeller Foundation Virus Laboratory. The virus was passed twice in chick embryo cell cultures and then through 3 alternating suckling mouse-*A. aegypti* passages before stock was prepared as a hundred-fold dilution of material harvested from a single infected mosquito.

Assay of Sindbis virus was performed by testing 0.5 ml aliquots of a series of 10-fold dilutions for plaque forming units (PFU) in the chick cell cultures. Diluting fluid consisted of growth medium with the $NaHCO_3$ content reduced to 0.084 g per 100 ml. Sindbis virus plaques appeared in 48 hours and the number did not increase after the third day when they were counted.

Mosquitoes. Aedes aegypti were derived from an established laboratory colony. Adults were maintained at 75°F and 70% relative

‡ We are indebted to Dr. Geoffrey Edsall and staff of Massachusetts Public Health Biological Laboratories for horse serum.

§ Difco Laboratories, Detroit, Mich.

‖ Trishydroxy Methyl Amino Methane, Fisher Scientific Co., Fair Lawn, N. J.

¶ Nutritional Biochemicals Corp., Cleveland, Ohio.

** Obtained from Long and Long, Belleville, N. J.

†† Mineralight-Ultraviolet Products, Inc., South Pasadena, Calif.

humidity in glass chimneys covered with netting. A dish containing water was fastened to the chimney bottom, and a cotton pledget wet with 5% glucose solution was placed on the netting.

Female mosquitoes were allowed to feed on 5-day-old white Swiss mice which had been inoculated intraperitoneally 2 days previously with approximately 150 PFU of Sindbis virus. Mosquitoes which became engorged after feeding were transferred to a separate chimney and maintained on glucose and water. At intervals, 2 mosquitoes were removed and stored at −65°C. Prior to titration they were thawed and individually ground in a chilled mortar containing 2 ml of diluting fluid. The suspension was centrifuged for 10 minutes at 1500 rpm, and the concentration of Sindbis virus in the supernate was determined by plaque assay. Mosquitoes which were killed immediately after feeding on an infected mouse contained 10^6-10^7 PFU of Sindbis virus. After 12 days, 98% of the surviving mosquitoes were found to be infected, and 50% transmitted lethal Sindbis virus infection to 3-day-old mice.

Testing of substances for capacity to stimulate A. aegypti *to probe and gorge.* Substances were tested by placing 5 ml in 10 × 35 mm plastic Petri dishes§§ which were covered with the membrane. Each dish, inserted into the bottom of a chimney, rested upon a heating pad regulated to maintain the contents of the dish at 33-34°C. Twenty to 25 female mosquitoes, not previously deprived of glucose or water, were exposed to the test substances for 10 minutes. The mosquitoes were then anesthetized with 100% CO_2 and the number which had gorged was determined by observing abdominal distention.

Results. Development of a cell culture system on which mosquitoes would feed. When offered any of several culture media covered by a membrane, female *A. aegypti* rarely probed and never appeared to gorge. Indeed, when tris-growth medium was provided as the only source of nutrient, mosquitoes survived 6 to 8 days, the same as controls from which food had been withheld. Under the same conditions, however, *A. aegypti* gorged avidly in response to heparinized chicken blood. Little probing or gorging followed exposure to red cells which had been separated from plasma by centrifugation, then washed, and suspended in phosphate buffered saline (PBS). If probing did occur, it was frequently associated with gorging. Fresh plasma or heated horse serum stimulated probing which was rarely followed by gorging. These observations suggested that both blood cells and plasma or serum contained active components affecting feeding by female *A. aegypti,* and that each played a role in normal probing and gorging activity. That feeding took place through the membrane, and not from its surface, was established visually and by tests with carmine particles and red cells which were recovered from the midgut.

Addition of ATP to serum resulted in increased probing and gorging. Peak feeding activity occurred with a serum concentration of 0.001 M ATP at which level more than 90% of exposed females rapidly gorged. The results of 3 experiments are summarized in Fig. 1.

A similar effect was observed when ATP was added to cell culture fluids. With tris-growth medium, peak activity occurred with 0.01 M ATP, and at this concentration 53% of mosquitoes gorged. Seventy percent gorged when the serum concentration was increased to 30%.

