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COMPARATIVE STUDIES ON THE INFECTION OF INVERTEBRATE  
AND VERTEBRATE CELL LINES WITH SOME ARBOVIRUSES.

BY

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DEPARTMENT OF ENTOMOLOGY  
LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE.

SEPTEMBER 1977.



## ABSTRACT.

The morphology and growth rates of cell lines from the mosquitoes Aedes aegypti (20A), Anopheles stephensi (43), Anopheles gambiae (55), Aedes malayensis (60), Aedes pseudoscutellaris (61), and from the toad Xenopus laevis (XTC-2) in various media were examined.

Minimum infectious dose experiments with eight arboviruses demonstrated that the 60 and 61 cells were the most highly sensitive to infection. The 20A, 43 and 55 cells were comparatively less sensitive, although the XTC-2 cells were comparable in sensitivity to monkey kidney cells.

Comparisons of the growth of 23 arboviruses in all the lines were made. The XTC-2 cells supported the growth of nearly all the viruses tested. Most of the viruses multiplied in the mosquito cells to varying degrees, but none of the tick-borne viruses tested grew in the mosquito cells.

Cytopathic effects (CPE) and plaques were produced by a number of viruses in the XTC-2 cells, and this cell line is routinely used for the low temperature assay of a range of viruses.

CPE in the mosquito cells was observed only in the 60 and 61 cells, and only with three flaviviruses. Attempts to produce plaques in all the mosquito cell lines under carboxymethylcellulose overlay were unsuccessful.

Virus carrier cultures were established and alterations in the properties of progeny virus were examined over long periods. The results suggest the presence of an interferon-like system in the XTC-2 cells, but not in the mosquito cells.

Comparison of the results obtained with published work shows a high degree of correlation. Aspects of virus-vector relationships in vitro are discussed, and some of the potential practical applications of these cell lines have been examined.

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## LIST OF CONTENTS

	PAGE.
Abstract.	3
List of Contents.	4
List of Tables.	9
List of Figures.	10
List of Plates.	11
List of Abbreviations.	13
1. INTRODUCTION AND REVIEW OF THE LITERATURE.	15
1.1. Establishment of cell lines from arthropods.	15
1.2. Infection of arthropod cell cultures with pathogens.	17
1.2.1. Infection of arthropod cell cultures with pathogens other than arboviruses.	18
1.2.2. Infection of primary mosquito and tick cell cultures with arboviruses.	18
1.2.3. Infection of mosquito cell lines with arboviruses.	20
1.2.3.1. Susceptibility of cell lines to infection.	20
1.2.3.2. Growth of arboviruses in mosquito cell lines.	24
1.2.3.3. Production of cytopathic effect and plaque formation by arboviruses in mosquito cell lines.	25
1.2.3.4. Persistent infections of mosquito cell lines by arboviruses.	27
1.2.3.5. Serial passage of arboviruses in mosquito cell lines.	33
1.2.3.6. Subculture level of mosquito cell lines and sensitivity to virus infection.	35
1.2.3.7. Effect of passage history of virus on growth in mosquito cells.	37
1.2.3.8. Ultrastructural studies of arboviruses in mosquito cells.	37

	PAGE.
1.2.4. The use of mosquito cell lines for the primary isolation of arboviruses.	39
1.2.5. The use of mosquito cell lines in serological tests.	41
1.2.6. Infection of mosquito cell lines with viruses other than arboviruses.	42
1.3. Cell cultures from poikilothermic vertebrates and their infection with arboviruses and other viruses.	43
1.3.1. Cell cultures from fishes	43
1.3.2. Cell cultures from amphibians and reptiles.	45
1.4. Aims of work.	
2. MATERIALS AND METHODS.	49
2.1. Cell lines and their maintenance.	49
2.1.1. Mammalian cell lines and their maintenance.	49
2.1.1.1. Mammalian cell lines.	49
2.1.1.2. Maintenance of mammalian cell lines.	50
2.1.2. Poikilothermic cell lines and their maintenance.	51
2.1.2.1. Nomenclature of poikilothermic cell lines.	51
2.1.2.2. Mosquito cell lines.	51
2.1.2.3. Lower vertebrate cell lines.	52
2.1.2.4. Maintenance of poikilothermic cell lines.	52
2.1.3. Determination of growth curves of the cell lines.	52
2.1.4. Isoenzyme analysis of cell lines.	53
2.1.5. Tests for mycoplasma contamination of cell lines.	53
2.2. Viruses and methods of infection.	53
2.2.1. Virus stocks.	53

2.2.2. Determination of virus growth curves and the amount of virus adsorbed to cell lines.	57
2.2.3. Minimum infectious dose experiments.	58
2.3. Virus assay.	59
2.3.1. Virus assay in tissue culture.	59
2.3.2. Virus assay in mice.	61
2.3.3. Accuracy of plaque assay system.	61
3. RESULTS.	62
3.1. Morphology and growth characteristics of cell lines.	62
3.1.1. 20A cells.	62
3.1.2. 43 cells.	62
3.1.3. 55 cells.	65
3.1.4. 60 cells.	68
3.1.5. 61 cells.	68
3.1.6. XTC-2 cells.	71
3.2. Infection of cell lines with viruses.	71
3.2.1. Adsorption of virus to cells.	71
3.2.2. Minimum infectious dose experiments.	73
3.2.2.1. SIN virus.	73
3.2.2.2. SF virus.	75
3.2.2.3. CHIK virus.	75
3.2.2.4. ONN virus.	75
3.2.2.5. WN virus.	77
3.2.2.6. YF virus.	77
3.2.2.7. BUN virus.	77
3.2.2.8. CE virus.	77
3.3. Growth curves of arboviruses in cell lines.	80
3.3.1. Growth of alphaviruses.	80
3.3.1.1. SIN virus.	80
3.3.1.2. NDU virus.	85
3.3.1.3. SF virus.	85
3.3.1.4. CHIK virus.	88
3.3.1.5. ONN virus.	89
3.3.1.6. URU virus.	92

2.2.2. Determination of virus growth curves and the amount of virus adsorbed to cell lines.	57
2.2.3. Minimum infectious dose experiments.	58
2.3. Virus assay.	59
2.3.1. Virus assay in tissue culture.	59
2.3.2. Virus assay in mice.	61
2.3.3. Accuracy of plaque assay system.	61
3. RESULTS.	62
3.1. Morphology and growth characteristics of cell lines.	62
3.1.1. 20A cells.	62
3.1.2. 43 cells.	62
3.1.3. 55 cells.	65
3.1.4. 60 cells.	68
3.1.5. 61 cells.	68
3.1.6. XTC-2 cells.	71
3.2. Infection of cell lines with viruses.	71
3.2.1. Adsorption of virus to cells.	71
3.2.2. Minimum infectious dose experiments.	73
3.2.2.1. SIN virus.	73
3.2.2.2. SF virus.	75
3.2.2.3. CHIK virus.	75
3.2.2.4. ONN virus.	75
3.2.2.5. WN virus.	77
3.2.2.6. YF virus.	77
3.2.2.7. BUN virus.	77
3.2.2.8. CE virus.	77
3.3. Growth curves of arboviruses in cell lines.	80
3.3.1. Growth of alphaviruses.	80
3.3.1.1. SIN virus.	80
3.3.1.2. NDU virus.	85
3.3.1.3. SF virus.	85
3.3.1.4. CHIK virus.	88
3.3.1.5. ONN virus.	89
3.3.1.6. URU virus.	92

	PAGE.
3.3.1.7. MID virus.	94
3.3.1.8. Other alphaviruses.	96
3.3.2. Growth of flaviviruses.	96
3.3.2.1. LGT and LI viruses.	96
3.3.2.2. WN virus.	96
3.3.2.3. JE virus.	99
3.3.2.4. ZIKA virus.	106
3.3.2.5. DEN-2 and YF viruses.	106
3.3.3. Growth of bunyaviruses.	110
3.3.3.1. BUN virus.	110
3.3.3.2. GER virus.	112
3.3.3.3. BWA virus.	114
3.3.3.4. CE virus.	114
3.3.3.5. SFS virus.	118
3.3.3.6. ANA virus.	118
3.3.4. Growth of rhabdoviruses.	121
3.3.4.1. CHP virus.	121
3.3.5. Growth of unclassified viruses.	121
3.3.5.1. QRF and ZIR viruses.	121
3.4. Cytopathic effect (CPE) and plaque formation by arboviruses.	123
3.4.1. CPE in mosquito cell lines.	123
3.4.2. Plaque production in mosquito cell lines.	124
3.4.3. CPE in XTC-2 cells.	124
3.4.4. Plaque production in XTC-2 cells.	126
3.5. Persistent virus infection of cell lines.	133
3.5.1. Establishment of a long-term persistently infected culture.	133
3.5.2. Studies on viral interference in persistently infected cultures.	135
3.5.3. Alterations in plaque size of virus produced in persistently infected cultures.	138
3.5.3.1. WN virus.	138
3.5.3.2. CHIK virus.	140
3.5.3.3. SF virus.	143
3.5.3.4. SIN virus	143

	PAGE.
4. DISCUSSION.	146
4.1. Growth characteristics of cell lines.	146
4.2. Contamination of cell cultures.	147
4.3. Culture conditions for infection of cells.	148
4.4. Virus adsorption.	148
4.5. Sensitivity of cell lines to viral infection.	149
4.6. Susceptibility of cell lines to viral infection.	149
4.7. Viral growth patterns.	157
4.8. CPE and plaque formation.	159
4.9. Persistent infection.	160
4.10. Alterations in virus progeny from persistently infected cells.	161
Acknowledgements.	163
List of References.	164
Appendix.	191

## LIST OF TABLES.

- Table 1. Details of virus stocks.
- Table 2. Virus adsorption to cells of different cell lines.
- Table 3. Minimum infectious dose of SIN virus.
- Table 4. Minimum infectious dose of SF virus.
- Table 5. Minimum infectious dose of CHIK virus.
- Table 6. Minimum infectious dose of ONN virus.
- Table 7. Minimum infectious dose of WN virus.
- Table 8. Minimum infectious dose of YF virus.
- Table 9. Minimum infectious dose of BUN virus.
- Table 10. Minimum infectious dose of CE virus.
- Table 11. Viruses and subculture levels of cells used in infection experiments.
- Table 12. Production of CPE in XTC-2 cells by various viruses.
- Table 13. Comparative plaque assay in XTC-2 and Vero cells.
- Table 14. Susceptibility of cell lines to infection with 23 arboviruses.
- Table 15. Comparison of the susceptibility of the 60 and 61 cell lines and Singh's Ae.albopictus cell line to infection with some arboviruses.
- Table 16. Comparison of the susceptibility of the 20A cell line and other Ae.aegypti cell lines to infection with some arboviruses.

## LIST OF FIGURES.

- Figure 1 a. Growth curves of 20A cells.  
Figure 1 b. Growth curves of 43 cells.  
Figure 2 a. Growth curves of 55 cells.  
Figure 2 b. Growth curves of 60 cells.  
Figure 3 a. Growth curves of 61 cells.  
Figure 3 b. Growth curves of XTC-2 cells.  
Figure 4 a, b. Growth curves of SIN virus.  
Figure 5 a, b. Growth curves of NDU virus.  
Figure 6 a, b. Growth curves of SF virus.  
Figure 7 a, b. Growth curves of CHIK virus.  
Figure 8 a, b. Growth curves of ONN virus.  
Figure 9 a, b. Growth curves of URU virus.  
Figure 10 a, b. Growth curves of MID virus.  
Figure 11 a, b. Growth curves of four tick-borne  
viruses in XTC-2 cells.  
Figure 12 a, b. Growth curves of WN virus.  
Figure 13 a, b. Growth curves of JE virus.  
Figure 14 a, b. Growth curves of ZIKA virus.  
Figure 15 a, b. Growth curves of DEN-2 virus.  
Figure 16 a, b. Growth curves of YF virus.  
Figure 17 a, b. Growth curves of BUN virus.  
Figure 18 a, b. Growth curves of GER virus.  
Figure 19 a, b. Growth curves of BWA virus.  
Figure 20 a, b. Growth curves of CE virus.  
Figure 21 a, b. Growth curves of SFS virus.  
Figure 22 a, b. Growth curves of ANA virus.  
Figure 23 a, b. Growth curves of CHP virus.  
Figure 24. Long-term production of WN virus  
from 60 carrier culture.  
Figure 25 a. Superinfection of 60WN carrier culture  
with WN and SF viruses.  
Figure 25 b. Superinfection of 61WN carrier culture  
with WN and SF viruses.  
Figure 26 a. Superinfection of long-term 60WN carrier  
culture with WN and SF viruses.  
Figure 26 b. Superinfection of XTC-2 WN carrier  
culture with WN and SF viruses.

## LIST OF PLATES.

- Plate 1. Monolayer of 20A cells.  
Plate 2. Monolayer of 43 cells.  
Plate 3. Monolayer of 55 cells.  
Plate 4. Monolayer of 60 cells.  
Plate 5. Monolayer of 61 cells.  
Plate 6. Monolayer of XTC-2 cells.  
Plate 7. Control XTC-2 cells grown on plastic.  
Plate 8. XTC-2 cells grown on plastic 2 days after infection with SIN virus.  
Plate 9. Control XTC-2 cells grown on plastic.  
Plate 10. XTC-2 cells grown on plastic 4 days after infection with WN virus.  
Plate 11. XTC-2 cells grown on plastic 11 days after infection with WN virus.  
Plate 12. Control 60 cells grown on glass.  
Plate 13. 60 cells grown on glass 4 days after infection with WN virus.  
Plate 14. Control 61 cells grown on plastic.  
Plate 15. 61 cells grown on plastic 5 days after infection with WN virus.  
Plate 16. Titration of BWA virus from infected 20A cells showing altered plaque morphology.  
Plate 17. Plaques produced in XTC-2 cells by the alphaviruses BEB, GET, URU and MAY.  
Plate 18. Plaques produced in XTC-2 cells by the bunyaviruses BUN, BAT and CVO.  
Plate 19. Plaques produced in XTC-2 cells by the rhabdoviruses PIRY, CHP and KC.  
Plate 20. Plaques produced in XTC-2 cells by the tick-borne viruses QRF, DUG and GAN.  
Plate 21. Plaques produced in XTC-2 cells by the tick-borne viruses HUG, PS and SOL.  
Plate 22. Plaques produced in XTC-2 cells by the tick-borne viruses KET, Puffin Island and SOL.  
Plate 23. CPE in XTC-2 cells produced under overlay by SFS virus at high virus concentrations.

- Plate 24. Titration of stock WN virus and 60WN virus in Vero cells showing altered plaque morphology of the carrier culture virus.
- Plate 25. Titration of stock WN virus and 60WN virus in XTC-2 cells showing altered plaque morphology of the carrier culture virus.
- Plate 26. Titration of stock CHIK virus in Vero cells.
- Plate 27. CHIK virus from the first passage of infected cells titrated in Vero cells.
- Plate 28. CHIK virus from the third passage of infected cells titrated in Vero cells.
- Plate 29. CHIK virus from the thirtieth passage of infected cells titrated in Vero cells.
- Plate 30. Titration of stock CHIK virus in XTC-2 cells.
- Plate 31. CHIK virus from the thirtieth passage of infected cells titrated in XTC-2 cells.

## LIST OF ABBREVIATIONS.

20A	Aedes aegypti cell line.
43	Anopheles stephensi cell line.
55	Anopheles gambiae cell line.
60	Aedes malayensis cell line.
61	Aedes pseudoscutellaris cell line.
ANA	Anopheles A virus.
AMT	Arumowot virus.
BAT	Batai virus.
BHK	Baby hamster kidney cell line.
BUN	Bunyamwera virus.
BWA	Bwamba virus.
CE	California encephalitis virus.
CMC	Carboxymethylcellulose.
CPD <sub>50</sub>	Dose of virus causing cytopathic effects in half of the cultures inoculated.
CPE	Cytopathic effect.
CVO	Calovo virus.
CHP	Chandipura virus.
CHIK	Chikungunya virus.
DEN-2	Dengue virus type 2.
DUG	Dugbe virus.
EDTA	Ethylenediaminetetra-acetic acid.
ENT	Entebbe bat virus.
EV	Extracellular virus.
FBS	Foetal bovine serum.
GAN	Ganjam virus.
GER	Germiston virus.
GET	Getah virus.
HUG	Hughes virus.
JE	Japanese encephalitis virus.
KC	Kern canyon virus.
KTR	Keterah virus.
L-15	Leibovitz L-15 medium.
LB	Lagos bat virus.
LD <sub>50</sub>	Dose of virus causing death in half the test animals inoculated.
LGT	Langat virus.

LJN Lanjan virus.  
LI Louping ill virus.  
MAY Mayaro virus.  
MEB Mount elgon bat virus.  
MID Middelburg virus.  
MK Modified Kitamura's medium.  
MM Mitsuhashi and Maramarosch's medium.  
MOD Modoc virus.  
MOI Multiplicity of infection.  
NDO Nyando virus.  
NDU Ndumu virus.  
ONN O'nyong-nyong virus.  
PFU Plaque forming units.  
PS Pig kidney cell line.  
PS Puntas Salinas virus.  
QRF Quaranfil virus.  
Sc Subculture.  
SFS Sicilian sandfly fever virus.  
SF Semliki forest virus.  
SIN Sindbis virus.  
SOL Soldado virus.  
TAH Tahyna virus.  
TEM Tembusu virus.  
UGS Uganda S virus.  
URU Uruma virus.  
Vero African green monkey kidney cell line.  
VP<sub>12</sub> Varma and Pudney's tick tissue culture medium.  
WHO World Health Organisation.  
WHAT Whatarhoa virus.  
WN West Nile virus.  
XTC-2 Xenopus laevis cell line.  
YF Yellow fever virus.  
ZIR Zirqa virus.

## 1. INTRODUCTION AND REVIEW OF THE LITERATURE.

### 1.1. Establishment of cell lines from arthropods.

Early literature on the culture of arthropod cells records only limited success in the long-term maintenance of surviving tissues, or primary cell cultures. The most significant advances in the techniques employed to establish cultures from arthropod tissues have taken place over the last 15-20 years. Pudney (1973), in a comprehensive review, has traced, in detail, the refinements in technique which led to the establishment of the first continuous cell lines from arthropods.

Initially, most success was obtained using tissues from non-bloodsucking arthropods, but subsequently, the same techniques were successfully applied to the culture of cells from bloodsucking arthropods also. Over the last 10 years a number of continuous cell lines have been established from various species of bloodsucking arthropods.

The first arthropod cell line to be established was from an insect, and was derived from pupal tissues of the moth Antherea eucalypti (Grace, 1962); and the first cell line from a bloodsucking arthropod was established from tissues of larvae, about to pupate, of the mosquito Aedes aegypti (Grace, 1966). Both these cell lines were maintained in media containing insect hemolymph as a supplement, but as hemolymph had to be extracted from An.eucalypti pupae this made both cell lines rather inconvenient to maintain. At this time insect hemolymph was considered to be essential in arthropod tissue culture techniques.

An important advance in technique was reported by Singh in 1967, when he described the establishment of two mosquito cell lines from first stage larval tissues of the mosquitoes Aedes aegypti and Aedes albopictus. Using a simple technique for surface sterilisation of mosquito eggs, he solved the problems

associated with obtaining sterile tissues for the initiation of cell cultures. In addition, commercially available foetal bovine serum, rather than insect hemolymph, was used as a supplement, thus demonstrating that insect hemolymph was not essential for the establishment and maintenance of mosquito cell cultures.

Subsequently, a number of additional mosquito cell lines have been established by various workers. These include cell lines from Aedes aegypti (Peleg, 1968 b; Varma and Pudney, 1969), Aedes albopictus (Bhat, 1973), Aedes malayensis and Aedes pseudoscutellaris (Varma, Pudney and Leake, 1974), Aedes novalbopictus (Bhat and Guru, 1973), Aedes vexans (Sweet and McHale, 1970), Aedes vittatus (Bhat and Singh, 1970), Aedes w-albus (Singh and Bhat, 1971), Culex molestus (Kitamura, 1970), Culex quinquefasciatus (Hsu, Mao and Cross, 1970), Culex tritaeniorhynchus (Schneider, 1971; Hsu, Li and Cross, 1972), Culiseta inornata (Sweet and McHale, 1970), Anopheles gambiae (Marhoul and Pudney, 1972), and Anopheles stephensi (Schneider, 1969; Pudney and Varma, 1971).

Attempts have also been made to establish cell lines from other bloodsucking arthropods, such as ticks and bugs, but until recently these efforts have been unsuccessful.

Pioneer work on tick cell cultures and their use for the study of viruses and rickettsiae has been reviewed by Rehacek (1971 a, b; 1972). Only limited success has been obtained with continuously subcultured cells. In 1972, Medvedeva et al described the subculture of embryonic cells from the tick Boophilus microplus twenty times before the cells died off. Using tissues from another species of tick Rhipicephalus appendiculatus Varma and Pudney (1973) only obtained limited growth initially; but subsequently, tissues from developing adults of this species were used to establish the first three tick cell lines (Varma, Pudney and Leake, 1975).

Culture of cells from bloodsucking triatomine bugs has also had limited success (Lüscher, 1947, Goodchild, 1954, Vago and Flandre, 1963). Primary cultures from embryonic tissues of the bug Triatoma maculata have been obtained (Varma and Pudney, 1967) and the culture of tissues of the bug Triatoma rubrofasciata was described by Bhat and Singh (1970). Recently, Pudney and Lanar (1977) described the establishment of a cell line from embryonic tissues of the bug Triatoma infestans. To our knowledge this is the first continuous cell line to be obtained from a bloodsucking bug, and preliminary studies have shown that these cells have applications in the study of trypanosomes in vitro (Lanar, in preparation).

A large number of invertebrate cell lines have been catalogued by Hink (1975). This list includes cell lines from bloodsucking arthropods as well as cell lines from non-bloodsucking arthropods as well as other invertebrate orders. At present this catalogue is under review, and an updated list of the established cell lines should be published in the near future.

#### 1.2. Infection of arthropod cell cultures with pathogens.

The development of continuous arthropod cell lines has proved useful in a number of fields of research. These cell lines are interesting from an academic point of view, and much of the early work was concerned with characterising the basic physiology of the cells in culture.

In this review the field of arbovirus-arthropod cell relationships is the prime consideration, but before this literature is examined it must be emphasised that arthropod cell lines from both bloodsucking and non-bloodsucking arthropods have also found applications in the study of a variety of pathogens of medical and economic importance.

1.2.1. Infection of arthropod cell cultures with pathogens other than arboviruses.

Arthropod cell cultures have been infected with a number of different pathogens and some examples are given below. Yunker (1971) and Rehacek (1972) reviewed work on the infection of both mosquito cell lines and primary tick cell cultures with rickettsial agents. Work has also been carried out on the in vitro development of trypanosomes in tsetse tissue cultures (Cunningham, 1973). The microfilaria Macacanema formosana was shown to develop in the presence of Grace's Ae. aegypti cells, although infective forms were not produced (Wood and Sutor, 1966), and the study of malarial parasites in mosquito cell cultures has been another major area of interest (for a review see Ball, 1972).

Arthropod cells have also been used to study various viruses. The establishment of cell lines from leafhoppers has enabled advances to be made in the study of plant viruses in their arthropod vector cells (for a review see Mitsuhashi, 1972). Arthropod cell cultures are now becoming increasingly important in research on insect viruses. It has been recognised that pathogenic insect viruses may play an important part in existing programmes of biological control of insect pests (for a review see Bailey, 1973). Insect virology is a rapidly expanding field in which comparatively little work has been done. Indeed, insect virology has been described (Paschke and Summers, 1975) as having 'a future and potential perhaps unparalleled as compared to other areas of virology.'

1.2.2. Infection of primary mosquito and tick cell cultures with arboviruses.

Whilst the availability of continuous cell lines has been a major advance in the in vitro study of arboviruses, early workers successfully demonstrated

in vitro multiplication of arboviruses using surviving tissues and primary cell cultures.

Trager (1938) demonstrated the multiplication of Western Equine Encephalitis (WEE) virus in surviving tissues of Ae.aegypti and subsequently, Peleg and Trager (1963) obtained growth of West Nile (WN) virus in surviving imaginal discs of Ae.aegypti. Extending these studies Peleg (1968 a) demonstrated the multiplication of Eastern Equine Encephalitis (EEE), Semliki Forest (SF) and WN viruses in primary cell cultures of Ae.aegypti. In 1969, Johnson successfully grew Venezuelan Equine Encephalitis (VEE) and EEE viruses in tissues from both Ae.aegypti and Ae. triseriatus. In addition, he demonstrated that serial passage of VEE virus in Ae.aegypti tissues did not result in any changes in the virulence of the virus for mice, or in the plaque size in vertebrate cells. In similar studies Fujita et al (1968) used primary cultures from C.pipiens molestus for the cultivation and serial passage of Japanese Encephalitis (JE) virus.

In spite of these studies there is a lack of extensive work using primary mosquito cell culture. This is probably due to the rapid establishment and availability of continuous cell lines. This is in contrast to studies using primary tick cell cultures as nearly all the work has been carried out using primary cultures.

The first report of the use of tick cells for the growth of an arbovirus was in 1960 when Rehacek and Pesek demonstrated the growth of the mosquito-borne EEE virus in surviving tissues from the ticks Dermacentor pictus and Ixodes ricinus. Subsequently (Rehacek, 1963) Tick-Borne Encephalitis (TBE) virus was grown in tissues from the ticks Dermacentor marginatus and Hyalomma asiaticum and it was shown (Rehacek, 1971) that serial passage of this virus in D.marginatus and Hyalomma dromedarii cultures did not result in any appreciable changes in the virulence of the virus for mice.

In further studies using H.dromedarii cultures Rehacek (1965 b) grew 15 tick-borne and mosquito-borne arboviruses without obtaining any signs of cytopathic effects. H.dromedarii cultures were later shown to support the multiplication of three further tick-borne viruses, Tribec (Rehacek et al, 1969; Rajcani et al, 1969), Lanjan (LJN) and Quaranfil (QRF) (Varma and Pudney, 1969). In one other report, Yunker and Cory (1969) demonstrated the growth of Colorado Tick Fever (CTF) virus in primary cultures of its tick vector Dermacentor andersoni.

There has only been one report on the use of a tick cell line for infection experiments with some arboviruses. Varma, Pudney and Leake (1975) successfully infected one of their cell lines from the tick R.appendiculatus with the mosquito-borne WN virus and the three tick-borne viruses Louping Ill (LI), Langat (LGT) and QRF.

#### 1.2.3. Infection of mosquito cell lines with arboviruses.

##### 1.2.3.1. Susceptibility of cell lines to infection.

Using Grace's Ae.aegypti cell line, Converse and Nagle (1967) demonstrated rapid growth of Yellow Fever (YF) virus; and later Rehacek (1968) showed that these cells were susceptible to infection with Murray Valley Encephalitis (MVE), Kunjin (KUN), JE and WN viruses, but that Sindbis (SIN), Bebaru (BEB), Edge Hill (EH), Kokobera (KOK) and SF viruses failed to infect the cells. Sweet and Unthank (1971) also found that St Louis Encephalitis (SLE) as well as JE virus would multiply in these cells at low levels, but that California Encephalitis (CE), Cache Valley (CV) and EEE viruses all failed to infect the cells.

Recently, Green and Charney (1971, 1973) have applied the technique of isoenzyme analysis to a number of arthropod cell lines. Their results strongly

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Recently, Green and Charney (1971, 1973) have applied the technique of isoenzyme analysis to a number of arthropod cell lines. Their results strongly

suggest that the strain of Grace's Ae.aegypti cell line that they tested was identical to Grace's An.eucalypti cell line. This implies that cross contamination of these lines had occurred. Bearing this in mind, the results obtained for the viral susceptibility of Grace's Ae.aegypti cells must be viewed with a certain amount of caution.

The application of these isoenzyme techniques should prove extremely valuable in the future, for work on the characterisation of newly established cell lines and also as a routine check on the identity of established cell lines.

There have been a number of studies examining the susceptibility of newly established mosquito cell lines to infection with arboviruses. In 1971, Sweet and Unthank found evidence of low level multiplication of JE and SLE viruses in their Ae.vexans and C.inornata cell lines, whereas three further mosquito-borne viruses EEE, CE and CV all failed to infect the cells. Bhat and Goverdhan (1972) successfully infected their Ae.vittatus cell line with Chikungunya (CHIK), Chandipura (CHP), JE and WN viruses, but they found that the mosquito-borne Dengue 2 (DEN-2) and the two tick-borne viruses Kyasanur Forest Disease (KFD) and Ganjam (GAN) would not multiply in their cells. In 1975, Buckley et al confirmed the multiplication of CHIK virus in this cell line and carried out further studies with this virus.

Similar results were obtained by Singh et al (1973) in studies on a cell line from Ae.w-albus. The cells were susceptible to infection with CHP, Dengue 1 (DEN-1), DEN-2, CHIK and JE viruses but were not infected with KFD or GAN viruses.

The cell line from C.quinquefasciatus established by Hsu et al (1970) was shown to be susceptible to infection with Sagiyama (SAG), DEN-2, Dengue 4 (DEN-4) Bunyamwera (BUN), JE and WN viruses, but not susceptible to infection with the alphaviruses EEE and

SIN (Hsu, 1971).

Infection studies have also been carried out on cell lines from anopheline mosquitoes. In 1971, Varma and Pudney found that Anopheles A (ANA), O'nyong-nyong (ONN), Calovo (CVO), and Bwamba (BWA) viruses, all isolated from anopheline mosquitoes, multiplied in their An.stephensi cell line. In further studies with these cells, Marhoul (1971) described the growth of Tahyna (TAH) virus, as well as CVO virus, but he failed to infect the cells with the tick-borne Uukuniemi (UUK) virus.

Buckley's (1971 c) studies on Schneider's An.stephensi cell line demonstrated replication of both CHIK and ONN viruses; although the cells were more sensitive to infection with ONN virus at low multiplicities of infection than CHIK virus. However, in 1973, Singh et al failed to infect these cells with CHIK virus, as well as with DEN-2, KFD and GAN viruses, although the cells were shown to be susceptible to infection with JE and CHP viruses.

Only one other anopheline cell line has been examined. This is an An.gambiae cell line which was found to be susceptible to infection with TAH virus but not CVO, TBE or Yaba-1 viruses (Marhoul, 1973). Yaba-1 virus, a member of the Turlock group, was originally isolated in Nigeria but has also been isolated in Czechoslovakia from Culex modestus mosquitoes (Malkova et al, 1972).

Apart from these studies, the majority of information on the susceptibility of mosquito cell lines to arbovirus infection has come from studies on well established cell lines derived from Aedes mosquitoes. Peleg's Ae.aegypti cell line was shown to be susceptible to infection with EEE, SF, WN and JE viruses (Peleg, 1968 b, 1971), SIN virus (Peleg and Stollar, 1974) and SLE, WEE and CE viruses (Sweet and Unthank, 1971). Peleg (1969) also demonstrated that these cells were susceptible to infection with naked RNA of both SF and WN viruses.

Varma and Pudney (1971) found that their Ae.aegypti cell line would not support the growth of ANA, ONN, CVO or BWA viruses, and it was also shown that TAH virus infected this cell line whereas the tick-borne UUK and TBE viruses failed to grow (Malkova and Marhoul, 1971; Marhoul, 1971).

The literature on the infection of mosquito cell lines with arboviruses is dominated by the amount of work that has been carried out on Singh's Ae.aegypti and Ae.albopictus cell lines. In a review, Singh (1972) lists the work of Singh and Paul (1968 a, b), Singh and Bhat (1968), Buckley (1969 a; 1971 a, b, c; 1972 a,b) Libikova and Buckley (1971), Mirchamsey et al (1970), Mugo and Shope (1972), Singh (1971), Stevens (1971), Sweet and Unthank (1971), Whitney and Deibel (1971), and Yunker (1971). Since this review was compiled other publications have been those of Artsob and Spence (1973), Buckley (1973), Buckley and Tignor, (1975) and Danielova (1973), as well as many other publications on other aspects of arbovirus growth in these cells.

More than 50 arboviruses have been tested in both these cell lines and the results have indicated that the Ae.albopictus cell line is more sensitive to a wider range of viruses than the Ae.aegypti cell line. Singh (1972) observed that, with the exception of CTF and GAN viruses, only those viruses that are naturally transmitted by mosquitoes, or infect mosquitoes experimentally, can multiply in one or both of these cell lines. Extending his argument Buckley (1972) has observed that the partially sodium deoxycholate resistant viruses (orbiviruses) can multiply in one or both of these cell lines, whereas the lipid solvent sensitive arboviruses isolated from ticks and phlebotomine flies cannot multiply in either cell line.

#### 1.2.3.2. Growth of arboviruses in mosquito cell lines.

Two types of virus growth curve have been observed in mosquito cell lines (Singh, 1972). In the first, the maximum observed virus titre never exceeds the inoculum by more than 1 dex (Haldane, 1960). This type of growth curve has only been observed for MVE, JE, WN, and KUN viruses in Grace's Ae.aegypti cell line (Rehacek, 1968). In the second type of growth curve the maximum virus titre exceeds the inoculum by 2-6 dex. Singh also noted that the growth curves obtained showed an initial fall in titre during the first 24 hours after infection, followed by a rise in titre to a peak between days 3 and 6 after infection. Subsequently virus titres fell gradually.

There have been several reports, however, of growth curves differing from this general pattern. In 1970, Stevens demonstrated that SIN virus reached a peak titre in Ae.albopictus cells around 24 hours after infection, but that in contrast the growth of DEN-2 virus was similar to the general pattern described by Singh. In 1973, Sinarachatanant and Olson also obtained similar results for the growth of DEN-2 virus in Ae.albopictus cells, but they also demonstrated that this growth pattern was similar to the growth of DEN-2 virus in vertebrate cells at 37°C.

Recently, detailed work has been carried out on Singh's Aedes cell lines with SF virus (Kelly and Dalgarno, 1971; Davey et al, 1973; Raghov et al, 1973 a; and Davey and Dalgarno, 1974), KUN virus (Kelly and Dalgarno, 1971; Davey et al 1973) and Ross River (RR) virus (Raghov et al, 1973 b; Dalgarno and Davey, 1973). These studies show that the two alphaviruses SF and RR rapidly reach a peak virus titre around 24 hours after infection in the Ae.albopictus cells, whereas the

flavivirus KUN grows more slowly reaching a peak 40 hours after infection. In contrast, in the Ae.aegypti cells the virus growth was slower, SF virus reaching a peak 60 hours after infection and KUN virus a peak 40-80 hours after infection.

1.2.3.3. The production of cytopathic effects (CPE) and plaque formation in mosquito cell lines.

Although many mosquito cell lines can be infected with arboviruses, and can produce virus titres comparable to mammalian cell lines, most of the mosquito cell lines, unlike mammalian cells, do not appear to suffer any harmful effects as a result of infection.

Until recently, the only mosquito cell line to show a CPE on infection with arboviruses has been Singh's Ae.albopictus cell line, but while these cells support the growth of a large number of arboviruses CPE is restricted to only a few viruses.

Singh and Paul (1968 a, b), described CPE produced in the Ae.albopictus cells by JE, WN, and DEN-1, 2, 3, and 4 viruses although CHIK, SIN, members of the Simbu, Piry and Bunyamwera groups and GAN and African Horse Sickness (AHS) viruses did not produce any CPE. The CPE observed was characterised by syncytia formation and was described in sequence by Paul et al (1969) as:

- 1) Cytolysis of individual small cells.
- 2) Development of large syncytial masses.
- 3) Gradual increase in the number of multinucleated giant cells.
- 4) Active phagocytosis of dead cells.
- 5) Ultimate recovery of the infected culture.

Subsequently, Dhandwate (1971, Personal communication in Singh, 1972) observed CPE with the ungrouped virus Minnall. The CPE differed from the syncytial type of response described above although no details were given.

Buckley (1969) confirmed the original observations of CPE with WN virus in the Ae.albopictus cells, but she failed to obtain CPE with JE, YF or SLE viruses. In contrast, Sweet and Unthank (1971) did obtain CPE

with SLE virus and confirmed the original findings for the Dengue virus serotypes.

In 1974, two new cell lines showing a CPE on infection with some arboviruses were described by Varma, Pudney and Leake. The cell lines were established from Ae.malayensis and Ae.pseudoscutellaris, two mosquito species closely related to Ae.albopictus. CPE was observed with WN, JE and DEN-2 viruses in the Ae.pseudoscutellaris cells and with JE and WN viruses in the Ae.malayensis cells. In their early subcultures both cell lines showed a syncytial type of response, but, in later subcultures, the Ae.malayensis cells showed a focal type of response consisting of rounding up of the cells followed by detachment of these cells from the surface of the culture vessel. Varma et al (1976) also observed this focal death in the Ae.pseudoscutellaris cell line when they used the cells for the isolation of YF virus from original field material.

The nature of the culture vessel appears to play an important part in the production of a CPE. Paul et al (1969) and Suitor and Paul (1969) have found that the CPE produced by DEN-2 virus in the Ae.albopictus cells is enhanced when the cells are grown on plastic tissue culture containers. These findings were supported and extended when Varma et al (1974) described enhanced effects with DEN-2, WN and JE viruses when their Ae.pseudoscutellaris and Ae.malayensis were grown on plastic surfaces.

