# Tissue cultures of blood-sucking Arthropods and their use for the cultivation of Viruses and Rickettsiae

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## Résumé

Le développement des cultures de tissus des Arthropodes hématophages et leur utilisation pour l'étude des microorganismes a grandement progressé depuis ces dernières années, principalement grâce à l'amélioration des techniques. L'établissement de plusieurs lignées continues de cellules de moustiques a aidé les virologues, en fournissant un milieu pour l'étude des réplications de virus.

Les cultures de tissus des Arthropodes hématophages sont issues de tiques, poux, punaises, puces, moustiques et mouches.

*Tiques :* Weyer, 1952, essaie d'utiliser des cultures d'organes de *Rhipicephalus* pour la culture de rickettsies mais sans succès. Rehacek, 1958 et Rehacek et Pesek, 1960 cultivent le virus EEE sur des cultures d'organes de différentes espèces de tiques et obtiennent une multiplication virale sur les tissus conjonctifs et de l'hypoderme. Hoffmann et Köhler, 1968, observent que des cultures d'organes de tiques *in vitro* restent vivantes plus de 2 mois.

Des cultures primaires de différentes espèces de tiques prises à l'état adulte à l'intérieur de nymphes gorgées sont décrites par Rehacek, 1958, 1962 et Martin et Vidler, 1962. Plus tard, la quantité d'acides aminés et de sucre utilisée par les cellules *in vitro* est déterminée par Rehacek et Brzostowski, 1969 *b* ; Varma et Pudnoy, 1969 *a.* Ces cultures sont utilisées avec succès pour la multiplication de virus divers transmis dans la nature par les tiques (Rehacek,

1965 b ; Yunker et Cory, 1967 ; Rehacek et al., 1969). Il a été montré qu'une souche de laboratoire du virus de l'encéphalite prélevé sur tiques se multiplie mieux dans les cellules de tiques que dans les cultures d'embryons de poulet ou par injection intracérébrale dans la souris. Les virus d'origines naturelles poussent également bien dans ces trois environnements (Rehacek et Kozuch, 1964, 1969). Les rickettsies pathogènes pour les vertébrés se multiplient in vitro dans les cellules de *Hyalomma dromedarii* (Rehacek et Brezina, 1964 ; Rehacek et al., 1968).

*Poux, punaises, puces :* Nauck et Weyer, 1941 utilisent des morceaux d'organes de poux, punaises et puces pour la culture de plusieurs rickettsies. Le seul organe ayant donné des résultats est l'estomac de pou, qui a pu être maintenu vivant pendant 40 jours. La croissance des rickettsies a été mise en évidence dans ces cultures d'estomac de poux.

Bien que les cultures d'organes aussi bien que les cultures primaires de *Heteroptera* soient réalisées (Vago et Flandre, 1963 ; Varma et Pudney, 1967), elles n'ont pas encore été utilisées pour l'étude des virus et des rickettsies.

*Mouches :* Trager, 1959 établit des cultures primaires de *Glossina palpalis*. Ces cultures sont utilisées pour la multiplication des Trypanosomes.

*Moustiques :* Parmi les cultures de cellules d'Arthropodes, celles de moustiques ont retenu l'attention de façon primordiale.

Des cultures d'organes, principalement des organes digestifs, sont maintenus in vitro pour la culture de Plasmodia (Ball, 1947, 1948 ; Schneider, 1968) et des fragments d'autres organes sont maintenus pour la culture de certains arbovirus (Trager, 1938 ; Haines, 1958). Les organes maintenus in vitro restent vivants comme le montrent les contractions. Des ovaires ont été maintenus pendant 80 jours (Gubler, 1968).

Les cultures de cellules primaires sont établies à partir de larves (Peleg et Trager, 1963 ; Peleg, 1965 ; Johnson, 1969), de pupes (Sweet et Dupree, 1968 ; Johnson, 1969), d'adultes (Gubler, 1968) et de tissus embryonnaires (Peleg, 1966 ; Singh et Bhat, 1969). La multiplication des cultures donne lieu à trois formations : tapis cellulaire, grandes vésicules sphériques creuses et longs tubes creux (Bhat et Singh, 1969). Les arbovirus West Nile, EEE, Semliki Forest (Peleg et Trager, 1963 ; Peleg, 1968) et les virus d'encéphalites japonaises (Fujita et al., 1968) poussent dans ces cultures de cellules.

La multiplication du virus de l'encéphalite japonaise est mise aussi en évidence par l'immunofluorescence. Johnson, 1969, trouve que la multiplication du VEE et du virus EEE dans des cultures type Maintland dérivées de larves et de pupes stériles hachées sont dépendantes de la température d'incubation, de l'agitation à la-

quelle les cellules sont soumises pendant l'incubation et de la concentration de virus. Deux lignées du VEE après 10 passages dans les tissus larvaires (souche virulente) et 5 passages (souche moins virulente) ne présentent aucun changement de virulence pour la souris ni de changement de taille des plages en culture de cellules.

L'établissement de la première lignée cellulaire continue d'insecte (Grace, 1962) à partir des papillons *Antheraea* et des moustiques *Aedes aegypti* (1966) met en évidence la possibilité d'obtention de telles lignées cellulaires à partir d'invertébrés et ouvre de nouvelles voies dans la recherche des cultures *in vitro* d'Arthropodes. Par la suite, des lignées cellulaires ont été obtenues à partir de tissus embryonnaires de moustiques (Peleg, 1968), de tissus larvaires (Singh, 1967 ; Varma et Pudney, 1969 *b*), de nymphes (Singh, 1967) de différentes espèces de moustiques *Aedes*.

Un large emploi des lignées cellulaires de Grace était limité par la difficulté d'obtenir de l'hémolymphe d'insecte. Ceci a été surmonté par l'adaptation des cellules à un milieu sans hémolymphe [Sweet et Dupree, 1968 ; Nagle, 1967 ; Suitor, 1968 (pers. com.)]. Suitor *et al.*, 1966, réussit à cloner des cellules en forme d'épingle à partir de la lignée cellulaire de Grace. Stanley et Vaughn, 1967, montrent que les lignées cellulaires ne tolèrent pas la Fungizone, préparation commerciale d'amphotéricine B.

La lignée de cellules de Grace est très utilisée pour la culture de certains arbovirus. Converse et Nagle, 1967, mettent en évidence la multiplication du virus de la fièvre jaune et Rehacek, 1968, observe la multiplication des virus West Nile, de l'encéphalite japonaise, de l'encéphalite de la vallée Murray et du virus Kunjin. Rehacek, 1968 *b*, réussit à établir un état porteur avec les virus de l'encéphalite japonaise. Filshie et Rehacek, 1968, décrivent la morphologie et la réplication de ces deux virus au moyen du microscope électronique. On découvre que les cellules de Grace sont contaminées par un virus latent inconnu d'insecte qui se multiplie quand les cellules sont infectées avec le virus de l'encéphalite japonaise Filshie *et al.*, 1967). Peleg, 1968 *a, b,* observe la multiplication des virus EEE, West Nile et de la maladie de la forêt Semliki dans les lignées de cellules d'embryons d'*Aedes aegypti*. Les lignées continues d'*Aedes albopictus* et d'*Aedes aegypti* réalisées par Singh, 1967, sont utilisées par Singh et Paul, 1968, pour la multiplication de différents arbovirus. Ces auteurs observent des différences marquées dans la multiplication des virus dans ces deux lignées cellulaires. L'effet cytopathologique marqué mis en évidence par les cytolyses, la formation de grandes masses syncitiales et les cellules géantes multinucléées sont décrites au cours de l'infection avec les virus de l'encéphalite japonaise, de West Nile et de la dengue dans les cellules d'*Aedes albopictus* seulement (Paul *et al.*, 1969). Cette lignée cellulaire supporte aussi la multiplication du virus de la Fièvre du Colorado (Yunker et Cory, 1969). Banerjee

et Singh, 1968, réussissent à établir un état porteur avec les virus
de l'encéphalite japonaise, de West Nile, de Chikungunya avec la
même lignée cellulaire.

Les différents aspects de l'application pratique des cultures de
tissus d'Arthropodes pour la multiplication des virus et des rickett-
sies sont discutés.

## *Summary*

The development of tissue cultures of blood-sucking arthro-
pods and their use for studies with microorganisms has progressed
greatly during the last few years. It has been achieved mainly by
basic improvement of cultivation techniques. The establishment
of many cell lines from mosquitoes aided virologists by providing
a medium for studies of virus replication.

Of the haematophagous arthropods, tissue cultures have been
derived from ticks, lice, bugs, fleas, mosquitoes and flies.

*Ticks :* Weyer, 1952, attempted to use organ cultures of *Rhi-
picephalus* ticks for the cultivation of rickettsiae without success.
Rehacek, 1958, and Rehacek and Pesek, 1960, cultivated EEE
virus on organ cultures of different tick species and accomplished
the propagation of virus on connective tissue together with hypo-
dermis. Hoffmann and Köhler, 1968, observed the viability of
tick organ cultures *in vitro* for more than 2 months.

Primary cultures derived from developing adults within engor-
ged nymphs of different tick species were described by Rehacek,
1958, 1962 and Martin and Vidler, 1962. Later the uptake of
amino acids and sugars by the cells *in vitro* was determined by
Rehacek and Brzostowski, 1969 *b ;* Varma and Pudney, 1969 *a.*
These cultures were used successfully for the propagation of various
viruses transmitted in nature by ticks (Rehacek, 1965 *b ;* Yunker
and Cory, 1967 ; Rehacek *et al.,* 1969). A laboratory strain of tick-
borne encephalitis virus was found to multiply better in tick cells
than in chick embryo cultures or intracerebrally injected mice.
Viruses from natural sources grew equally well in all three media
(Rehacek and Kozuch, 1964, 1969). Rickettsiae pathogenic for
vertebrates were found to multiply in *Hyalomma dromedarii* cells
*in vitro* (Rehacek and Brezina, 1964 ; Rehacek *et al.,* 1968).