The effect of ATP was also observed when it was added to tris-agar medium. The response of female mosquitoes which had been offered medium containing varying ATP and serum concentrations is shown in Fig. 2. Over 90% gorged on tris-agar medium containing 0.01 M ATP and 20% serum. This effect was maintained for 6 hours at 36°C without measurable loss of activity.

The possible effect of ATP on chick embryo cells and on development of Sindbis virus plaques was investigated. Samples of tris-agar medium containing 10-fold dilutions of ATP were added to monolayer cultures which had been infected 30 minutes earlier with 10-16 PFU of Sindbis virus. Plaques failed to develop in cultures containing concentrations of 0.01 M ATP or greater. At these concentra-

§§ Disposable sterile Petri dishes—No. PD 3510, Falcon Plastics, Los Angeles, Calif.

tions the pH of the cultures was lower than in those containing less or no ATP. With an overlay containing 0.001 M ATP, the appearance of the cell sheet and the clarity and number of plaques did not differ from that of infected cultures which lacked ATP. When a thin layer of tris-agar medium containing an otherwise toxic concentration (0.01 M) of ATP was added to the surface of a culture, near maximum mosquito stimulating effect was achieved without apparent adverse effect on the cells or viral plaque development.

The method finally adopted for preparing these cultures was as follows (Fig. 3). Four ml of tris-agar medium were added to the plastic Petri dish and permitted to solidify. Next, 0.5 ml of tris-agar and tris-growth medium containing 6×10^7 chick cells was introduced. After the cell suspension had hardened, 0.5 ml of a third layer was added which comprised 5 parts tris-agar medium, 4 parts heated horse serum and 1 part 0.1 M ATP. This layer (mosquito stimulating layer) induced mosquitoes to feed.

Before exposing the culture to mosquitoes, the surface was covered by the membrane which was held in place by a cover with a center hole 3 cm in diameter. Mosquitoes readily probed through the membrane and retracted the labial sheath as in feeding on an animal host. The membrane protected the underlying material from microbial contamination. After exposure, the membrane was removed, the culture was covered with an intact lid, inverted, and incubated at 36°C in humidified air. This system is designated the chick cell agar-suspension (CAS) culture.

Exposure of CAS cultures to infected mosquitoes. Eight groups each composed of 3 or 4 infected *A. aegypti* were confined over separate, freshly prepared CAS cultures. Ninety percent of the mosquitoes gorged and plaques which resembled those made by Sindbis virus subsequently appeared in all but one of the cultures. The plaques, visible after 36 to 40 hours incubation, reached a maximum size of 4-8 mm after 72 hours (Fig. 4-7), and remained apparent for 6 to 8 days. The number of plaques developing in each culture varied from 2 to more than 20 and was related to the observed mosquito feeding activity.

Plaques of this type were not observed in cultures fed upon by uninfected mosquitoes. However, frequently in such control cultures small clear areas 1 to 1.5 mm in diameter appeared at some of the feeding sites within 20 hours after exposure. Similar areas were less frequently seen after cultures were exposed to infected mosquitoes. These minute clear spots which did not increase in size were easily differentiated from Sindbis plaques which developed more slowly, became larger, and were more opaque.

Subculture of Sindbis virus from infected CAS cultures. Two methods were employed for recovery of Sindbis virus from CAS cul-