Initially it appeared that the use of mosquito cells for the plaque assay of arboviruses would be of little practical use, as so few viruses caused a CPE in the cells. However, Suitor (1969) successfully demonstrated that JE virus would produce plaques in Ae.albopictus cells when they were incubated under an agarose overlay at 28°C.

In 1972, Cory and Yunker then described further experiments with the Ae.albopictus cells, and by using similar techniques they confirmed the original findings with JE virus and also obtained plaques with WN, DEN-1, 2 and 4 and with the Indiana strain of Vesicular Stomatitis (VSI) virus. Powassan (POW), Kemerovo (KEM), Itaqi (ITQ) and SLE viruses all failed to produce plaques. The significant finding in this study was that VSI virus produced plaques in the cells although previously there had been no evidence to suggest that the virus produced CPE in the Ae.albopictus cells.

Extending this study, Yunker and Cory (1975) tested 124 virus strains for plaque formation in the Ae.albopictus cells under agarose overlays employing incubation temperatures of 35-37°C. They found that a number of viruses which did not produce CPE in the cells would produce plaques under these conditions. A total of 30 viruses produced plaques, and all these viruses were proven, or presumed to be, mosquito-borne viruses. Yunker and Cory concluded that this technique offers a means of direct assay of arboviruses in mosquito cells and that plaque formation in these cells may indicate that a given virus is mosquito-borne.

Other cell lines are now being studied for plaque formation, and recently Hsu et al (1975,a) reported preliminary results on the successful production of plaques in C.tritaeniorhynchus cells by JE virus. In this laboratory Pudney (personal communication) has also obtained plaques with several viruses in the Ae.pseudoscutellaris cell line, using an incubation temperature of 28°C.

#### 1.2.3.4. Persistent infection of mosquito cells by arboviruses.

It has been frequently found that once a mosquito cell line has been infected with an arbovirus the cells

become persistently infected, and that virus continues to be released over extended incubation periods. The growth rates of these persistently infected cells appears to be unaffected by the virus infection and the cells can be readily subcultured over extended periods. These cultures can thus be easily studied as virus carrier cultures.

Carrier cultures of Ae.albopictus cells have been established with JE, WN and CHIK viruses (Banerjee and Singh, 1968, 1969; Buckley, 1971, and Buckley et al, 1975), AHS virus (Mirchamsey et al, 1970), KEM virus (Libikova and Buckley, 1971), Palyam, Lebombo and Irituia viruses (Buckley, 1972), SF virus (Dalgarno and Davey, 1973), DEN-2 virus (Sinarachatanant and Olson, 1973), CE virus (Kascsak and Lyons, 1974), Cocal virus (Artsob and Spence, 1974), TAH virus (Otova and Krobova, 1974) and VEE virus (Esparaza and Sanchez, 1975).

Other carrier cultures have been established in Peleg's Ae.aegypti cell line with SF virus (Peleg, 1969) and with SIN virus (Shenk et al, 1974; Peleg and Stollar, 1974 and Stollar et al, 1974), as well as carrier cultures of DEN-2 virus in Ae.pseudoscutellaris cells (Varma et al, 1974) and JE virus in C.tritaeniorhynchus cells (Hsu et al, 1975 b).

Whilst this phenomenon has been frequently seen little evidence has been obtained to explain how these persistent infections become established, or how they are regulated. There have, however, been several studies designed to examine various aspects of persistent virus infections.

Using immunofluorescent techniques, Whitney and Deibel (1971) found that only a small number of cells were infected in both Ae.aegypti and Ae.albopictus cells infected with CE virus. Similar results were obtained by Libikova and Buckley (1971), who found that only 1.0-3.8% of the cells were infected in Ae.albopictus carrier cultures of KEM virus as measured by plaque assay and fluorescent methods. In 1974, Otova

and Krobova found only 2.4% of infected cells in Ae.albopictus cultures persistently infected with TAH virus, and in 1969, Peleg described detailed studies on SF virus infection of his Ae.aegypti cell line which showed a fall from 2.8-8.0% of infected cells at the height of infection to as low as 0.01-0.09% of infected cells as measured by infectious centre assay.

Esparaza and Sanchez (1975) showed that in Peleg's Ae.aegypti and Singh's Ae.albopictus cells infected with VEE virus some 10-15% of cells were infected 10-15 hours after infection as measured by infectious centre assay. This percentage then fell dramatically to be maintained at levels as low as 0.01-0.05% of infected cells. They also carried these studies one step further by establishing subpopulations of both cell lines and testing each population for infection with the virus. The different subpopulations showed considerable differences in susceptibility to infection with VEE virus and it was shown that a high proportion of the subpopulations were unable to support replication of the virus to detectable levels, and only a small proportion (5.5% for Ae.aegypti and 1.9% for Ae.albopictus) were able to support efficient virus replication. This led the authors to suggest that a persistent infection may be a reflection of a balance between susceptible and refractory cells within a single culture.

This theory seems quite plausible when it is considered that the majority of insect cell lines have been established from larval or ovarian tissues containing several cell types. It seems that this theory can only be verified when mosquito cell lines are cloned for virus infection experiments as suggested by Singh (1972).

In further studies with SF virus in Ae.albopictus cells Davey and Dalgarno (1974) found that up to 81% of cells were infected, as measured by infectious centre assay, and 100% when measured by immunofluorescent

techniques, 12 hours after infection. A steep fall was then observed until, 48 hours after infection, only 2% of the cells were infected as measured by infectious centre assay, and most of the cells did not fluoresce. They found that the fall in the number of infectious centres appeared to be a reflection of a marked shut-down in virus-specific RNA synthesis at around 12-15 hours after infection.

Later, Buckley *et al* (1975) obtained similar results with the alphavirus CHIK in the *Ae.albopictus* cells. 80-100% of the cells were infected in the first passage of infected cells, but this fell sharply to only 1.4-3.2% of infected cells by the tenth passage.

From all these studies it has been concluded that the establishment of a persistent infection is independent of the initial multiplicity of virus infection, cannot be related to the release of interferon-like activity from the cells, or to the presence of anti-viral activity in infected cells or medium, and cannot be related to changes in total levels or subcellular distribution of the lysosomal enzyme acid phosphatase.

This last conclusion is in contrast to the results obtained by Raghov *et al* (1973, a, b). In electron microscope studies of SF and RR virus infections in *Ae.albopictus* cells it was observed that virus-specific cytoplasmic inclusions lost electron dense material thus forming microvesiculated vacuoles. This occurred at a time when the extracellular virus titre was falling rapidly and the cell membrane appeared free of virus. This was interpreted as the digestion of the inclusion body contents as a result of fusion with lysosomal microvesicles during the establishment of a persistent infection. It was pointed out, however, that virus within inclusion bodies probably did not give rise to extracellular infectivity anyway, and that the fall in the number of infected cells may be

the result of a single effect which is reflected in the two, apparently separate, responses of the formation of multivesicular vacuoles and the absence of virus from the cell membrane.

Further support for the studies suggesting a rapid blocking of virus-specific RNA synthesis was obtained by Artsob and Spence (1974). They found that treatment of Ae.albopictus cells persistently infected with Cocal (COC) virus with actinomycin D resulted in increased virus yields, but that the yield in newly infected cells was unaffected. They suggested that the virus may be present in partially replicated form, and that actinomycin D may be countering a cellular block to viral replication at the ribosomal level, possibly by upsetting a competitive balance between host cell and viral synthetic processes. As a possible alternative they also suggest that their results could be interpreted on the basis of actinomycin D inhibiting the synthesis of interferon-like activity. However, the available literature on viral interference in mosquito cells does not support this view as homologous interference has been observed in mosquito carrier cultures, but little evidence has been found for heterologous interference (Peleg, 1969, 1972; Peleg and Stollar, 1974 and Stollar and Shenk, 1973), or for the production of anti-viral substances such as interferon by infected cells (Peleg, 1969, Murray and Morahan, 1973, Kascsak and Lyons, 1974)

In 1973, Enzmann did describe the production of an interferon-like substance in SIN virus infected Ae.albopictus cells. However, the possibility of homologous virus interference was not examined and attempts to infect carrier cultures with heterologous viruses were not made.

In the most detailed study to date Kascsak and Lyons (1974) used a synthetic double-stranded ribopolymer POLY I: POLY C on a stable carrier culture of CE virus in Ae.albopictus cells. POLY I: POLY C has been shown to be a potent inducer of interferon production in vertebrate cell systems (Field et al, 1967, 1968), but in the mosquito cells no evidence of interference with the replication of the heterologous SF, SIN or VEE viruses was found indicating the absence of interferon-mediated interference. Their results led the authors to suggest that the establishment of a persistent infection in the Ae.albopictus cell line, and by analogy the mosquito vector, may be explained by the absence of an interferon type of defence mechanism.

Other mechanisms of viral interference have also been examined in attempts to explain persistent virus infections in mosquito cells. These mechanisms are auto-interference and interference by temperature sensitive virus strains.

Auto-interference has been observed in vertebrate cells and is a property of defective-interfering (DI) particles, which may be produced in vertebrate cells by serial undiluted passage of virus. The DI particles interfere with normal virus replication by competing for the replication of viral RNA. Although DI particles of SIN and SF viruses have been produced in vertebrate cells (Schlesinger et al, 1972; Levin et al, 1973) they have not been demonstrated in mosquito cells. Indeed, the available evidence suggests that DI particles do not interfere with virus replication in mosquito cells. Igarashi et al (1975) found that when Ae.albopictus cells were infected with DI particles of SIN virus from vertebrate cells they appeared to be either inert or not recognised as defective by the mosquito cells.

Similar results have also been obtained by Eaton (1975)

who found that DI particles of SF virus produced in vertebrate cells did not interfere with standard virus RNA synthesis in Ae.albopictus cells.

The production of temperature sensitive (ts) mutant strains of virus in carrier cultures may also interfere with the yield of standard virus by competing for the replication of viral RNA. Stollar and Shenk (1973) have demonstrated homologous interference between a small plaque (SP) variant of SIN virus produced in Ae.aegypti and Ae.albopictus carrier cultures and the large plaque (LP) wild type strain of SIN virus. It was then demonstrated that this SP variant was temperature sensitive (Shenk et al, 1974; Stollar et al, 1974).

SP variants have been produced in mosquito cells with a number of viruses (see section 1.2.3.5.) but they seem to appear after some time, certainly well after the initial fall in virus titre. It seems unlikely therefore that this type of interference can explain the establishment of a persistent infection although it may play some role in the later modulation of virus production in persistently infected cells.

#### 1.2.3.5. Serial passage of arboviruses in mosquito cells.

Whilst the full nature of persistent virus infections in mosquito cells remains to be determined carrier cultures have been used to study virus production over long periods of time. In addition, virus has been serially passaged through uninfected mosquito cells and the effects on the virus examined.

The value of such studies was first demonstrated by Banerjee and Singh (1969), who obtained CHIK virus from an Ae.albopictus carrier culture. After the seventh serial subculture of infected cells the virus failed to produce death in mice and by the thirty-fifth

passage only traces of virus could be detected in mice. However, virus was readily detected by assay in vertebrate cell culture, so the virus was used to immunise mice. These mice were then subsequently challenged with adult mouse-adapted virus and they withstood the challenge fairly well indicating that the passaged strain of virus had offered some immunological protection.

In another study using CHIK virus Buckley (1971, b) assayed virus produced in serially subcultured Ae.albopictus carrier cultures, in parallel, in mice and vertebrate cell culture. She found a loss of virulence of the virus for mice which was correlated with the appearance in tissue culture of an SP variant. Mice which were inoculated with the SP strain and subsequently challenged with the original LP strain showed increased average survival times (10.8 days) compared to control animals (3.5 days), again indicating some measure of protection.

Extending these studies, Buckley et al (1975) infected numerous vertebrate and invertebrate cells with the LP and SP variants of CHIK virus. Their results indicated that in three mosquito carrier cultures of Ae.albopictus, Ae.aegypti and Ae.w-albus the virus produced in cultures infected with the SP variant continued to resemble the SP type whereas virus produced in the cultures infected with the LP type tended to modify towards the SP type.

In other studies, Sinarachatanant and Olson (1973) described the attenuation of DEN-2 virus in Ae.albopictus carrier cultures and showed a correlation with altered plaque morphology in vertebrate cells.

SP variants of SF virus have been found in Ae.aegypti cells (Peleg, 1971), and in Ae.albopictus cells (Davey and Dalgarno, 1974), and SP variants of SIN virus have also been found in both these cell lines (Peleg and Stollar, 1974; Stollar et al, 1974; Shenk et al, 1974).

Shenk et al (1974) demonstrated that their SP variant of SIN virus was temperature sensitive but they also found that temperature sensitive mutants could be produced in vertebrate cells by undiluted passage of virus at low incubation temperatures. This led them to suggest that the production of SP temperature sensitive variants in mosquito cells may not be a property of the mosquito cell per se, but rather an expression of the low incubation temperature of mosquito cells.

In correlation with these studies on SIN virus Brown and Gleidman (1973) have observed the progeny virus produced in SIN virus infected Ae.albopictus cells using the electron microscope. Three types of virion were found. The first type resembled the original strain and was termed normal, but the other types were smaller and were divided into two groups, mediums, and smalls. Further studies to isolate the three types were under way.

#### 1.2.3.6. Serial subculture of mosquito cells and sensitivity to virus infection.

As mentioned in section 1.2.3.4. the majority of mosquito cell lines have been established from larval or ovarian tissues and contain a mixed cell population which may have widely differing susceptibility to virus infection. This was first recognised by Peleg (1972) when he proposed that EEE and SF viruses may replicate in different target cells within a single culture of his Ae.aegypti cells. Esparaza and Sanchez (1975) also found that different subpopulations of Ae.albopictus cells differed widely in their susceptibility to VEE virus (see section 1.2.3.4.).

It follows that as mosquito cell lines contain mixed cell types a continuous process of adaptation to the culture conditions is taking place which results in the selection of a population of cells best suited

to the culture conditions. The sensitivity and susceptibility of a given culture to virus infection therefore depends on whether the cells best suited to the culture conditions are also susceptible to infection.

The effects of serial passage of cells on virus susceptibility was demonstrated by Johnson (1973) who showed that an Ae.aegypti cell line serially passaged in a mammalian tissue culture medium showed a reduced sensitivity to infection with WN virus. Initially the cells were infected with 1.5 pfu of WN virus, whereas after 70 serial passages infection could not be established with an inoculum of 250 pfu of WN virus.

Other studies have shown that while the cells remain susceptible to infection their sensitivity, as reflected by a cytopathic response to infection, has or is in the process of being altered. Murray and Morahan (1973) observed that WN virus produced little or no CPE in high passage level (sc 176-210) Ae.albopictus cells, although the kinetics of virus growth appeared similar to earlier reports. Similarly, Sinarachatanant and Olson (1973) did not see CPE with DEN-2 virus in Ae.albopictus cells although the growth kinetics were typical. In our laboratory, the development of CPE in the Ae.pseudoscutellaris cells is more clearly defined in low passage level cells than in high passage level cells (Pudney, personal communication).

The value of Singh's (1972) suggestion that cloning of the more promising mosquito cell lines should be attempted was emphasised by Porterfield and deMadrid (1973). They infected a number of clones from an Ae.aegypti cell line with WN virus and found considerable differences in both the virus yield and the production of haemagglutinin between the clones tested.

1.2.3.7. The effect of passage history of arboviruses on virus growth in mosquito cells.

Arboviruses, in nature, are maintained by a cycle between invertebrate and vertebrate hosts, and it is reasonable to assume that there is a heterogenous mixture of virus populations varying in their avidity for vertebrate and invertebrate tissues. Therefore, under laboratory conditions, continuous passage of virus in one, say the vertebrate system, will tend to select a population showing reduced or modified growth characteristics in the other (invertebrate) system.

The only published work to support this is by Igarashi et al (1973). They demonstrated considerable differences in the rates of growth of JE virus strains, which had been maintained by continuous passage in vertebrate tissue culture or in mouse brains, when grown in Singh's Aedes cell lines and also in Peleg's Ae.aegypti cell line.

In addition, extensive studies in our laboratory have demonstrated that the passage history of the virus is an important factor in the growth and production of CPE by arboviruses in our mosquito cell lines.

1.2.3.8. Ultrastructural studies of arboviruses in mosquito cells.

Mosquito cell lines appear to be particularly useful for ultrastructural studies of arboviruses in mosquito cells, as attempts can be made to correlate growth kinetics with observed ultrastructural changes. Although a number of studies have been carried out on the course of virus infection in whole mosquitoes, the major difficulty has been the sequential nature of infection throughout the mosquito. After ingestion of a blood meal containing virus the first cells to become infected are the gut cells, and the last are the salivary

gland cells. In the ultrastructural studies that have been performed on whole mosquitoes (for a review see Murphy et al, 1975) salivary gland infection takes place asynchronously and over a long period of time, and it is not possible to correlate particular changes in ultrastructure with stages in virus replication.

The first report of the use of a mosquito cell line in ultrastructural studies was in 1968 when Filshie and Rehacek studied JE and WN viruses in Grace's Ae. aegypti cells.

More recently, detailed studies have been carried out by a number of workers. Raghow et al (1973 a, b) worked with SF virus in the Ae. albopictus cells and also carried out comparative studies on RR virus growth in both Ae. albopictus and Vero cells. The results in mosquito cells indicated that RR virus matured within large cytoplasmic inclusions and at the cell membrane, whereas for SF virus only a small percentage of cells contained virus-specific structures. This was interpreted to mean that SF virus maturation and release was relatively efficient compared to RR virus.

In 1973, Lyons and Heyduk also described studies on CE virus growth in Ae. albopictus cells. Virus assembly appeared to take place exclusively at internal cytomembrane interfaces, the Golgi complex appearing as the initial assembly site. In a low proportion (7%) of the cells prominent granulofibrillar cytoplasmic masses were seen.

Later, Gleidman et al (1975) reported aspects of SIN virus morphogenesis in Ae. albopictus cells. In virtually all the infected cells maturation occurred within complex vesicular structures. Free nucleocapsids were only rarely seen in the cytoplasm, and complete virions were so rarely seen budding from the cell surface that this could not account for the amount of virus produced by infected cells. The majority of extracellular virus was produced by fusion of the

vesicular structures with the plasma membrane and release of the vesicle contents.

In addition to studies on morphogenesis and release of various viruses, electron microscope studies should also be able to give important information on the early stages of virus infection regarding entry of the virus into the cell. It is worth mentioning a recent study by Webb *et al* (1976) on the pathology of an insect virus Mosquito Iridescent Virus (MIV), in both Singh's and Peleg's *Ae.aegypti* cells. It was observed that virus attached to the cells and was taken into the cell by a process of viropexis (phagocytosis) within 15 minutes after infection. No other process of virion entry was observed. Other insect viruses have been shown to enter the cell by fusion exclusively, or by a combination of viropexis and fusion (for a review see Dales, 1973). Other mechanisms that have been observed in vertebrate cells have been direct passage of the virion through the plasma membrane of HeLa cells by Adenovirus 7, by disintegration of the virion and contiguous plasma membrane with influenza virus in chick fibroblast cells, and via breaks or by dissolution of the plasma membrane with Rauscher Leukaemia virus in mouse embryo fibroblast cells (Dales, 1973). Whether the entry of arboviruses into mosquito cells is solely by viropexis, or whether other mechanisms are important remains to be seen.

#### 1.2.4. The use of mosquito cell lines for the primary isolation of arboviruses.

The absence of a CPE in the majority of mosquito cell lines infected with arboviruses has limited the use of these cell systems for primary isolation work. However, several successful attempts have been made to use those cell lines which do show a CPE for virus isolation from original field materials.

In 1969, Singh and Paul used the Ae.albopictus cell line to isolate the four serotypes of DEN virus from both human sera and mosquitoes, on the basis of the characteristic CPE produced by these viruses in the cells. Singh (1972) stated that these cells had proved a very convenient tool for the rapid isolation and identification of the DEN virus serotypes during epidemics in India.

DEN-2 virus was also isolated from infected human serum in the Ae.pseudoscutellaris cells (Varma et al, 1974) and subsequently, these cells were also used for the isolation of YF virus strains that failed to produce CPE in Ae.albopictus cells (Varma et al, 1976).

Some data has been obtained that suggests that mosquito cells may be superior to other systems for the isolation of certain viruses. In a laboratory study, Paul and Singh (1969) compared the sensitivity of the Ae.albopictus and Ae.aegypti cell lines to both Vero cells and suckling mice for the infection with a number of viruses. The Ae.albopictus cells proved to be slightly more sensitive to infection with CHIK, JE, WN, and DEN-2 viruses but less sensitive to infection with Batai (BAT) and CHP viruses than the vertebrate systems. The Ae.aegypti cell line proved to be the least sensitive system.

Similar findings were obtained with field isolations by Chappell et al (1971). They found that the Ae.albopictus cells were superior to both infant mice and LLC-MK2 cells for the isolation of DEN virus from human blood and mosquitoes during the 1969 epidemic in Puerto Rico.

In 1976, Varma et al also found that the Ae.pseudoscutellaris cell line was superior to both Vero cells and mice for the isolation of YF virus strains from field materials.

1.2.5. The use of mosquito cell lines in serological tests.

Neutralisation tests (NT) in tissue culture depend on the production of a CPE or plaques in the cell system by the virus under study. The production of CPE or plaque formation can then be inhibited or prevented by pre-treatment of the virus with specific antisera.

In practice neutralisation tests by inhibition of CPE in mosquito cells are restricted as only a few viruses will produce CPE in mosquito cells. With selected viruses however, promising results have been obtained. Paul *et al* (1969) inhibited the production of CPE in Ae.albopictus cells infected with JE virus by treatment with JE virus antiserum. Similarly, Varma *et al* (1974) completely blocked CPE production by WN virus in Ae.malayensis and Ae.pseudoscutellaris cells by treatment with specific antisera.

In the only field trial however, Singh and Paul (1969) failed to identify the DEN virus serotypes by NT in Ae.albopictus cells due to the fact that heterologous neutralisation occurred between the serotypes by antisera prepared against each virus.

In 1975, Yunker and Cory successfully carried out plaque reduction NT in Ae.albopictus cells and currently this would appear to be the system of most promise for NT in mosquito cells.

In 1969, Pavri and Ghosh demonstrated that tissue culture fluid from DEN virus infected Ae.albopictus cells could be used directly in complement fixation (CF) tests. Subsequently, Singh and Paul (1969) used this method during the primary isolation and identification of the DEN virus serotypes.

CF antigens of CHP and JE viruses have been found in infected Ae.albopictus cells and also Ae.aegypti cells (Ghosh *et al*, 1973). In the JE virus infected cells the CF antigens were found in both intracellular

and extracellular fluid, whereas in the CHP virus infected cells CF antigens were retained intracellularly. Infection with higher doses of CHP virus resulted in the production of less CF antigen, but this was not observed with higher doses of JE virus.

Ajello *et al* (1975) also demonstrated CF antigen of WN virus in an acetone extracted fluid phase from infected Ae.albopictus cells.

Specific haemagglutinin was detected, after extraction, in primary cell cultures of Ae.aegypti infected with EEE virus, but not with SF or WN viruses (Peleg, 1968). Similar studies with Ae.albopictus cells (Ghosh and Bhat, 1971) showed that uninfected cells also produced haemagglutinating activity. This was exclusively extracellular and the authors concluded that this problem precluded the use of the Ae.albopictus cell line in this technique.

Johnson (1973) described a potential application of mosquito cells for use in serological surveys. He investigated the growth of infectious virus-antibody complexes in both mosquitoes and mosquito cell lines, and his results indicated that these complexes grew significantly less well than controls. Further, this inhibition of growth was very sensitive to antibody in test sera, and he suggested that this system could be used to screen test sera that had proved negative in mammalian systems. Also from these studies he was able to make some interesting speculations on the epidemiology of the WN-JE-SLE group of flaviviruses and, in particular, to suggest possible interactions between DEN and YF viruses.

#### 1.2.6. Infection of mosquito cell lines with mammalian pathogenic viruses other than arboviruses.

Several attempts have been made to infect mosquito cell lines with vertebrate pathogenic viruses, but consistently the cells have been found to be refractory to infection with all the viruses tested.

Peleg (1969) demonstrated that his Ae.aegypti cell

line was not infected with naked RNA from polio type 1 or encephalomyocarditis (EMC) viruses.

In his 1972 review, Singh quotes the negative results obtained after attempted infection of his Aedes cell lines with polio, EMC, coxsackie B 5 and hepatoencephalocarditis viruses (Singh and Paul, 1968), with Lassa virus (Buckley and Casals, 1970) and with Junin and Portillo viruses (Mettler and Buckley, 1971).

Of particular interest is the attempted infection of Ae.aegypti cells with human type 5 adenovirus by Shortridge, Pudney and Varma (1972). They detected levels of adenovirus soluble antigen in the mosquito cells comparable with levels found in HeLa cells. However, these potential capsid components were exclusively intracellular in the mosquito cells and no nucleoprotein was detected. This led the authors to suggest that this abortive infection of a mosquito cell line by adenovirus type 5 may be of use to study the mechanism of virion assembly and that the exclusive intracellular concentration of soluble antigens may suggest that similar studies in other mosquito cell lines may require re-evaluation.

### 1.3. Cell cultures from poikilothermic vertebrates and their infection with arboviruses and other viruses.

#### 1.3.1. Cell cultures from fishes.

The culture of tissues from fishes is, like arthropod tissue culture, a relatively recent development. In 1957, Wolf and Dunbar demonstrated that tissues from three species of trout, and from the goldfish Carassius auratus could be grown in Eagle's mammalian tissue culture medium. This was an important development and led to the subsequent establishment of several cell lines from freshwater fishes.

The first studies on the culture of cells from marine fishes were carried out by Clem et al (1961),

using tissues from the yellow striped grunt and several other species. However, to date relatively few cell lines have been established from marine species (Lee and Loh, 1975).

There have only been a few studies on the infection of fish cell lines with arboviruses. Officer et al (1964) showed that a cell line from rainbow trout gonads (RTG) supported the growth of VEE and EEE viruses with the production of a CPE. EEE virus produced more extensive CPE than VEE virus, and a persistent infection was obtained with VEE virus, but not with EEE virus.

In 1970, Nims et al infected cell lines from steelhead trout embryos (STE-137) and chinook salmon embryos (CHSE-114) with WEE virus amongst a range of other non-arboviruses. Both cell lines were normally maintained at between 18° and 23°C, but at this temperature WEE virus multiplication was very limited. At the elevated temperature of 26°C WEE virus multiplied quite well producing CPE in both cell lines.

Further reports have been along the lines of initial characterisation of cell lines, and usually only one or two arboviruses have been tested along with other representative non-arboviruses. For example, Solis and Mora (1970) found that SIN virus multiplied in the fathead minnow cell line along with many other viruses

Similar studies on marine fish cell lines have shown that these cell lines appear to be refractory to infection with all the mammalian viruses tested including some arboviruses (Lee and Loh, 1975).

Continuous fish cell lines have been used mainly for the examination of the growth of fish viruses. The first isolation of a viral agent from fishes was that of infectious pancreatic necrosis (IPN) virus from trout (Wolf et al, 1960). To date, there have only been a few additional agents isolated from fishes and these are; channel catfish virus (CCV) (Wolf and Darlington, 1971), a small cytoplasmic polyhedral virus of eels (EV-1) (Wolf and Quimby, 1973) and five

rhabdoviruses; viral haemorrhagic septicaemia (VHS) of rainbow trout (Zwillenberg et al, 1971), also known as Egtved virus, infectious haematopoietic necrosis (IHN) (Amend et al, 1969), spring viraemia of carp (SVC) (Fijan et al, 1971), swim bladder inflammation of cyprinids (SBI) (Bachmann and Ahne, 1973), and pike fry disease (PFD) (DeKinkelin et al, 1973).

In a recent report Hill et al (1975) present data that suggests that SVC and SBI viruses are, in fact, identical. Further studies with these rhabdoviruses (Roy et al, 1975; Clerx et al, 1975; Hill et al, 1975) in fish cell lines have provided the first comparative data for these viruses to the results obtained for the growth of rhabdoviruses of the Rabies and VSV serogroups in mammalian systems.

In 1974, Clark and Soriano found that SVC, IHN and VHS viruses would multiply in a number of mammalian, reptilian and fish cell lines. They suggested that the efficiency of fish rhabdovirus replication in BHK-21 cells could offer a system for comparative study on nucleic acid and protein metabolism, as well as virion morphogenesis, as BHK-21 cells are the type most often used for the study of the VSV and Rabies group rhabdoviruses.

#### 1.3.2. Cell cultures from amphibians and reptiles.

Unlike arthropod and fish cell culture, cell culture of amphibians and reptiles has a history as old as the history of tissue culture itself, as frog embryonic tissues were used for the first studies on the in vitro culture of cells (Harrison, 1907). Pudney (1973) has traced in detail the early and recent history of cell culture in this field, and so this section of the review will deal solely with aspects of the infection of amphibian and reptilian cells with various viruses.

Studies of the growth of viruses in these poikilothermic vertebrate cell lines are of particular interest since Reeves (1961, 1974) pointed out that these vertebrates may have a role in the overwintering

of arboviruses in temperate regions.

In 1967, Clark and Karzon carried out studies with arboviruses and other viruses in a cell line from the box turtle Terrapene caroline. They successfully propagated VSV serially in these cells at 37°C, and obtained evidence of autointerference. SIN virus caused non-passageable cytopathic or cytotoxic effects, and nine amphibian viruses all produced extensive CPE at 23°C.

Later (Clark et al, 1970), 11 cell lines which had been established from lizards and turtles were tested with a range of viruses including SIN and VSV. SIN virus produced CPE in six of the reptilian cell lines tested and VSV in seven. In addition, the results indicated that the reptile cell lines were highly susceptible to infection with a range of other non-arboviruses.

The first cell line from a snake was established by Zeigel and Clark (1971), from a large myxofibroma of the spleen of the viper Vipera russelli. The cell line (VSW) possessed many properties of tumour cells and produced large quantities of C-type virions (Zeigel and Clark, 1971; Gildeen et al, 1970). Another cell line (VH2) was subsequently established from the heart of a normal female viper (Clark et al, 1973), and comparative studies on these two cell lines showed that rabies virus and VSV replicated more efficiently in the VSW cell line than in the VH2 cell line. The VSW cell line was also shown to be refractory to several viruses that grew in the VH2 cell line.

Extending his studies on rabies virus, Clark (1972) found little or no evidence for rabies virus growth in several fish or amphibian cell lines. Five reptilian cell lines tested ranged in susceptibility from the unsusceptible iguana line (IgH2) to the highly susceptible viper cell line (VSW). Rabies virus serially passaged in VSW cells became virtually apathogenic for mice, but remained highly immunogenic. Comparable passage of rabies virus in gecko cells (GL1) showed similar attenuation by passage 40, but a reduced

immunogenicity was also observed.

In 1974, Michalski et al also studied the infection of gecko cells with several viruses, and observed a CPE with VSV and SIN virus, as well as transformation of the cells induced by SV 40 virus.

The growth of three tick-borne arboviruses, QRF, LI and LGT in primary cultures from the frog Rana temporaria was demonstrated by Pudney and Varma (1971). No CPE was seen in the cells and serial passage of LI virus over 11 passages did not result in any change in the pathogenicity of the virus for mice.

In 1975, Leake described preliminary results on the use of a cell line from the toad Xenopus laevis for the plaque assay of four arboviruses SIN, SF, ONN and CHIK. This cell line has subsequently been used for the plaque assay of a range of arboviruses and for virus assay by CPE production (Leake, Varma and Pudney, 1977). To our knowledge this is the first time that amphibian cells have been successfully used for arbovirus assay.

Other studies, on tortoise cells, have shown the likely presence of an interferon-like inhibitor. Falcoff and Fauconnier (1965) detected the presence of a viral inhibitor in these cells using parainfluenza type 1 as an inducing virus. Galabov et al (1973) also induced interferon-like activity in tortoise cells using SIN and WN virus, and in one other report Clark (1972) found that the VSW viper cell line failed to produce interferon on infection with rabies virus.

In general, cell lines from poikilothermic vertebrates are proving very useful in the developing study of amphibian and reptilian viruses. Due, in some cases, to their wide susceptibility, these cell lines provide 'an opportunity for the virologist to study the growth of homeothermic vertebrate viruses in cells of hosts far removed phylogenetically from the normal host, and at temperatures below those of their normal hosts' (Clark and Karzon, 1969).

#### 1.4. Aims of the work.

Research in this laboratory, over the last ten years, has resulted in the establishment, and routine maintenance, of a number of cell lines from various arthropod and poikilothermic vertebrate species. Various studies have been carried out on these cell lines, but the work presented in this thesis has been essentially concerned with the growth of arboviruses in some of these cell lines, with the comparative susceptibility of the cell lines to infection with a range of arboviruses, and with certain applied aspects of arbovirus infection of these cell lines.

Much of the published information on the infection of mosquito cell lines has come from studies on relatively few cell lines derived mainly from Aedes species mosquitoes. There was thus a need to carry out comparative studies in other mosquito cell lines, as pointed out by Singh (1972). Since both Aedine and Anopheline cell lines had been established in this laboratory, I had the opportunity to carry out such comparative studies.

As poikilothermic vertebrates may play a role in the overwintering of arboviruses in nature, it is surprising that cell lines from poikilothermic vertebrates have not been extensively used for in vitro studies. An amphibian cell line was therefore included in this work, to provide information on the application of amphibian cells to arbovirus studies, and also to provide a vertebrate system for comparison to the arthropod cell lines used.

## 2. MATERIALS AND METHODS.

### 2.1. Cell lines and their maintenance.

#### 2.1.1. Mammalian cell lines and their maintenance.

##### 2.1.1.1. Mammalian cell lines.

For virus assay three mammalian cell lines were used:

A) PS cells. These are a stable line of pig-kidney cells originally supplied to Dr B. K. Johnson, at the School, by Dr J. S. Porterfield, National Institute for Medical Research, Mill Hill, London. The cells are routinely maintained in Leibovitz L-15 medium (Leibovitz, 1973), supplemented with 3% or 5% foetal bovine serum (FBS), which had been heat inactivated at 56°C for thirty minutes. The cells are subcultured by a split ratio of 1:4 to 1:15 as required.

In this study the PS cells were used for the assay of a number of the flaviviruses, and also for the assay of Sandfly Fever virus.

B) Vero cells. The African green monkey kidney (Vero) cell line was also supplied by Dr J. S. Portfield. The cells are maintained in L-15 medium with 5% FBS and are subcultured by a split of 1:4 to 1:12 as required.

These cells were used for the assay of the majority of the alphaviruses studied, some of the flaviviruses, most of the bunyaviruses, and the rhabdovirus CHP.

C) BHK-21 cells. The baby hamster kidney (BHK-21) cell line was supplied by Ms Hilary Way, Microbiological Research Establishment, Porton, Wilts. In this laboratory the cells were maintained in L-15 medium with 5% FBS, and were subcultured by a split of up to 1:20. This cell line was not used extensively as, after a number of subcultures, confluent monolayers were not produced.

The cells were used solely for the assay of the alphavirus ONN, as they proved more sensitive than Vero

cells for the assay of this virus.

#### 2.1.1.2. Maintenance of the mammalian cell lines.

Handling of the routine cell cultures was carried out in a Microflow (Microflow Ltd, Fleet, Hants) positive pressure work station, or in a Microflow recirculation work station.

All the mammalian cell lines were grown at 37°C, in 250ml flat glass prescription bottles with screw caps, in approximately 20ml of complete growth medium (see below).

Powdered L-15 medium (Flow Laboratories Ltd, Ayrshire, Scotland) was reconstituted with glass distilled water, and sterilised by positive pressure filtration through Millipore 47mm or 142mm filtration systems (Millipore Corporation, Bedford, Massachusetts, USA), using a filter with a pore size of 0.22 $\mu$ . This stock liquid was then supplemented with 10% tryptose phosphate broth (TPB) (Gibco-Biocult Ltd, Glasgow, Scotland), and this working solution was then stored at 4°C. Prior to use the required concentration of FBS (Gibco-Biocult Ltd), and antibiotics (penicillin 100 units/ml and streptomycin 100  $\mu$ g/ml) were added.

Subculture of the mammalian cells was carried out by first decanting the medium from a bottle containing a confluent cell sheet. The cell sheet was then rinsed with 20ml of warmed phosphate buffered saline (PBS) (Dulbecco A saline, without calcium or magnesium salts, Oxoid Ltd, London). This was then decanted, 16ml of 0.025% trypsin/ versene solution (see Appendix) added, and the cells incubated at 37°C for about two minutes. The trypsin/ versene solution was then decanted and the bottle returned to the incubator for up to fifteen minutes by which time the cells had detached from the glass. The cells were then suspended in the required volume of fresh growth medium, dispensed into clean bottles, and returned to the incubator.

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2.1.2. Poikilothermic cell lines and their maintenance.

2.1.2.1. Nomenclature of poikilothermic cell lines.

Recently, the code numbers of the cell lines used in this study have been re-designated along the guidelines suggested by the Tissue Culture Association Committee on Nomenclature. Throughout this thesis, the cell lines are referred to by their original code numbers (see below), and the new code numbers are given here for future reference:

Ae.aegypti (20A) re-designated LSTM-AA-20A.