*Lice, bugs and fleas :* Nauck and Weyer, 1941, used minced
organs from lice, bugs and fleas for the cultivation of several
rickettsiae. The only susceptible organ was found to be louse sto-
mach which was maintained for 40 days. Growth of rickettsiae
was demonstrated in these louse stomach organ cultures.

Although the organ culture as well as primary cell cultures of
*Heteroptera* were developed (Vago and Flandre, 1963 ; Varma and

Pudney, 1967) they have not yet been used for virus and rickettsial studies.

*Flies:* Primary cultures were established from *Glossina palpalis* by Trager, 1959. These cultures have been used for the propagation of Trypanosomes.

*Mosquitoes:* Mosquito cultures have received primary attention in studies of arthropod cell cultures by most investigators in this research area.

Organ cultures, primarily digestive organs, have been maintained *in vitro* for the cultivation of Plasmodia (Ball, 1947, 1948 ; Schneider, 1968) and fragments of other organs maintained for cultivation of certain arboviruses (Trager, 1938 ; Haines, 1958). The organs maintained *in vitro* remain viable as evidenced by contractibility. Ovary has been maintained for 80 days (Gubler, 1968).

Primary cell cultures have been established from larvae (Peleg and Trager, 1963 ; Peleg, 1965 ; Johnson, 1969), pupae (Sweet and Dupree, 1968 ; Johnson, 1969), adult tissue (Gubler, 1968) and from embryonal tissues (Peleg, 1966 ; Singh and Bhat, 1969). Growth of cultures have been characterized by three types : the establishment of a cell sheet, large hollow spherical vesicles, and elongate hollow tubes (Bhat and Singh, 1969). The arboviruses, West-Nile, EEE, Semliki Forest (Peleg and Trager, 1963 ; Peleg, 1968), and Japanese Encephalitis viruses (Fujita et al., 1968) were grown in these cell cultures. The propagation of Japanese encephalitis virus was also described by immunofluorescence. Johnson, 1969, found the multiplication of VEE and EEE viruses in Maintland type cultures derived from minced germ-free larvae and pupae to be dependent on the temperature of incubation, shaking of cells during incubation, and on the virus concentration used. Two strains of VEE virus passed in larval tissue ten times (virulent strain) and five times (less virulent strain) caused no changes in virulence for mice or in plaque size in cell culture.

The establishment of the first insect cell lines (Grace, 1962) from *Antheraea* butterflies and *Aedes aegypti* mosquitoes (1966) demonstrated the possibility of deriving cell lines from invertebrates and opened new areas of *in vitro* arthropod research. Further cell lines have been derived from embryonic mosquito tissue (Peleg, 1968) and from larval tissue (Singh, 1967 ; Varma and Pudney, 1969 *b*), from pupae (Singh, 1967) from different species of *Aedes* mosquitoes.

Wider use of Grace's cell lines was limited by lack of availability of insect haemolymph. This was overcome by the adaptation of cells to media without haemolymph [Sweet and Dupree, 1968; Nagle, 1967 ; Suitor, 1968 (pers. com.)]. Suitor, *et al.,* 1966, succeeded in cloning spindle-shaped cells from Grace's cell line.

Stanley and Vaughn, 1967, found this cell line to be intolerant to Fungizone, a commercial preparation of the polyene amphotericin B.

Grace's cell line has been used extensively for the cultivation of certain arboviruses. Converse and Nagle, 1967, demonstrated the propagation of Yellow Fever virus and Rehacek, 1968 *a*, observed the multiplication of West Nile, Japanese Encephalitis, Murray Valley Encephalitis and Kunjin viruses. Rehacek, 1968 *b*, succeeded in the establishment of a carrier state with Murray Valley Encephalitis and Japanese Encephalitis viruses. Filshie and Rehacek, 1968, described the morphology and replication of both viruses by means of electron microscopy. It was discovered that Grace's cells were contaminated with an unknown latent insect virus which multiplied following the infection with Japanese Encephalitis virus Filshie *et al.*, 1967). Peleg, 1968 *a, b*, observed the multiplication of EEE, West-Nile and Semliki Forest disease viruses in the *Aedes aegypti* embryo cell line. The cell line from *Aedes albopictus* and *Aedes aegypti* derived by Singh, 1967 has been used by Singh and Paul, 1968, for the propagation of different arboviruses. These authors observed marked differences in propagation among viruses in both cell lines. Marked cytopathic effect demonstrated by cytolysis, formation of large syncytial masses and multinucleated giant cells has been described following infection with Japanese Encephalitis, West-Nile and Dengue viruses in *Aedes albopictus* cells only (Paul et al., 1969). This cell line also supported the propagation of Colorado Tick Fever virus (Yunker and Cory, 1969). Banerjee and Singh, 1968, succeeded in the establishment of a carrier state with Japanese Encephalitis, West-Nile and Chikungunya viruses with the same strain of cells.

Practical application of arthropod tissue cultures for the propagation of viruses and rickettsiae from various aspects is discussed.

The development of tissue cultures of blood-sucking arthropods and their use for studies with microorganisms has progressed greatly during the last few years. It has been achieved mainly by basic improvement of cultivation techniques. The establishment of many cell lines from mosquitoes aided virologists by providing a medium for studies of virus replication.

Of the haematophagous arthropods, tissue cultures have been derived from ticks, lice, bugs, fleas, mosquitoes, and flies.

**Ticks.**

The first experiments on maintenance of tick organs *in vitro* were done by Weyer, 1952, in an attempt to cultivate rickettsiae. However, he succeeded only in keeping

the organs viable for a very short time. The uninfected tick organs kept together with infectious louse stomach *in vitro* did not become infected.

Organ culture as a medium for the cultivation of Eastern equine encephalomyelitis (EEE) virus was used by Rehacek and Pesek, 1960. Various organs of half engorged females *Ixodes ricinus, Dermacentor pictus* and *D. marginatus* maintained in test tubes at 25°C in TC 199 medium were infected with various doses of virus. The tissues of organs such as ovaries, malpighian tubes, salivary glands and guts manifested an almost identical slightly protective effect on virus in medium in comparison with virus in cell-free medium. The multiplication of virus occurred only in the presence of hypodermis and connective tissues of *D. pictus* and *I. ricinus* ticks, the virus increment being of the order of 4 logarithmic units of $TCID_{50}$ of virus.

The organ and haemolymph cultures of *D. pictus* and *I. ricinus* females were used for the study of the *Coxiella burneti* development from its filterable particles. The typical *C. burneti* was found in the tissues of organs after 14 to 16 days in quantities considerably smaller than in ticks injected with the same inoculum (Kordova and Rehacek, 1959).

A series of experiments with the organ cultures of adult *Rhipicephalus appendiculatus* ticks were performed by Martin and Vidler, 1962, then with organs of *Boophilus microplus* (Rehacek, 1966, unpublished results) and other several hard tick species (Hoffmann and Köhler, 1968). These experiments demonstrated only a long time survival of different organs as evidenced primarily by contractile or peristaltic movements.

More attention has been paid to primary tick tissue cultures which seem to be a more convenient medium for the cultivation of microorganisms. The method of their establishment was described by Rehacek, 1958, 1962, 1965 *a* ; Martin and Vidler 1962, Yunker and Cory, 1965, 1967 and Varma and Wallers, 1965. These cultures originate from the developing tissues of adults from engorged nymphs undergoing metamorphosis. The tissues consist of oesophagus, salivary glands, gonadal and other genital tissues, neural ganglion, nerve trunks, muscles and elements of the hypoderm of the preimaginal stage. The cultures are used in the form of an explant of the body content *in toto* or dispersed to separate cells by mechanical or chemical means, or with both methods. The best results were achieved with the medium composed of the mixture of Eagle's medium (Eagle, 1955), and the medium of Vago and Chastang (Vago and Chastang, 1960), in a ratio of 2 : 1 plus 0,1 % of a fraction from dried calf serum (Rehacek, 1965 *a*) ; (the latter component can also be foetal bovine serum) (Varma and Wallers, 1965), and by Martin and Vidler, 1962, which consisted of Hank's balanced salt solution to which the amino acids and vitamins of Eagle's medium and 20 % ox serum were added. Yunker and Cory, 1967, cultivated the viscera of ticks successfully in a medium composed of Lactalbumin hydrolysate in in Hank's balanced salt solution to which 10 % heat inactivated normal rabbit serum, 10 % whole chicken-egg ultrafiltrate and bovine plasma albumin were added. The cells cultivated in these media multiplied very well for a few months, often establishing

the monolayers on the coverglasses or walls of the containers in which they were cultivated.