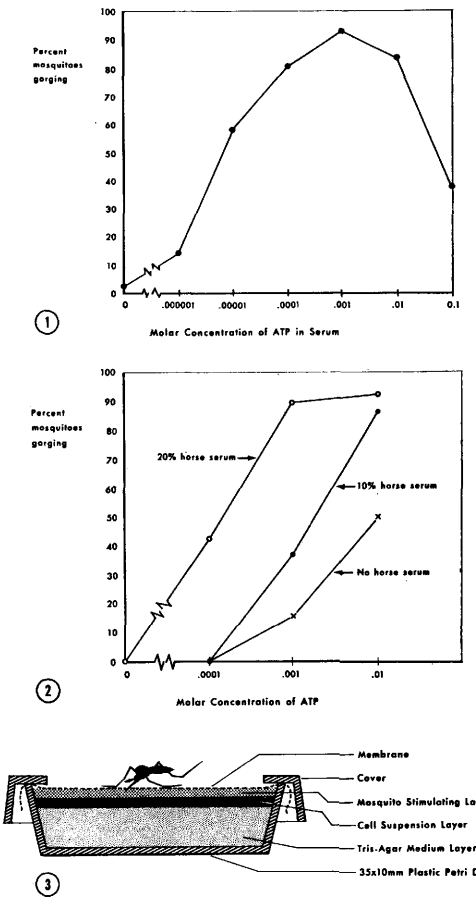

FIG. 1. The feeding stimulative effect on female *Aedes aegypti* of various concentrations of adenosine triphosphate in horse serum.

FIG. 2. The feeding stimulative effect on female *Aedes aegypti* of various concentrations of adenosine triphosphate and horse serum in tris-agar medium.

FIG. 3. Cross section through the chick embryo cell agar suspension culture.

tures which had been inoculated by infected mosquitoes. Individual plaques were removed after 72 hours incubation with a 10 mm cork borer and ground in a mortar with 1 ml diluting fluid. Alternatively, the culture was frozen and thawed so that fluid was readily expressed from the altered agar. Material obtained by either method was centrifuged at 1000 rpm for 10 minutes, and then the supernate was titrated for virus content. Fluid harvested by freezing from a culture containing 20 plaques contained 5.2×10^6 PFU of Sindbis virus. Materials from 2 plaque cores removed by the cork-borer contained 8×10^5 and 2×10^6 PFU of Sindbis virus respectively, while no virus was recovered from a core obtained from a nearby area that appeared normal.