An.stephensi (43) re-designated LSTM-AS-43.

An.gambiae (55) re-designated LSTM-AG-55.

Ae.malayensis (60) re-designated LSTM-AM-60.

Ae.pseudoscutellaris (61) re-designated LSTM-AP-61.

Xenopus laevis (XTC-2) re-designated LSTM-XL-2.

2.1.2.2. Mosquito cell lines.

Five mosquito cell lines were used during this study:

- A) A cell line (20A) established from Aedes aegypti larval tissues (Varma and Pudney, 1969).
- B) A cell line (43) established from Anopheles stephensi larval tissues (Pudney and Varma, 1971).
- C) A cell line (55) established from Anopheles gambiae larval tissues (Marhoul and Pudney, 1971).
- D) A cell line (60) established from Aedes malayensis larval tissues (Varma, Pudney and Leake, 1974).
- E) A cell line (61) established from Aedes pseudoscutellaris larval tissues (Varma, Pudney and Leake, 1974).

The Aedine cell lines are maintained in MM/VP<sub>12</sub> medium (Varma and Pudney, 1969) and the Anopheline cell lines in MK/VP<sub>12</sub> medium (Pudney and Varma, 1971) (Appendix).

#### 2.1.2.3. Lower vertebrate cell lines.

Only one lower vertebrate cell line was used. This cell line (XTC-2) was established from a metamorphosing tadpole, with four legs and a half-digested tail, of the South African clawed toad Xenopus laevis (Pudney, Varma and Leake, 1973). The cell line is maintained in L-15 medium with 10% FBS.

#### 2.1.2.4. Maintenance of poikilothermic cell lines.

The techniques used for the maintenance of these cell lines have already been described in detail in the original publications. The only alteration in technique is that enzymatic dispersion of the cells is not employed during routine maintenance.

All the cell lines are grown at 28°C in 1oz flat glass prescription bottles closed with silicone rubber bungs. Weekly subcultures are carried out by scraping the cells from the glass, into the required volume of fresh growth medium, using a rubber policeman. Effective breakdown of the cell clumps is achieved by vigorous pipetting of the cell suspension with a pasteur pipette. The required volume of cell suspension is then dispensed into clean bottles containing fresh medium to make up a final volume of 3ml.

#### 2.1.3. Determination of growth curves of cell lines.

The six poikilothermic cell lines were subcultured into 1oz bottles containing either routine growth medium or L-15 medium with 10% FBS. On each day after seeding the medium was decanted from two bottles from each series, and the cells were treated with trypsin/ versene solution as described in section 2.1.1.2. to assist breakdown of the cell clumps. The cells were then suspended in suitable volumes of PBS or medium and then counted in a Neubauer haemocytometer chamber. Mean cell numbers were calculated from at

least ten sets of counts taken from each bottle, and the total number of cells per bottle was then calculated.

#### 2.1.4. Isoenzyme analysis of poikilothermic cell lines.

Isoenzyme analysis of the cell lines used in this study, and of other cell lines maintained in this laboratory, was carried out by Dr Mary Pudney of the Department, in collaboration with Dr Parr and Ms C. Swindlehurst of the London Hospital Medical School.

The results show that all the cell lines can be readily distinguished by their individual isoenzyme patterns (Pudney, personal communication).

#### 2.1.5. Tests for contamination of the poikilothermic cell lines with mycoplasmas.

All the poikilothermic cell lines were tested by Dr R. H. Leach of the Mycoplasma Reference Laboratory, Colindale, London. Using standard techniques for the detection of vertebrate mycoplasmas and additional test incubations at 28°C, all the cell lines were found to be free from detectable mycoplasma contamination.

### 2.2. Viruses and methods of infection.

#### 2.2.1. Virus stocks.

A total of 47 viruses were used during this study and details of these viruses, including strain, passage level, and titre of the stock material in cell culture are shown in Tables 1 a, 1 b, and 1 c. Stock materials are held at -70°C, as suckling mouse brain suspension, tissue culture fluid or original field materials.

TABLE 1 a.  
DETAILS OF VIRUS STOCKS.

VIRUS	ABBR.	STRAIN	PASS LEVEL	TITRE IN CULTURE	TISSUE DEX/ML	CELL SYSTEM
ANOPHELES A	ANA	Prototype	17	6.8 pfu		Vero
ARUMOWOT	AMT	AR 1284-64	3	8.2 CPD <sub>50</sub>		XTC-2
BATAI	BAT	MM 2222	17	7.6 pfu		XTC-2
BEBARU	BEB	MM 2354	13	9.1 pfu		Vero
BUNYAMWERA	BUN	Original	20	8.9 pfu		Vero
BWAMBA	BWA	M 459	1	7.1 pfu		Vero
CALIFORNIA ENCEPHALITIS	CE	BFS 283	12	6.9 pfu		Vero
CALOVO	CVO	184	9	7.6 pfu		Vero
CHANDIPURA	CHP	I 653514	1	9.2 pfu		Vero
CHIKUNGUNYA	CHIK	E 103	4	6.0 pfu		XTC-2
DENGUE-2	DEN-2	New Guinea	34	6.7 pfu		PS
DUGBE	DUG	AR 1792	12	6.5 CPD <sub>50</sub>		XTC-2
ENTEbbe BAT	ENT	IL 30	1	7.8 pfu		PS
GANJAM	GAN	G 619	10	5.2 CPD <sub>50</sub>		XTC-2
GERMISTON	GER	AR 1050	12	9.2 pfu		Vero
GETAH	GET	MM 2021	8	8.7 pfu		XTC-2
JAPANESE ENCEPHALITIS	JE	Nakayama	1	8.9 pfu		PS

All abbreviations and antigenic groups of the viruses used are listed in the 'International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates', (1975, Ed. T.O.BERGE.).

TABLE 1 b.  
 DETAILS OF VIRUS STOCKS

VIRUS	ABBR.	STRAIN	PASS LEVEL	TITRE IN TISSUE CULTURE DEX/ML	CELL SYSTEM
KERN CANYON	KC	M 206	4	7.7 CPD <sub>50</sub>	XTC-2
KETERAH	KTR	P6-1361	8	6.8 pfu	XTC-2
LAGOS BAT	LB	Original	13	7.5 CPD <sub>50</sub>	XTC-2
LANGAT	LGT	TP 64	6	8.9 pfu	PS
LANJAN	LJN	TP 94	6	3.5 CPD <sub>50</sub>	XTC-2
LOUPING ILL	LI	369T2	4	8.2 pfu	PS
MAYARO	MAY	TR 4675	11	8.4 pfu	XTC-2
MIDDELBURG	MID	AR 749	16	7.8 CPD <sub>50</sub>	XTC-2
MODOC	MOD	M 544	10	8.0 pfu	PS
MOUNT ELGON BAT	MEB	BP 846	7	4.9 CPD <sub>50</sub>	XTC-2
NDUMU	NDU	AR 2211	9	7.9 pfu	XTC-2
NTAYA	NTA	Original	5	6.8 CPD <sub>50</sub>	XTC-2
NYANDO	NDO	MP 401	7	5.7 pfu	Vero
O'NYONG-NYONG	ONN	Ahero	12	6.7 pfu	BHK
PIRY	PIRY	An 24232	1	9.0 pfu	Vero
PONGOLA	PGA	AR 1	8	5.7 pfu	Vero
PUFFIN ISLAND	*	*	3	5.4 pfu	XTC-2

\* Unregistered virus isolated in this laboratory from  
Ornithodoros maritimus ticks.

TABLE 1 c.  
 DETAILS OF VIRUS STOCKS.

VIRUS	ABBR.	STRAIN	PASS LEVEL	TITRE IN CULTURE	TISSUE DEX/ML	CELL SYSTEM
QUARANFIL	QRF	AR 1095	23	6.9 pfu		XTC-2
SANDFLY FEVER	SFS	Sicilian	1	6.2 pfu		PS
SEMLIKI FOREST	SF	DB 478 MH	13	8.0 pfu		Vero
SINDBIS	SIN	AR 339	1	7.2 pfu		XTC-2
TAHYNA	TAH	CZ 92	18	7.4 pfu		Vero
TEMBUSU	TMU	*	*	10.2 pfu		61
UGANDA S	UGS	Original	12	7.8 pfu		PS
URUMA	URU	‡	‡	7.2 pfu		Vero
WEST NILE	WN	E 101	10	7.4 pfu		Vero
WHATAROA	WHA	M 78	6	8.5 CPD <sub>50</sub>		XTC-2
YELLOW FEVER	YF	17D	24	7.4 pfu		Vero
ZIKA	ZIKA	MR 766	4	7.6 pfu		PS
ZIRQA	ZIR	A 2070-1	4	6.1 pfu		XTC-2

\* Primary isolation in mosquito cells.

‡ URU virus is now considered to be a strain of MAYARO virus (Russell.P.K. Personal communication in the International Catalogue of Arboviruses. p.470.).

2.2.2. Determination of virus growth curves and of the amount of virus adsorbed to the cells.

For infection experiments, the mosquito and toad cells were subcultured into 1oz bottles containing 3ml of L-15 medium with 10% FBS as growth medium. The split ratios of the various cell lines were adjusted so that all the cell lines would produce confluent monolayers simultaneously, ready for infection. The L-15 medium was then removed from confluent cell sheets and replaced with 2.7ml of fresh L-15 medium with 2% FBS as maintenance medium. L-15 with 2% FBS was used throughout the infection experiments for the maintenance of the infected cells.

Stock virus suspensions were diluted decimally in maintenance medium, and 0.3ml of the required virus dilution was added to duplicate bottles of each cell line, and the bottles were gently rocked to disperse the virus suspension over the cells. In all cases part of the inoculum was stored at  $-70^{\circ}\text{C}$  for later titration.

Virus adsorption was allowed to proceed for three hours at  $28^{\circ}\text{C}$ , after which the medium was discarded and the cell sheets thoroughly rinsed, three times, with 3ml volumes of PBS or maintenance medium. In a number of experiments the third wash was stored at  $-70^{\circ}\text{C}$  for later titration of residual virus. Finally, all the bottles were replaced with 3ml of fresh maintenance medium, and returned to the  $28^{\circ}\text{C}$  incubator.

To determine the amount of adsorbed virus, the final part of this infection technique was modified. After the cells had been rinsed three times, they were scraped down into 1ml of PBS using a rubber policeman. The cell suspension was then rapidly frozen in a methanol-dry ice bath and thawed at  $37^{\circ}\text{C}$ . Three cycles of freezing and thawing were performed, and this effectively disrupted the cells releasing adsorbed virus for assay.

To determine the amount of extracellular virus (EV) produced by infected cultures the maintenance medium was harvested at intervals over 14 or 21 day incubation periods. Harvesting was carried out by removing all the medium from infected cultures and replacing with fresh maintenance medium. The harvested material was stored at  $-70^{\circ}\text{C}$  in plastic ampoules or bijou bottles. Cell associated virus (CAV) was not assayed.

Uninfected control cultures were harvested and replaced in the same way, and at irregular intervals medium from control cultures was assayed in cell culture or mice to ensure that the cell lines were free from contaminating viral agents.

During the earlier infection experiments harvests were carried out on days 1, 2, 3, 5, 7, 9, 11, 14, 17, and 21 after infection, but this was simplified in later experiments to days 1, 2, 3, 6, 8, 10 and 14. For uniformity, the virus titres shown in the graphs have been plotted up until 14 days. Values beyond this time are given in the text.

#### 2.2.3. Minimum infectious dose experiments.

Cells prepared as described above were infected with serial decimal dilutions of virus. The range of the virus dilutions tested was estimated from the titres of the stock virus suspensions in Tables 1 a-c, and the highest dilution of virus inoculated was always 1 dex higher than the terminal dilution indicated in the tables. For example, in experiments with SF virus (see Table 4) the virus titre in Vero cells was 8.0 dex pfu per ml. The highest virus dilution used to inoculate the cells was therefore  $10^{-9}$ . Bottles were harvested only on days 3 and 10 after inoculation and the results of virus assay of these harvests were compiled into composite tables (see section 3.2.2.).

### 2.3. Virus assay.

#### 2.3.1. Virus assay in tissue culture.

Virus was assayed by the method of deMadrid and Porterfield (1969). Decimal dilutions of virus suspensions or harvested tissue culture fluid were prepared in ice-cold medium, and 0.2ml aliquots of the dilutions placed into wells of ultra-violet light sterilised standard World Health Organisation haemagglutination (HA) trays. Aliquots of fresh medium were added to wells as controls.

Cell suspensions of the mammalian cell lines were prepared as described earlier. The concentration of cells in suspension was estimated by counting in a Neubauer haemocytometer chamber, and the number of cells in the suspension adjusted to give a final concentration of  $3 \times 10^5$  cells per ml for PS and BHK-21 cells, and  $4 \times 10^5$  cells per ml for Vero cells.

0.2 ml aliquots of the required cell suspension were then added to each tray well, the trays were placed in sterile gamma-irradiated (Irradiated Products Ltd, Swindon, Wilts) polythene bags, and incubated at  $37^{\circ}\text{C}$  for at least three hours. After this initial incubation period, the trays were removed from the polythene bags and 0.4ml of overlay medium (see Appendix) was added to each well. The trays were then placed in fresh bags and returned to the incubator.

This technique, using HA trays, was used for the assay of WN, JE, YF, SF, SIN, and ONN viruses. Subsequently, and for a major part of the work, virus titrations were performed using plastic disposable tissue culture trays. These trays, Linbro Dispo-trays no. FB-16-24-TC (Linbro Chemical Co. Ltd. New Haven, Conn, USA) contain 24 wells, and are supplied sterile, with a plastic lid. These trays possess a number of advantages over HA trays, with excellent optical properties and larger well capacity and can be easily opened and closed using the plastic lid. These factors combine to give improved ease of handling, reducing chances of contamination and

gives results more easily and consistently.

Using Linbro trays, the unit volume of the assay technique was increased to 0.5ml for both virus and cell suspensions. 1ml of overlay medium was added to each well, resulting in a final volume of 2ml per well.

Both HA trays and Linbro trays were stained to reveal plaques as described by deMadrid and Porterfield (1969). The trays were placed in specially designed perspex boxes and flooded with 0.85% saline to remove the overlay from the cells. The saline was then decanted and naphthalene-black stain (see Appendix) was added. The stain was allowed to remain over the cells for at least thirty minutes, it was then decanted, the trays rinsed in tapwater, and allowed to air-dry. Trays were examined for the presence of plaques, and the virus titres calculated as described by deMadrid and Porterfield (1969). Titres are expressed as dex plaque forming units (pfu)/ml unless otherwise indicated.

Incubation periods for the assay of different viruses was essentially similar to those determined by other workers (deMadrid and Porterfield, 1969; Stim, 1969; Johnson, 1973).

HA trays were re-used by removing the stained cells from the perspex using undiluted sodium hypochlorite (Chlorox) solution, followed by routine washing for tissue culture glassware. Linbro trays were usually discarded but could be re-used by soaking the trays overnight in a 1% solution of Decon 90 (Decon Laboratories Ltd. Brighton), followed by an overnight soak in a solution of a domestic washing powder 'Ariel', followed by routine washing. Both HA and Linbro trays were sterilised by exposure to ultra-violet light, and placed in sterile polythene bags ready for use.

### 2.3.2. Virus assay in mice.

Some virus samples were titrated by intracerebral inoculation of 0.01ml aliquots of decimally diluted virus suspensions into 2-3 day old mice. The LD<sub>50</sub> was calculated by the method of Thompson's moving averages (Thompson, 1947) and titres are expressed as dex LD<sub>50</sub>/0.01ml.

### 2.3.3. Accuracy of the plaque assay system.

To determine the accuracy of the plaque assay system, titrations were performed in parallel in HA and Linbro trays, using SF virus as a marker. Numerous replicate samples were titrated, and the lowest dilution of virus in which plaques could be counted easily was taken. This was usually the penultimate dilution in which plaques were present. The titres obtained were expressed in dex pfu per ml and the results were analysed statistically.

Using HA trays with 25 0.4ml samples, the virus titre was calculated to be 7.97 dex pfu per ml, with a standard error of 0.85 dex. The comparable result in Linbro trays with 25 1ml samples was 8.09 dex pfu per ml, with a standard error of 0.69 dex.

### 3. RESULTS.

#### 3.1. Morphology and growth characteristics of the cell lines.

Since all the infection experiments were performed using cells grown in L-15 medium, an initial comparison was made between cells grown in their usual growth medium and L-15 medium.

##### 3.1.1. 20A cells.

The 20A cells grown in MM/VP<sub>12</sub> medium have an epithelial-type morphology and form clumpy cell sheets (Plate 1) which, even after 14 days growth, remain firmly attached to the glass. Transfer into L-15 medium resulted in a change to a fibroblast-type appearance similar to that described by Johnson (1973). After 14 days growth in L-15 medium the cells tend to detach more readily, and large clumps of apparently healthy cells float freely in the medium.

Cells at sc 302 were studied for growth rates in the two media, and the results are shown in Fig 1 a. In both media growth was faster than that obtained by Johnson (1973) using cells at sc 136. A maximum number of  $18 \times 10^6$  cells per bottle was obtained in MM/VP<sub>12</sub> medium, some 2-3 times higher than that observed by Johnson. My results with L-15 medium confirm Johnson's finding that the growth of the 20A cells was poorer in this medium than in MM/VP<sub>12</sub>. The peak cell number in L-15 was about  $8.5 \times 10^6$  cells per bottle.

In the logarithmic phase of growth, cells in both media had a population doubling time of about 24 hours.

##### 3.1.2. 43 cells.

The 43 cell line is grown in MK/VP<sub>12</sub> medium and has a mixed population of epithelial and fibroblast-type cells which form dense clumpy cell sheets (Plate 2). Transfer into L-15 medium did not result in any changes

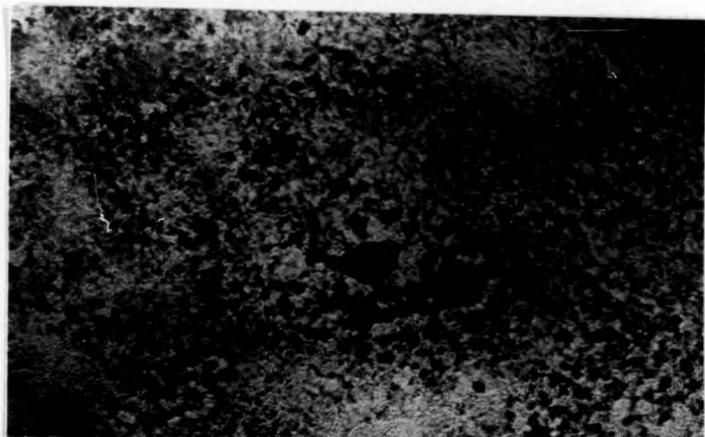


Plate 1. 20A cells, sc 302 6 days after seeding into a glass bottle. Live x 85.

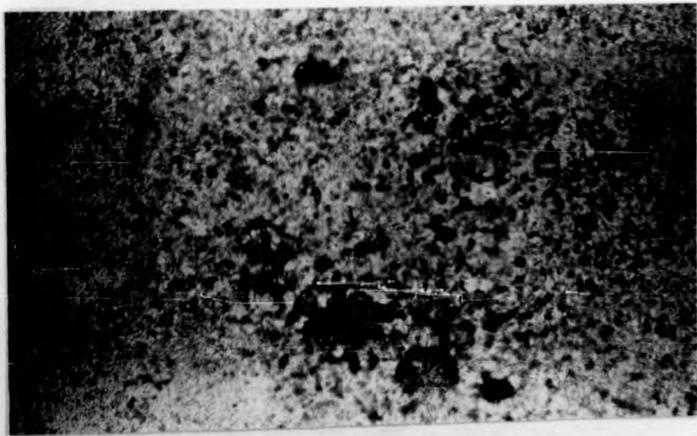


Plate 2. 43 cells, sc 263 5 days after seeding into a glass bottle. Live x 85.

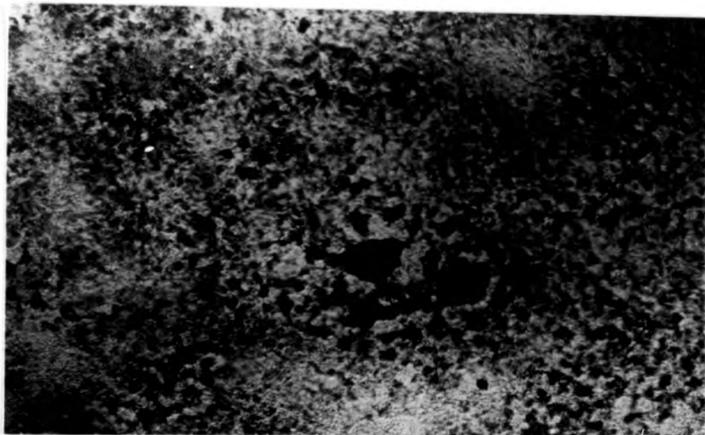


Plate 1. 20A cells, sc 302 6 days after seeding into a glass bottle. Live x 85.

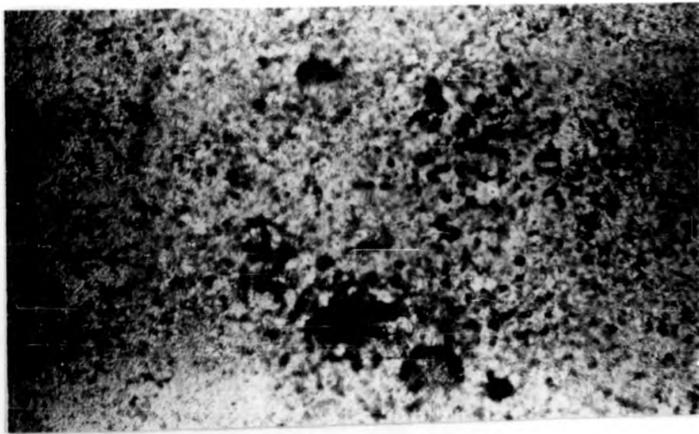


Plate 2. 43 cells, sc 263 5 days after seeding into a glass bottle. Live x 85.

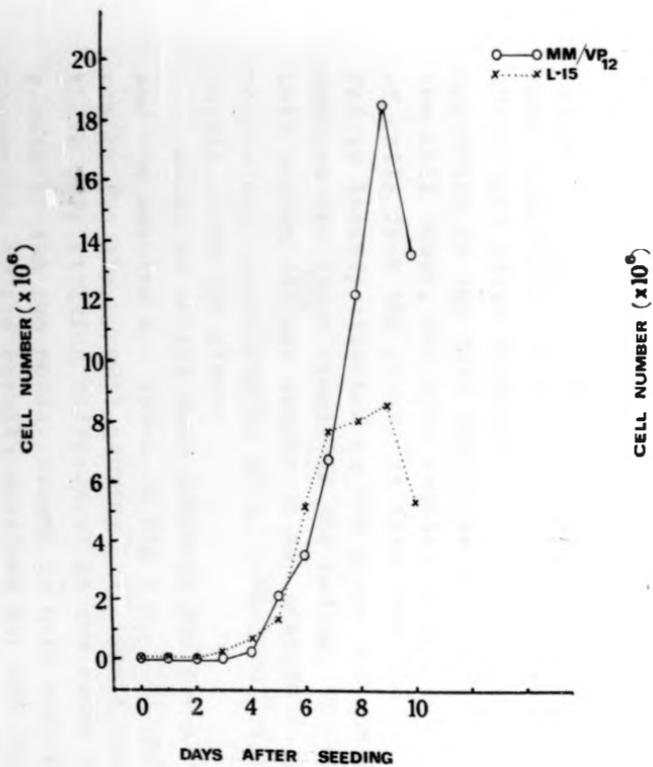


Figure 1 a. Growth curves of the 20A cell line in two media.

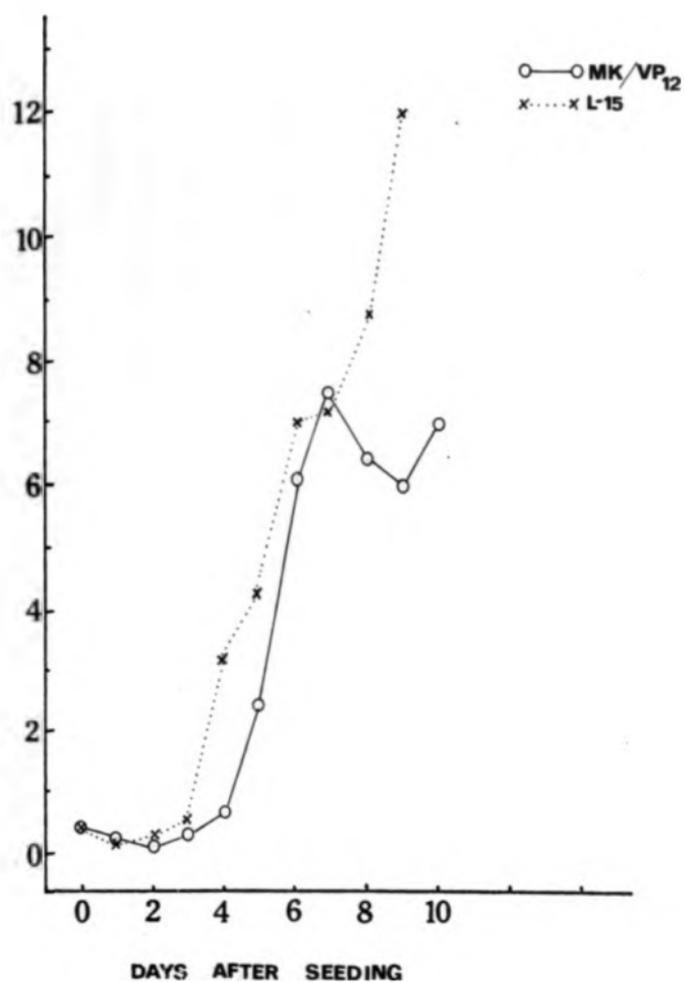


Figure 1 b. Growth curves of the 43 cell line in two media.

in morphology, a finding similar to that of Johnson (1973). The cells remain firmly attached to the glass for at least 14 days, and there never appears to be many floating cells in the medium.

Cells at sc 262 were studied for growth rates, and the results are shown in Fig 1 b. The peak cell number in MK/VP<sub>12</sub> medium was approximately  $7.0 \times 10^6$  cells per bottle, which was similar to the results obtained earlier by Pudney and Varma (1971). In L-15 medium, however, a maximum cell number of  $12 \times 10^6$  cells per bottle was obtained after a similar incubation period, confirming Johnson's (1973) observation that these cells grow faster in L-15 medium.

Population doubling times in both MK/VP<sub>12</sub> and L-15 medium was about 15 hours.

### 3.1.3. 55 cells.

The 55 cells grown in MK/VP<sub>12</sub> medium have an epithelial-type morphology, and they form dense very clumpy cell sheets (Plate 3). Incubation for 10-14 days after seeding results in large gaps appearing in the cell sheet due to retraction of the cell sheet, and also results in the detachment of cells from the glass. At this time the cells are fairly loosely attached to the glass and considerable numbers may float freely in the medium. Transfer into L-15 medium did not result in any changes in cell morphology, although the cells tended to detach more readily from the glass.

Cells at sc 192 were examined for growth rates, and the results are shown in Fig 2 a. This cell line was by far the fastest growing cell line studied, and there were striking differences in the rates of growth in the two media. Growth in L-15 medium was comparable to the results obtained for the 20A and 43 cells, with a peak cell number of  $13 \times 10^6$  cells per bottle. In contrast, growth in MM/VP<sub>12</sub> was very

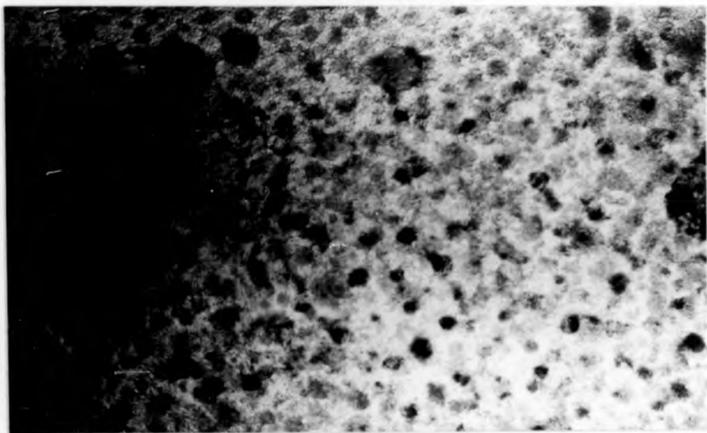


Plate 3. 55 cells, sc 192 5 days after seeding into a glass bottle. Live x 85.

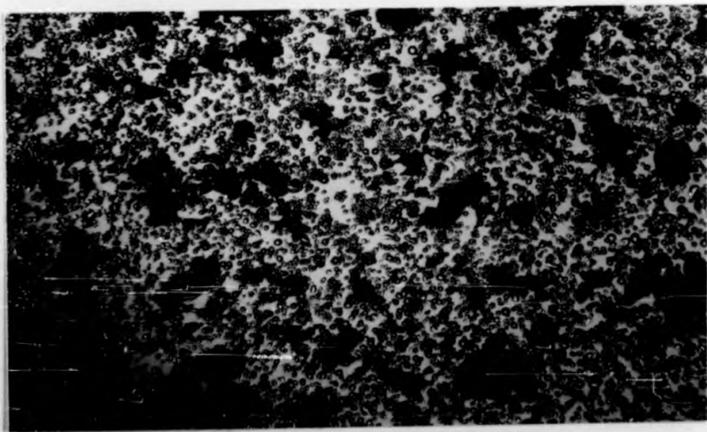


Plate 4. 60 cells, sc 188 11 days after seeding into a plastic flask. Live x 85.

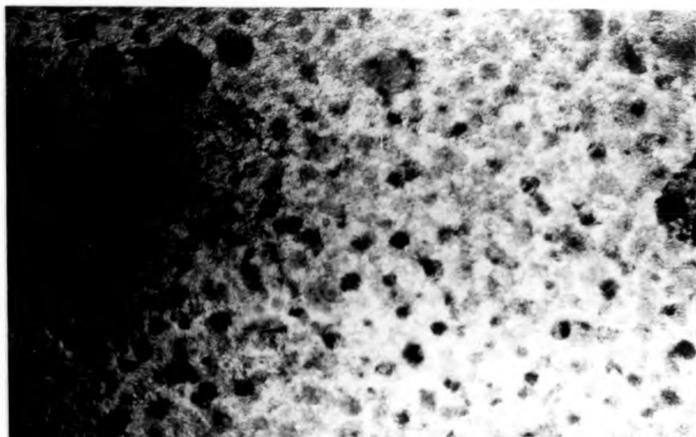


Plate 3. 55 cells, sc 192 5 days after seeding into a glass bottle. Live x 85.

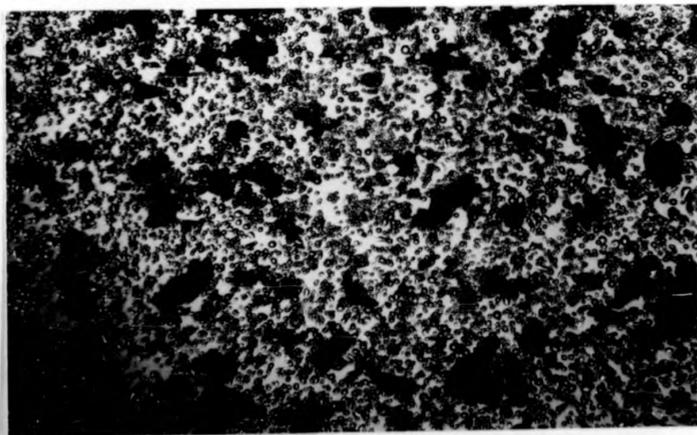


Plate 4. 60 cells, sc 188 11 days after seeding into a plastic flask. Live x 85.

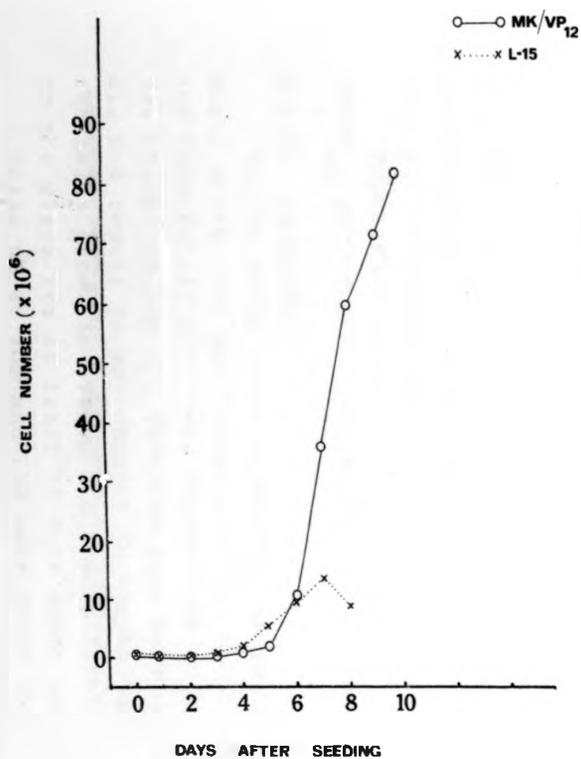


Figure 2 a. Growth curves of the 55 cell line in two media.

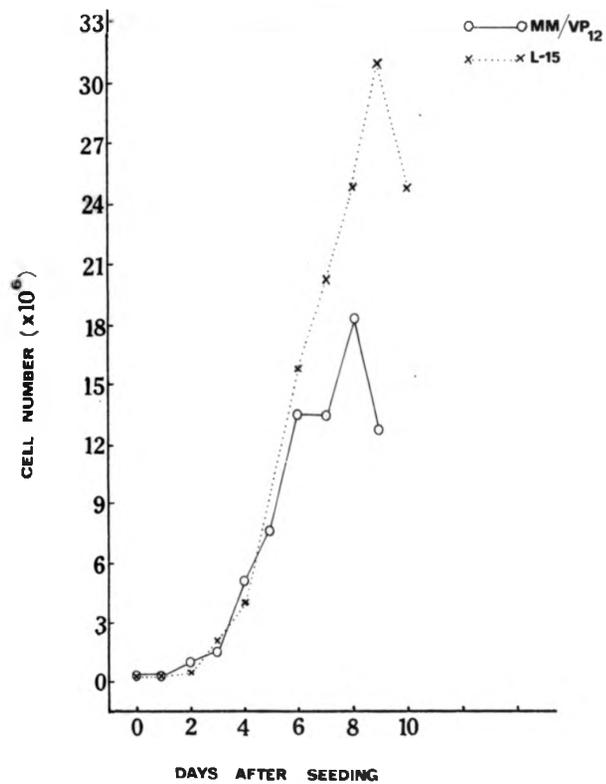


Figure 2 b. Growth curves of the 60 cell line in two media.

rapid, showing a 300-fold increase in cell number over a 10 day incubation period, giving a peak cell number of  $81 \times 10^6$  cells per bottle.

Population doubling times in MK/VP<sub>12</sub> medium were as low as 6 hours, compared to about 18 hours in L-15 medium.

#### 3.1.4. 60 cells.

The 60 cells grown in MM/VP<sub>12</sub> medium have an epithelial-type morphology, and form a dense often very clumpy cell sheet (Plate 4). Transfer into L-15 medium did not result in any changes in morphology although there appeared to an increase in the numbers of cells floating in the medium. Incubation for up to 14 days after seeding results in large numbers of cells, and cell clumps, floating freely in the medium.

Cells at sc 109 and 134 were studied for growth rates in both media, and the results are shown in Fig 2 b. The results confirm Johnson's (1973) findings that these cells grow better in L-15 medium as a maximum cell number of  $31 \times 10^6$  cells per bottle was obtained in L-15 medium, compared with only  $18 \times 10^6$  cells per bottle in MM/VP<sub>12</sub> medium.

Population doubling time was about 36 hours in MM/VP<sub>12</sub> medium and 32 hours in L-15 medium.

#### 3.1.5. 61 cells.

The 61 cells grown in MM/VP<sub>12</sub> medium have a mixed epithelial and fibroblast-type morphology, and form fairly even cell sheets with relatively few clumps (Plate 5). Transfer into L-15 medium did not result in any changes in morphology, and the cells grown in both media remain firmly attached to the glass for at least 14 days after seeding.