The last improvement on the composition of medium was accomplished by Rehacek and Brzostowski, 1969 *a*. This is based on the chemical analysis of amino acids, sugars and salts of haemolymph of engorged *Boophilus microplus* females. Medium was tested on the cells of *Rhipicephalus sanguineus* ticks and showed growth and viability of cells for nine months. The same authors (Rehacek and Brzostowski, 1969 *b*) described the utilization of the amino acids and sugars in the medium by cells of the same tick species (Fig. 1-4). They observed the uptake of several amino
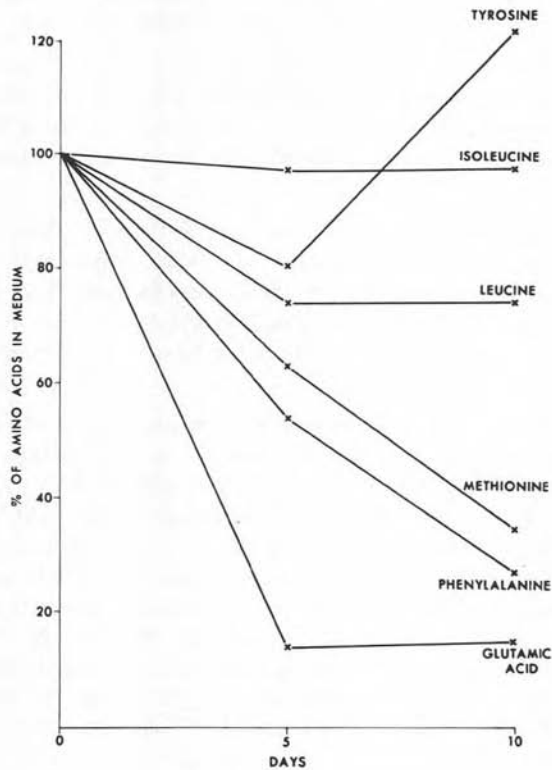


Fig. 1

acids such as leucine, methionine, threonine, phenylalanine, proline, glutamic and aspartic acid to a significant extent while the other amino acids occurring in the medium showed no change in concentration. Both sugars, glucose and inositol, were used by cells in cultures to a great extent; this being confirmed later by Varma and Pudney, 1969 *a*. All these studies were done to further improve the composition of the media.
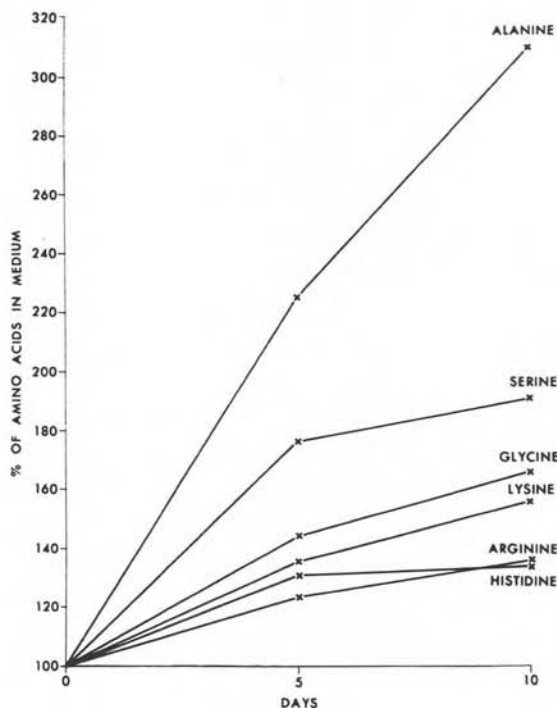
FIG. 2

As a sufficient amount of *in vitro* tick cells became available (Fig. 5), cultivation experiments of different microorganisms could be tried. It was found that tick-borne encephalitis virus multiplies in tick cells very well (Rehacek, 1962, 1963) (Fig. 6 and 7) and this was an impetus for testing other viruses. For this purpose the cultures of *Hyalomma dromedarii* ticks were used (Rehacek, 1965 *b*). It was found that of group A arboviruses, Western equine encephalomyelitis, EEE, Sindbis and Semliki Forest viruses multiplied very well in cultures (Fig. 8). The cultivation of group B arboviruses yielded varied results: Langat (TP 21), Japanese encephalitis (JE), St. Louis encephalitis (SLE) and yellow fever viruses multiplied in tick tissue cultures poorly. Kyasanur Forest Disease, Powassan, Omsk haemorrhagic fever, and West Nile viruses multiplied relatively well and tick-borne encephalitis (western type), Russian-spring summer encephalitis and louping-ill viruses multiplied excellently (Fig. 9 and 10). The highest titers of the viruses were obtained with small inocula, the virus increment being approximately of the order of 0,5-1 logarithmic units per day. The viruses not belonging to the arbovirus group, such as-EMC, polio, vaccinia, vesicular stomatits, NDV and pseudorabies did not multiply in tick tissue cultures and their titers in samples taken at two days intervals for 8 days of cultivation were practically the
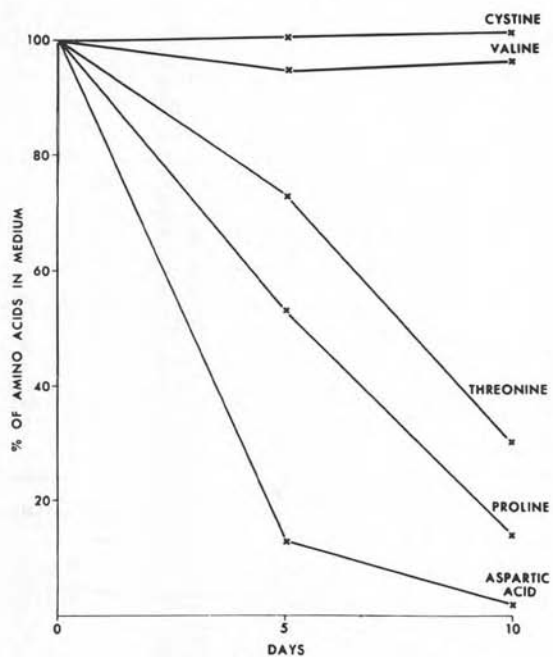
FIG. 1, 2, 3. — Utilization of amino acids by tick cells
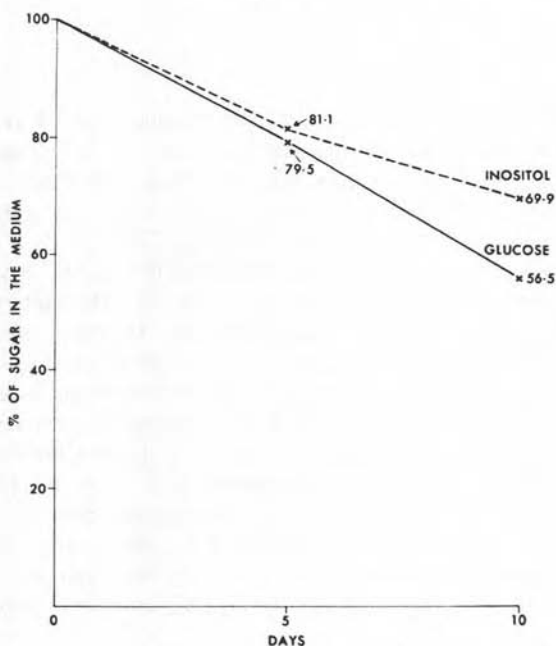*in vitro*



FIG. 4. — Utilization of glucose and inositol by tick cells
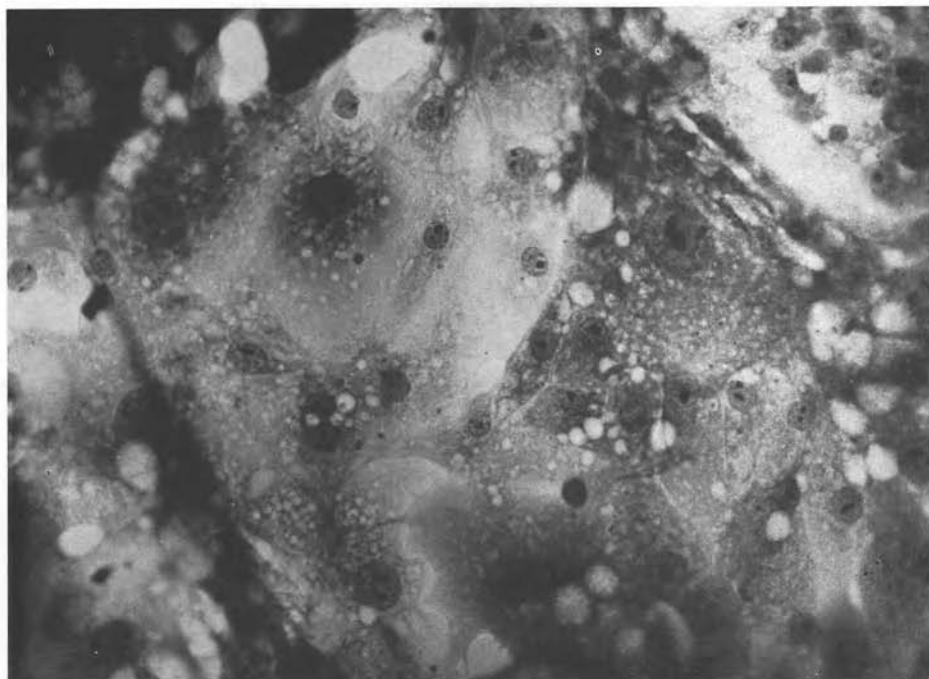*in vitro*

FIG. 5. — Monolayer of *Hyalomma dromedarii* cells after 10 days cultivation.
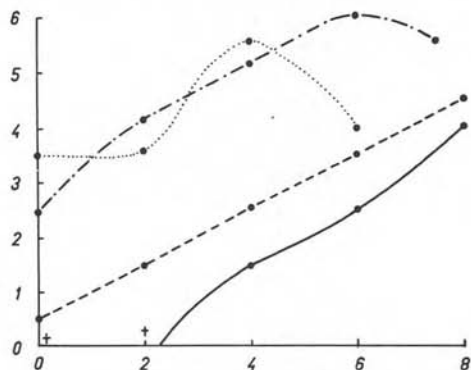


FIG. 6. — Cultivation of tick-borne encephalitis
virus in *Hyalomma asiaticum* cells *in vitro*

same as in the control-cell free medium. An exception was the LCM virus which mul-
tiplied in tick cells.