Discussion. It is anticipated that the cell

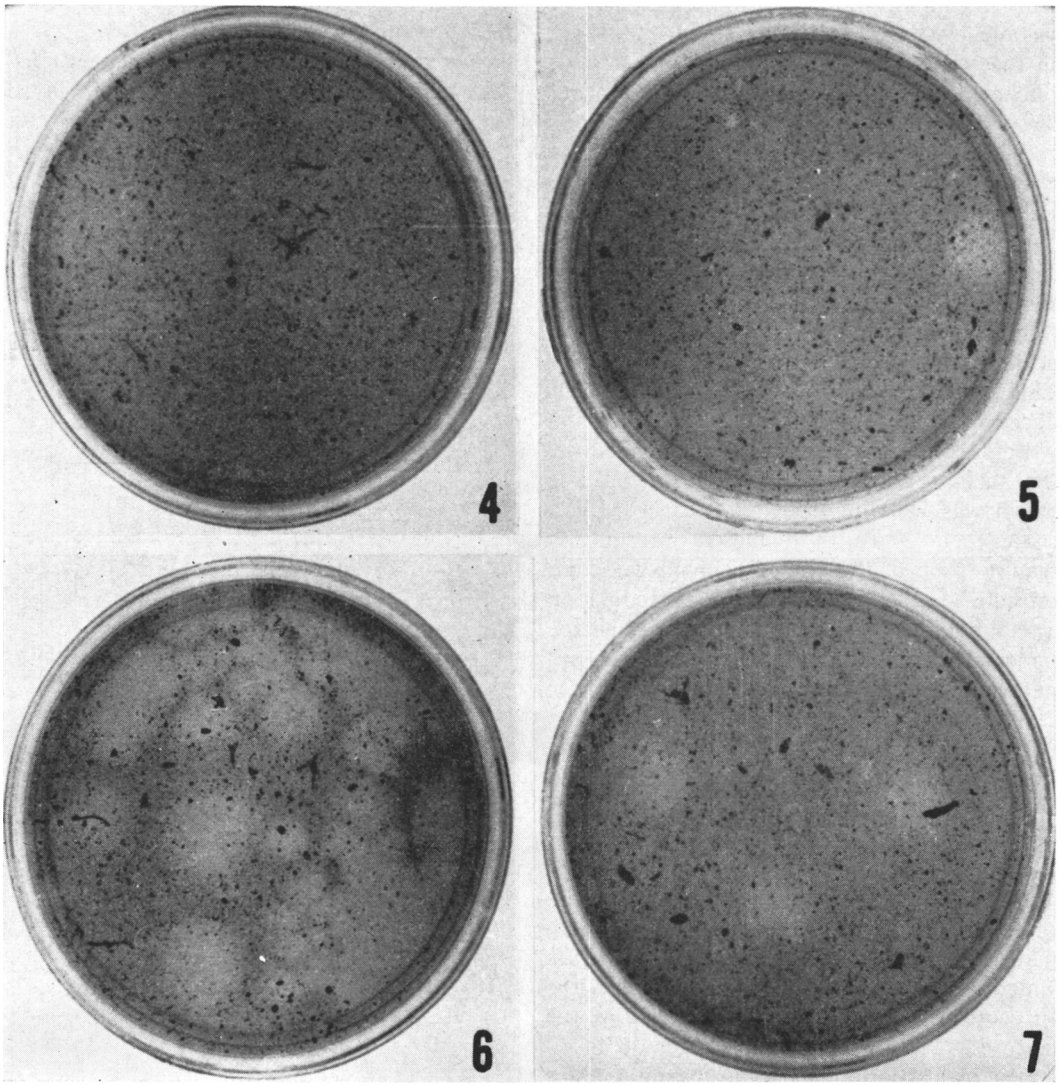

FIG. 4. A chick cell agar-suspension culture 3 days after exposure to 3 uninfected female *Aedes aegypti*.

FIG. 5. A similar culture 3 days after exposure to 3 *A. aegypti* infected with Sindbis virus. Two plaques are visible.

FIG. 6. A similar culture 3 days after exposure to a second group of 3 *A. aegypti* infected with Sindbis virus. More than 20 plaques, some of which are confluent, are visible.

FIG. 7. Three plaques which were produced by stabbing through the mosquito stimulating layer with a fine wire which had been dipped in Sindbis virus suspension (8×10^6 PFU/ml).

culture system developed during these experiments may prove useful in the study of arthropod-borne virus transmission in the field. Further work is planned to explore applications of the method and its limitations. The major obstacle in the development of the CAS culture system was the incorporation of substances which would attract and stimulate mosquitoes to feed but were not harmful to cells or virus. Serum only partially fulfilled these requirements. Blood cells, which might have provided the additional needed feeding stimulus, were undesirable because of impaired visualization of viral plaques.

A variety of chemicals appears to attract arthropods or to stimulate them to feed. Hosoi(4) identified adenylic acid from ox blood cells as a feeding stimulant for *Culex pipiens*. He concluded that adenosine phosphates in blood cells were the main stimulus promoting gorging of the mosquito on a blood meal. Galun *et al*(5) observed that the effectiveness of the adenine nucleotides increased with each addition of a phosphate group. Adenosine tetraphosphate possessed the greatest activity and was closely followed by ATP. We determined that inclusion of ATP in the CAS culture provided an excellent blood cell substitute. Certain other biological compounds such as lysine(9,10) and steroids(11) have been reported to be attractive to female mosquitoes. The attractiveness of these substances in CAS cultures is being studied.

The findings presented here might be of potential use in another context. The Mediterranean fruit fly, *Ceratitis capitata*, was specifically eradicated from Florida through the combination of a synthetic attractant Trimedlue(12) and an insecticide. This suggests the possibility of a similar approach to the control of *A. aegypti* and other mosquito species.

Summary. A chick embryo cell culture containing adenosine triphosphate and covered with an animal-derived membrane was developed. Female *A. aegypti* fed upon these cultures readily. After exposure of the cultures to mosquitoes infected with Sindbis virus, plaques appeared within 72 hours and virus was recovered from the plaques. This culture system may be useful in the study of arbovirus ecology.

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Serum Iron Levels in Patients with Malignant Disease.* (29956)

A. CLARK GRIFFIN, SEBRON C. DALE, DOROTHY WELLINGTON AND VIRGINIA C. WARD
University of Texas, M. D. Anderson Hospital and Tumor Institute, Departments of Medicine, Epidemiology, and Biochemistry, Houston

Iron and porphyrin metabolism may be affected considerably during the development of malignant neoplastic disease. Price(1) has reviewed the subject of anemia in cancer, and Nakahara and Fukuoka(2) have covered other aspects of tumor-host relations involving iron and porphyrin compounds. Several investiga-

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