Cells at sc 105 and 130 were grown in both media, and the results are shown in Fig 3 a. There was better growth in MM/VP<sub>12</sub> medium, the cells reaching a peak

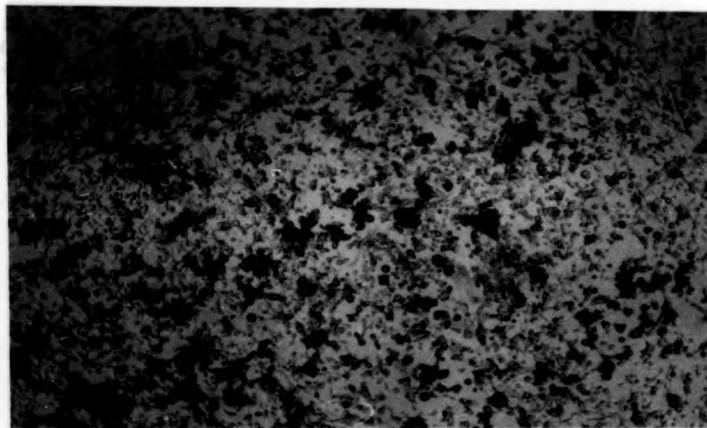


Plate 5. 61 cells, sc 139 16 days after seeding into a plastic flask. Live x 85.

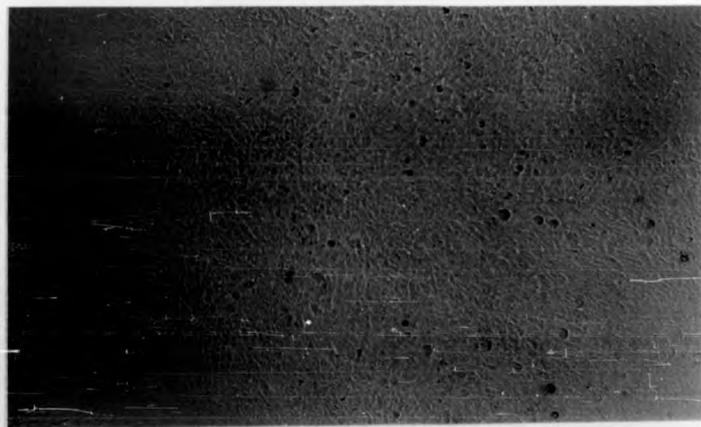


Plate 6. XTC-2 cells, sc 277 5 days after seeding into a plastic tray. Live x 85.

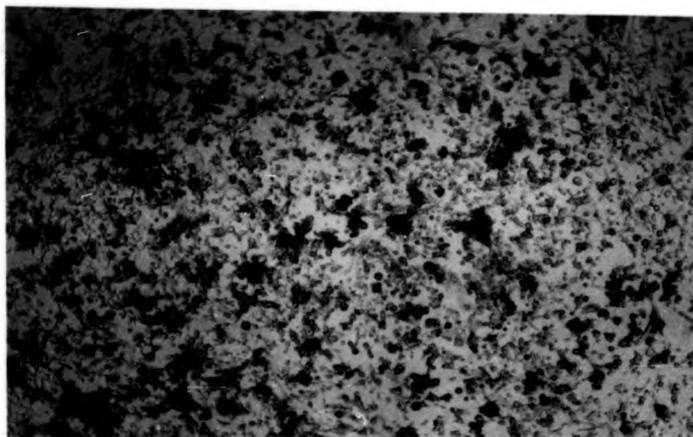


Plate 5. 61 cells, sc 139 16 days after seeding into a plastic flask. Live x 85.

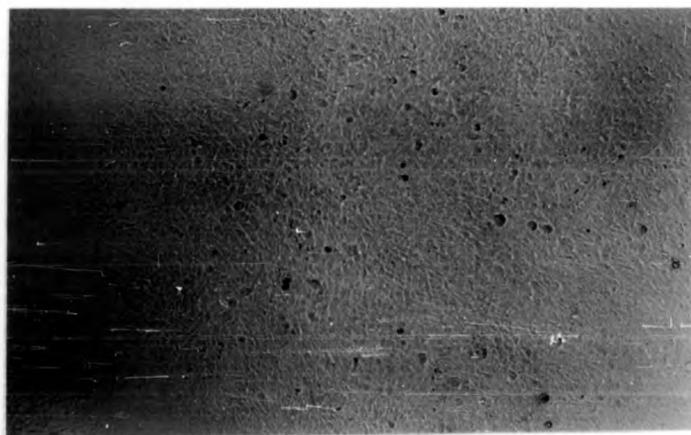


Plate 6. XTC-2 cells, sc 277 5 days after seeding into a plastic tray. Live x 85.

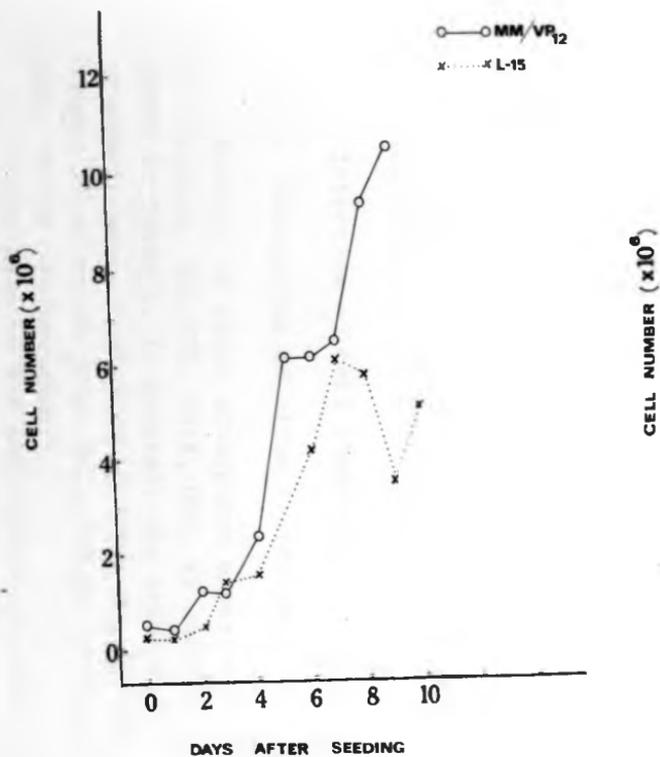
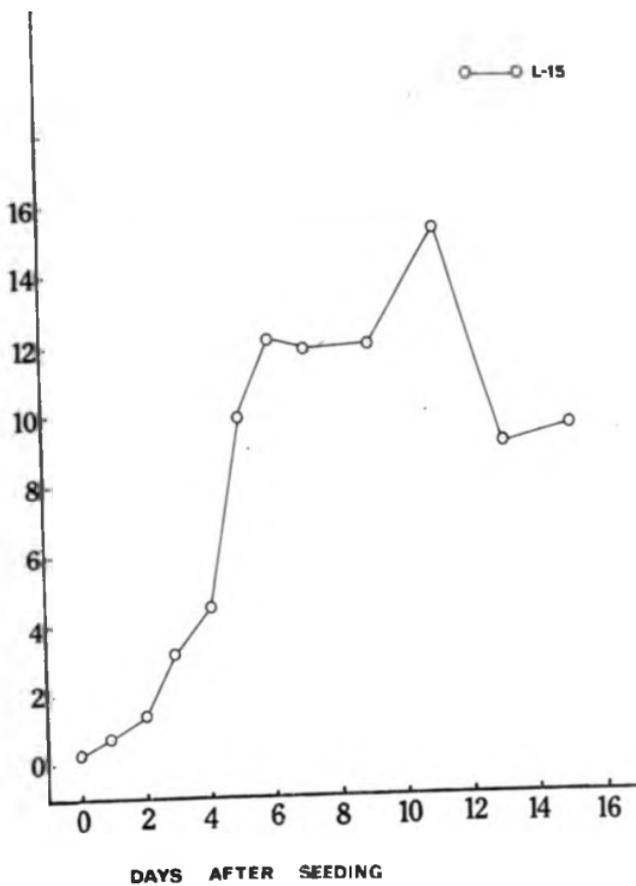


Figure 3 a. Growth curves of the 61 cell line in two media.



70

Figure 3 b. Growth curve of the XTC-2 cell line in L-15 medium.

number of  $10.5 \times 10^6$  per bottle compared to only  $6 \times 10^6$  cells per bottle in L-15 medium.

Population doubling time in MM/VP<sub>12</sub> was about 36 hours, and in L-15 about 44 hours.

#### 3.1.6. XTC-2 cells.

The XTC-2 cells are routinely grown in L-15 medium and the morphology is variable. As the cells start to grow out they appear fibroblast-like, but when a confluent monolayer has formed the closely packed cells take an epithelial-type appearance (Plate 6). Only a few clumps are formed on extended incubation, but there is a tendency for the cell sheet to roll up at the edges and lift off the glass as a whole.

Cells at sc 271 were grown in L-15 medium, and the results are shown in Fig 3 b. Comparison with earlier results is difficult, as the cells were originally established and grown in an amphibian culture medium (NCTC-109). Growth in L-15 medium was found to be better than in the original medium and so the cells were subsequently maintained solely in L-15 medium.

A maximum cell number of  $15 \times 10^6$  per bottle was obtained in my experiments, and the population doubling time was about 24 hours.

### 3.2. Infection of cell lines with viruses.

#### 3.2.1. Adsorption of virus to the cells.

Results of virus adsorption experiments with six viruses, SF, SIN, CHIK, ONN, YF and LGT, are shown in Table 2. In general, the results are in agreement with those of other workers, in that the amount of adsorbed virus detected was usually less than 10% of the inoculum.

In the case of SIN and YF viruses, with the exception of YF in the 60 cells, no virus was detected adsorbed to the cells after a three hour incubation period at 28°C. Later studies however (see section 3.3.2.5),

TABLE 2.

## VIRUS ADSORPTION TO CELLS OF DIFFERENT CELL LINES.

VIRUS	SAMPLE	CELL LINES					
		20A	43	55	60	61	XTC-2
SF	Inoculum	2.9	2.9	2.9	2.9	2.9	2.9
	3rd Wash	0	0	0	0	0	0
	Adsorbed	0.7	0.4	0.7	0	0	0
SIN	Inoculum	2.5	2.5	2.5	2.5	2.5	2.5
	3rd Wash	0	0	0	0	0	0.4
	Adsorbed	0	0	0	0	0	0
CHIK	Inoculum	3.0	3.0	3.0	3.0	3.0	3.0
	3rd Wash	-	-	-	-	-	-
	Adsorbed	2.9	1.1	2.7	1.4	2.1	2.0
ONN	Inoculum	2.9	2.9	2.9	2.9	2.9	2.9
	3rd Wash	0	0	0	0.7	0.4	0.4
	Adsorbed	0	1.9	1.7	1.4	1.4	1.4
YF	Inoculum	3.9	3.9	3.9	3.9	3.9	3.9
	3rd Wash	0	0	0	0	0	0
	Adsorbed	0	0	0	1.0	0	0
LGT	Inoculum	2.1	2.1	2.1	2.1	2.1	2.1
	3rd Wash	-	-	-	-	-	-
	Adsorbed	1.4	1.9	1.4	1.0	1.0	1.0

The set of three figures for each virus reflect the total amount of virus, expressed as dex pfu, in 0.3ml of inoculum, 3ml of the third wash, and 1ml of disrupted cell suspension.

showed that in many cases the cells were productively infected with these viruses. Presumably adsorbed virus had already been taken into the cells and had been uncoated, and was therefore not detected.

The results suggest that measurement of adsorbed virus in this manner is of limited value. There appeared to be no correlation between the amount of adsorbed virus detected, and the ability of the virus to multiply in a given cell line. For example, with LGT virus there was measured virus adsorption to all the cells, although LGT virus multiplied only in the XTC-2 cells (see section 3.3.2.1.). High adsorption figures may therefore reflect passive virus adsorption also, and not the total amount entering each cell system.

The absence of detectable adsorbed virus in cells that were productively infected, suggests that the time course of the infection process is considerably shorter than the three hour period that was employed.

In all further experiments the calculated total inoculum per bottle, and not the amount of adsorbed virus, was used as a baseline. However, a reduction of this figure by 1/10 as an estimate of the amount of virus actually adsorbed to the cells would not be unreasonable, and even this figure could well be high.

### 3.2.2. Experiments to determine minimum infectious dose.

Tests were carried out on eight viruses, SIN, SF, CHIK, ONN, WN, YF, BUN and CE, and the results are shown in Tables 3-10.

#### 3.2.2.1. SIN virus (Table 3).

The 60 and 61 cells were markedly more sensitive to infection with SIN virus than the other cell lines, and were infected with a dilution of at least  $10^{-9}$  of the stock virus suspension. The XTC-2 cells were

TABLE 3.  
MINIMUM INFECTIOUS DOSE OF SIN VIRUS.

VIRUS DILUTION INOCULATED	CELL LINES					
	20A	43	55	60	61	XTC-2
10 <sup>-5</sup>	-	-	+	+	+	+
-6	-	-	-	+	+	+
-7	-	-	-	+	+	-
-8	-	-	-	+	+	-
-9	-	-	-	+	+	-

Titre of stock material in Vero cells

7.0 dex pfu/ml.

+ Growth.

- No Growth.

TABLE 4.  
MINIMUM INFECTIOUS DOSE OF SF VIRUS.

VIRUS DILUTION INOCULATED	CELL LINES					
	20A	43	55	60	61	XTC-2
10 <sup>-5</sup>	+	-	+	+	+	+
-6	-	-	+	+	+	+
-7	-	-	-	+	+	+
-8	-	-	-	+	+	+
-9	-	-	-	+	+	-

Titre of stock material in Vero cells

8.0 dex pfu/ml.

+ Growth

- No Growth.

infected at a dilution of  $10^{-6}$  and the 55 cells at a dilution of  $10^{-5}$ . The 20A and 43 cells failed to become infected at a dilution of  $10^{-5}$  and so an additional experiment was carried out using a  $10^{-2}$  dilution of stock virus suspension. There was still no detectable virus growth in either cell line.

#### 3.2.2.2. SF virus (Table 4).

The 60 and 61 cells were again more sensitive to SF virus than the other cells. The highest dilution tested ( $10^{-9}$  of stock virus suspension) readily infected both these cell lines. The XTC-2 cells were infected at a dilution of  $10^{-8}$  and were therefore comparable to Vero cells. In contrast, the 20A and 55 cells were much less sensitive, requiring dilutions of  $10^{-5}$  and  $10^{-6}$  respectively to become infected. The 43 cells failed to be infected with the lowest dilution used ( $10^{-5}$ ), and also failed to become infected in additional experiments using inocula at a dilution of  $10^{-2}$  of the stock virus suspension.

#### 3.2.2.3. CHIK virus (Table 5).

With this virus, the 60, 61 and XTC-2 cells were all infected with a  $10^{-7}$  dilution of virus, the highest dilution tested. The 20A cells were infected at a dilution of  $10^{-5}$ , and the 55 cells at a dilution of  $10^{-4}$ , but the 43 cells were not infected at this dilution. An additional test using a  $10^{-2}$  dilution of stock virus suspension also failed to establish an infection in the 43 cells.

#### 3.2.2.4. ONN virus (Table 6).

With ONN virus, the 61 cells appeared to be the most sensitive as they were infected with a dilution of  $10^{-7}$  of the stock virus suspension. The 60 and XTC-2 cells were both infected with a dilution of  $10^{-6}$ , and the 55 cells at  $10^{-5}$ , but neither the 20A or the 43 cells were infected at the lowest dilution tested ( $10^{-5}$ ). In an additional test both

TABLE 5.  
MINIMUM INFECTIOUS DOSE OF CHIK VIRUS.

VIRUS DILUTION INOCULATED	CELL LINES					
	20A	43	55	60	61	XTC-2
10 <sup>-4</sup>	+	-	+	+	+	+
-5	+	-	-	+	+	+
-6	-	-	-	+	+	+
-7	-	-	-	+	+	+

Titre of stock material in Vero cells

6.7 dex pfu/ml.

+ Growth.

- No Growth.

TABLE 6.  
MINIMUM INFECTIOUS DOSE OF ONN VIRUS.

VIRUS DILUTION INOCULATED	CELL LINES					
	20A	43	55	60	61	XTC-2
10 <sup>-1</sup>	-	+	+	+	+	+
-5	-	-	+	+	+	+
-6	-	-	-	+	+	+
-7	-	-	-	-	+	-
-8	-	-	-	-	-	-

Titre of stock material in XTC-2 cells

6.8 dex pfu/ml.

+ Growth.

- No Growth.

cell lines were inoculated with  $10^{-1}$  virus suspension but only limited growth was detected, and then only in the 43 cells.

3.2.2.5. WN virus (Table 7).

The 60 and 61 cells were markedly more sensitive to infection with WN virus, and were infected with  $10^{-9}$ , the highest dilution tested. All the other cell lines were infected but only at a dilution of  $10^{-6}$  of the stock virus suspension.

3.2.2.6. YF virus (Table 8).

An interesting finding with this virus was that only the three Aedes cell lines, 20A, 60 and 61, were infected with the virus, whereas the 43, 55 and XTC-2 cells could not be infected even at a  $10^{-2}$  dilution of the stock virus suspension. Of the three susceptible lines both the 60 and 61 cells were infected with a dilution of  $10^{-8}$ , the highest dilution tested, whereas the 20A cells were less sensitive being infected with a dilution of only  $10^{-6}$ .

3.2.2.7. BUN virus (Table 9).

With BUN virus, the XTC-2 cells were the most sensitive, and the highest dilution tested ( $10^{-9}$ ) readily infected the cells. All the other cell lines were infected by the virus but at different dilutions, the 20A and 60 cells at  $10^{-7}$ , the 61 cells at  $10^{-6}$  and the 43 and 55 cells at  $10^{-5}$ .

3.2.2.8. CE virus (Table 10).

With CE virus, the 60 cells were the most sensitive, being infected with a  $10^{-7}$  dilution of the stock virus suspension, the highest dilution tested. All the other cell lines were infected with CE virus, the 61 cells at a dilution of  $10^{-6}$ , the 20A and 43 cells at a dilution of  $10^{-5}$ , and the 55 and XTC-2 cells at a dilution of  $10^{-4}$ . However, when tested at 10 days, tissue culture fluid from the 55 cells inoculated with  $10^{-3}$  and  $10^{-4}$  dilutions of the virus

TABLE 7.

## MINIMUM INFECTIOUS DOSE OF WN VIRUS.

VIRUS DILUTION INOCULATED	CELL LINES					
	20A	43	55	60	61	XTC-2
$10^{-6}$	+	+	+	+	+	+
-7	-	-	-	+	+	-
-8	-	-	-	+	+	-
-9	-	-	-	+	+	-

Titre of stock material in Vero cells

6.7 dex pfu/ml.

+ Growth.

- No Growth.

TABLE 8.

## MINIMUM INFECTIOUS DOSE OF YF VIRUS.

VIRUS DILUTION INOCULATED	CELL LINES					
	20A	43	55	60	61	XTC-2
$10^{-5}$	+	-	-	+	+	-
-6	+	-	-	+	+	-
-7	-	-	-	+	+	-
-8	-	-	-	+	+	-

Titre of stock material in Vero cells

7.4 dex pfu/ml.

+ Growth.

- No Growth.

TABLE 9.

## MINIMUM INFECTIOUS DOSE OF BUN VIRUS.

VIRUS DILUTION INOCULATED	CELL LINES					
	20A	43	55	60	61	XTC-2
10 <sup>-5</sup>	+	+	+	+	+	+
-6	+	-	-	+	+	+
-7	+	-	-	+	-	+
-8	-	-	-	-	-	+
-9	-	-	-	-	-	+

Titre of stock material in Vero cells

8.9 dex pfu/ml.

+ Growth.

- No Growth.

TABLE 10.

## MINIMUM INFECTIOUS DOSE OF CE VIRUS.

VIRUS DILUTION INOCULATED	CELL LINES					
	20A	43	55	60	61	XTC-2
10 <sup>-3</sup>	+	+	+	+	+	+
-4	+	+	+	+	+	+
-5	+	+	-	+	+	-
-6	+	+	-	+	+	-
-7	-	-	-	+	-	-

Titre of stock material in Vero cells

6.9 dex pfu/ml.

+ Growth.

- No Growth.

were negative, and this probably reflects the very poor growth of CE virus in these cells (see section 3.3.3.4.).

### 3.3. Growth of arboviruses in the cell lines.

Infection of the cell lines was attempted with 23 viruses from different serological groups. The viruses and the subculture levels of the cell lines used in these experiments are shown in Table 11.

The 20A cells were used from sc 231 to 373, the 43 cells from sc 199 to 335, the 55 cells from sc 128 to 264, the 60 cells from sc 32 to 180, the 61 cells from sc 35 to 176, and the XTC-2 cells from sc 208 to 342.

Due to the variability of the results obtained for virus adsorption, the total inoculum per bottle has been calculated, expressed in dex pfu, and plotted on all the graphs as the 0 day figure as a base line. The remaining points are expressed in dex pfu/ml. In most cases the inoculum used provided a multiplicity of infection (MOI) of approximately  $1 \times 10^{-3}$  to  $1 \times 10^{-4}$  pfu per cell, and only in one or two cases, such as the infection of the XTC-2 cells with LGT and LI viruses, were higher MOI employed.

#### 3.3.1. Growth of alphaviruses.

##### 3.3.1.1. SIN virus.

An inoculum of 2.5 dex pfu per bottle was used for infection, and the results are shown in Fig 4 a,b.

The 20A cells did not support replication of SIN virus, there being no detectable extracellular virus, and additional minimum infectious dose experiments with higher doses of virus have also failed to establish infection. Additional experiments were carried out in the 20A cells with negative results but, in one experiment, replication of the virus was demonstrated. Cells at sc 392 were infected with an inoculum of 3.1 dex pfu per bottle, and a marked rise in virus titre was observed from 1.6 dex

TABLE 11.  
 VIRUSES AND SUBCULTURE LEVELS OF THE CELL LINES  
 USED IN INFECTION EXPERIMENTS.

GENUS	VIRUS	CELL LINES AND SUBCULTURE LEVELS					
		20A	43	55	60	61	XTC-2
ALPHAVIRUS	SIN	288	250	179	94	90	256
	NDU	368	330	259	136	132	298
	SF	288	250	179	94	90	256
	CHIK	311	272	201	117	113	279
	ONN	289	250	179	94	90	256
	URU	368	330	259	136	132	298
	MID	368	330	259	136	132	298
FLAVIVIRUS	LGT	297	258	187	103	99	265
	LI	297	258	187	103	99	265
	JE	257	218	147	63	59	220
	WN	231	199	128	32	35	208
	ZIKA	373	335	264	180	176	342
	DEN-2	349	311	240	156	152	318
	YF	297	258	187	103	99	265
BUNYAVIRUS	BUN	349	311	240	136	132	298
	GER	367	329	258	136	132	298
	BWA	349	311	240	156	152	318
	CE	349	311	240	156	152	318
	ANA	338	300	229	145	141	307
SFS	361	323	252	168	164	330	
RHABDOVIRUS	CHP	368	300	229	145	141	307
UNGROUPED	ZIR	338	300	229	145	141	307
	QRF	338	300	229	145	141	307

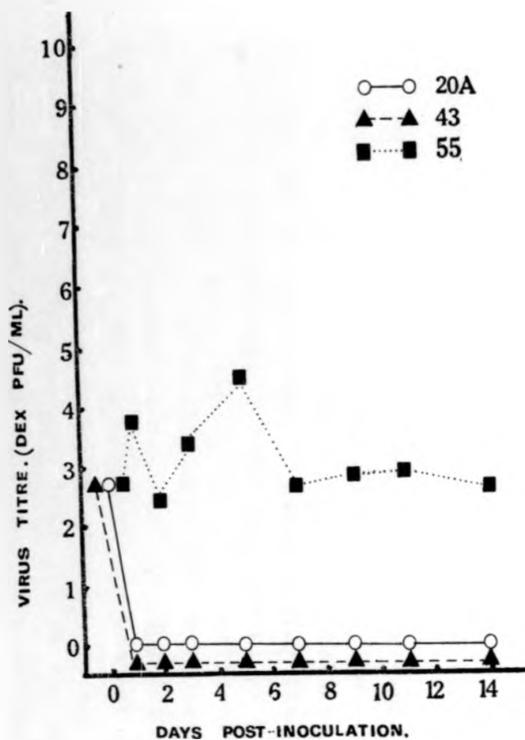


Figure 4 a.

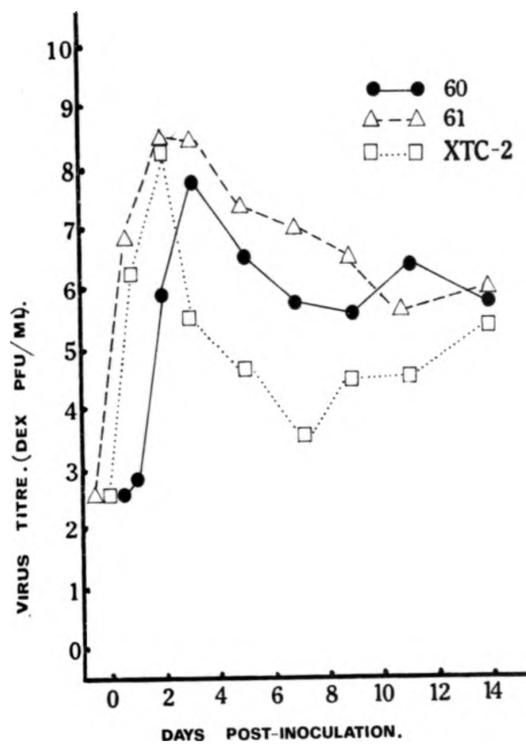


Figure 4 b.

Growth of SIN virus in various cell lines.

pfu per ml on day 1, to 8.0 dex pfu per ml by day 9. Subsequently the titre was maintained at above 6.5 dex pfu per ml until 21 days.

The 43 cells also failed to support replication of the virus, and even inocula of about 5.0 dex pfu per ml failed to infect the cells. In contrast, the other anopheline cell line, 55, supported replication of the virus, although the growth pattern was different from that observed in the 60, 61 and XTC-2 cells. A peak titre of 4.4 dex pfu per ml was reached by day 5, and subsequently the titre was maintained at a uniform level of about 2.8 dex pfu per ml until day 21.

In the 60 cells there was a rapid rise in virus titre, reaching a peak of 7.8 dex pfu per ml by day 3; this dropped slowly and was maintained at about 6.0 dex pfu per ml over a 21 day incubation period. A similar growth pattern was observed in the 61 cells, although the initial rise in titre was more rapid reaching 8.4 dex pfu per ml by day 2. As with the 60 cells, the titre then fell gradually to be maintained at about 6.0 dex pfu per ml over a 21 day incubation period.

In the XTC-2 cells there was also a rapid rise in titre to 8.4 dex pfu per ml by day 2, but this was then followed by a marked decline, falling to 3.4 dex pfu per ml by day 7. Subsequently the titre rose steadily reaching 5.7 dex pfu per ml by day 21.

A CPE was seen only in the XTC-2 cells with SIN virus. By day 2, there was extensive cell death, with a considerable amount of cell debris floating in the medium (Plates 7, 8). Subsequently, there was further detachment of cells from the glass, but by day 7 new cells were beginning to grow out, and by day 14 this process was complete. At this time, the cultures, while still infected, appeared normal.

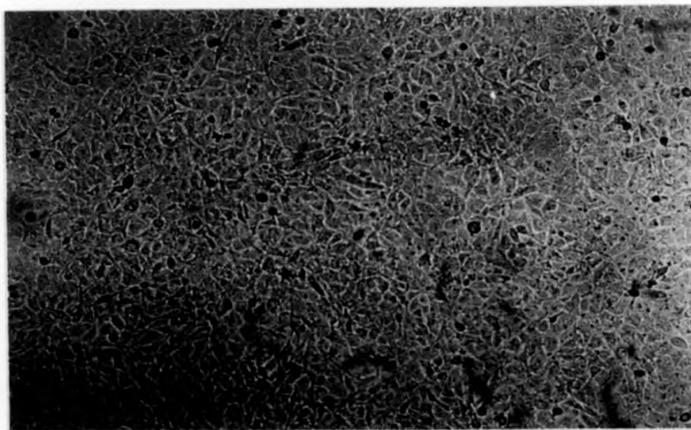


Plate 7. Control XTC-2 cells, sc 195 grown on plastic.



Plate 8. XTC-2 cells grown on plastic 2 days after infection with SIN virus.

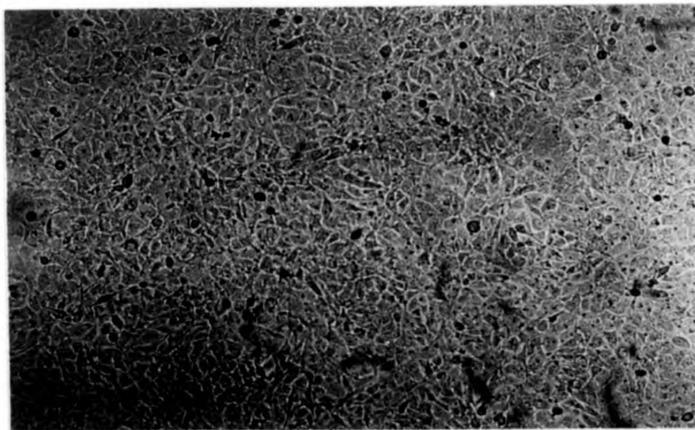


Plate 7. Control XTC-2 cells, sc 195 grown on plastic.

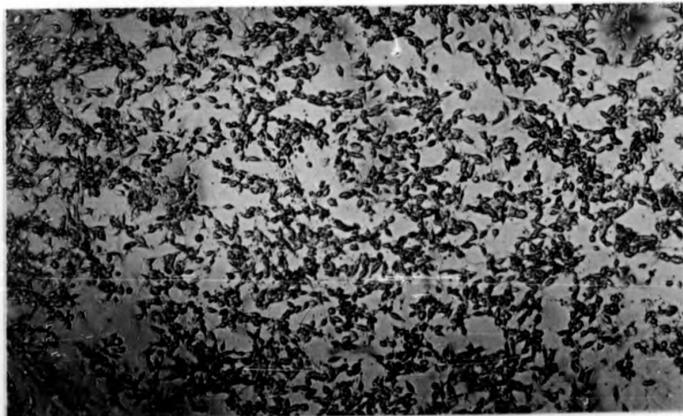


Plate 8. XTC-2 cells grown on plastic 2 days after infection with SIN virus.

### 3.3.1.2. NDU virus.

The 20A, 43 and 55 cells were infected with an inoculum of 5.4 dex pfu per bottle, and the 60, 61 and XTC-2 cells with an inoculum of 2.4 dex pfu per bottle. The results are shown in Fig 5 a, b.

The two anopheline cell lines, 43 and 55, did not support replication of the virus, there being no detectable virus over a 14 day incubation period. In contrast, in the 20A cells the titre rose to more than 6.0 dex pfu per ml by day 5, and remained at this fairly high level, only falling to 4.3 dex pfu per ml by day 14.

In the 60 cells a steady rise in titre was seen reaching a peak of 7.0 dex pfu per ml by day 6. As with SIN virus, this titre was maintained with a drop of only about 1 dex until day 14.

In contrast, the growth pattern in the 61 cells was similar to SIN virus. There was a rapid rise in titre to a peak of 8.4 dex pfu per ml by day 3, followed by a gradual fall to 6.7 dex pfu per ml by day 14.

A growth pattern similar to SIN virus was also seen in the XTC-2 cells, with a peak titre of 7.6 dex pfu per ml being reached by day 2, followed by a marked fall to 3.6 dex pfu per ml by day 6. On completion of the experiment at 14 days the titre had risen to 6.0 dex pfu per ml.

As with SIN virus, a CPE was only observed in the XTC-2 cells. By day 2 there was fairly extensive cell death with a fair amount of floating debris. However, the CPE was not as pronounced as with SIN virus, and recovery of the cultures was complete by day 8.

### 3.3.1.3. SF virus.

An inoculum of 2.9 dex pfu per bottle was used to infect all the cell lines ; the results are shown in Fig 6 a, b.

Only the 43 cells failed to support replication of SF virus, there being no detectable virus over a

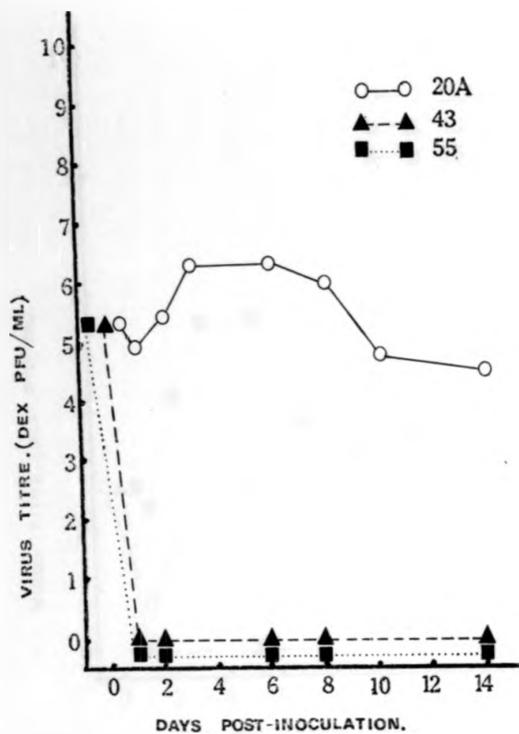


Figure 5 a.

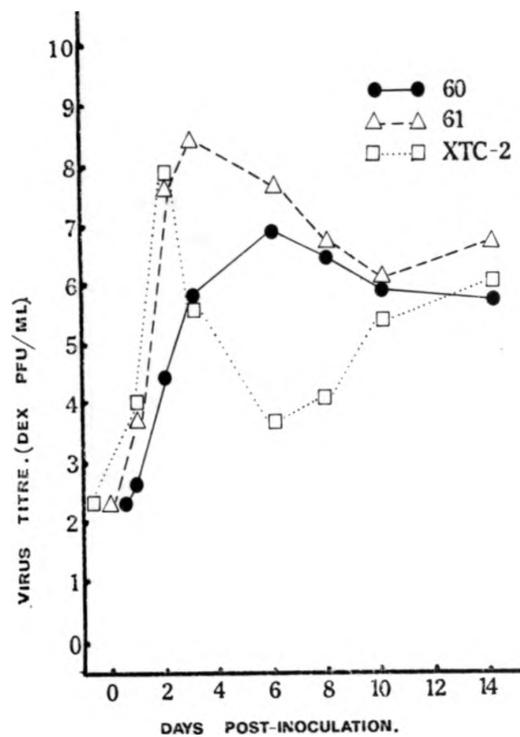


Figure 5 b.

Growth of NDU virus in various cell lines.

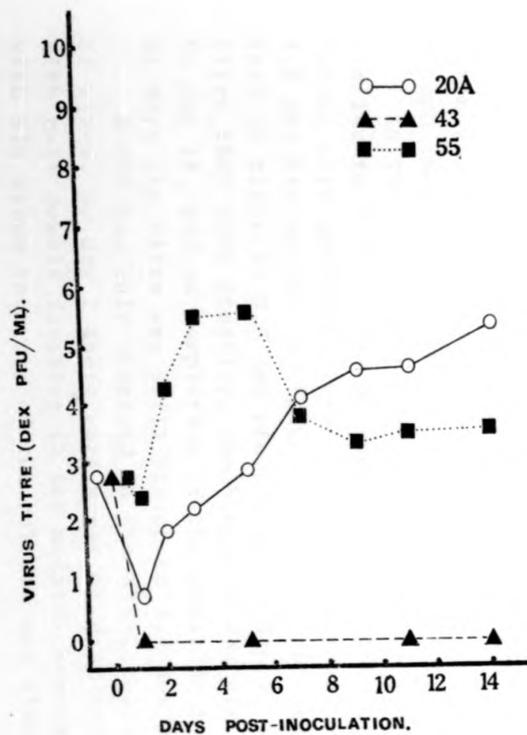


Figure 6 a.

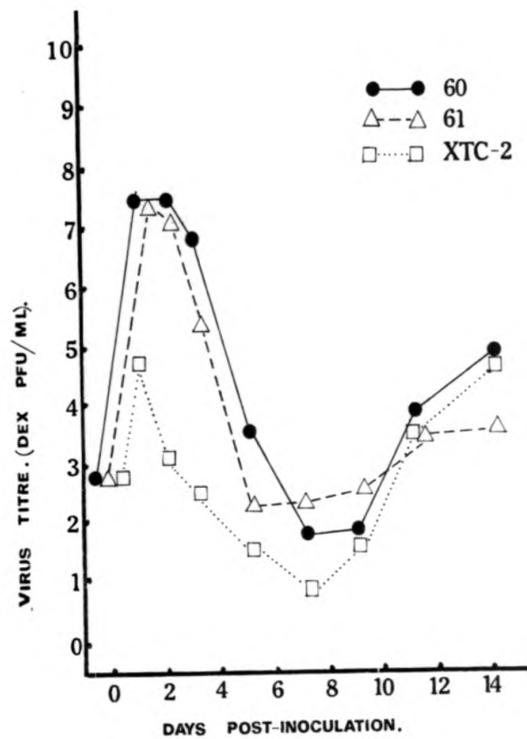


Figure 6 b.

Growth of SF virus in various cell lines.

21 day incubation period. This finding confirmed the minimum infectious dose experiments, where an inoculum of about 6.0 dex pfu per bottle failed to establish an infection.

In the 20A cells there was a slow, steady rise in titre reaching a peak of 5.1 dex pfu per ml by day 14, followed by a fall to 3.9 dex pfu per ml by day 21.

In the 55 cells the rise in virus titre was more rapid reaching 5.4 dex pfu per ml by day 5, before falling to be maintained at about 3.5 dex pfu per ml until 21 days.