The growth of Colorado Tick Fever virus was studied in primary tissue cultures
of its vector, *Dermacentor andersoni,* by Yunker and Cory, 1967. After a latent
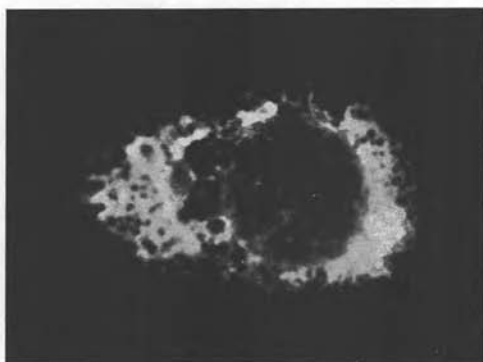
FIG. 7. — Tick-borne encephalitis virus in *Hyalomma dromedarii* cells *in vitro* (5 days after the infection) by fluorescent method
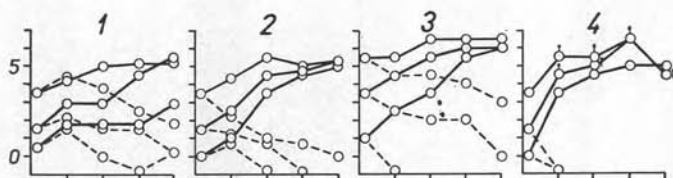


FIG. 8. — Multiplication of WEE (1), EEE (2), Sindbis (3) and Semliki forest (4) viruses in tick tissue cultures
———— tick tissue cultures ; - - - - - controls
Abscissa : days after inoculation ; ordinate : virus titers in log $TCID_{50}/0,1$ ml values
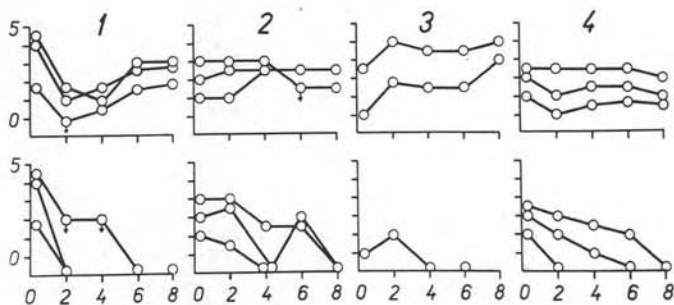


FIG. 9. — Multiplication of Japanese encephalitis (1), St. Louis encephalitis (2), West Nile (3), and yellow fever (4) viruses in tick tissue cultures
top row : tick tissue cultures,
bottom row : controls,
Abscissa : days after inoculation,
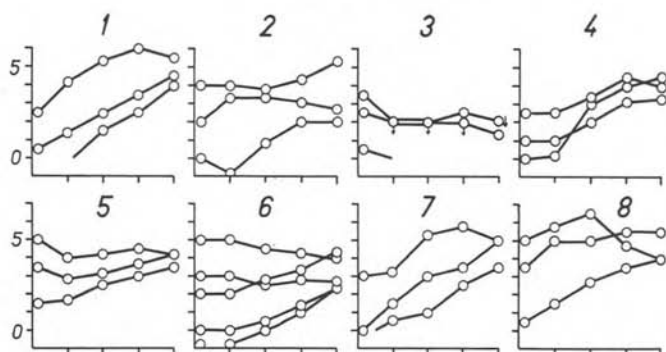Ordinate : virus titers in log mouse $LD_{50}/0,03$ ml values.

FIG. 10. — Multiplication of European tick-borne encephalitis (1), KFD (2), Langat (3), louping-ill (4), Omsk haemorrhagic fever (5), Powassan (6), Russian spring-summer encephalitis (7), and LCM (8) viruses in tick tissue cultures. For explanation see fig. 9

period of 6-10 days, a 4 ½ logarithmic increase of extracellular virus at 4-5 weeks followed. Virus was then isolated successfully in small quantities for as long as 159 days in medium and for 166 days in triturated tissues. It was shown that less than 0,1 of a suckling mouse intracerebral $LD_{50}$ can be taken up and propagated to higher levels by tick cells *in vitro*.

Rehacek *et al.*, 1969, described the propagation of Tribec virus in *Hyalomma dromedarii* tissue cultures. The titers reached $10^{4.5}$ mouse intracerebral $LD_{50}/0.1$ ml from 4-14 days after infection (Fig. 11). Fluorescent antibody studies revealed the viral antigen in the form of confluent and brightly fluorescing granules in the cytoplasm of infected cells from the second day following inoculation (Fig. 12).

In all these experiments, the tick tissues or cells were not affected by viral infection.

Because tick-borne encephalitis is spread in different countries in Europe, more attention was paid to the relationship of this virus in tick cells *in vitro*. It was shown that two strains of different origins (one in the 48th mouse brain passage, the second one adapted to HeLa cells) multiplied equally well in the cultures of *D. marginatus* and *H. dromedarii* ticks (Rehacek, 1963). In comparing the susceptibility of small doses of this virus to chick embryo cells and to tick primary cultures of *H. dromedarii*, Rehacek and Kozuch, 1964, found that with inocula of 30 and 3 $IFD_{50}$, the percentage of infected chick embryo cells and tick cell cultures was about equal. With inocula containing 0.3 and 0.03 $IFD_{50}$ of virus, the respective percentage of infected tick cell cultures was 76 and 18, whereas that of chick embryo cultures only 34 and 3. Following this, the tick cells were compared in isolation experiments from different materials, i.e. suspensions of rodent brains, blood samples and suspensions of ticks from nature. Altogether 187 samples were tested and from this number 5 strains of the virus were isolated. however. all 5 strains were obtained in tick cells, in chick embryo cells, as well as in mice injected intracerebrally. This indicated that the
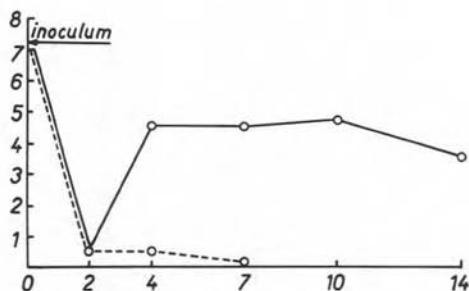
Fɪɢ. 11. — Multiplication of Tribec virus in tick
tissue cultures
Abscissa : days after inoculation
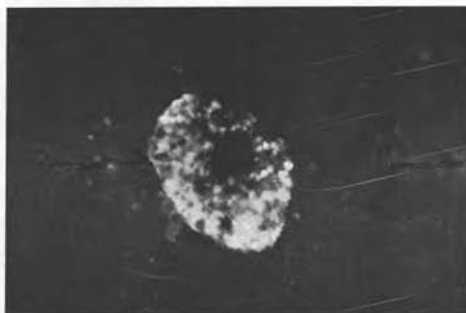Ordinate : virus titers in log mouse $LD_{50}$/
0,01 ml values



Fɪɢ. 12. — Tribec virus in tick cells *in vitro*
(7 days after the infection) by fluorescent method

sensitivity of tick cells *in vitro* to small doses of wild strains of tick-borne encephalitis virus is at least equal to that of chick embryos cells and mice (Rehacek and Kozuch, 1969).

It is well known that ticks are biological vectors of various rickettsial diseases, and it was interesting to determine whether the tick cells *in vitro* would also be able to propagate the rickettsiae. It was found that *Coxiella burneti* was propagated in cultures of *H. dromedarii* very well, reaching at 6 days a titer of $10^6$ $ID_{50}$/ml in yolk sacs (Rehacek and Brezina, 1964). (Fig. 13), *Rickettsia prowazeki, R. mooseri, R. conori* and *R. akari* also multiplied in tick cells. Simultaneous titrations of rickettsial strains in tick cell cultures and in chick embryos revealed higher sensitivity of tick cell cultures to *R. conori* and *R. akari. R. prowazeki* formed chains and threads in the cells (Fig. 14), *R. mooseri* formed segmented chains around the vacuoles, *R. conori* and *R. akari* multiplied in the form of single rods and sometimes in that of diplobacilli. *R. conori* and *R. akari* were found occasionally in the cell nuclei.
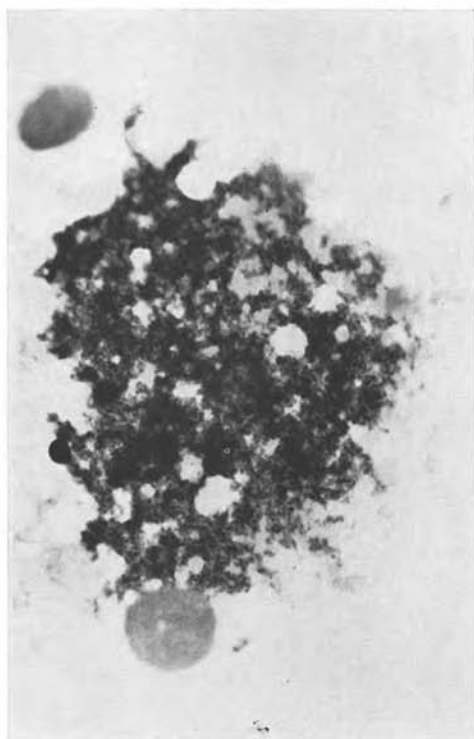
FIG. 13. — *Coxiella burneti* in tick tissue culture,
4 days after the infection

## Lice.

Lice are well known as vectors of several rickettsial diseases, especially epidemic typhus fever and trench fever. Nauck and Weyer, 1941, and Weyer, 1952, used this tissue *in vitro* for the cultivation of rickettsiae. From the different organs tested, the digestive organs were shown to have the longest survival time (30-40 days).

The explants of different tissues of *Pediculus humanus* lice were used for the cultivation of rickettsiae by the method of hanging drops of human or rabbit plasma at 31-32°C. The positive results were obtained only with explants from stomachs of lice infected more than 5 days before the explantation and which had excreted the rickettsiae in their feces. In these cases the propagation of *Rickettsia quintana*, *R. prowazeki* and *R. mooseri* was observed.

## Bugs.

Experiments on the cultivation of different organs of the bed bug *Cimex lectularius in vitro* were done by Nauck and Weyer, 1941, in order to find a medium for

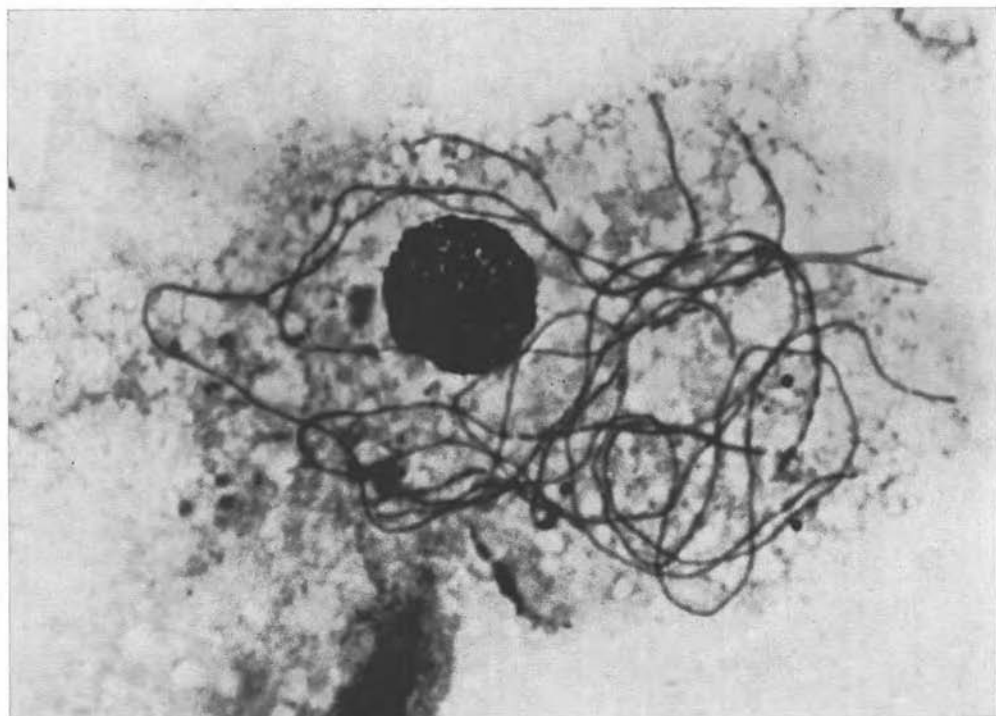FIG. 14. — *Rickettsia prowazcki* in tick cells *in vitro,* 8th day of cultivation

the cultivation of rickettsiae. Unfortunately, tissues survived only about 10 days *in vitro*. This insect was omitted from further investigation.

A very interesting method for the observation of growth of invertebrate cells and tissues *in vitro* has been elaborated by Lüscher, 1947, 1948. The method is based on the fact that epithelial cells after injury will grow along any surface and that if a tissue injury is covered with glass, the cells will spread on the surface of the glass. This has been accomplished with the hind leg of the fifth stage nymph *Rhodnius prolixus* which was amputated in the distal part of the tibia and a glass tube introduced into the opening of tibia. By the use of this method many cell divisions in the migrating haemocytes in the first day were seen together with the epithelial cells which formed a sparse network of cells interconnected by protoplasmic processes.

Later Vago and Flandre, 1963, observed the mitoses in fibroblast-like cells which migrated out from the explants of ovaries and malpighian tubes of *Triatoma infestans* cultured in plasma clots and a special medium.

The promising primary cultures of *Heteroptera* which might be used for the cultivation of microorganisms were derived by Varma and Pudney, 1967. The embryonic cells of the bug *Triatoma maculata* were used. After setting up the cultures, the outgrowth of fibroblast-like cells occurred at the periphery of the cell clumps. The

cells then joined together to form a loose network. The epithelial cells were included in the networks, and most cells remained healthy up to about 40 days when the degeneration slowly occurred.

## Flies.

The blood-sucking fly, *Glossina palpalis,* species was employed in experiments for cultivation *in vitro* (Trager, 1959). Preimaginal tissues obtained from pupae and the different organs of the adult were used. Various modifications of media based mostly on Wyatt, 1956 and Grace, 1958, were successfully used. The outgrowth of cells was observed to occur from the brain, optic lobe, proventriculus and hind gut. The viability of organ explants was demonstrated by their contractibility. Imaginal buds were found to be the best source for the cells of different types. The formation of confluent sheets and networks of cells with mitoses occurred, and some of the cell cultures were found in excellent condition up to 35-43 days.

## Fleas.

The use of the cells and tissues of fleas would be helpful in the explanation of the relation between the vector cells and the agents transmitted by them. Nauck and Weyer, 1941, tried to develop a culture of viscera of the adult *Ctenophthalmus segnis* fleas but it was not successful.

## Mosquitoes.

The culturing of mosquitoes has been a prime concern of most investigators of arthropod cell cultures.

Explant cultures have been derived from different organs of various mosquito stages. These cultures survived for limited periods and sometimes demonstrated limited migration of epithelial or fibroblast-like cells.

The first notable experiments of this kind were done by Trager, 1938, who used the explants of larvae, pupae and adults of *Aedes aegypti* for the propagation of the WEE virus. The best results were obtained with the tissues of the midgut of full-grown larvae and ovaries of newly emerged females. The larvae tissue survived at least 30 days, the ovary tissue up to 47 days. The various mosquitoes tissues supported virus propagation. Best results were obtained with subcultures which were started in larval thoracic tissue, then passed in pupal head tissues, passed again in larval thoracic tissues and finally in pupal head tissues. After 28 days, including 4 passages at 7 days intervals, the titer was $10^5$ times higher than that of the original suspension.

In further experiments, salivary glands, thorax muscles and gut of *Anopheles maculipennis* and *Stegomyia fasciata* were cultivated in chick plasma containing larval mosquito extract. Migration of cells was observed with little cell multiplication (Gavrilov and Cowez, 1941).

Ragab, 1948, 1949, derived a method for the cultivation of isolated mosquito gut in which a continuous flow of nutrient medium was passed over the isolated

gut, however no marked increase in the survival time of the explanted organs was obtained. A saline solution prepared by Bradford and Ramsay, 1949, based on the analysis for sodium and potassium of the midgut and malpighian tubules of *Aedes aegypti*, was found suitable for the survival of mosquito organs *in vitro*.

In 1956, Beckel did experiments on mosquito egg development *in vitro*. The extirpated ovaries of *Aedes hexodontus, Aedes aegypti* and *A. communis* were maintained *in vitro* ; the muscle tissue of *A. hexodontus* and *A. communis* ovaries showed contraction for as long as 7 days, the *A. aegypti* tissue demonstrated contraction for as long as 60 days. In both cases there was no development of eggs.

Various tissues of the pupae, larvae and eggs of *A. aegypti* mosquitoes were used *in vitro* for the cultivation of EEE virus by Haines, 1958. Embryonic tissue was maintained in medium TC 199 for 5-21 days, pupal ovary tissues up to 10 days and fourth instar larval midgut tissues up to 23 days. The latter was chosen for the experiments on the cultivation of EEE virus but only the short time virus survival was found.

The mosquito stomachs, surviving *in vitro*, were of interest and convenient substrates for the study on the development of *Plasmodium oocysts*. Ball, 1947, 1948, used for this purpose the stomachs of *Culex tarsalis* whose hindgut showed contractibility for as long as 7 days. Unsatisfied with the results on the stomach cultivation, in relation to previous results on the amino acids analysis of the culicid tissues, Ball, 1954, modified his medium. Although contraction was observed for as long as 5 weeks, the epithelial and muscle cells of the midgut almost degenerated and were unrecognizable by this time.

More recently, Gubler, 1968, cultured organs of the female *Culex pipiens molestus*. In a simple medium consisting of salts, sugars, few organic acids, lactalbumin hydrolysate and fetal bovine serum, explanted ovaries were maintained without loss of shape, size, and contraction for over 80 days. The gut was maintained for 47 days. Explants of midguts of mosquitoes (*A. aegypti*) infected with *Plasmodium gallinaceum* oocysts were cultivated by Schneider, 1968. She found the slightly modified medium of Grace, 1958, most suitable.

Maitland-type tissue culture system from minced germ-free larvae and pupae of the mosquitoes *A. aegypti* and *A. triseriatus* have been developed by Johnson, 1967, 1969. Minced tissues maintained in suspensions were cultivated in Clements medium. Johnson used these cultures for the cultivation of VEE and EEE viruses. Both of the viruses multiplied in cultures very well and Johnson described in detail several factors concerning this propagation. He found the adsorbtion rate appeared to be nearly constant at temperatures from 4-30°C, but at 37°C the values dropped off sharply. The agitation of the cultures during the virus adsorption process showed no significant effect on the amount of virus adsorbed but it was an important factor in increasing the yield of virus. Maximum yield of viruses were found also to be dependent on the amount of virus present in the adsorption mixtures and incubation temperatures. The amount of mosquito tissue seeded per ml of culture fluid showed little effect over the range from 20 to 200 larvae per ml. The titer of the Trinidad strain of VEE virus was essentially constant and a pH range of 7-8 gave a slightly depressed

growth rate. It was found the supernatant fluid of mosquito cultures contained less
virus about 0.5 logarithmic units than the cells. Similar results as with
*A. aegypti* have been reached with *Aedes triseriatus* and, in general, with EEE virus.
The virulent strain Trinidad of VEE was serially passed ten times in *A. aegypti* larval
tissue cultures and five times in pupal tissue cultures showing only a slight increase
in the peak titers attained with virus in both culture types. No change in the mouse
virulence or the plaque characteristics of the virus was observed. The less virulent 9th
strain of VEE virus showed no changes in growth rate or peak titers after five serial
passages in the same type of cultures.

*Mosquito primary culture.*

Primary mosquito cultures have been established from all developmental stages.
Three categories of insect cell growth are known (Larsen, 1967) and all of them were
found in mosquito tissue cultures (Singh, 1967 ; Bhat and Singh, 1969). This growth
can be characterized by the formation of cell sheet, by establishment of large hollow
spherical vesicles and by means of elongate hollow tubes.

The first experiments on the cultivation of the mosquito primary cultures were
done by Peleg and Trager, 1963, who used the imaginal discs of fourth instar larvae
of *A. aegypti*. Using Trager's medium (Trager, 1959) with calf serum replacing the
sheep serum, they noticed the establishment of a single layer within a boundary of its
limiting membrane followed by cell migration through the membrane. The cells were
maintained as long as 40 days. Enriching of medium with 10 % chick embryo extract
had considerable beneficial effect on cell growth. These types of cultures supported
the multiplication of West Nile virus resulting in an increase of 2 logarithmic units of
views during a period of 25 days. The authors also observed the propagation of virus
in mosquito tissues during four serial passages. No cytopathic effect was noted in the
infected cultures.