The growth patterns of SF virus in the 60 and 61 cells differed considerably from the results obtained in the 20A and 55 cells. In both cell lines there was a rapid rise in titre to about 7.4 dex pfu per ml by day 1, followed by a marked fall to about 2.0 dex pfu per ml by day 7. Subsequently, the titre rose to about 4.5 dex pfu per ml in the 60 cells by day 14, and was maintained at this level until 21 days, whereas in the 61 cells this rise in titre was slower reaching 3.7 dex pfu per ml by day 14 and then falling to 2.9 dex pfu per ml by day 21.

In the XTC-2 cells the growth pattern was similar to that in the 60 and 61 cells, although the titres were generally lower. An initial peak of 4.9 dex pfu per ml was reached on day 1 followed by a fall in titre to 0.7 dex pfu per ml on day 7. The titre then rose steadily, reaching 4.4 dex pfu per ml by day 14, and on completion of the experiment at 21 days the titre was still rising to 5.4 dex pfu per ml.

A CPE was only observed in the XTC-2 cells with SF virus. By day 2 there were areas of dead cells with cell debris floating in the medium. However, as with SIN virus recovery of the culture was almost complete by 10 days.

#### 3.3.1.4. CHIK virus.

Infection of all the cell lines was carried out with an inoculum of 3.0 dex pfu per bottle, and the

results obtained are shown in Fig 7 a, b.

The 43 cells did not support the replication of CHIK virus, and this confirmed the findings of minimum infectious dose experiments, where inocula of 5.0 dex pfu per bottle also failed to establish an infection.

Similar virus growth curves were seen in the 20A and 55 cells, with a rise in titre to about 5.0 dex pfu per ml by day 6 in both cell lines. Titres from between 3.9 and 4.9 dex pfu per ml were then seen for up to 21 days.

Markedly different growth curves were seen in the 60 and 61 cells. As with SF virus there was an early rise in titre reaching a peak on days 1 and 2, but subsequently the fall in titre was not as marked as with SF virus and the titres were maintained at fairly steady levels until 21 days.

#### 3.3.1.5. ONN virus.

The 20A, 43 and 55 cells were infected with a high inoculum of 6.2 dex pfu per bottle, and the 60 61 and XTC-2 cells with an inoculum of 2.9 dex pfu per bottle. The results are shown in Fig 8 a, b.

The 20A cells failed to support replication of the virus, and in the 43 cells only low levels of virus were detected of less than 1.0 dex pfu per ml until day 9 and none subsequently. These levels may suggest limited replication of the virus, but could also be explained on the basis of elution of adsorbed virus only.

In contrast, the other anopheline cell line, 55, supported replication of the virus with a growth pattern similar to that obtained with CHIK virus, although the titres observed with ONN virus were generally higher.

In the 60, 61 and XTC-2 cells the growth patterns were rather different from those obtained with CHIK virus, as there was no rapid rise in virus titre; rather a steady rise reaching a peak in the XTC-2 cells on day 2, and later in the 60 and 61 cells. The

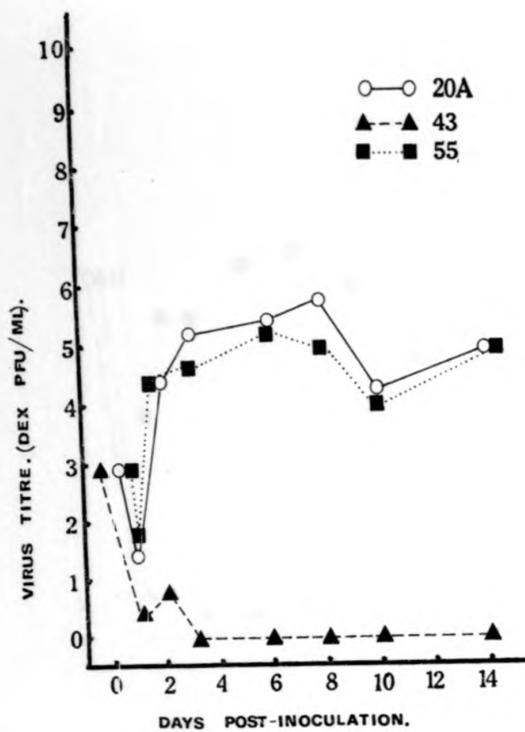


Figure 7 a.

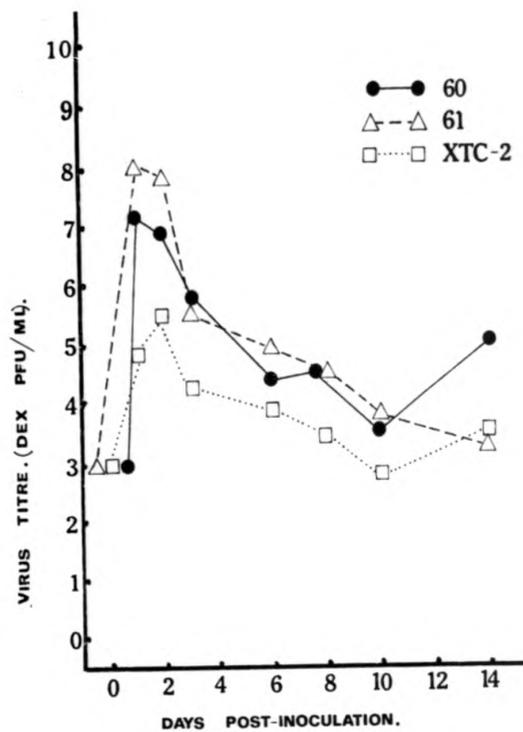


Figure 7 b.

Growth of CHIK virus in various cell lines.

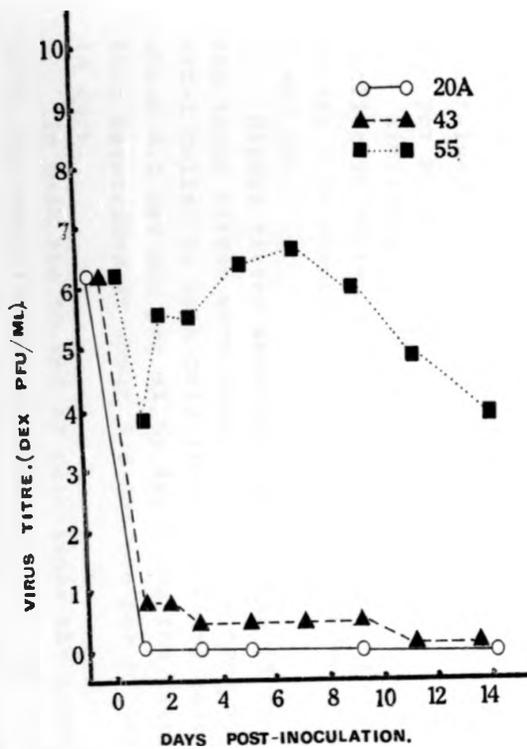


Figure 8 a.

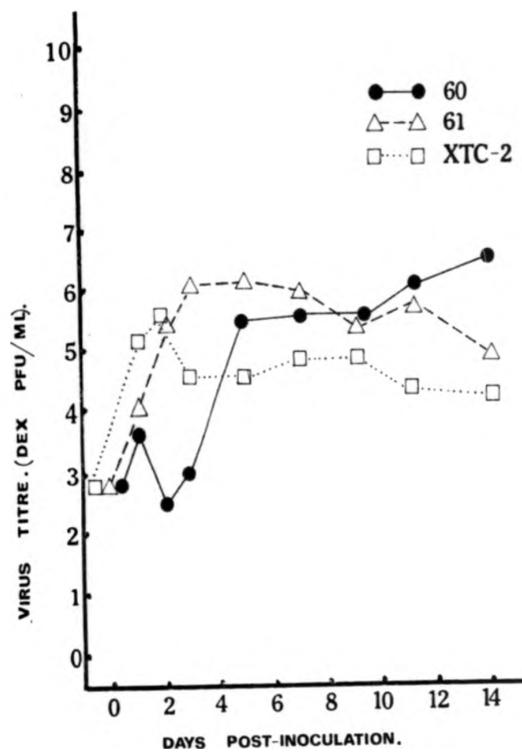


Figure 8 b.

Growth of ONN virus in various cell line.

titres were then maintained at fairly high levels, 5.0-6.5 dex pfu per ml, compared to the initial inoculum.

A CPE was observed only in the XTC-2 cells. With low inocula a gradual accumulation of floating cell debris was noticed, but in experiments using higher inocula effects similar to that for SF virus were seen. By day 2 distinct focal sites of cell death were seen, with cell debris floating in the medium. Again new cells had grown out by days 8-10.

#### 3.3.1.6. URU virus.

The 20A, 43 and 55 cells were infected with an inoculum of 4.5 dex pfu per bottle, and the 60, 61 and XTC-2 cells with an inoculum of 2.2 dex pfu per bottle. All the cell lines supported replication of the virus to varying degrees, and the results are shown in Fig 9 a, b.

In the 20A cells there was an initial rise in titre to 5.0 dex pfu per ml by day 2. This titre was maintained until 8 days before falling to 3.1 dex pfu per ml by day 14.

Similarly, in the 43 cells there was a steady decline in titre from a peak of 4.8 dex pfu per ml on day 2 to about 2.8 dex pfu per ml between days 8 and 14.

Higher titres were observed in the 55 cell line, and these titres were similar to those seen in the XTC-2 cells. In both cell lines the titre rose to about 6.5 dex pfu per ml by day 3 and the titres were then maintained at about 5.5 dex pfu per ml until 14 days.

In both the 60 and 61 cell lines the growth patterns were different reaching quite high titres. In the 61 cells there was a rapid rise to 9.2 dex pfu per ml by day 2. This level was maintained until day 6, after which there was a sharp drop to between 5.0 and 6.0 dex pfu per ml by day 14. In contrast, there was no rapid rise in titre in the 60 cells, where a

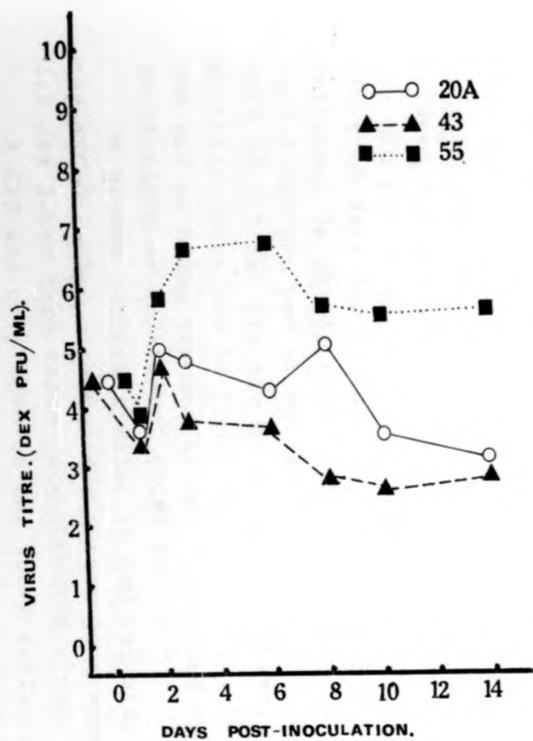


Figure 9 a.

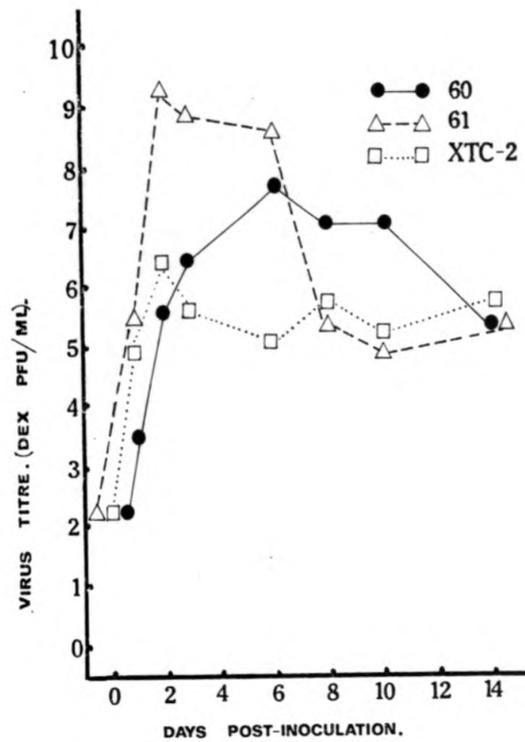


Figure 9 b.

Growth of URU virus in various cell lines.

level of 7.7 dex pfu per ml was reached by day 6. The pattern of growth of URU virus in the 60 cells closely resembles the growth of NDU virus.

Limited CPE was observed with URU virus only in the XTC-2 cells. By day 2 there was a fair number of floating cells in the medium, and by day 3 there were a few patches of dark rounded cells on the glass. However, by day 6 the cells appeared normal with little cell debris remaining.

#### 3.3.1.7. MID virus.

The 20A, 43 and 55 cells were infected with an inoculum of 3.3 dex pfu per bottle, and the 60, 61 and XTC-2 cells with an inoculum of 1.4 dex pfu per bottle. The results are shown in Fig 10 a, b.

The two anopheline celllines, 43 and 55, did not support replication of the virus, but attempts to establish infection with higher inocula were not made. In contrast, the three aedine cell lines, 20A, 60 and 61 all supported replication of the virus and the growth curves were similar to those obtained with URU virus.

In the 20A cells the virus titre fluctuated between 5.0 dex pfu per ml by day 3 to about 3.2 dex pfu per ml by day 10. No results were obtained after 10 days due to fungal contamination of the cultures. In the 61 cells, the titre rose rapidly to 7.3 dex pfu per ml by day 2, followed by a steady fall to 2.7 dex pfu per ml by day 14, whereas in the 60 cells there was no rapid rise in titre, 4.8 dex pfu per ml being reached by day 8 followed by maintenance at this level until day 14.

A growth pattern similar to URU was also seen in the XTC-2 cells, although the titres seen were about 1.5 dex lower than those seen with URU virus.

A CPE was only seen in the XTC-2 cells, and this was restricted to only a few foci of dead cells by day 3. By day 6 only a few dark cells could be seen on top of a healthy monolayer, and by day 8 the cells appeared normal.

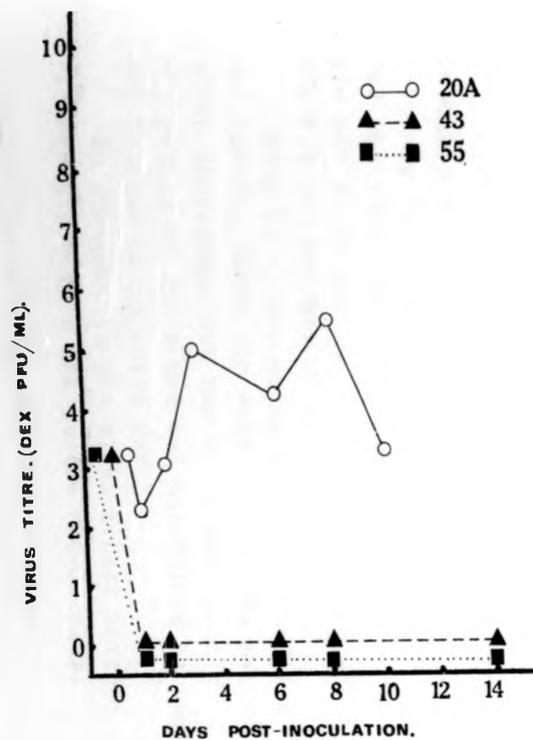


Figure 10 a.

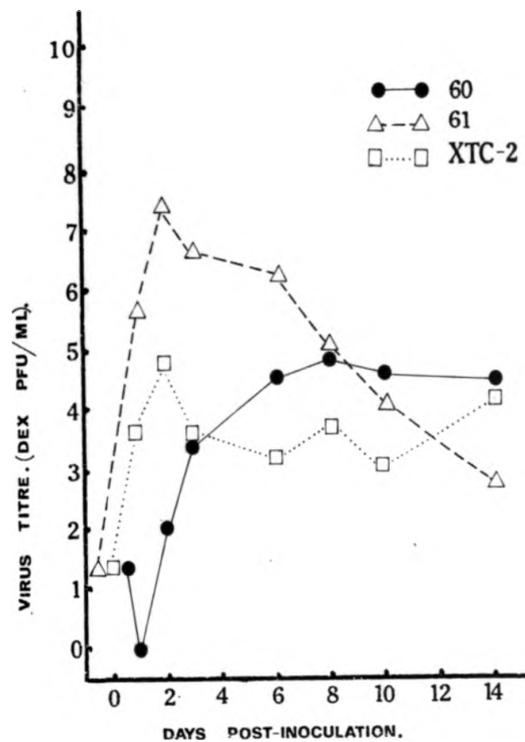


Figure 10 b.

Growth of MID virus in various cell lines.

### 3.3.1.8. Other alphaviruses.

Preliminary work has shown that the 60, 61 and XTC-2 cells will support the replication of GET and BEB viruses. The highest titres for both viruses were observed in the 61 cells of at least 8.0 dex pfu per ml, and CPE was only seen in the XTC-2 cells. With BEB virus there was extensive cell death, leaving only a few patches of cells attached to the glass by day 2. By day 14, however, these cells had grown out to form a sparse layer. GET virus produced less marked effects, and only a few patches of dead cells were seen on day 3.

### 3.3.2. Growth of flaviviruses.

#### 3.3.2.1. LGT and LI viruses.

Only the XTC-2 cells supported the growth of these two tick-borne viruses, and the results are shown in Fig 11 a. In several experiments, using high inocula, none of the mosquito cell lines could be infected with these viruses.

With LGT virus, the titre dropped from an inoculum of 5.0 dex pfu per bottle to an undetectable level by day 3. Virus was not detected until day 10, and the titre then rose steadily reaching 1.8 dex pfu per ml by day 14, 3.0 dex pfu per ml by day 17 and 4.2 dex pfu per ml by day 21.

With LI virus, using an inoculum of 6.6 dex pfu per bottle, there was only 0.3 dex pfu per ml 2 days after infection, but the titre then rose to 2.6 dex pfu per ml by day 6 and was maintained at about 2.0 dex pfu per ml up until day 21.

In contrast to the alphaviruses neither of these viruses produced a CPE in the XTC-2 cells.

#### 3.3.2.2. WN virus.

All the cell lines were infected with inocula of 2.5 dex pfu per bottle, and all the lines supported replication of the virus with similar results that are shown in Fig 12 a, b.

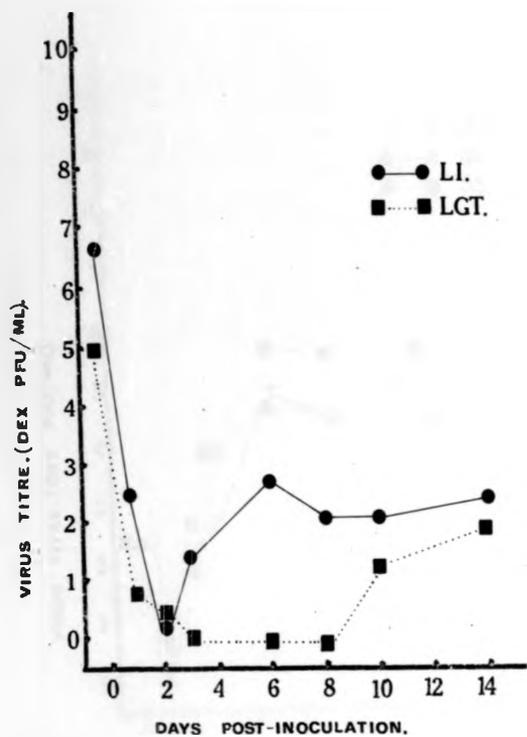


Figure 11 a.

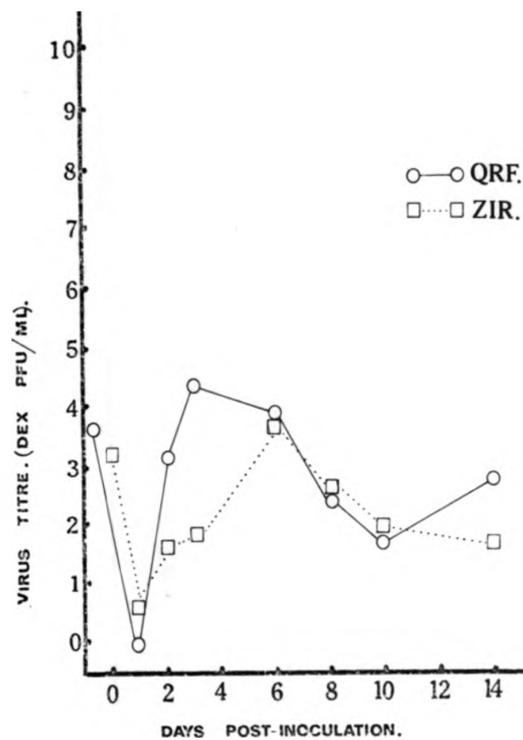


Figure 11 b.

Growth of four tick-borne viruses in XTC-2 cells.

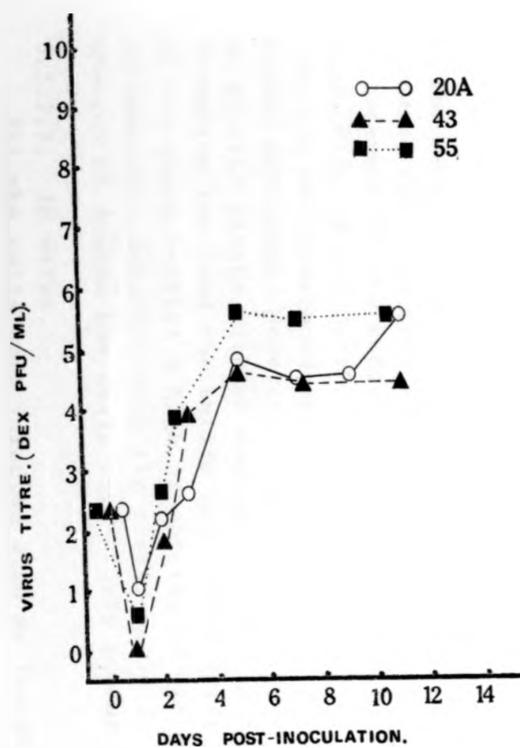


Figure 12 a.

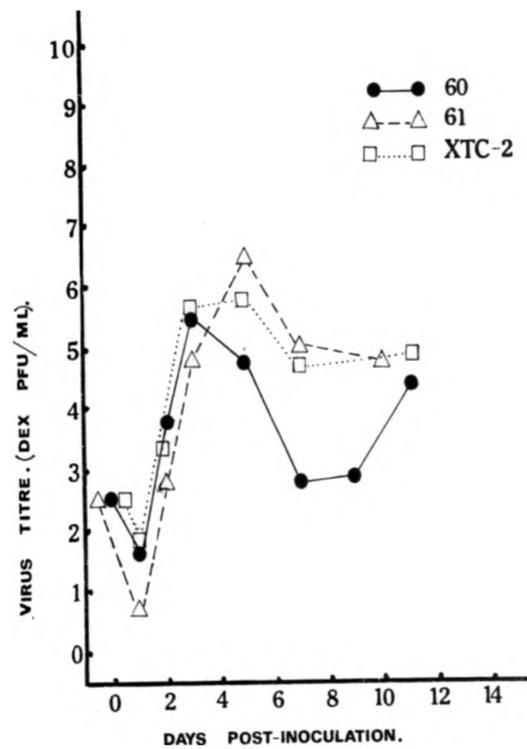


Figure 12 b.

Growth of WN virus in various cell lines.

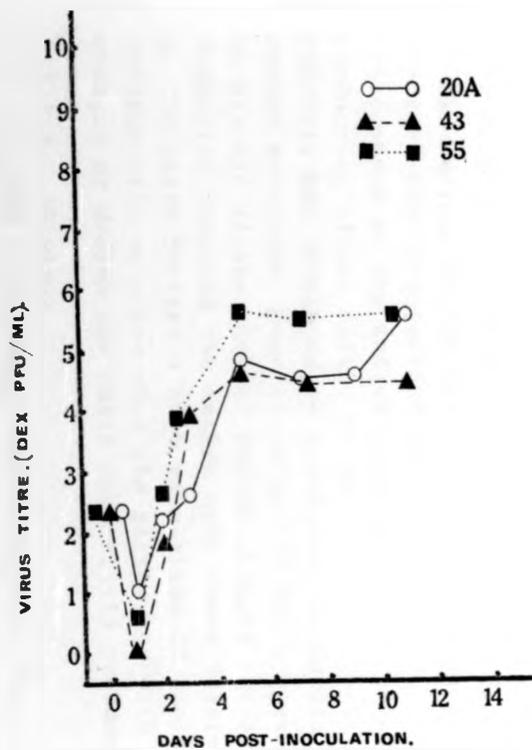


Figure 12 a.

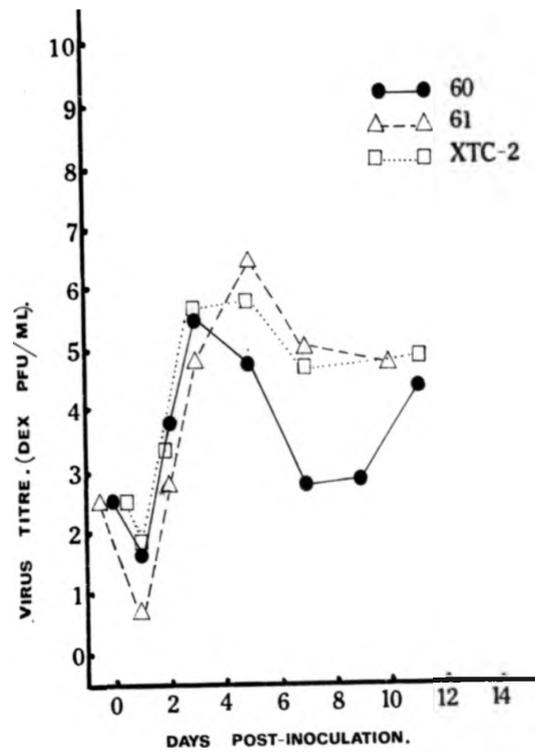


Figure 12 b.

Growth of WN virus in various cell lines.

In contrast to the alphaviruses, the initial rise in virus titre was slow, reaching a peak by about 5 days, ranging from 4.7 dex pfu per ml in the 43 cells to 6.4 dex pfu per ml in the 61 cells. There was no marked fall in titre after this, except in the case of the 60 cells, and titres were maintained at a fairly steady level for up to 21 days.

A CPE was observed in the XTC-2 cells, and also in the two mosquito cell lines, 60 and 61. In the XTC-2 cells the development of a CPE was slow compared to the alphaviruses. By day 5 there were a few dark rounded cells attached to the glass, becoming more evident by day 7. By day 11 most of the cells were affected, and by day 17 many had detached from the glass. When the XTC-2 cells were grown on plastic trays without regular changes of medium this effect was more pronounced (Plates 9, 10, 11.).

The CPE in the two mosquito cell lines was similar to that described by Varma *et al* (1974). In the 60 cells the effect was quite severe, and by day 4 there were quite a considerable number of cells floating in the medium, whilst the cells attached to the glass appeared dark and rounded (Plates 12, 13.). Regular replacement of the medium resulted in the steady production of new cells. In the 61 cells small syncytia had developed by day 4, but these did not become extensive. However, when the cells were grown on plastic flasks without medium changes the syncytia formation involved the entire cell sheet (Plates 14, 15.). In the glass bottles a high proportion of the cells subsequently detached from the glass, but with regular changes of medium new cells were slowly produced.

#### 3.3.2.3. JE virus.

All the cells were infected with an inoculum of 3.4 dex pfu per bottle, and all the cells supported replication of the virus as shown in Fig 13 a, b.

In the 43 cells replication was limited, showing only a small peak of 2.4 dex pfu per ml on day 3, followed by a progressive fall to undetectable levels

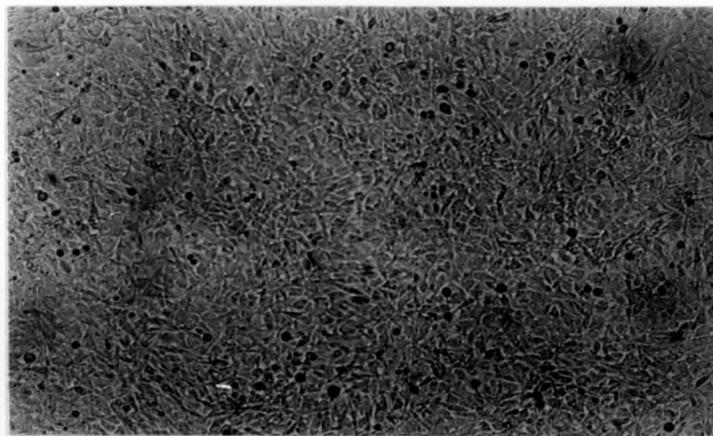


Plate 9. Control XTC-2 cells , sc 178 grown on plastic.



Plate 10. XTC-2 cells grown on plastic 4 days after infection with WN virus.

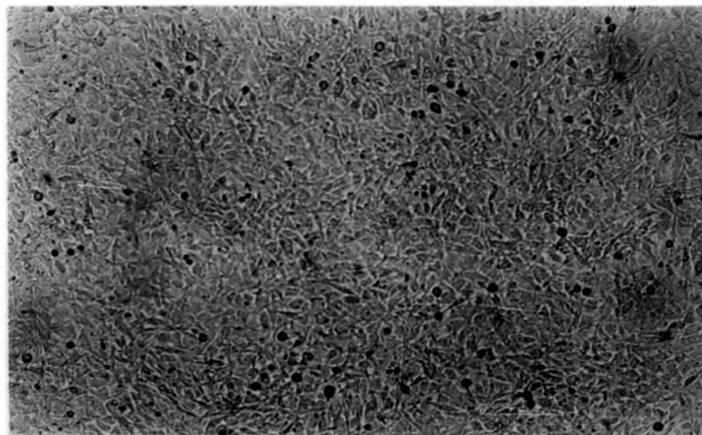


Plate 9. Control XTC-2 cells , sc 178 grown on plastic.

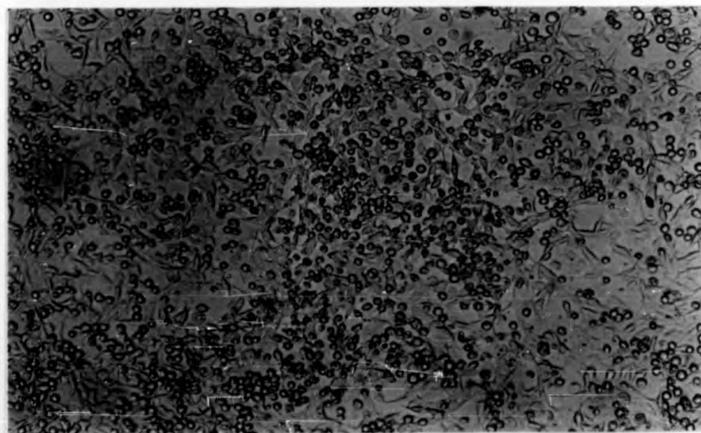


Plate 10. XTC-2 cells grown on plastic 4 days after infection with WN virus.

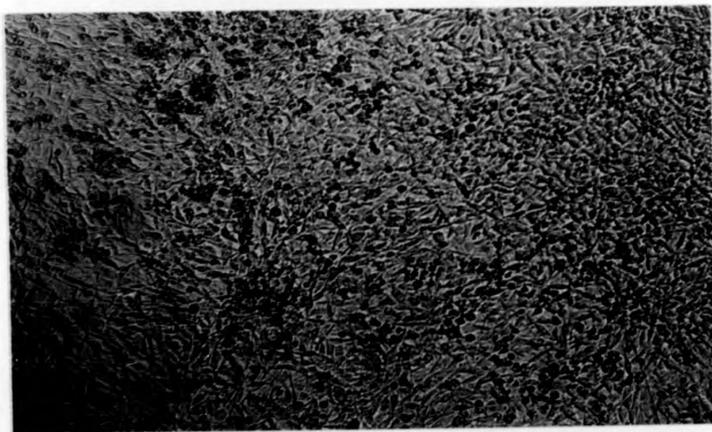


Plate 11. XTC-2 cells grown on plastic 11 days after  
infection with WN virus.

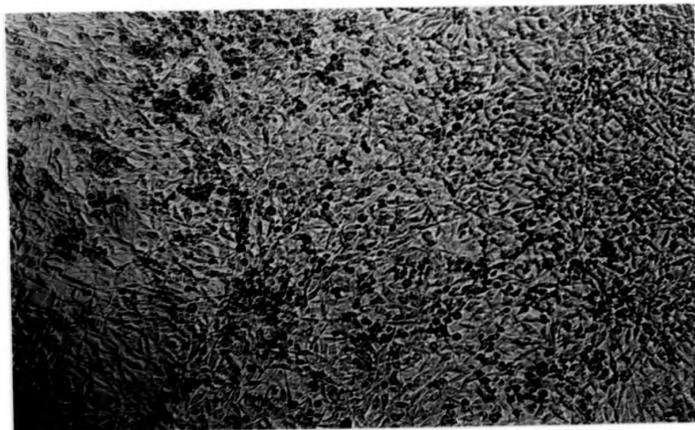


Plate 11. XTC-2 cells grown on plastic 11 days after  
infection with WN virus.

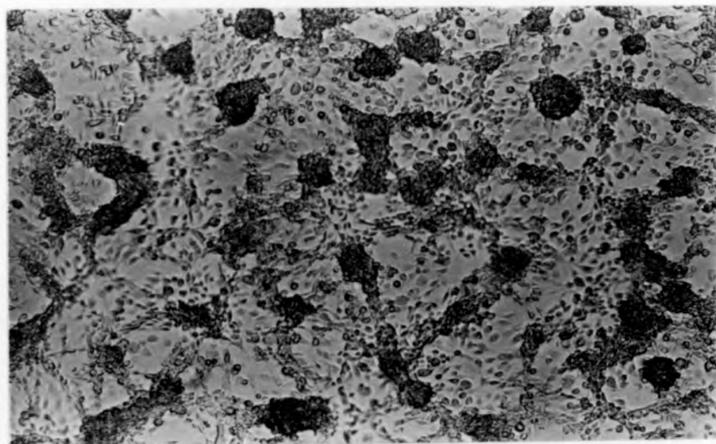


Plate 12. Control 60 cells, sc 43 grown on glass.

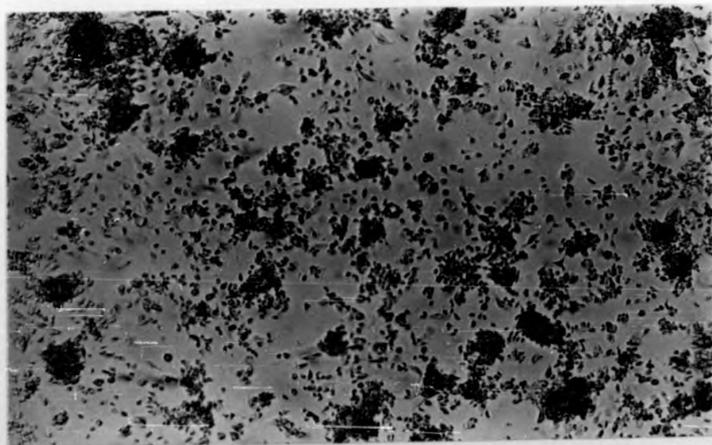


Plate 13. 60 cells grown on glass 4 days after infection with WN virus.

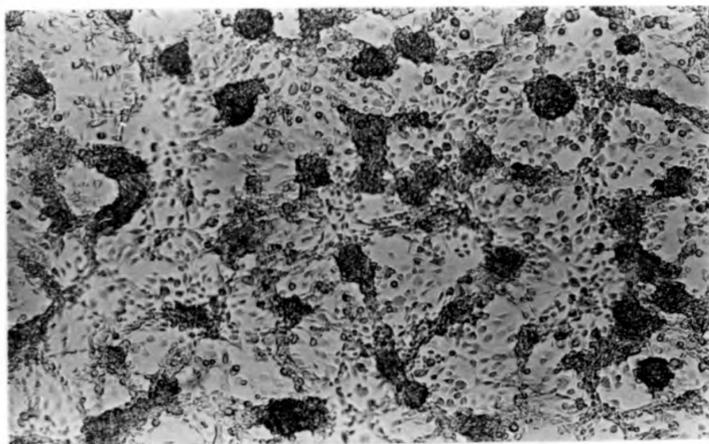


Plate 12. Control 60 cells, sc 43 grown on glass.