Later Peleg, 1965, markedly improved the growth of mosquito cells *in vitro* by
enriching Trager's medium with 20 % calf serum and 1 % chick embryo extract. In
these experiments he used the cells prepared from whole larvae. Under this condition
5 months growth of cells was noted. Later, Peleg, 1966, continued the improvement of
primary mosquito cultures using the cells from wholly or partially disintegrated *A.
aegypti* embryos. The eggs, after careful surface sterilization for 5 minutes in 0.05 %
$HgCl_2$ in 70 % ethyl alcohol and successive washing in distilled water, were pressed
through steel grid and collected. They were then suspended in the modified Trager's
medium, where in addition to an altered salt composition, yeast extract was replaced
with the vitamins of Eagle's medium (Eagle, 1955). In this medium with 2 % chick
embryo extract added, whole microscopic fields were seen to be covered with new
cells and many mitotic figures could be demonstrated on the 5th and 6th days.

Kitamura, 1964, successfully cultivated cells derived from ovarian tissues of
*Culex pipiens* var. *molestus*. A simple and convenient medium was used. NaCl 0.65 g,
KCl 0.05 g, $CaCl_2$ 0.02 g, $NaHCO_3$ 0.01 g, glucose 0.8 g, lactalbumin hydrolysate
1.0 g, distilled water 100 ml, chicken plasma 10 ml, antibiotics. Dividing cells were

seen for more than 3 weeks when the medium was changed every 4th day. Kitamura, 1965, further improved the medium composition with mostly an enrichment with 20 % of synthetic mixture No. 199. The ovaries were found to be contracting and the outgrowth of cells continued for more than three months when the culture medium was replaced every sixth day. Kitamura, 1966, further continued the improvement of the cultivation technique in an effort to investigate the origin of growing cells and to subculture these cells. Different cell types were found to grow from the ovaries of three species of mosquitoes, *Culex molestus, Aedes albopictus* and *Aedes aegypti,* for long time periods, often forming a cell sheet. Decrease of cell yield was observed up to the 8th subculture.

Gubler, 1968, described a method for the preparation of primary mosquito cultures from adult *Culex pipiens* var. *molestus* ovaries and guts, when the peritrophic membrane of the latter was extracted before the seeding of the organ explants. Ovarian and midgut tissues showed good migration of epithelial cells followed by the formation of cell sheets for about 3 weeks. The principal cells of the sheet were epithelial in appearance. Fibroblast-like cells were observed in young cultures, giant cells and syncytia occurred in older cultures. Subculturing has been unsuccessful.

A sufficient amount of primary cultures has been used for the cultivation of different viruses. Peleg, 1968 a, cultivated EEE, Semliki Forest (SF) and West Nile viruses in primary tissue cultures of *Aedes aegypti* embryos. All these viruses were multiplying very well. Differences in the growth pattern were found in the release of virus from cells. The release of SF virus began about 8 hours, West Nile virus after 36-48 hours after infection. SF virus increased more rapidly reaching a peak titer in 2 days, while West Nile increased significantly slower reaching a peak titer 8-10 days after infection. On the other hand SF virus declined the fastest with two pronounced falls in titer in comparison with West Nile virus in which the decline in titer was slower and longer. EEE virus occupied an intermediate position between these viruses tested. The cultures infected with West Nile virus yielded positive results with the direct staining method of the immunofluorescence technique. Slight morphologic changes have been found in a few cultures infected with EEE and SF viruses, nevertheless, their attribution to virus infection is questionable.

Japanese Encephalitis (JE) virus was cultivated in primary cultures of ovaries of *Culex molestus* by Fujita *et al.,* 1968. An increase of titer at the 4th day of incubation reaching a maximum ($10^{4.5}$ per 0.02 ml in mouse i.c. $LD_{50}$) at the 7th day was shown. The virus was found up to 65 days in the medium with some variation in titer. No apparent cytopathic effect was seen in the infected cells. The positive reactions of immunofluorescence staining were found 1-3 hours after the inoculation of virus. At about 36 hours they appeared again, increasing in intensity with lapse of time, when caused by the propagated virus. The fluorescence was noted first in the juxtanuclear area and then spread out over the whole cytoplasm sometimes as a number of tiny brilliant spots or condensed masses appearing mainly in the peripheral parts of the cells. The virus was serially transmitted through mosquito cultures, the original virus inoculum was diluted $10^{27}$ times during 164 days and it retained its

mouse infectivity throughout these cultures, holding mouse $LD_{50}$ titers of about $10^3$ to $10^4$ per 0.02 ml.

Primary cell cultures from the embryos of *Anopheles stephensi* mosquitoes were established by Singh and Bhat, 1969. The explanted cells and fragments multiplied very well and at about the tenth day most of the vessel surface was covered with them. Four different types of cells were observed, the predominant cells being muscle cells, followed by fibroblast-like cells, rounded cells and epithelial like cells. Also a large number of vesicles were observed in cultures. Amitotic divisions were seen in muscle cells. Subculturing of cells was unsuccessful, however, the cultures were maintained for more than five months in good condition.

*Mosquito stable cell lines.*

Stable lines of arthropod cells *in vitro* were established first by Grace, 1962, from ovarian tissue of the Saturniid *Antheraea eucalypti*. The same author (Grace, 1966) derived the first stable cell line from blood-sucking insect *A. aegypti* larval tissues. This cell line is maintained in Grace's medium (Grace, 1966) which is now produced commercially. Suitor *et al.*, 1966, adapted this line to medium containing Grace's medium plus 10 % fetal bovine serum with 0.5 % *Philosamia cynthia pryeri* haelmolymph, and succeeded in cloning the spindle-shaped cells. Mosquito cells were grown in suspension in a special modified medium, designated No. 35 (Nagle, 1969) which was improved by increasing the level of choline in the medium, with an optimal concentration of 50 mg/l. Grace's cell line was found to be intolerant to Fungizone, a commercial preparation of the polyene amphotericin B (Stanley and Vaughn, 1967). The mosquito cells exhibited cytopathology in 1 µg/ml and no mosquito cultures tolerated 3 µg/ml.

The adaptation of Grace's line to haemolymph-free media (Nagle *et al.*, 1967 : Sweet and Dupree, 1968, unpublished results ; Suitor, 1968, unpublished results ; and Nagle, 1969) enabled the wide use of cells for the cultivation of different micro-organisms. *Plasmodium gallinaceum* oocysts (Schneider, 1967 and 1968), microfilariae *Macacanema formosana* (Wood and Suitor, 1966) and viruses were cultivated in a specially modified medium.

The first set of experiments concerning the determination on sensitivity of Grace's mosquito cells to different arboviruses were done by Rehacek, 1968 a. The cells, cultivated at 28°C, supported the propagation of JE, Murray Valley Encephalitis (MVE), Kunjin and West Nile virus (Fig. 15-18) but not Sindbis, SF, Bebaru, Edge Hill and Kokoberra viruses. The viruses which multiplied showed no greater growth than 1 logarithmic unit per day, but they multiplied for a long time period. Only a slightly unclear cytopathic effect was found in cells infected with JE virus. The Asibi strain of yellow fever virus could be propagated in this cell line. The virus exhibited a 2 log. increase in titer within 24 hours, followed by lowered titers on subsequent days (Conserve and Nagle, 1967). Filshie and Rehacek, 1968, described the replication and morphology of MVE and JE viruses in mosquito cells by means of electron microscopy. The viral particles were seen in initially infected cells and in persistently infected cells. MVE virus particles were first observed in acutely infected cells 6 days
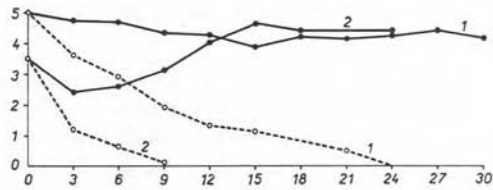
FIG. 15. — Growth curves of Murray Valley Encepha-
litis virus
Abscissa : days P.I. ; ordinate : virus titer in log PFU/
ml values
——— Mosquito cell cultures : 1-all medium changed.
2-2/3 of medium changed
- - - - - Controls : 1-1/3 of medium changed ; 2-2/3
of medium changed



FIG. 16. — Growth curves of Japanese encephalitis
virus
Abscissa and ordinate as in fig. 15
——— Mosquito cell cultures (all medium changed)
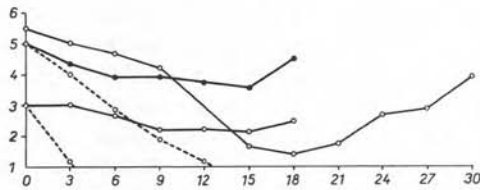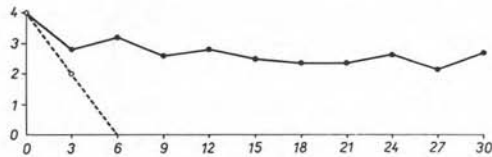- - - - - Controls (1/3 of medium changed)



FIG. 17. — Growth curves of Kunjin virus
Abscissa and ordinate as in fig. 15
——— Mosquito cell culture (all medium changed)
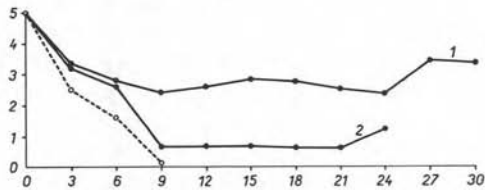- - - - - Control (2/3 of medium changed)



FIG. 18. — Growth curves of West Nile virus
——— Mosquito cell cultures ; 1-all medium changed ;
2-2/3 of medium changed
- - - - - Control (2/3 of medium changed)

after inoculation and in persistently infected cultures in the 5th passage and the 6th day after seeding. In infected cells, mature particles are always contained within vacuoles or elements of the endoplasmic reticulum, never free in the cytoplasm. From the 6th day, large vacuoles frequently contained single particles and crystalline groups of particles in addition to other debris apparently ingested from the culture medium by phagocytosis. On the 9th day, about 5 % of cells contained virus particles.