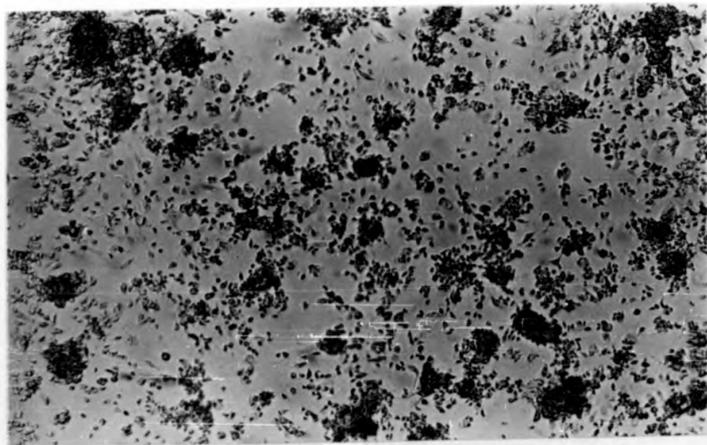


Plate 13. 60 cells grown on glass 4 days after infection  
with WN virus.



Plate 14. Control 61 cells, sc 41 grown on plastic.

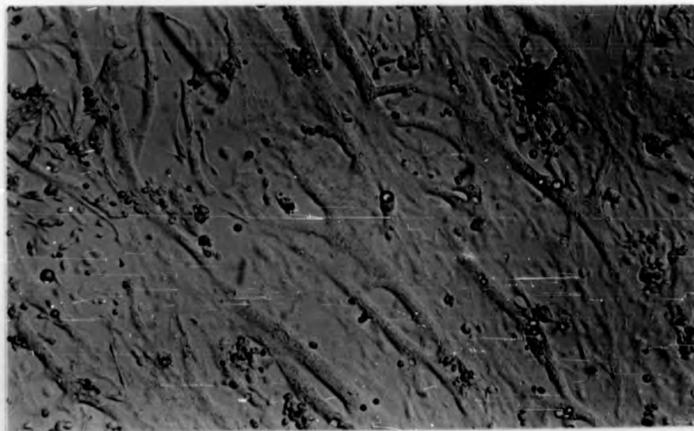


Plate 15. 61 cells grown on plastic 5 days after infection with WN virus.



Plate 14. Control 61 cells, sc 41 grown on plastic.

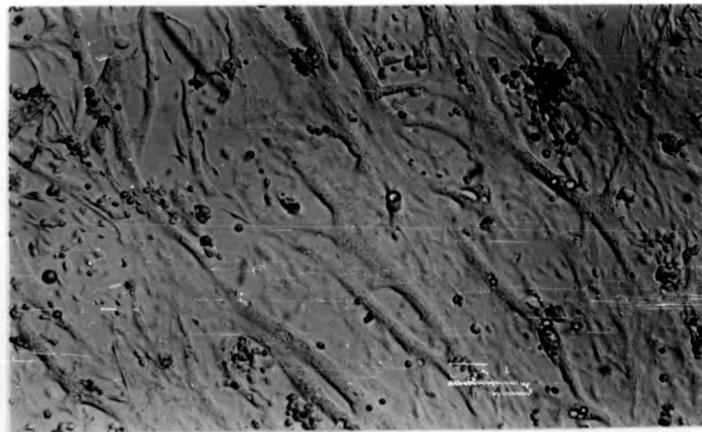


Plate 15. 61 cells grown on plastic 5 days after infection with WN virus.

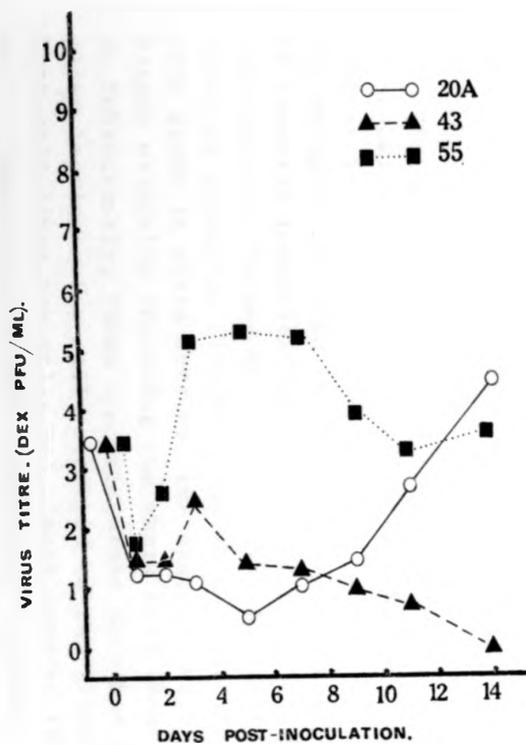


Figure 13 a.

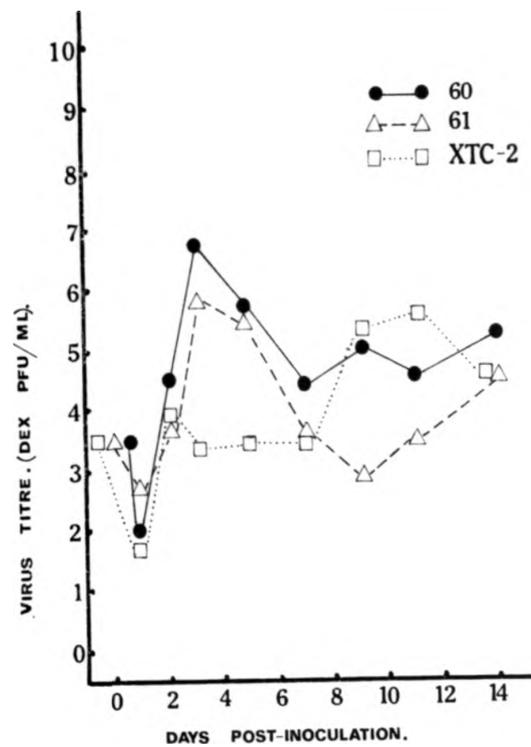


Figure 13 b.

Growth of JE virus in various cell lines.

by day 14. Low titres were also seen in the 20A cells of only about 1.0 dex pfu per ml up until day 6. Then there was a steady rise in titre reaching 4.4 dex pfu per ml on day 14 and 5.1 dex pfu per ml by day 21.

In contrast, the 55 cells supported more rapid growth of the virus with a rise to 5.1 dex pfu per ml by day 3, which was maintained until day 7 before falling to 3.5 dex pfu per ml by 21 days.

There was also a more rapid rise in titre in the 60 and 61 cells, reaching 6.7 dex pfu per ml in the 60 cells and 5.7 dex pfu per ml in the 61 cells by day 3. The titre then fell in the 60 cells to be maintained at about 5.0 dex pfu per ml between days 8 and 21, whereas in the 61 cells a more marked fall to 2.8 dex pfu per ml was seen by day 9 followed by maintenance at about 4.4 dex pfu per ml between days 14 and 21.

In the XTC-2 cells the virus titre was maintained at about the inoculum level until day 8 and then rose to over 5.0 dex pfu per ml by day 11. Between days 14 and 21 levels of about 4.4 dex pfu per ml were reached.

CPE, seen in the 60 and 61 cells but not in the XTC-2 cells or the other mosquito cell lines, was similar to that described by Varma et al (1974). In the 60 cells, as with WN virus, there was no evidence of syncytia formation, but in the 61 cells small syncytia were formed by day 3. Unlike the restricted syncytia formation with WN virus when the 61 cells were grown in glass bottles, the syncytia with JE virus became extensive involving the whole cell sheet by day 5. Subsequently, these syncytial areas detached from the glass leaving a few cells attached to the glass. In both cell lines new cells had almost covered the glass at the completion of the experiment at 21 days.

Whilst there was no evidence of CPE in the XTC-2 cells, separate experiments ( see section 3.4.4.) have indicated some general CPE under CMC overlay at high virus concentrations, but no clear plaques

have been seen.

#### 3.3.2.4. Zika virus.

All the cell lines were infected with an inoculum of 3.7 dex pfu per bottle, and the results are shown in Fig 14 a, b.

The virus multiplied in the mosquito cell lines but not in the XTC-2 cells. Low titres not exceeding 2.7 dex pfu per ml were seen in the two anopheline cell lines, 43 and 55, and growth was particularly poor in the 43 cells.

In contrast, the virus multiplied well in the three aedine cell lines. In the 20A cells the titre rose to about 4.0 dex pfu per ml by day 3 and reached 5.7 dex pfu per ml by day 14. Titres in the 60 and 61 cells were higher reaching about 6.0 dex pfu per ml by day 3, but the levels then fell steadily being below that of the 20A cells by 14 days.

No CPE was observed in any of the cell lines with this virus.

#### 3.3.2.5. DEN-2 and YF viruses.

Infection of the cells with DEN-2 virus was attempted with an inoculum of 3.9 dex pfu per bottle, and with 3.8 dex pfu per bottle for YF virus. The results for DEN-2 virus are shown in Fig 15 a, b, and in Fig 16 a, b, for YF virus. With both viruses replication was only seen in the three aedine cell lines.

Similar titres of DEN-2 virus were found in both the 60 and 61 cells, showing a steady rise in virus titre to a peak from about 6-8 days and with titres never exceeding 4.0 dex pfu per ml in both cell lines. In contrast, in the 20A cells there was no detectable virus until day 10, when 2.0 dex pfu per ml was found. Assay of the 14 day tissue culture fluid suggested that virus was being maintained at this level, but in separate experiments using higher inocula and longer incubation periods only trace amounts of virus were detected on days 17 and 21. These results must be considered as inconclusive.

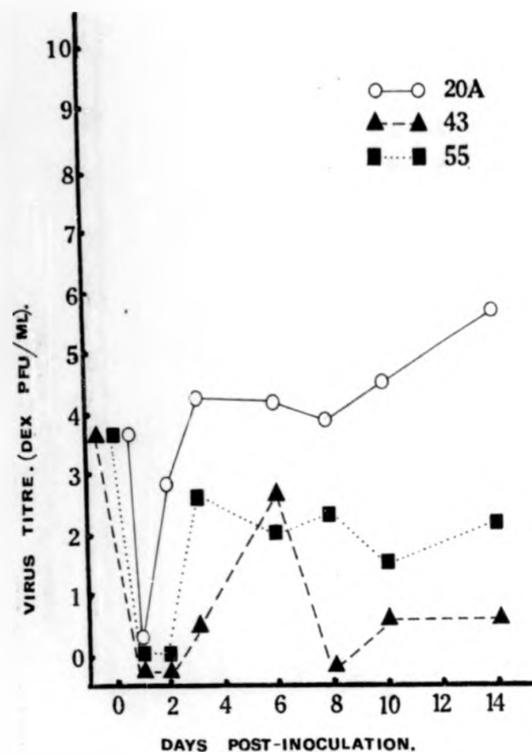


Figure 14 a.

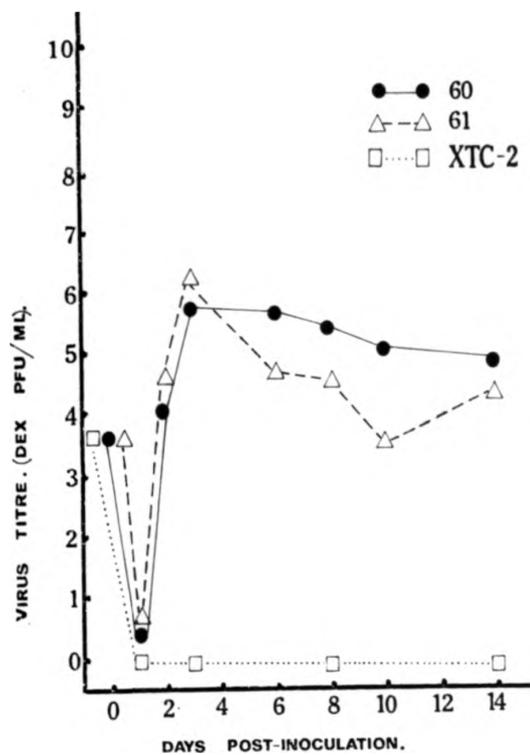


Figure 14 b.

Growth of ZIKA virus in various cell lines.

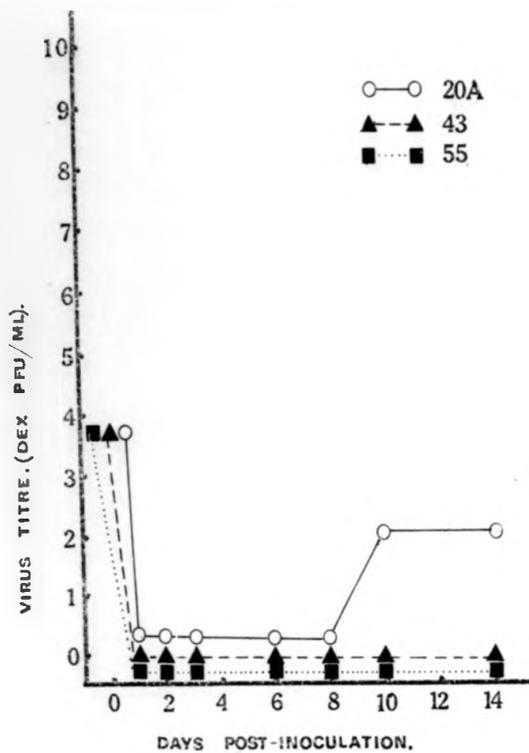


Figure 15 a.

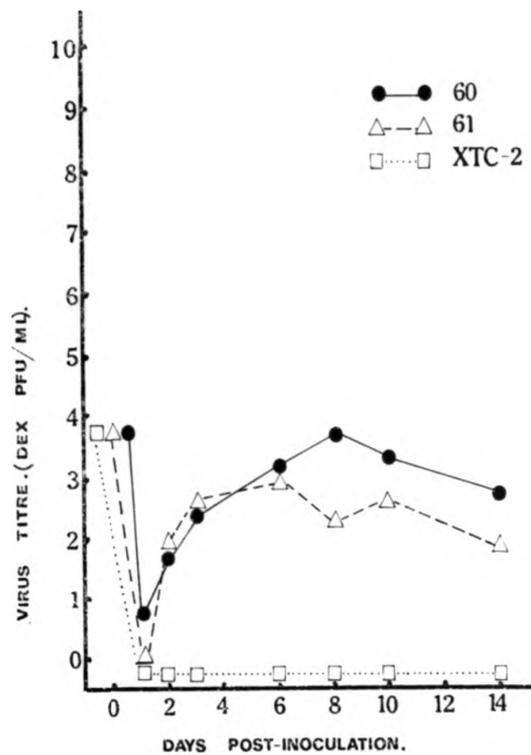


Figure 15 b.

Growth of DEN-2 virus in various cell lines.

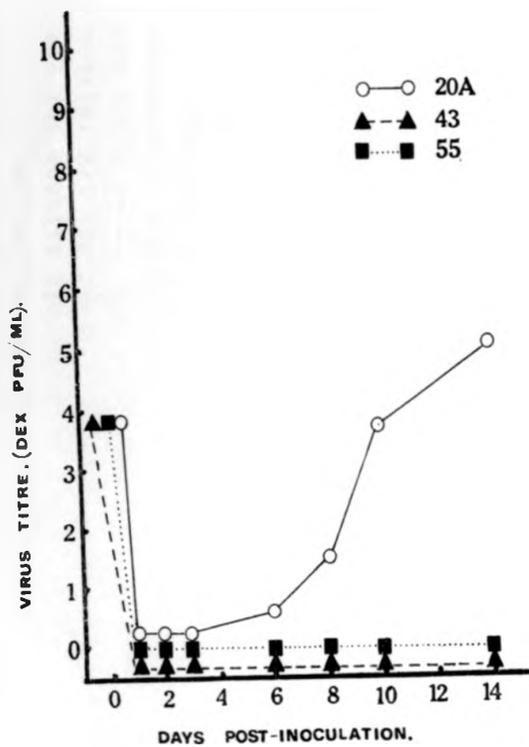


Figure 16 a.

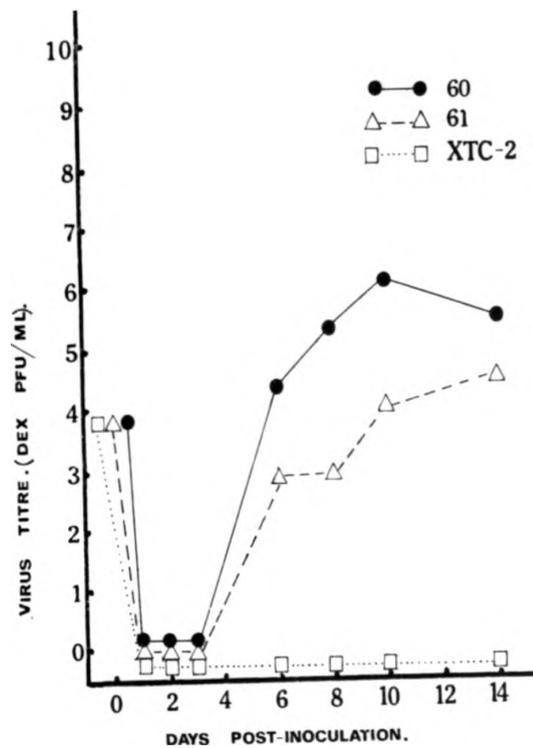


Figure 16 b.

Growth of YF virus in various cell lines.

With YF virus higher titres were observed in all three cell lines, although there was no detectable virus in any of the cultures for the first three days. Titres then rose quite sharply in the 60 cells, and more slowly in the 61 and 20A cells. A peak titre of 6.0 dex pfu per ml was seen in the 60 cells 10 days after infection, and this fell progressively reaching 3.7 dex pfu per ml by day 21. In the the 61 cells a lower titre of 4.5 dex pfu per ml was reached by day 14 followed by a fall to 3.7 dex pfu per ml by day 21 compared to 5.0 dex pfu per ml in the 20A cells on day 14, 5.3 dex pfu per ml on day 17 and 3.7 dex pfu per ml by day 21.

A CPE was observed in the 60 and 61 cell lines, but only with DEN-2 virus. In the 60 cells the effects were rather indistinct, and by day 6 the cell sheet was very clumpy with a lot of dark granular cells attached to the glass between the clumps. By day 8 most of these dark cells had detached from the glass leaving the clumps still attached. In contrast, extensive syncytia involving the entire cell sheet were formed in the 61 cells from about 3 days. These remained attached to the glass for about a week but many had detached from the glass by 10 days, leaving new cells to grow out on the glass.

### 3.3.3. Growth of bunyaviruses.

#### 3.3.3.1. BUN virus.

The 20A, 43 and 55 cells were infected with an inoculum of 3.8 dex pfu per bottle, and the 60, 61 and XTC-2 cells with an inoculum of 3.5 dex pfu per bottle. All the lines supported growth of the virus and the results are shown in Fig 17 a, b.

In the 20A cells there was a steady rise in titre from 3.5 dex pfu per ml on day 1 to 5.6 dex pfu per ml by day 14, compared to lower titres in the anopheline cell lines. In the 43 cells titres were maintained at about 3.0 dex pfu per ml until day 8 before falling to about 2.0 dex pfu per ml by day 14, whereas in the

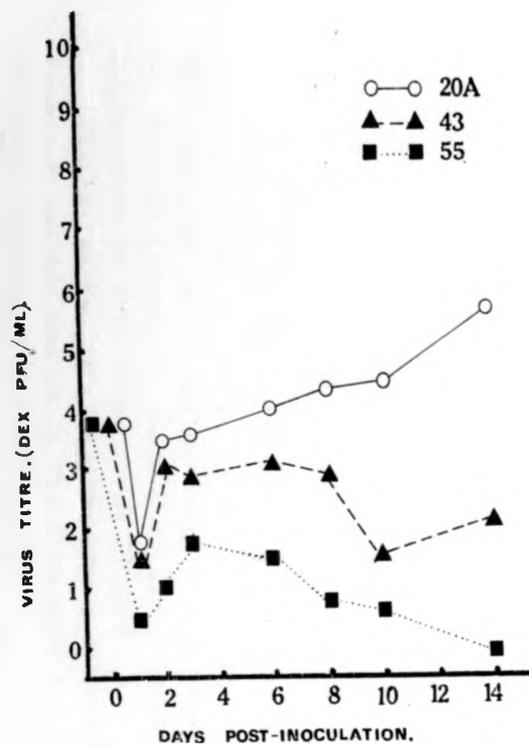


Figure 17 a.

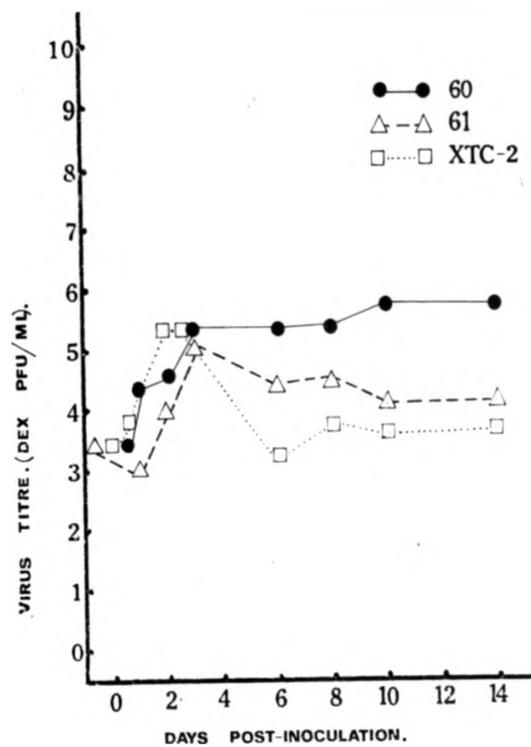


Figure 17 b.

Growth of BUN virus in various cell lines.

55 cells the titre fell from only 1.8 dex pfu per ml on day 3 to undetectable levels by day 14.

The growth patterns in the 60, 61 and XTC-2 cells were all quite similar, showing a rise in all three lines to about 5.0 dex pfu per ml by day 3, and then falling only slightly in the 60 and XTC-2 cells by day 14.

A CPE was seen only in the XTC-2 cells with BUN virus. By day 2 there were extensive areas of dead cells, and by day 8 considerable numbers of dead cells were floating in the medium. By day 10 there was some growth of new cells, but large numbers of floating cells were still present on completion of the experiment at 14 days.

#### 3.3.3.2. GER virus.

The 20A, 43 and 55 cells were infected with an inoculum of 4.7 dex pfu per bottle, and the 60, 61 and XTC-2 cells with an inoculum of 2.0 dex pfu per bottle. All the lines supported replication of the virus and the results are shown in Fig 18 a, b.

In the 20A and 55 cells similar growth patterns were seen, with fairly low titres ranging from 2.0 to 3.0 dex pfu per ml over a 14 day incubation period. In contrast to these results, and the results with the previous viruses, there was a marked rise in titre in the 43 cells to 6.4 dex pfu per ml by day 3 followed by a progressive fall to 3.7 dex pfu per ml by 14 days.

Similar patterns were seen in the 60 and 61 cells, although titres in the 61 cells were about 1 dex lower than those in the 60 cells, where a peak titre of 6.3 dex pfu per ml was reached by day 6 followed by maintenance at this level until 14 days.

Titres in the XTC-2 cells were much lower falling from 3.7 dex pfu per ml on day 2 to only 1.5 dex pfu per ml by day 14.

As with BUN virus a CPE was seen only in the XTC-2 cells. This was not extensive, and by day 3 there were only a few foci of dead cells. By day 6 only a few dark rounded cells could be seen on top of a healthy cell

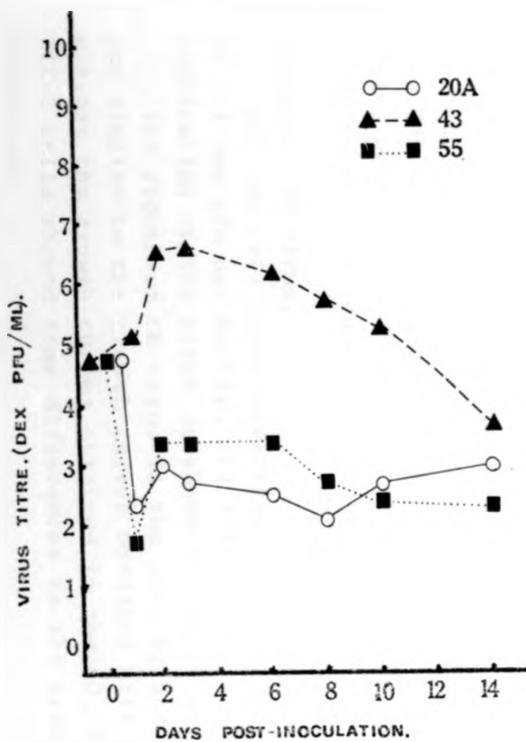


Figure 18 a.

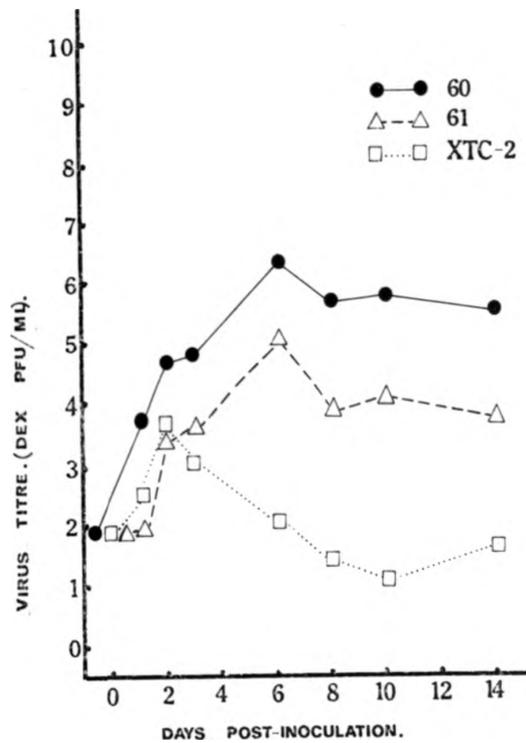


Figure 18 b.

Growth of GER virus in various cell lines.

sheet, and by day 10 most of these cells had detached and were floating in the medium.

#### 3.3.3.3. BWA virus.

All the cells were infected with an inoculum of 3.1 dex pfu per bottle and the results are shown in Fig 19 a, b. With the exception of the 55 cells, all the cell lines supported replication of the virus although low titres were seen, not exceeding the level of the inoculum. This finding is in marked contrast to the results obtained with some of the other bunyaviruses.

The results obtained in the 20A cells were of particular interest. Titration of the harvested tissue culture fluid on days 1, 2, 3 and 6 revealed no detectable virus. However, on day 8 a faint plaque type could be seen compared to the original stock virus that produced distinct plaques in Vero cells (Plate 16). Dead cells could be seen in stained Vero cells inoculated with a  $10^{-3}$  dilution of the 14 day harvest, but a definite plaque enumeration proved impossible due to the nature of the plaques.

No CPE was seen in any of the cell lines, but separate experiments (see section 3.4.4.) have shown that BWA virus will produce faint plaques in XTC-2 cells, but only at a dilution of  $10^{-4}$  of the stock virus suspension compared to  $10^{-7}$  in Vero cells.

#### 3.3.3.4. CE virus.

All the cell lines were infected with an inoculum of 2.4 dex pfu per bottle, and all the cells supported replication of the virus as shown in Fig 20 a, b.

The growth of CE virus in the 20A, 43 and 55 cells was similar to the growth curves obtained with BUN virus, whereas the growth curves obtained in the 60, 61 and XTC-2 cells showed some differences to the growth of BUN virus.

In the 60 cells the titre never exceeded 4.0 dex pfu per ml throughout the incubation period of 14 days, whereas in the 61 cells a titre of 6.7 dex pfu per ml was reached by day 6 before falling steadily to 5.0 dex pfu per ml on day 14. In the XTC-2 cells titres were

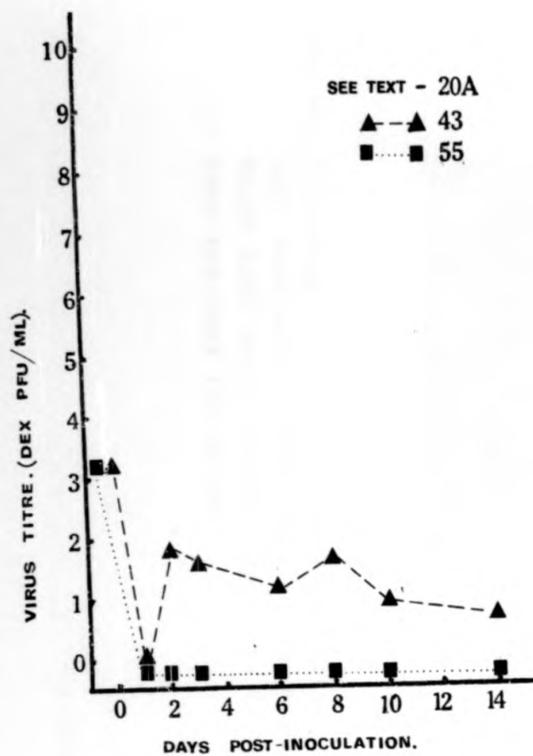


Figure 19 a.

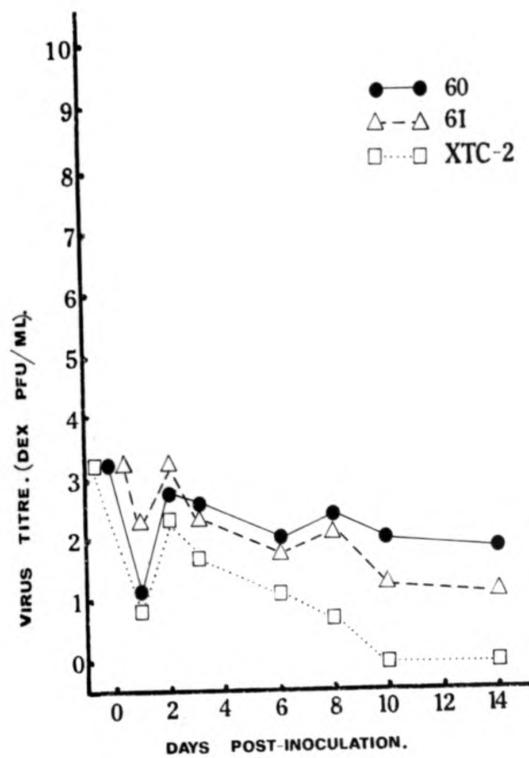


Figure 19 b.

Growth of BWA virus in various cell lines.



Plate 16. Titration of BWA virus in Vero cells.  
Left hand well, stock virus plaque morphology.  
Right hand well, plaque morphology of virus  
from infected 20A cells.



Plate 16. Titration of BWA virus in Vero cells.  
Left hand well, stock virus plaque morphology.  
Right hand well, plaque morphology of virus  
from infected 20A cells.

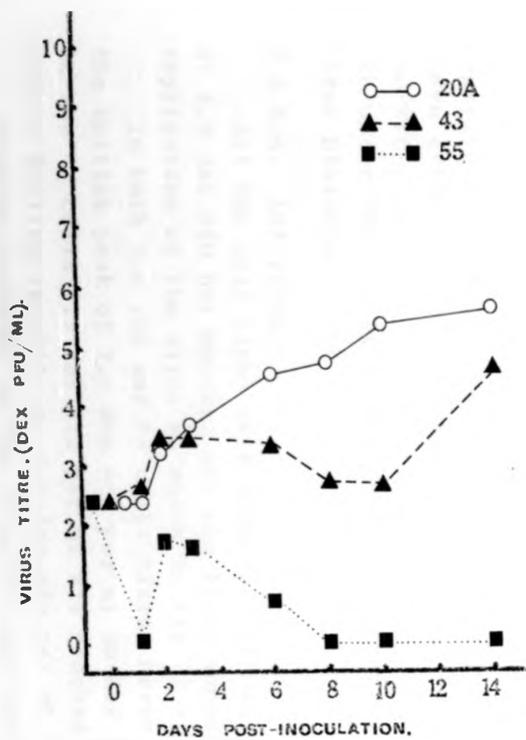


Figure 20 a.

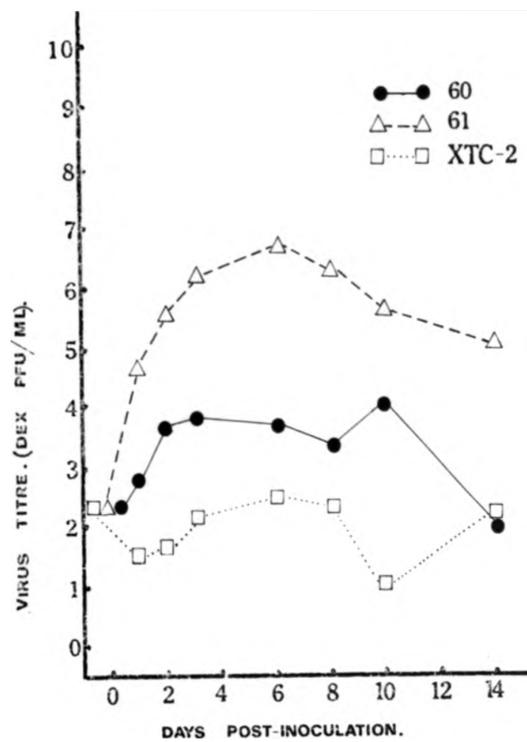


Figure 20 b.

Growth of CE virus in various cell lines.

even lower than the 60 cells, never exceeding 2.4 dex pfu per ml over the 14 day uncubation period.

No CPE was observed in any of the cell lines with CE virus. However, separate experiments (see section 3.4.3.) have shown that a low grade CPE is present in the XTC-2 cells, appearing as a few clumps of dark rounded cells on top of a healthy monolayer. This effect was only seen at high virus concentrations.

#### 3.3.3.5. SFS virus.

All the cell lines were infected with an inoculum of 4.8 dex pfu per bottle, and the results are shown in Fig 21 a, b. Only limited virus titres were seen, and then only in the XTC-2 and the 43 cells. Small peaks of 2.8 dex pfu per ml on day 2 in the 43 cells, and 2.1 dex pfu per ml on days 2-6 in the XTC-2 cells, were followed by a fall to undetectable levels. Such low levels could also be explained on the basis of survival and elution of adsorbed virus and so the results must be considered inconclusive.

No CPE was seen in any of the cell lines, although separate experiments (see sections 3.4.3., 3.4.4.) have shown evidence of a CPE, suggesting virus growth, in the XTC-2 cells in plastic trays and also a generalised CPE under CMC overlay rather than the appearance of clear plaques.

#### 3.3.3.6. ANA virus.

All the cell lines were infected with an inoculum of 4.8 dex pfu per bottle, all the lines supported replication of the virus as shown in Fig 22 a, b.

In both the 20A and 55 cells titres never exceeded the initial peak of 2.6 dex pfu per ml on day 2, whereas in the 43 cells 5.9 dex pfu per ml was reached by day 3 before falling steadily to 3.3 dex pfu per ml by day 14.

Similar patterns to the 43 cells were seen in the 60 and 61 cells but in the XTC-2 cells the titres fell progressively until 3 days and then levels of 1.0 dex pfu per ml were found up until 14 days.

As with SFS virus no CPE was seen with ANA virus,

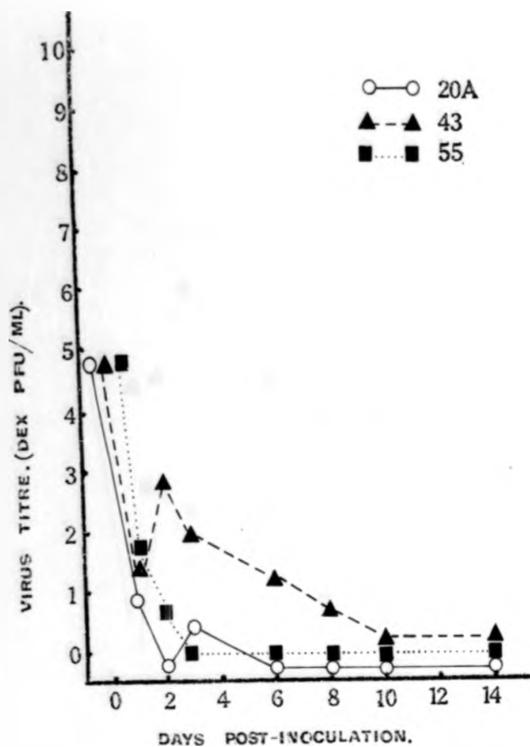


Figure 21 a.

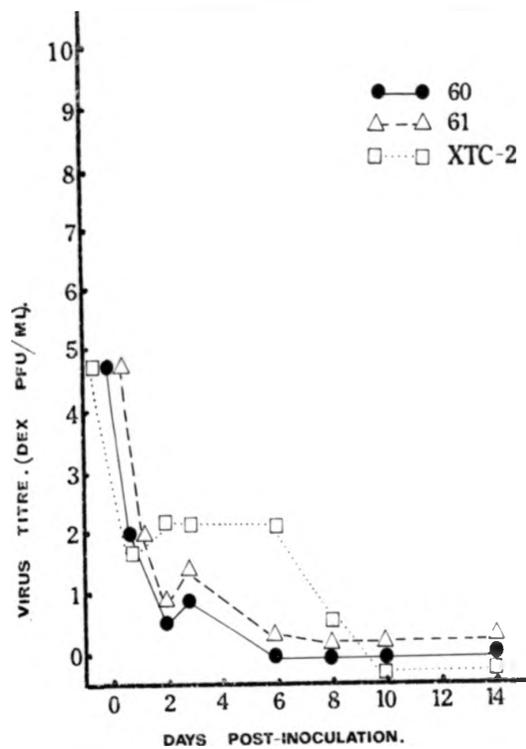


Figure 21 b.