Rehacek, 1968 b, described the persistent infection of MVE and JE viruses in Grace's cells. After almost 21 weekly passages for 210 days, the different qualitative and quantitative relations and the properties of viruses and infected cells were followed. A slow and permanent loss of virus content was found with an increase in the number of passages ; the higher amount of virus particles always being present in the cells at 1-2 logs higher than in the medium. There was no difference in the growth rates of cells infected with MVE virus and uninfected cells, but there was a significant decrease of cells infected with JE virus when $5.2 \times 10^5$ cells/ml were used for seeding of the passage. The infected cells of 15th passage, cultivated in the presence of specefic antibodies, lowered gradually their virus amount and no virus was detected in the cells infected with MEV virus after the 4th and in JE virus infected cells after the 3rd subsequent passage, when the medium was changed twice during each weekly passage. The infected cells could be superinfected with $10^4$ PFU/ml of Kunjin virus at their 13th and 18th passages. During the course of these experiments the size and appearance of plaques was studied, but no significant change was observed with either MVE or with JE viruses. In the 4th, 10th and 20th passage the virus yields were titrated by the inoculation of infant mice intracerebrally and subcutaneously and by the plaque method. The results showed almost no change in pathogenicity for mice for both the viruses tested at the 4th and 10th passage and a decrease of pathogenicity at the 20th passage when inoculated subcutaneously.

Singh, 1967, succeeded in establishing further cell lines-three lines of freshly hatched larvae of *Aedes albopictus* and two of *Aedes aegypti* mosquitoes. The culture medium of Mitsuhashi and Maramorosch, 1964, was used without haemolymph. The cells were maintained in the medium with 10 % fetal bovine serum. Mainly three morphological types of cells were seen in *A. albopictus* cultures. The predominant type was round and 6-20 μ in diameter. The second type was spindle-shaped, 7-10 μ wide and 15-90 μ long. The third type of cell was binucleated, round and 37-53 μ in diameter. On the other hand, the growth pattern of the *A. aegypti* cultures was different in that they developed from the cells of hollow vesicles. These cells consisted mainly of an epithelial type of cell and even after many subcultures still had a tendency to form hollow vesicles and occassionally tube-like structures. Both cell lines have been used extensively for the cultivation of different viruses.

Singh and Paul, 1968 a, used the cell lines from the 22nd to the 29th passage for the cultivation of Chikungunya, Sindbis, Kyasanur Forest Disease (KFD), JE, dengue 1, dengue 2, dengue 3, dengue 4 viruses. Chikungunya, Sindbis, JE and West Nile viruses, all mosquito-borne viruses, grew well in *A. albopictus* cell cultures.

Approximately a $10^4$-$10^6$ fold increase over the original inoculum was observed. All the dengue types of viruses, which are also mosquito-borne viruses, multiplied in the same cultures but the maximum titers reached did not exceed $10^{2.0}$ to $10^{3.0}$ $LD_{50}$. On the other hand, *A. aegypti* cell line supported the multiplication of only Chikungunya and West Nile viruses. Neither of the cell lines supported the growth of KFD virus, which is a tick-borne virus. Cytopathic effect was observed with JE, West Nile, dengue 1, dengue 2 and dengue 4 viruses in only *A. albopictus* cells. Paul *et al.*, 1969, described in detail, the cytopathic effect in cells infected with West Nile, JE and dengue 2 viruses. In the case of the West Nile virus infection, mostly a slight thinning out of cell population with fewer mitotic figures was seen on the second day in comparison with uninfected cells. No cytological changes were seen except the withdrawal of ectoplasm by a small proportion of small cells as evidenced by the stretching of intercellular bridges across and increased pericellular space. Some of the small cells were rounded up with their cytoplasm showing increased basophilia in the vicinity of the nucleus. On the fourth day a gross reduction was noted in the total cell population. A very large number of small cells showed a withdrawal of ectoplasm and ectoplasmic bridges, followed by the formation of syncytial masses and a large number of multinucleate cells. Active phagocytosis of small cells was seen in the cultures. From the fifth to tenth day basically the same changes were noted except that they were much advanced, including disintegration of cell sheets into an amorphous acellular debris, cytolysis with phagocytosis of the small cells. On the twelfth to thirtieth day post inoculation the cellular debris disappeared through phagocytosis or from detachment from the glass walls and the cultures were composed of an increased population of cytologically healthy looking small cells and multinucleate cells of various shapes and sizes with variable numbers of nuclei. No intracellular or cytoplasmic inclusions were seen in any of the infected cultures. The cytopathic changes with JE and dengue 2 viruses were essentially similar.

Singh and Paul, 1968 *b* continued in the cultivation of several viruses in both mosquito cell lines. Of those cultivated were Sathuperi, Batai (Chittoor), Ganjam, African horse sickness (AHS), Chandipura, Dhori, Kaisodi, KFD, EMC, polio I., coxsackie B 5 and mouse-hepato-encephalitis (HE) viruses. Sathuperi, Batai, Ganjam, AHS and Chandipura viruses multiplied in *A. albopictus* cells as compared with *A. aegypti* cells where only Chandipura virus multiplied. In this cell line AHS virus was not cultivated. No cytopathic effect was seen in the cells of both cell lines infected with any of the above mentioned viruses.

As with Grace's *A. aegypti* cells also with *A. albopictus* cells the carrier state of viruses was described (Banerjee and Singh, 1968). These viruses included JE, West Nile and Chikungunya viruses. The carrier culture infected with JE virus were at the time of the publication in the 13th passage, West Nile virus in 8th passage and Chikungunya in the 7th passage. The JE virus titers in different passages ranged from $10^{6.0}$ to $10^{8.0}$ $LD_{50}$/ml in adult mice, the virus titers of the West Nile from $10^{5.1}$ — $10^{6.0}$ $LD_{50}$/ml in adult mice and the virus produced by the Chikungunya infected cultures was at a substantially lower level than both those mentioned.

Suitor and Paul, 1969, confirmed the cytopathic effect caused by dengue 2 virus cultivation in *A. albopictus* cells and described the cell syncytia formation when the infected cells were cultivated in plastic containers. These syncytia often have wavy or striated patterns containing large numbers of nuclei. The monolayer often becomes one large syncytial mass by the eighth to tenth day. Comparative endpoints of type 2 dengue virus pool on PS/Y 15 (porcine kidney) cells and in suckling mice showed that the formation of syncytia in *A. albopictus* cells is more sensitive than plaque formation or tests in suckling mice.

Another virus successfully cultivated in *A. albopictus* cell lines was Colorado Tick Fever (CTF) virus which multiplied with titers of 2.2-2.6 logs during the 3rd-4th weeks following inoculation without causing cytopathic effects (Yunker and Cory, 1969). This was the first report of the multiplication of a virus transmitted in nature by ticks in insect cells *in vitro*.

Paul and Singh, 1969, compared the sensitivity of mosquito cell lines to infection with arboviruses with that in infant mice and the VERO cell line. Six viruses (Chikungunya, West Nile, JE, dengue 2, Batai and Chandipura) were used in these experiments. It was shown that *Aedes aegypti* cells supported the growth of only Chikungunya, West Nile and Chandipura viruses and are much less sensitive to infection with arboviruses than *A. albopictus* cell line which is equally or slightly more sensitive to Chikungunya, West Nile and JE viruses, slightly less sensitive to Batai and Chandipura viruses as compared to infant mice and VERO cells, however, it is 100 times more sensitive than VERO and 40 times more sensitive than infant mice to infection with dengue 2 virus.

*Aedes albopictus* cell line has been used also by Yunker and Cory, 1968, for testing of a few arboviruses. The authors found that St. Louis Encephalitis (SLE), JE and West Nile viruses propagate to significant levels above the amounts inoculated, but Powassan and Langat which occur in the same arbovirus group but are tick-borne members, do not propagate.

Comprehensive studies on the sensitivity of Singh's cell lines to infection with arboviruses were done by Buckley, 1969 to determine whether the *A. albopictus* and *A. aegypti* cell lines could efficiently serve as *in vitro* assay systems for arboviruses. Twenty three different arboviruses were screened for their ability to multiply in mosquito cells at 30°C and 35°C. Buckley found cytopathic effects only in *A. albopictus* cells infected with West Nile virus. Both *Aedes* cell lines demonstrated infections with Chikungunya, EEE, SF, VEE, SLE, West Nile, yellow fever and vesicular stomatitis viruses. CTF multiplied only in *A. albopictus* cells but not in *A. aegypti* cells while the remaining 13 viruses (Cowbone Ridge, Modoc, Sicilian and Naples sandfly fever virus, Langat, Farallon, Silverwater, Chenuda, Johnston Atoll, Qualyub, Bandia, C 558 and URB-TMA 1381) failed to multiply in either mosquito cell line.

Sweet and Dupree, 1968, derived cell lines from female *Culiseta inornata* and pupal tissues of *Aedes vexans*. The cells of both lines grow as free floating cells rather than as monolayers. *C. inornata* lines were at the beginning of September, 1969 in

the 62nd passage and *A. vexans* at the 54th passage (Sweet, personal communication). Both cell lines resemble morphologically, by cultural characteristics and by immunological criteria the *Antheraea* and *A. aegypti* lines derived by Grace. Both cell lines have been used for the cultivation of SLE, EEE, Cache Valley and California viruses. Using a low multiplicity input and not changing the media, and holding for a period of 14 days, no significant viral multiplication above the input level occurred. SLE virus demonstrated a low level multiplication, or represented residual virus that was more stable in the presence of the mosquito cells (Sweet *et al.,* 1968). In a further study, Sweet and unthank, 1969, observed the multiplication at low levels of SLE and JE viruses over a 6 weeks period in the cells of *C. inornata, A. vexans* and Grace's *A. aegypti* cells.

Further cell lines have been derived from embryonic tissues of *Aedes aegypti* by Peleg, 1968 *b* and successfully used in 8-22 cell passages for the cultivation of EEE, SF and West Nile viruses. All of these viruses multiplied, culminating in a 100 to 10,000-fold increase in titer. Peak titers were attained by SF and EEE viruses in 2 or 3 days and West Nile virus increased at 6-7 days following infection. SF virus was isolated at 210 days, EEE at 70 and West Nile at 145 days from the cultures following infection. No cytopathic changes or differences in the lifespan of infected and normal cultures which could be due to the propagation of viruses tested were observed. The same author, Peleg, 1968 *c*, studied whether the mosquito cells *in vitro* could be infected with viral RNA. The results showed that the embryo cells in 24-35 passages inoculated with RNA of SF and West Nile arboviruses yielded virus progenies but those of EMC and polio viruses did not. Peleg explained that differences in the growth behavior of RNA could be related to intracellular factors.

Three further cell lines have been established from the first instar larvae of *Anopheles stephensi* by Schneider, 1969. Modified Grace's medium was used. In contrast to other insect lines, the cells of these lines are diploid having predominantly 6 chromosomes. The results of these cells used for the cultivation of any pathogen have been not as yet published.

The recently described cell lines were established from *Aedes aegypti* larvae by Varma and Pudney, 1969 *b*, who derived three different cell lines, and the growth of arboviruses in these cell lines is being studied.

**Conclusions.**

Tissue cultures of lice and fleas which might be used for the cultivation of different microorganisms are not yet available and therefore, these media are as yet of no concern for arbovirologists.

The development of cultures from bugs, flies and ticks is now in the stage of reasonable primary cultures, nevertheless, their wider use for microbial studies is

limited by the few cultures available and difficult preparation. Despite these troubles, tick tissue cultures have been used successfully for the cultivation of different viruses and rickettsiae and the results obtained can serve for possible criteria in diagnosis of various virus groups. The results indicate several viruses and rickettsiae have a high affinity to tick cells ; for example tick-borne encephalitis, Colorado Tick Fever virus and *Coxiella burneti, Rickettsia conori* and *R. akari*. These cells could be used in the isolating of these agents from nature.

The establishment of many cell lines from mosquitoes, mostly of the *Aedes* species, has allowed many workers to use them in virus studies. Most of the studies are concerned with their sensitivity to arboviruses multiplication and classification of virus groups. The very high sensitivity of these cells to several arboviruses also offers a convenient medium for their isolation nature. Cells for the multiplication of various arboviruses might be used in routine arbovirus work in laboratories investigating various properties and morphology of these viruses.

The first step in arbovirus and rickettsiae studies in arthropod cells *in vitro* has been the establishment and determination of their sensitivity to pathogens. The second step now follows, i.e. the comparison of the sensitivity to vertebrate cells *in vivo* and *in vitro* and the application of results.

The question is open on the use of arthropod cells *in vitro* for the propagation of agents which might be useful in biological control of arthropods. Such studies have not yet been accomplished. Studies by Filshie *et al.,* 1967, on the latent infection of an unknown virus in mosquito cells emerging arbovirus infection (fig. 19) may lead to solving this problem, but no data is yet available.

It would be difficult to interpret the results of the propagation of pathogens in arthropod cells *in vitro* for conclusions on their biology in arthropods. Such results, as for example with the Japanese Encephalitis and St. Louis Encephalitis viruses which are not ordinarily transmitted by *Aedes vexans, A. aegypti* or *Culiseta inornata* in nature have been shown to multiply in the cells of these arthropods *in vitro*. On the other hand, Cache Valley and California encephalitis viruses have been isolated from *C. inornata* mosquito and do not multiply in the cell lines of this species (Sweet and Unthank, 1969) support this premise.

Despite this slightly pessimistic conclusion, we suggest that tissue cultures of arthropods are a convenient tool, especially for arbovirologists. Their wide application for studies of the arthropod cell and virus relationships will follow.
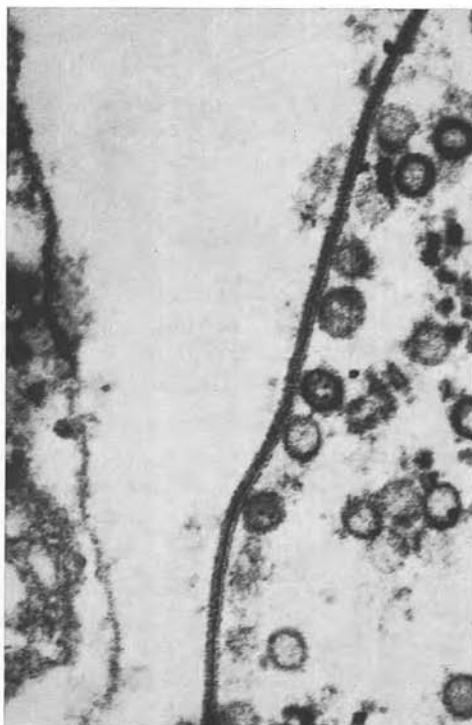
FIG. 19. — Structurally identical particles in the
cytoplasm of *Aedes* mosquito cell, 9 days after
infection with Murray Valley Encephalitis virus.
Most particles appear « hollow » but a few have
centrally disposed dense granules. Particles are
interspersed with ribosomes, R. 120000 ×

**Table I.** *Survey on mosquito cell lines.*

| SPECIES | STAGE | AUTHOR |
|---|---|---|
| *Aedes aegypti* ........ | larval tissues | Grace, 1966 |
| *Aedes albopictus* ..... | larval tissues | Singh, 1967 |
| *Aedes aegypti* ........ | larval tissues | Singh, 1967 |
| *Aedes aegypti* ........ | embryonic tissues | Peleg, 1968 *b* |
| *Culiseta inornata* ..... | adult female tissues | Sweet and Dupree, 1968 |
| *Aedes vexans* ........ | pupal tissues | Sweet and Dupree, 1968 |
| *Anopheles stephensi* ... | larval tissues | Schneider, 1969 |
| *Aedes aegypti* ........ | larval tissues | Varma and Pudney, 1969 *b* |

**Table II.** *Survey on virus cultivation in mosquito cell lines.*

| LINE | VIRUSES TESTED | VIRUSES PROPAGATED | CPE | AUTHOR |
|---|---|---|---|---|
| *Aedes aegypti* (Grace, 1966) | JE, MVE | JE, MVE | JE | Filshie and Rehacek, 1968 |
| | YF | YF | | Converse and Nagle, 1967 |
| | JE, WN, MVE, Kunjin, Sindbis, SF, EH, Bebaru, Kokoberra | JE, WN, MVE, Kunjin | JE | Rehacek, 1968 *a* |
| | EEE, SLE, JE, Cache Valley, CE | SLE, JE | | Sweet and Unthank, 1969 |
| *Aedes albopictus* (Singh, 1967) | Chikungunya, JE, Sindbis, WN, KFD, dengue 1, 2, 3, 4 | Chikungunya, JE, Sindbis, WN, dengue 1, 2, 3, 4 | JE, WN, dengue 1, 2, 4 | Singh and Paul, 1968 *a* |
| | Sathuperi, AHS, Batai, Ganjam, Chandipura, KFD, Dhori, Kaisodi, EMC, polio. I., HE, coxsackie B5 | Sathuperi, Batai, Ganjam, AHS Chandipura | | Singh and Paul, 1968 *b* |
| | CTF | CTF | | Yunker and Cory, 1969 |
| | JE, SLE, WN, Langat, Powassan | JE, SLE, WN | | Yunker and Cory, 1968 |
| | WN, Chikungunya, EEE, SF, VEE, SLE, YF, VS, CTF, Modoc, CR, SNSF, Langat, Farallon, Silverwater, Chenuda, Johnston Atoll, Qualyub, Bandia C 558, URB-TMA 1381 | WN, Chikungunya, EEE, SF, VEE, SLE, YF, VS, CTF | WN | Buckley, 1969 |

| Line | Viruses tested | Viruses Propagated | CPE | Author |
|------|----------------|--------------------|-----|--------|
| *Aedes aegypti* (Singh, 1967) | Chikungunya, WN, Sindbis, JE, KFD, dengue 1, 2, 3, 4 | Chikungunya, WN, Sindbis ? | | Singh and Paul, 1968 *a* |
| | Sathuperi, Batai, Ganjam, Kaisodi, Chandipura, Dhori, KFD, EMC, polio I., coxsackie B5, HE | Chandipura | | Singh and Paul, 1968 *b* |
| | WN, Chikungunya, EEE, SF, VEE, SLE, YF, VS, CTF, Modoc, CR, SNSF, Langat, Farallon, Silverwater, Chenuda, Johnston Atoll, Qualyub, Bandia, C 558, URB-TMA 1381 | WN, Chikungunya, EEE, SF, VEE, SLE, YF, VS | | Buckley, 1969 |
| *Aedes aegypti* (Peleg, 1968 *b*) | EEE, SF, WN | EEE, SF, WN | | Peleg, 1968 *b* |
| | RNA of WN, FS, EMC, polio | WN, SF | | Peleg, 1968 *c* |
| *Culiseta inornata* and *Aedes vexans* (Sweet and Dupree, 1968) | SLE, EEE, CE Cache Valley | SLE | | Sweet et al., 1968 |
| | SLE, EEE, JE, CE, Cache Valley | SLE, JE | | Sweet and Unthank, 1969 |

Glossary of abreviations used :

JE - Japanese encephalitis ; MVE - Murray Valley Encephalitis ; YF - Yellow fever ; WN - West Nile ; SF - Semliki Forest ; EH - Edge Hill ; SLE - St. Louis Encephalitis ; CE - California Encephalitis ; KFD - Kyasanur Forest Disease ; AHS - African horse sickness ; EMC - Encephalomyocarditis ; HE - Mouse-hepato-encephalomyelitis ; CTF - Colorado Tick Fever ; VEE - Venezuelan Equine Encephalomyelitis ; EEE - Eastern Equine Encephalomyelitis ; VS - Vesicular Stomatitis ; CR - Cowbone Ridge ; SNSF - Sicilian and Naples sandfly fever.

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