Growth of SFS virus in various cell lines.

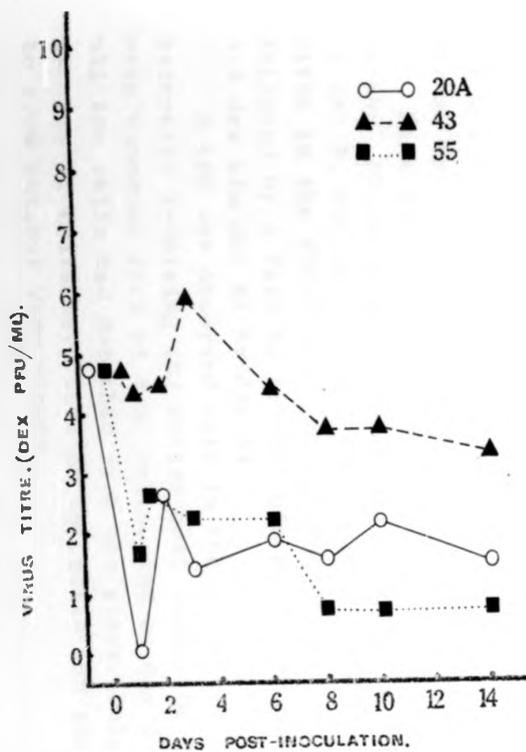


Figure 22 a.

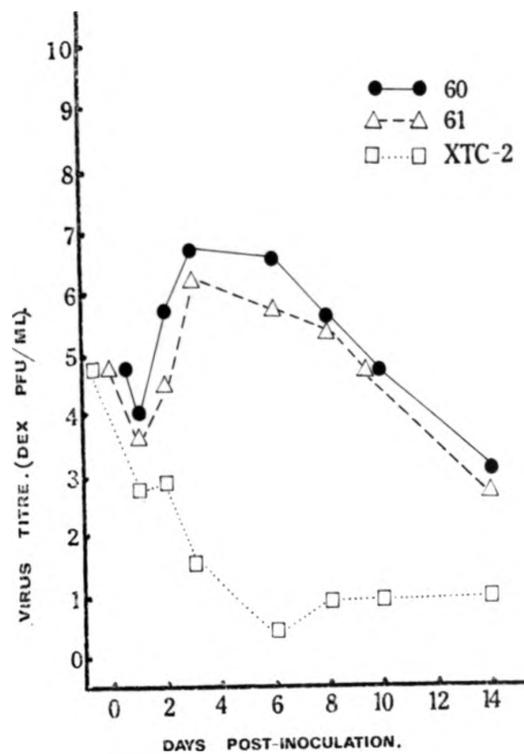


Figure 22 b.

Growth of ANA virus in various cell lines.

but separate experiments (see section 3.4.4.) have shown a generalised CPE under overlay at high virus concentrations.

### 3.3.4. Growth of rhabdoviruses.

#### 3.3.4.1. CHP virus.

CHP virus was the only rhabdovirus tested in the cell lines, and an inoculum of 2.9 dex pfu per bottle was used to infect the cells. This was sufficient to establish an infection in all the cell lines except for the 20A cells, but subsequently an infection was established in the 20A cells using an inoculum of 5.4 dex pfu per bottle. The results are shown in Fig 23 a, b.

In the 43 cells there was a rapid rise in titre to about 7.0 dex pfu per ml by day 3, and titres were then maintained at a high level until day 14, whereas in the 55 cells the rise in titre was more gradual reaching a peak of 6.5 dex pfu per ml by day 14. Titres in the 20A cells were lower than this reaching 2.2 dex pfu per ml on day 14 and only rising to 4.4 dex pfu per ml by day 14.

Peak titres of 7.0 dex pfu per ml were reached in the 60 cells and 5.9 dex pfu per ml in the 61 cells by day 8, but in contrast there was a rapid rise in titre in the XTC-2 cells to 8.8 dex pfu per ml by day 2, followed by a fall to 4.0 dex pfu per ml by day 8 and 3.3 dex pfu per ml by day 14.

A CPE was observed only in the XTC-2 cells, this was extensive involving the entire cell sheet. On day 1 there were numerous foci of dead cells, and by day 2 nearly all the cells had detached from the glass. Only a few patches of cells were left on the glass and these began to grow out, but very slowly.

### 3.3.5. Growth of unclassified viruses.

#### 3.3.5.1. QRF and ZIR viruses.

Replication of these two tick-borne viruses was only obtained in the XTC-2 cells using inocula of 3.1 dex pfu

but separate experiments (see section 3.4.4.) have shown a generalised CPE under overlay at high virus concentrations.

### 3.3.4. Growth of rhabdoviruses.

#### 3.3.4.1. CHP virus.

CHP virus was the only rhabdovirus tested in the cell lines, and an inoculum of 2.9 dex pfu per bottle was used to infect the cells. This was sufficient to establish an infection in all the cell lines except for the 20A cells, but subsequently an infection was established in the 20A cells using an inoculum of 5.4 dex pfu per bottle. The results are shown in Fig 23 a, b.

In the 43 cells there was a rapid rise in titre to about 7.0 dex pfu per ml by day 3, and titres were then maintained at a high level until day 14, whereas in the 55 cells the rise in titre was more gradual reaching a peak of 6.5 dex pfu per ml by day 14. Titres in the 20A cells were lower than this reaching 2.2 dex pfu per ml on day 14 and only rising to 4.4 dex pfu per ml by day 14.

Peak titres of 7.0 dex pfu per ml were reached in the 60 cells and 5.9 dex pfu per ml in the 61 cells by day 8, but in contrast there was a rapid rise in titre in the XTC-2 cells to 8.8 dex pfu per ml by day 2, followed by a fall to 4.0 dex pfu per ml by day 8 and 3.3 dex pfu per ml by day 14.

A CPE was observed only in the XTC-2 cells, this was extensive involving the entire cell sheet. On day 1 there were numerous foci of dead cells, and by day 2 nearly all the cells had detached from the glass. Only a few patches of cells were left on the glass and these began to grow out, but very slowly.

### 3.3.5. Growth of unclassified viruses.

#### 3.3.5.1. QRF and ZIR viruses.

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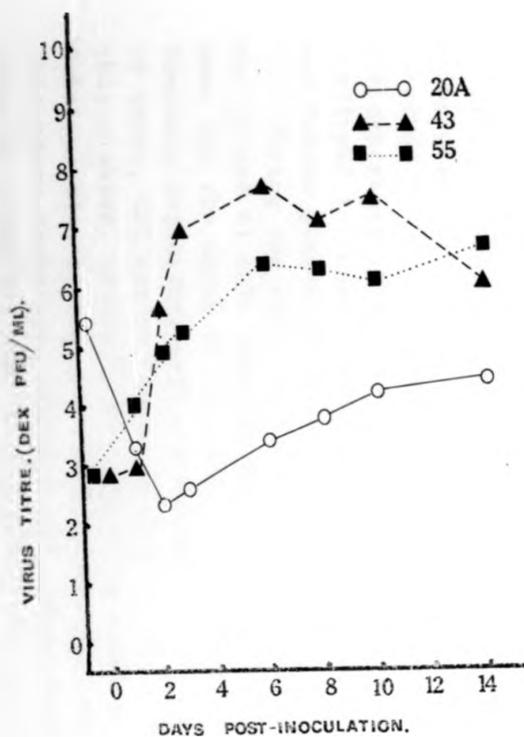


Figure 23 a.

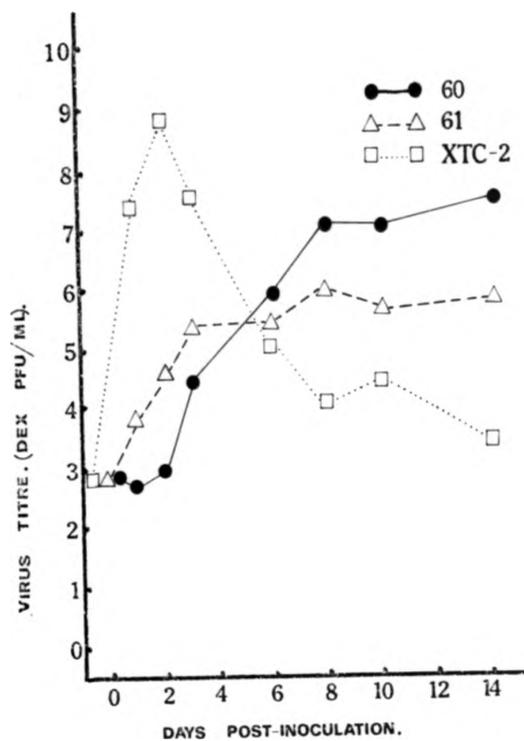


Figure 23 b.

Growth of CHP virus in various cell lines.

per bottle for QRF virus and 3.6 dex pfu per bottle with ZIR virus. The results obtained are shown in Fig 11 b.

QRF virus appeared to grow faster than ZIR virus reaching a peak of 4.3 dex pfu per ml by day 3, but ZIR reached a peak of 3.6 dex pfu per ml only by day 6.

There was evidence of a CPE caused by both viruses consisting of small areas of dark rounded cells, either detaching from the glass or resting on top of a healthy cell sheet. This effect was more noticeable with QRF virus.

#### 3.4 Cytopathic effect (CPE) and plaque formation by arboviruses.

##### 3.4.1. CPE in mosquito cells.

CPE was observed in the 60 and 61 cells, but only with three viruses, all flaviviruses, WN, JE and DEN-2. The CPE, which has been described in the previous section, was similar to that described by Varma et al (1974), although all my experiments were performed using cells grown in glass bottles. Varma et al (1974) observed that the surface on which the cells are grown influences the production of clear CPE, and this was confirmed in separate studies using both Linbro trays and Falcon plastic flasks.

During the early part of studies on CPE production the 60 and 61 cell lines grown in Linbro trays were used for the assay of the three flaviviruses mentioned. However, coinciding with the delivery of a new batch of trays, CPE was no longer obvious in cells grown on this plastic, although cell growth appeared normal. Subsequently, Linbro trays have proved useless for this assay technique, although the plaque assay of virus in mammalian and XTC-2 cells in these trays has shown no alterations. It must be assumed that the failure of the mosquito cells to respond to infection with these flaviviruses by the production of a CPE is due to some change in the manufacturing process of

the plastic trays, or is due to alterations in the raw materials used to fabricate the trays.

#### 3.4.2. Plaque production in mosquito cells.

Numerous attempts were made to produce plaques in all the cell lines using WN as a test virus. The cells were infected as monolayers or in suspension in Linbro trays, overlaid with CMC overlay, and incubated at 28°C. The cells were stained in the usual manner with naphthalene-black stain.

The results obtained were consistently negative with all the cell lines.

Attempts were made to produce plaques by incorporating 10ug/ml of trypsin into the overlay as described for influenza viruses by Appleyard and Maber (1974), but again without success.

A few pilot experiments using the 60 and 61 cells grown in Falcon plastic flasks gave inconclusive results, and parallel experiments using cells grown in glass bottles were discontinued as the cells tended to detach from the glass under the overlay.

#### 3.4.3. CPE in XTC-2 cells.

Of the 23 viruses tested for growth in these cells CPE was observed with 14, and in additional experiments with CE, ANA, JE and SFS viruses evidence was obtained of a low grade CPE, consisting of a few dark rounded cells on top of a healthy cell sheet, when the cells were grown in Linbro trays.

These studies were extended to include a number of other viruses, and the results obtained with 38 viruses are summarised in Table 12.

In general, the alphaviruses tested formed a fairly homogenous group producing extensive CPE usually commencing from about day 2 after infection. In contrast, the 11 flaviviruses tested generally failed to produce CPE in the cells, with the exception of WN and NTA viruses. In the case of the three mosquito-borne flaviviruses DEN-2, YF and ZIKA no replication was demonstrated in the cells, but the

TABLE 12.

## PRODUCTION OF CPE IN XTC-2 CELLS BY VARIOUS VIRUSES.

VIRUS	EXTENT CPE	DAY CPE BEGINS	DAY CPE ENDPOINT	TITRE DEX CPD <sub>50</sub> /ML	TITRE IN
					MAMMALIAN CELLS (DEX PFU/ML) OR MICE (LD <sub>50</sub> /ML)
BEB	4	2	4-5	7.7	*
CHIK	1-2	2	5-6	6.0	6.0 (Vero)
GET	4	2	4-5	8.5	*
MAY	4	2	5-6	8.5	*
MID	2-3	4	5-6	6.8	*
NDU	2-3	3	5	7.5	*
ONN	2-3	2	4-5	6.4	4.9 (Vero)
SF	3-4	2	4-5	7.5	8.0 (Vero)
SIN	3-4	2	3-4	6.5	7.0 (Vero)
WHAT	4	2	5	7.5	7.0 (Vero)
DEN-2	No	-	-	-	*
ENT	No	-	-	-	7.8 (PS)
JE	No	-	-	-	8.9 (PS)
LGT	No	-	-	-	7.6 (PS)
LI	No	-	-	-	8.2 (PS)
MOD	No	-	-	-	8.0 (PS)
NTA	2-3	4	5-6	5.8	5.5 (Mice)
TEM	No	-	-	-	7.6 (PS)
WN	2-3	4	6-8	6.5	*
YF	No	-	-	-	*
ZIKA	No	-	-	-	7.6 (PS)
GER	3-4	4	5-7	8.2	*
PGA	No	-	-	-	5.5 (Mice)
CE	+	4	4-6	4.4	*
TAH	2	2	5-7	5.2	*
AMT	3-4	4	4-5	7.2	6.4 (Mice)
SFS	2-3	2	5-6	5.9	6.2 (PS)
DUG	2-3	4	5-6	5.5	6.7 (Mice)
GAN	1-2	4	5-6	4.2	5.3 (Mice)
LJN	1-2	4	5-6	2.5	6.7 (PS)
CHP	4	1	3-5	7.9	*
PIRY	4	1	3-5	6.5	*
KC	2-3	3	5-7	6.7	*
LB	2-3	5	6-9	6.5	6.6 (Vero)
MEB	+	5	6-8	3.9	5.9 (Mice)
ZIR	1-2	4	5-8	5.8	5.9 (Mice)
QRF	1-2	4	5-7	7.0	4.7 (Mice)
KET	2-3	4	5-8	6.7	6.8 (Mice)

\* See Table 13 for titre in Vero cells.

two tick-borne flaviviruses LGT and LI were shown to multiply in the cells (see section 3.3.2.), but CPE was not seen.

All the bunyaviruses tested produced CPE, except with PGA virus. This was usually not very extensive, and was rather slow to appear, being particularly evident with the tick-borne bunyaviruses DUG, GAN and LJN, where the first signs of CPE were apparent from about 4 days after infection.

Similarly, the unclassified tick-borne viruses ZIR, QRF and KET also produced CPE starting about 4 days after infection.

All the rhabdoviruses tested produced CPE which was severe with CHP and PIRY viruses, causing extensive cell death from 1 day after infection. In contrast, the three other rhabdoviruses tested, KC, LB and MEB, did not produce such extensive cell death. Two other rhabdoviruses, Obodhiang and Kotonkhan, which are not listed in Table 12, failed to produce CPE in the XTC-2 cells, although virus replication has yet to be examined.

#### 3.4.4. Plaque production in XTC-2 cells.

The XTC-2 cell line was readily adapted to the plaque titration method of deMadrid and Porterfield (1969), using a routine incubation temperature of 28°C. Preliminary tests showed that it was very important to trypsinise the cells before seeding into Linbro plastic trays, in order to ensure the formation of a uniform monolayer of cells. The techniques have been described in section 2 for the use of mammalian cells for plaque assay. For the XTC-2 cells the concentration of cells used for seeding the trays was approximately  $4 \times 10^5$  cells per ml, and L-15 medium with 10% FBS was used throughout. It was found later that the concentration of FBS could be reduced to 2%, thus affording considerable savings in serum.

The first virus tested which produced plaques was the flavivirus WN (Plate 25), and subsequently a large number of viruses were found to produce plaques

in the XTC-2 cells. Examples of the plaque types obtained are shown in Plates 17-23 and in section 3.5.

Comparative plaque assays were performed in XTC-2 and Vero cells with 32 viruses, and the results obtained are shown in Table 13. With the exception of the flaviviruses, a high proportion of the viruses tested produced plaques in the XTC-2 cells.

In general, the alphaviruses formed clear plaques, 1-2mm in diameter, with endpoints usually occurring from 2-3 days after infection, but 4 days in the case of MID and NDU viruses. ONN virus, which is not listed in Table 13, produced small faint plaques on day 3 after infection, but staining of trays on 4 days revealed no signs of plaques and the cells appeared normal. To date, all the alphaviruses that I have tested have produced plaques in the XTC-2 cells.

In contrast to these results with the alphaviruses only one flavivirus, WN, produced plaques in the XTC-2 cells. With JE virus a general CPE was seen under the overlay at high concentrations of virus, and this was similar to the results obtained with the bunyaviruses ANA and SFS (Plate 23).

The majority of the bunyaviruses tested produced plaques in the cells, but the plaque size was usually smaller than those seen with the alphaviruses, and the endpoints varied from as short as 3 days with GER virus to about 7 days with BWA virus. Several tick-borne bunyaviruses produced plaques in the XTC-2 cells, as did the unclassified tick-borne viruses (Plates 20, 21 and 22), plaques were usually small and appeared from about 5-7 days after infection.

The three rhabdoviruses tested, CHP, PIRY and KC, all produced clear plaques with endpoints on day 3 after infection (Plate 19).

The bunyavirus CE was the only virus that produced a low grade CPE in the cells without producing plaques, and no virus produced plaques whilst failing to produce CPE.

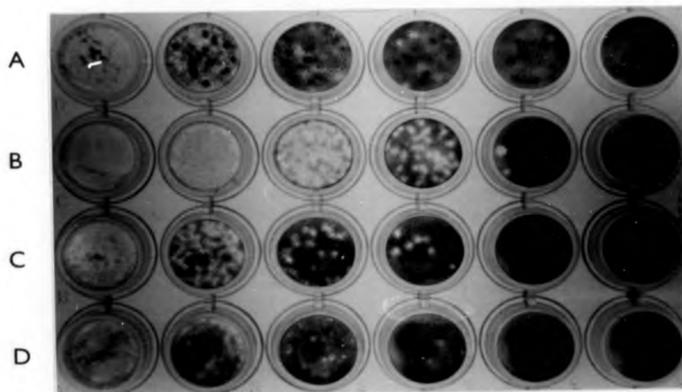


Plate 17. Plaques produced in the XTC-2 cells by four alphaviruses. A) MAY. B) URU. C) GET. D) BEB.

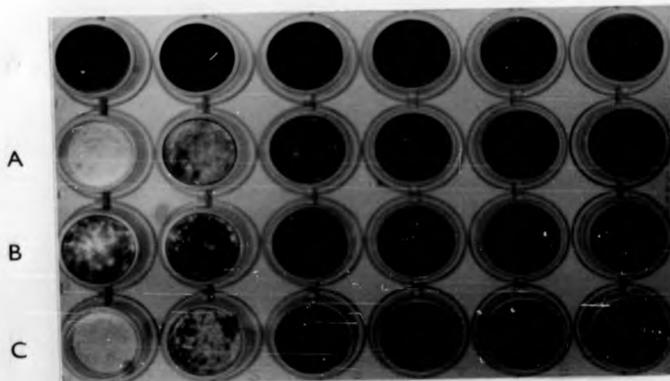


Plate 18. Plaques produced in the XTC-2 cells by three bunyaviruses. A) CVO. B) BAT. C) BUN.

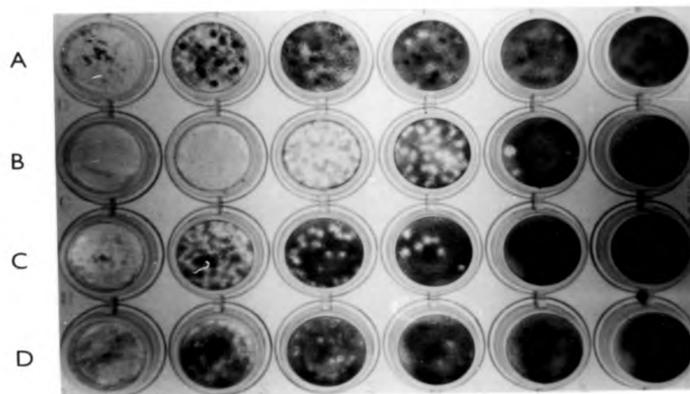


Plate 17. Plaques produced in the XTC-2 cells by four alphaviruses. A) MAY. B) URU. C) GET. D) BEB.

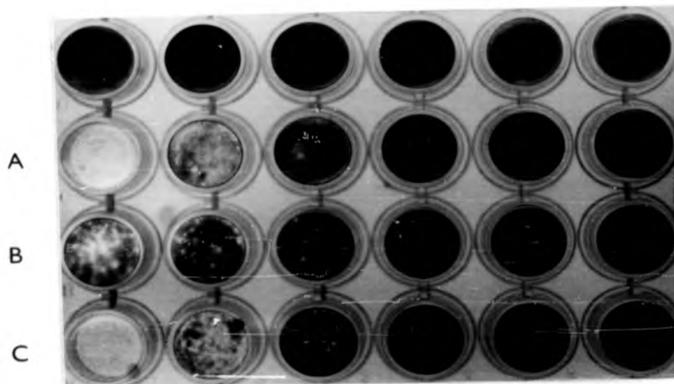


Plate 18. Plaques produced in the XTC-2 cells by three bunyaviruses. A) CVO. B) BAT. C) BUN.

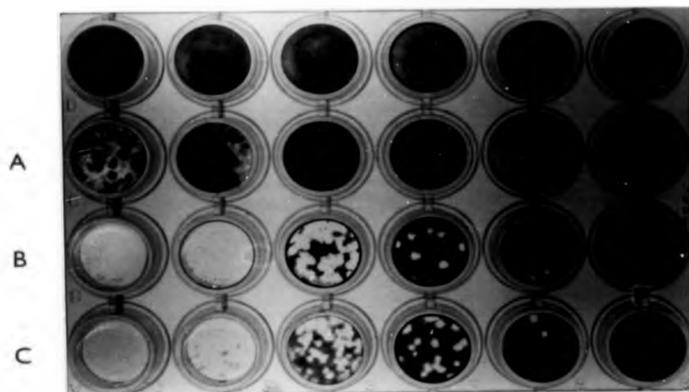


Plate 19. Plaques produced in the XTC-2 cells by three rhabdoviruses. A) KC. B) CHP. C) PIRY.

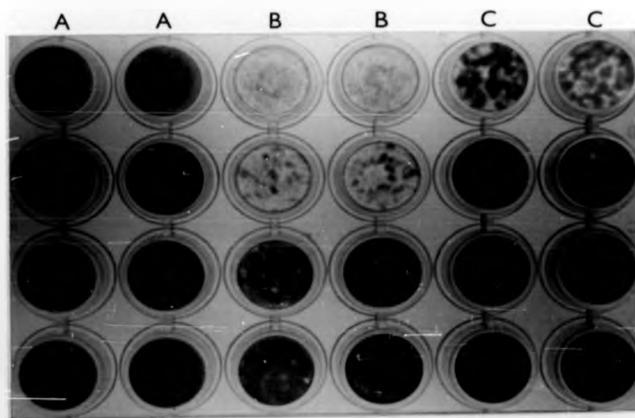


Plate 20. Plaques produced in the XTC-2 cells by three tick-borne viruses. AA) QRF. BB) DUG. CC) GAN

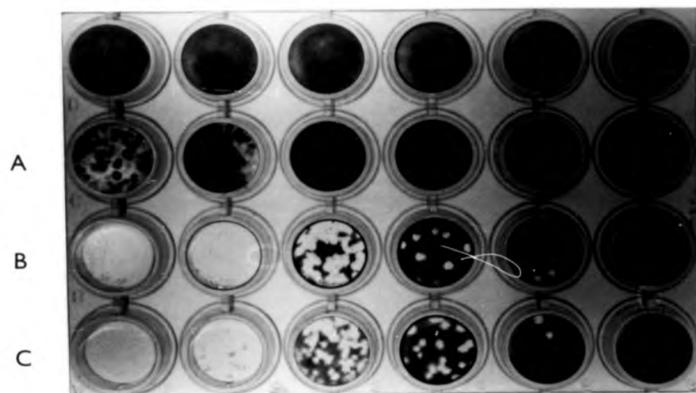


Plate 19. Plaques produced in the XTC-2 cells by three rhabdoviruses. A) KC. B) CHP. C) PIRY.

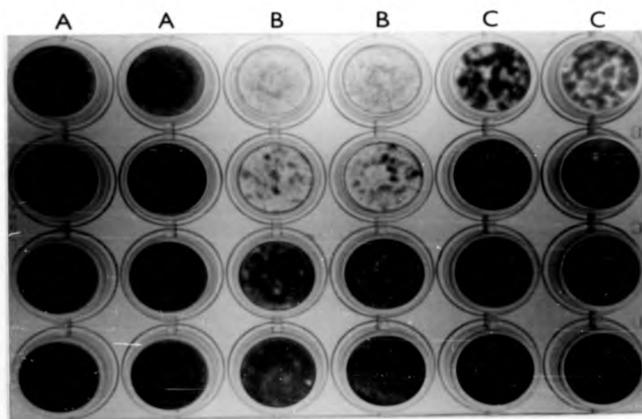


Plate 20. Plaques produced in the XTC-2 cells by three tick-borne viruses. AA) QRF. BB) DUG. CC) GAN

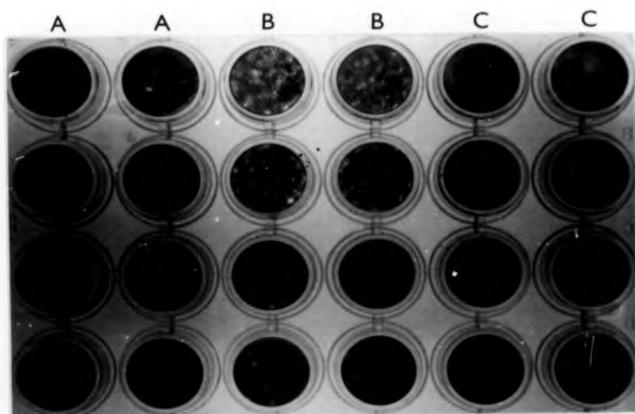


Plate 21. Plaques produced in the XTC-2 cells by three tick-borne viruses. AA) HUG. BB) PS. CC) SOL.

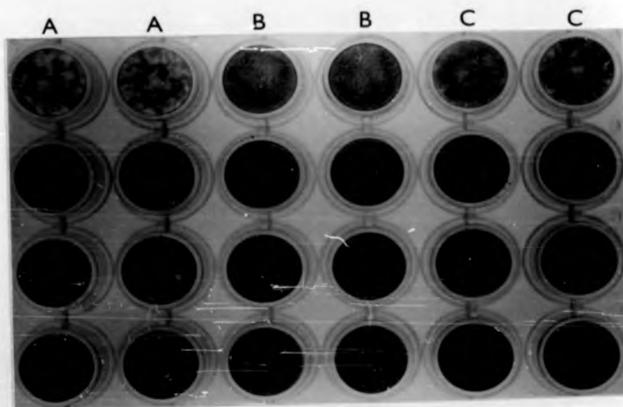


Plate 22. Plaques produced in the XTC-2 cells by three tick-borne viruses. AA) KET BB) Puffin Island. CC) ZIR.

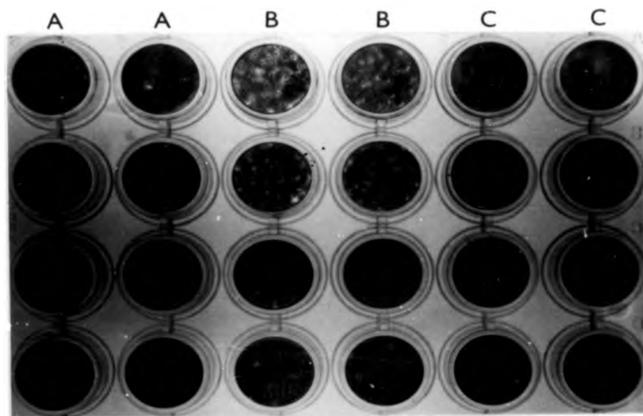


Plate 21. Plaques produced in the XTC-2 cells by three tick-borne viruses. AA) HUG. BB) PS. CC) SOL.

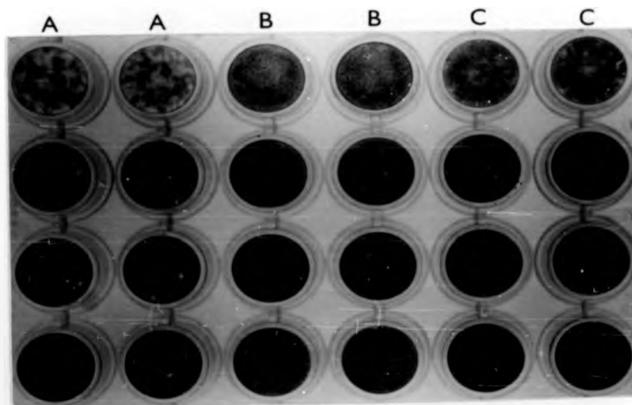


Plate 22. Plaques produced in the XTC-2 cells by three tick-borne viruses. AA) KET BB) Puffin Island. CC) ZIR.

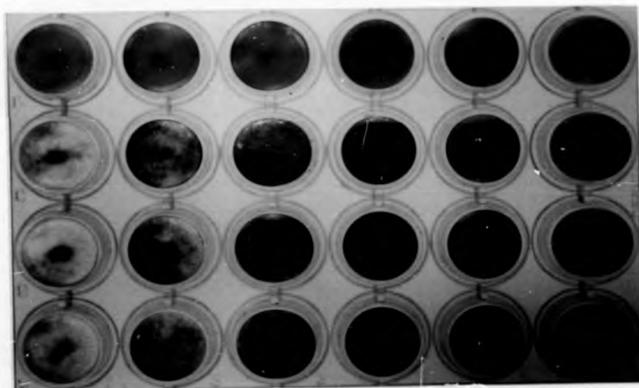


Plate 23. CPE in the XTC-2 cells produced under CMC overlay by SFS virus at high virus concentrations. No clear plaques are evident.

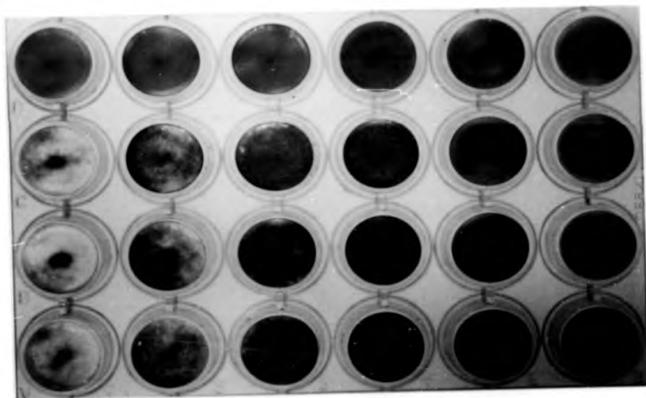


Plate 23. CPE in the XTC-2 cells produced under CMC overlay by SFS virus at high virus concentrations. No clear plaques are evident.

TABLE 13.  
COMPARATIVE PLAQUE ASSAY IN XTC-2 AND VERO CELLS.

VIRUS	PLAQUES IN XTC-2	PLAQUE SIZE IN mm	TITRE		TITRE		DAY	
			IN XTC-2	PFU/ML	IN VERO	PFU/ML	READ IN XTC-2	READ IN VERO
BEB	CLEAR	1	8.2		9.1		2	3
GET	CLEAR	1-2	8.7		8.1		2	3
MAY	CLEAR	1-2	8.4		7.2		3	3
MID	CLEAR	1-2	6.2		5.3		4	4
NDU	FAINT	1-2	7.9		7.9		4	4
URU	CLEAR	2	6.9		7.2		3	3
DEN-2	No	-	-		5.7		-	8
ENT	No	-	-		NT		-	NT
JE	CPE	-	-		NT		-	NT
LGT	No	-	-		NO PLAQUES		-	
LI	No	-	-		NO PLAQUES		-	
MOD	No	-	-		NO PLAQUES		-	
UGS	No	-	-		NT		-	NT
WN	CLEAR	1-2	8.6		7.4		7	5
YF	No	-	-		7.4		-	5
ZIKA	No	-	-		NT		-	NT
BUN	CLEAR	<1	8.9		8.9		4	4
BAT	FAINT	1-2	7.6		5.1		4	5
CVO	CLEAR	1	5.9		7.6		3	5
GER	CLEAR	<1	8.6		9.2		3	3
BWA	FAINT	2	4.0		7.1		7	6
CE	No	-	-		6.9		-	4
TAH	FAINT	<1	6.6		7.4		4	5
SFS	CPE	-	-		NO PLAQUES		-	
ANA	CPE	-	-		6.8		-	4
CHP	CLEAR	2-3	8.4		9.2		3	3
PIRY	CLEAR	1-2	6.9		9.0		3	3
KC	CLEAR	<1	6.5		4.0		3	10
NDO	No	-	-		5.7		-	6
ZIR	CLEAR	1	6.1		NO PLAQUES		7	
QRF	FAINT	<1	6.9		NO PLAQUES		6	
KET	CLEAR	1	6.8		NO PLAQUES		5	

NT Not tested.

Comparison of the results in both XTC-2 and Vero cells show that Vero cells are the system of choice for the assay of the flaviviruses, and most of the bunyaviruses tested. However, the results with the alphaviruses were comparable in both cell lines, and the XTC-2 cells appeared to be particularly useful for the assay of the non-flavi tick-borne viruses, several of which failed to produce plaques in Vero cells. In addition, the availability of an assay system grown at 28°C was particularly useful for the study of temperature sensitive virus strains produced in persistently infected cell cultures (see section 3.5.).

### 3.5 Persistent virus infections of cell lines.

#### 3.5.1. Establishment of a long-term persistently infected culture.

From the results on virus growth in the cell lines it can be seen that in many cases the infected cultures were still producing virus at the termination of the experiments at 14 or 21 days. This range was extended by infecting the 60 cells with WN virus and monitoring the production of virus over a period of 65 days, by harvesting the entire medium and replacing with fresh medium regularly after the initial 21 day incubation period. At 65 days, the cells were very clumpy, so they were re-seeded to break up these clumps and re-distribute the cells. After repeated re-seeding one bottle was selected and subcultured at weekly intervals from day 123 over 29 subcultures, the total period being 309 days after infection. The tissue culture fluid from each subculture was stored and assayed in PS cells.

The results (Fig 24) show that virus was produced at a fairly steady level throughout this long period, with titres varying from 5.7 dex pfu per ml 86 days after infection to 2.4 dex pfu per ml at the end of the experiment.

The persistent infection of a cell line, and the

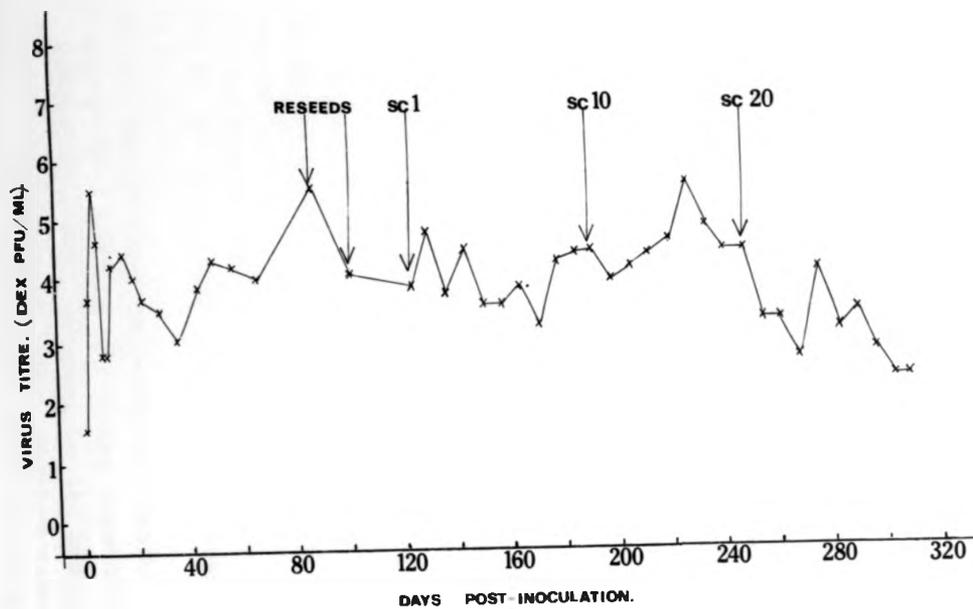


Figure 24. Long-term production of WN virus from a 60 carrier culture.

ease with which persistently infected cells could be maintained, enabled an examination of viral interference in persistently infected cells, and the changes in properties of virus produced from persistently infected cells to be carried out. For these experiments cells infected with WN virus and the alphaviruses CHIK, SF and SIN were used.

### 3.5.2. Studies on viral interference in persistently infected cultures.

In 60, 61 and XTC-2 cells infected with WN virus for these experiments, the virus growth curves were similar to those described earlier ( see section 3.3.2.). CPE was seen in all the lines , but within two weeks after infection the cultures were fully recovered and they were subcultured. Replicates of the first passage of infected cells were prepared in glass bottles for study 7 days after seeding. Two bottles were used as controls, two for superinfection with the homologous WN virus, and two for superinfection with the heterologous SF virus. In addition to these newly infected cells, replicates of the 60WN carrier culture described in section 3.5.1.were also used. These cells were at the sixty-sixth subculture.

The inocula used for superinfection were 4.1 dex pfu per bottle for WN virus, and 5.3 dex pfu per bottle for SF virus. Tissue culture harvests were carried out on days 1, 2, 3, 6, 8, 10 and 14 and the fluids were assayed in PS cells for WN virus and in Vero cells for SF virus. The results are shown in Fig 25 a, b, 26 a, b.

There was no evidence of enhanced yields of WN virus in any of the cultures following superinfection with the homologous virus, whereas in the mosquito cultures the heterologous SF virus reached titres comparable with the results obtained in section 3.3.1.3. for the growth of SF virus in uninfected cells, suggesting a lack of any heterologous interference. In the XTC-2 culture, however, there was a marked fall in the titre of SF virus which is in marked contrast to the growth

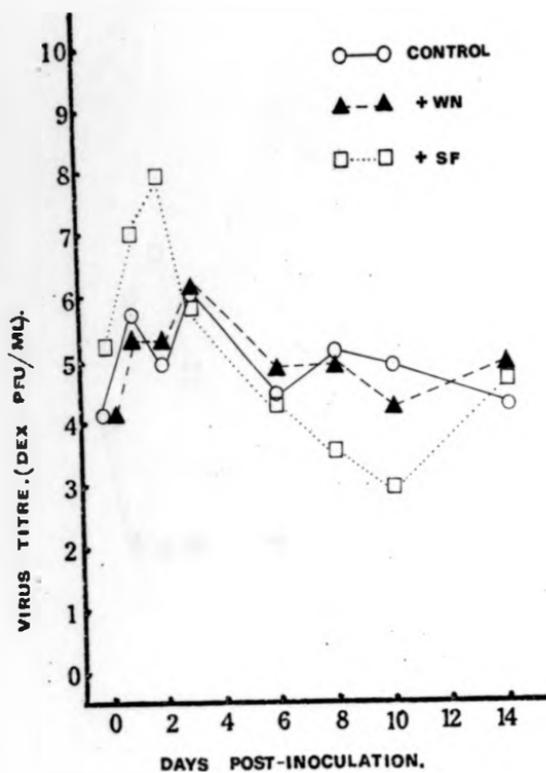
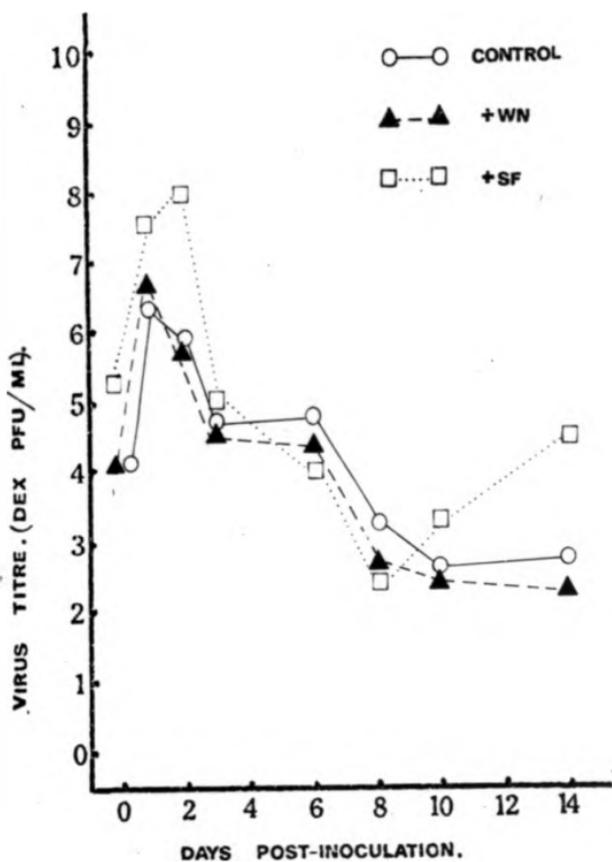


Figure 25 a. Superinfection of 60WN carrier culture with WN and SF viruses.



136

Figure 25 b. Superinfection of 61WN carrier culture with WN and SF viruses.

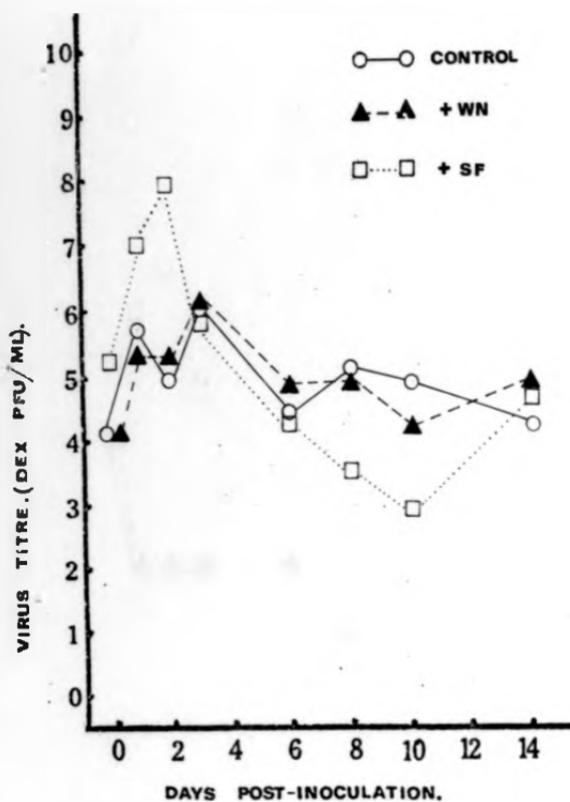


Figure 25 a. Superinfection of 60WN carrier culture with WN and SF viruses.

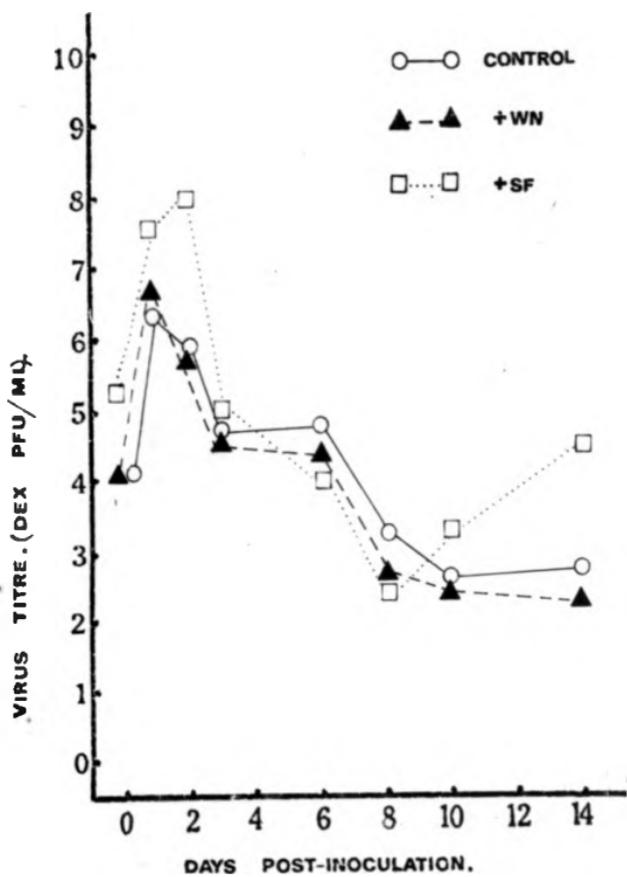


Figure 25 b. Superinfection of 61WN carrier culture with WN and SF viruses.

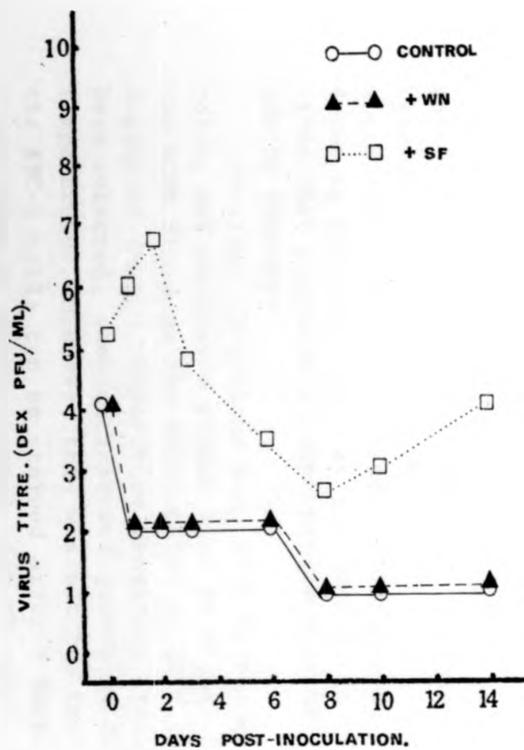


Figure 26 a. Superinfection of long-term 60WN carrier culture with WN and SF viruses.

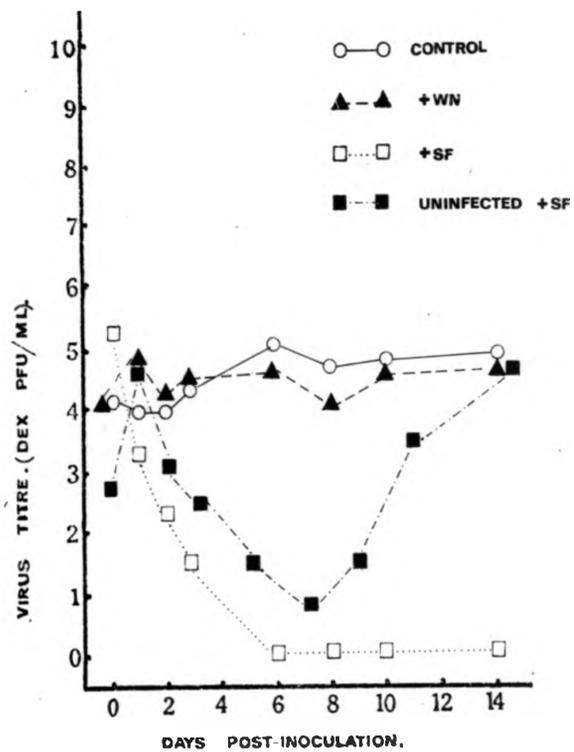


Figure 26 b. Superinfection of XTC-2WN carrier culture with WN and SF viruses.

of SF virus in uninfected cells (the growth curve for SF virus growth in uninfected cells has been copied from Fig 6 b, for comparison), and indicates the action of a heterologous interfering mechanism. No further studies to examine the nature of the interfering agent in the XTC-2 cells were carried out.

### 3.5.3. Alterations in plaque size of viruses produced in persistently infected cultures.

#### 3.5.3.1. WN virus.

Continuous subculture of the 6OWN carrier culture described in section 3.5.1. was finally terminated when the culture had reached in excess of 80 subcultures. Tissue culture fluids stored at the termination of this experiment, 699 days after infection, and the original stock virus suspension were then compared by titration in Vero cells.

Usually Vero cells can be stained 5 days after infection to reveal plaques produced by WN virus, but after 5 days incubation no plaques were present with the 6OWN fluid. The titration was repeated, and allowed to proceed for 7 days before staining, and this gave the results shown in Plate 24. The original stock virus suspension produced large plaques from 4-6mm in diameter, whereas the 6OWN fluid contained virus that produced a faint plaque type of less than 1mm in diameter.

Parallel titrations were also performed in XTC-2 cells, and increased plaque size or higher titres of the 6OWN fluid in the XTC-2 cells at 28°C would have suggested that a temperature sensitive strain had been selected, showing improved growth at lower temperatures. However, this was not the case. Normally the XTC-2 cells can be stained after 7 days to reveal clear 1-2mm diameter plaques with WN virus, but the incubation period had to be increased to 9 days before faint plaques of less than 1mm diameter could be seen in the 6OWN fluid (see Plate 25), and there was no

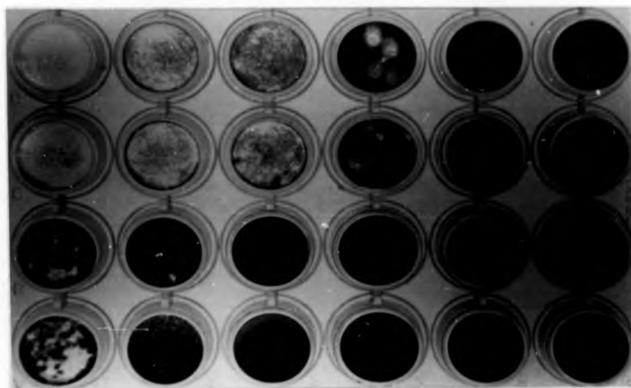


Plate 24. Stock WN virus (top 2 rows) and WN virus from 60 carrier culture (bottom two rows) titrated in Vero cells, showing altered plaque morphology of the carrier culture virus at 37°C.

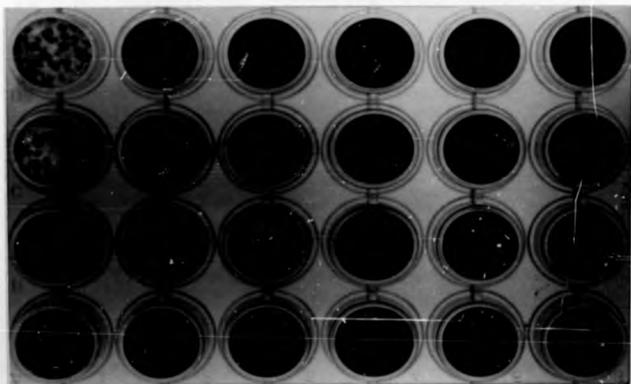


Plate 25. Stock WN virus (top 2 rows) and WN virus from 60 carrier culture (bottom two rows) titrated in XTC-2 cells, showing altered plaque morphology of the carrier culture virus at 28°C.

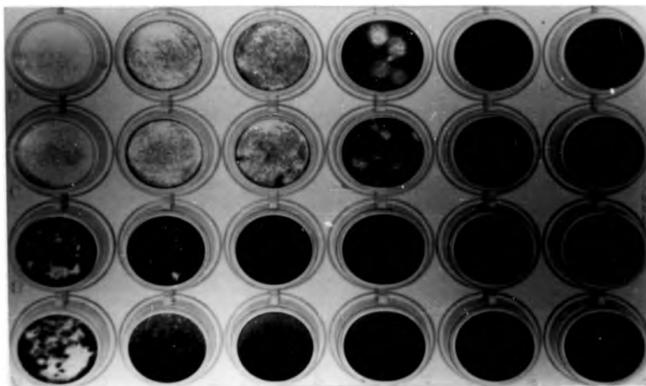


Plate 24. Stock WN virus (top 2 rows) and WN virus from 60 carrier culture (bottom two rows) titrated in Vero cells, showing altered plaque morphology of the carrier culture virus at 37°C.

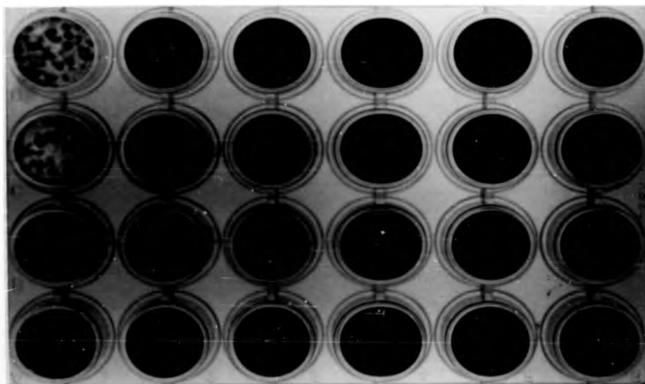


Plate 25. Stock WN virus (top 2 rows) and WN virus from 60 carrier culture (bottom two rows) titrated in XTC-2 cells, showing altered plaque morphology of the carrier culture virus at 28°C.

noticeable change in the virus titre.

These results suggest that a slow growing strain of WN virus had been selected by continuous passage of the infected cells. Unfortunately, the early samples from the 60WN culture had been discarded so it was not possible to determine whether the changes in plaque size took place gradually over a number of passages, or whether the small plaque type appeared suddenly.

#### 3.5.3.2. CHIK virus.

60, 61 and XTC-2 cells were infected with CHIK virus as described in section 3.3.1.4. CPE was only seen in the XTC-2 cells, but recovery was complete by 14 days after infection and all the infected cells were subcultured at this time. Subsequently, the infected cultures were subcultured at weekly intervals over 30 serial subcultures and the tissue culture fluids from each subculture were assayed in parallel in Vero and XTC-2 cells.

Plate 26 shows the plaque type present in the stock CHIK virus suspension when titrated in Vero cells, and Plate 27 shows the plaque types present in tissue culture fluids from the first passage of infected cells. The plaque types produced by the 60 (Row C) and the 61 (Row B) cells are similar to the original material, whereas in the XTC-2 cells (Row A) a small plaque type had appeared to the total exclusion of the original plaque type.

Titration of the fluid from the third passage of infected cells (Plate 28) showed that in the 60 and 61 cells two plaque types were present, one resembling the original large plaque type, with the other being smaller. This small plaque type was slightly larger than the type seen in the XTC-2 cells at this time.

By 30 passages the situation appeared to have stabilised. In both the 60 and XTC-2 cells counting of the individual plaques was not possible, as the plaques were too small (Plate 29), whereas in the

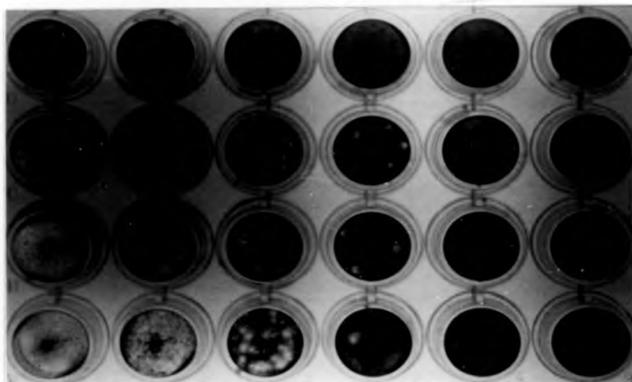


Plate 26. Stock CHIK virus titrated in Vero cells.

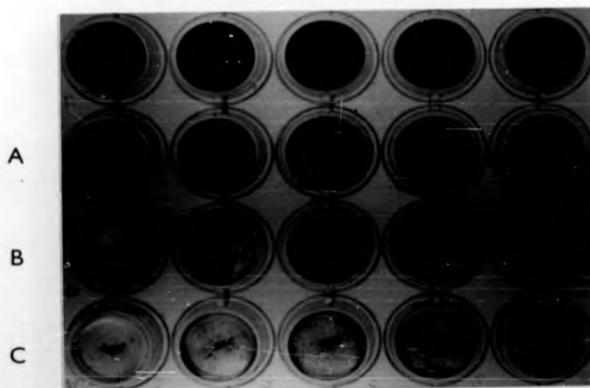


Plate 27. CHIK virus from the first passage of infected cells titrated in Vero cells. A) XTC-2. B) 61 C) 60.

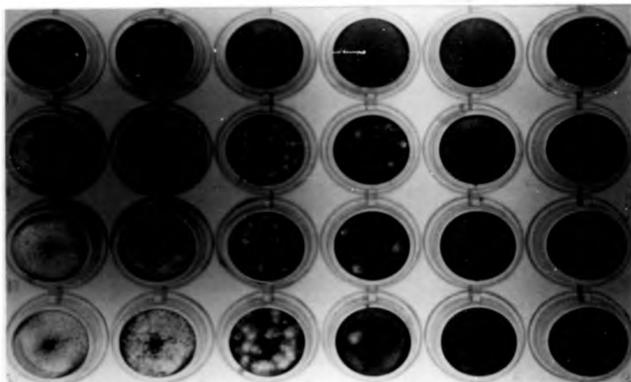


Plate 26. Stock CHIK virus titrated in Vero cells.

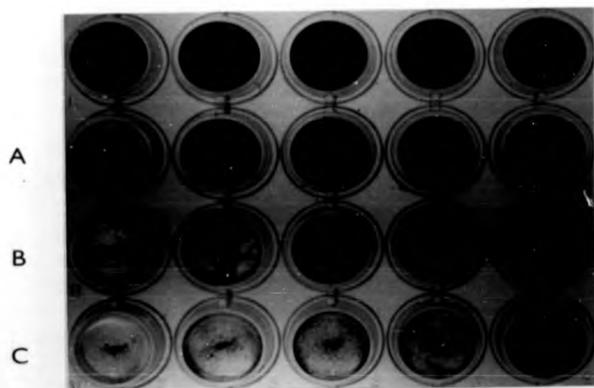


Plate 27. CHIK virus from the first passage of infected cells titrated in Vero cells. A) XTC-2. B) 61  
C) 60.

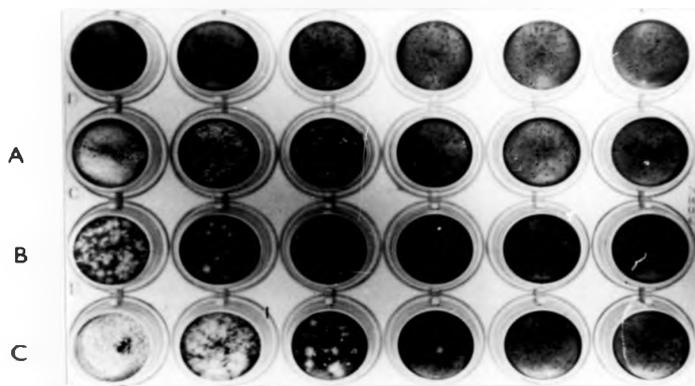


Plate 28. CHIK virus from the third passage of infected cells titrated in Vero cells. A) XTC-2. B) 61 C) 60.

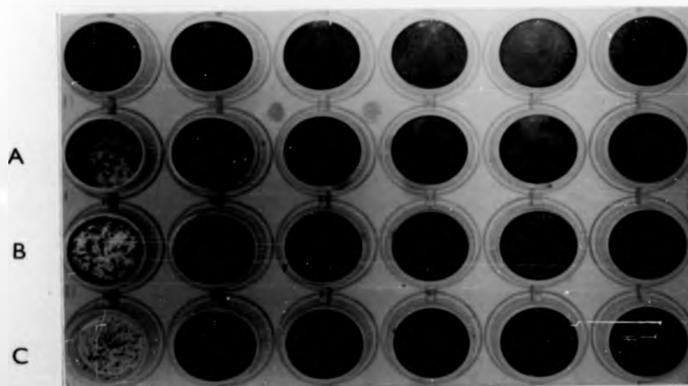


Plate 29. CHIK virus from the thirtieth passage of infected cells titrated in Vero cells. A) XTC-2. B) 61 C) 60

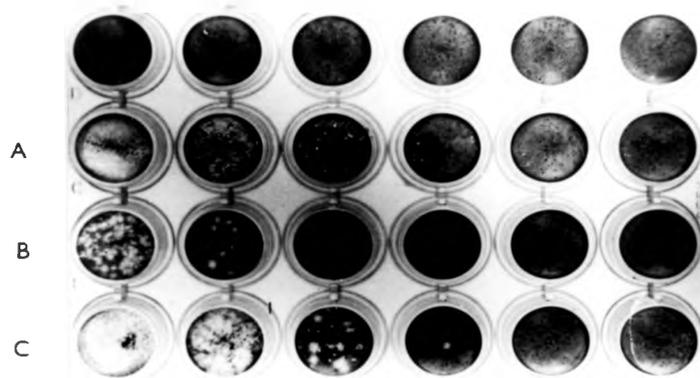


Plate 28. CHIK virus from the third passage of infected cells titrated in Vero cells. A) XTC-2. B) 61  
C) 60.

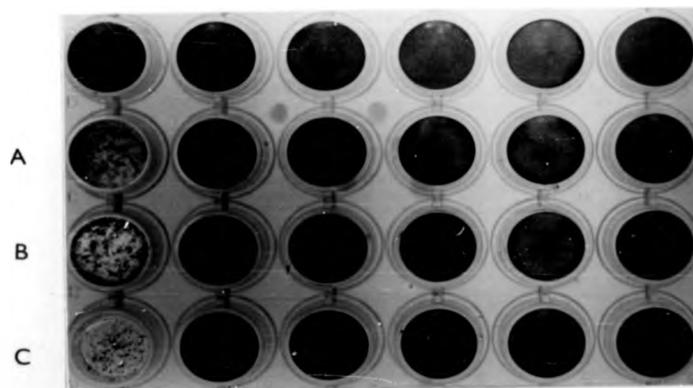


Plate 29. CHIK virus from the thirtieth passage of infected cells titrated in Vero cells. A) XTC-2. B) 61  
C) 60

61 cells an intermediate sized plaque type was predominant.

Titration of the stock virus suspension and the 30th passage material in XTC-2 cells gave interesting results. The stock virus suspension formed clear plaques (Plate 30) whereas faint plaques were present with the 30th passage material from all three cultures (Plate 31). The XTC-2 fluid titrated in XTC-2 cells reached about 5.0 dex pfu per ml, and this was higher than the titres obtained in Vero cells (about 1.0 dex pfu per ml). This suggests that this strain from persistently infected XTC-2 cells showed improved growth at the reduced temperature, or an increased affinity for the XTC-2 cells.

#### 3.5.2.3. SF virus.

The 60, 61 and XTC-2 cells were infected with SF virus as described in section 3.3.1.3., and after 14 days the cultures were subcultured serially twenty times at weekly intervals. Titration of the harvested tissue culture fluids gave results similar to those obtained with CHIK virus. The 20th passage fluids from the 60 and 61 cells contained a plaque type of about 1mm in diameter compared to 2-3mm diameter of the stock virus suspension, titrated in Vero cells. In the XTC-2 tissue culture fluid a small plaque type of less than 1mm in diameter was present.

Titration in XTC-2 cells showed faint plaque types with fluids from all three cultures. The titre of the XTC-2 fluids in the XTC-2 cells was about 4.0 dex pfu per ml compared to only 2.0 dex pfu per ml in Vero cells, again suggesting improved growth of this strain.

#### 3.5.2.4. SIN virus.

The 60, 61 and XTC-2 cells were infected with SIN virus as described in section 3.3.1.1. and the cultures were subcultured twenty times as described

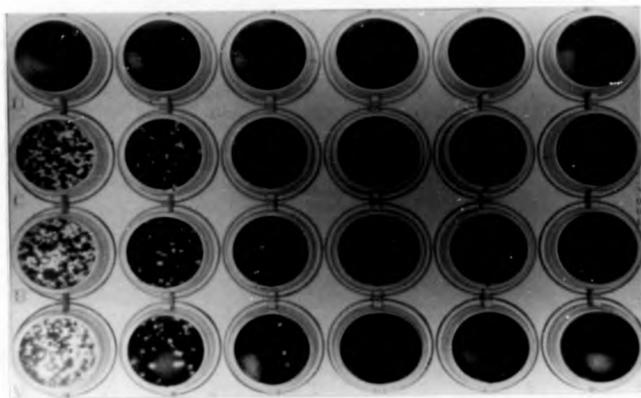


Plate 30. Stock CHIK virus titrated in XTC-2 cells.

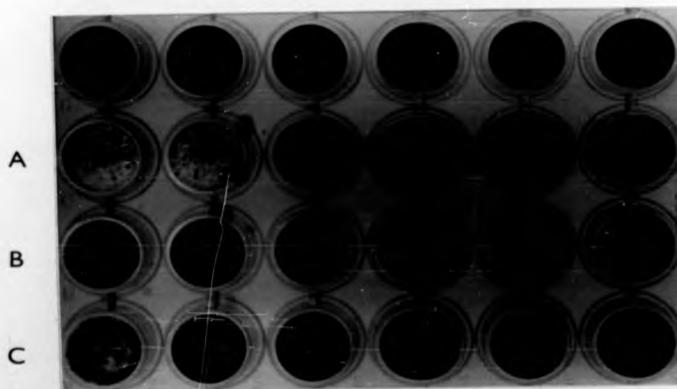


Plate 31. CHIK virus from the thirtieth passage of infected cells titrated in XTC-2 cells. A) XTC-2. B) 61. C) 60.

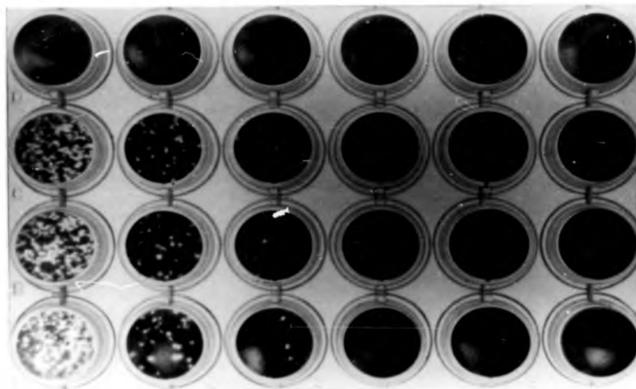


Plate 30. Stock CHIK virus titrated in XTC-2 cells.

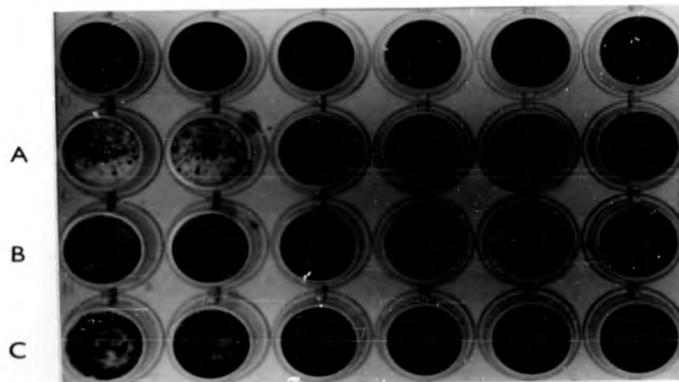


Plate 31. CHIK virus from the thirtieth passage of infected cells titrated in XTC-2 cells. A) XTC-2. B) 61. C) 60.

above with SF virus.

Titration of the 20th passage fluids in Vero cells showed two plaque types in the 60 cell fluids one measuring about 1.5mm in diameter and the other being very small, less than 0.5mm in diameter; compared to 2-3mm plaque types in the stock virus suspension. Intermediate plaque types were present in the 61 fluids ranging around 1.5mm in diameter, and in the XTC-2 cells a mixed population of plaque types from less than 1mm to about 1.5mm in diameter were present.

When the fluids were titrated in the XTC-2 cells the 60 fluids contained plaque types up to 2mm in diameter whereas in the 61 fluids 2-3mm diameter plaques, similar to the stock virus type, were seen. Two plaque types were seen in the XTC-2 fluids, one being about 2mm in diameter and the other less than 1mm in diameter.

The titre of the virus in both Vero and XTC-2 cells was comparable for fluids from all the infected cultures.

#### 4. DISCUSSION.

##### 4.1. Growth characteristics of the cell lines.

The cell lines were distinguishable on the basis of morphology, individual growth characteristics in culture and on their isoenzyme patterns.

The rates of growth of the mosquito cell lines in their routine growth medium of MM/VP<sub>12</sub> or MK/VP<sub>12</sub> and in L-15 medium were comparable for about 5-6 days after seeding, but subsequently, higher cell numbers were observed in the routine media with the 20A, 55 and 61 cells; whereas the 43 and 60 cell lines reached peak cell numbers in L-15 medium.

The fastest growing mosquito cell line was the 55 cell line, in which a peak cell number of  $81 \times 10^6$  cells per bottle was observed 10 days after seeding in MK/VP<sub>12</sub> medium. During the logarithmic phase of growth the population doubling time of these cells was estimated to be only 6 hours. In comparison, the next fastest cell line was the 43 cell line with a population doubling time of about 15 hours in both MK/VP<sub>12</sub> and L-15 media.

The slowest growing mosquito cell line was the 61 cell line where peak cell numbers of about  $10.5 \times 10^6$  cells were seen in MM/VP<sub>12</sub> medium. These results were similar to those obtained for the XTC-2 cell line which reached a peak number of  $15 \times 10^6$  cells per bottle.

My results on the growth of the 20A, 43 and 60 cells show good agreement with Johnson's (1973) data using these cells.

In general, all the cell lines showed a continuous process of adaptation to the culture conditions during the study, and this was reflected in a steadily increasing split ratio. For example, this was particularly evident in the 60 cell line where, between the 109th and 180th subcultures, the split ratio increased from 1:17 to 1:70.

#### 4.2. Contamination of cell cultures.

All the cell lines were shown to be free of vertebrate mycoplasma contamination when tested by standard isolation techniques, and by additional tests at 28°C.

Occasional bacterial or fungal contamination of the cultures was readily detected optically, and the cultures were usually discarded without resort to treatment with additional antibiotics or fungicides.

Throughout the study, at irregular intervals, tissue culture fluids from control cultures of various ages were tested in PS and Vero cells for latent or extrinsic virus contamination using the plaque assay method. In addition, tests were also carried out in the 6O, 61 and XTC-2 cells for CPE production at 28°C, and in one series of tests tissue culture fluids were also inoculated intracerebrally into suckling mice. No contaminating agents were detected by any of these assay methods.

These findings do not, however, preclude the possibility that cell-associated agents were, or indeed are, present in these cell lines. For example, Pudney et al (1971) described a rod-shaped virus-like particle in the 43 cells which was only detected during ultrastructural studies. They further demonstrated this particle in the mosquito colony from which the cell line was established, implying that this particle had been present in the cells over many subcultures. Such detailed studies of the other cell lines remains to be carried out, but it must be considered possible that other virus-like particles may be found.

My studies confirm the findings of a number of workers (see section 1.2.3.3.) that arbovirus infections of mosquito cell lines do not usually result in the production of a CPE. Extrinsic contamination of mosquito cell lines could thus go unnoticed, and increasing inter-laboratory co-operation could result in the transfer of contaminated cells to new laboratories. This danger has recently been emphasised in papers on

the isolation of CHIK virus contaminating an Ae.albopictus cell culture (Cunningham et al, 1975), and a flavivirus-like agent contaminating Peleg's Ae.aegypti cells (Stollar and Thomas, 1975). In both cases contamination was inapparent under routine culture conditions.

Routine screening of cell lines, in particular, mosquito cell lines for viral contamination must therefore be considered as highly desirable.

#### 4.3. Culture conditions for infection of cells.

Cultures were prepared for infection by growing the cells in L-15 medium with 10% FBS. Prior to infection the cells were changed into maintenance medium of L-15 with 2% FBS, and this was used for the duration of the experiment. This procedure had a number of advantages. Firstly, for comparative purposes all the cells would be infected under similar culture conditions. Secondly, this change of culture media may serve to stress the cells, thereby enhancing the production of CPE by the infecting virus, as suggested by Varma et al (1974). Finally, and a point of particular practical importance, was the size of the infection experiments. These involved the use of large volumes of media. Large scale preparation of the individual culture media would have been very time consuming and technically difficult, whereas L-15 medium was an ideal choice as it is available commercially as a liquid, or in powdered form for large volume preparation.

#### 4.4. Virus adsorption.

Throughout the work a virus adsorption period of 3 hours was used, after which time adsorbed virus was measured. Webb et al (1976) found that viropexis of mosquito iridescent virus by Peleg's Ae.aegypti cells was complete only 15 minutes after infection. Therefore, if we assume that arbovirus infection of mosquito cells follows a similar time course, the measurement of adsorbed virus at 3 hours may reflect only passively adsorbed virus. Treatment of the cells

with specific antisera would have eliminated this possibility, but this was not attempted.

It is obvious that the early stages of infection of mosquito cells requires more study, particularly coupled with ultrastructural examination.

#### 4.5. Sensitivity of cell lines to viral infection.

Minimum infectious dose experiments with 8 viruses showed that there was a varying level of sensitivity of all the cell lines to infection. The 60 and 61 cell lines were the most highly sensitive, followed by the XTC-2 cells. In comparison, the 20A, 43 and 55 cells were relatively insensitive.

The results suggest that the sensitivity of the 60 and 61 cells may be useful for the primary isolation of viruses from field material, and indeed the 61 cells have been used for the primary isolation of DEN-2 and YF viruses (Varma *et al* 1974, 1976). In laboratory studies the 60 and 61 cells proved more sensitive than mice in studies with WN, JE and DEN-2 viruses (Varma *et al*, 1974), and the 61 cells have been shown (Varma *et al*, 1976) to be superior to suckling mice, Ae.albopictus cells and marginally better than Vero cells, for the isolation of YF virus strains from field material.

Johnson (1973) suggested that the 20A cells could be used for the screening of survey sera for antibody on the basis of restricted growth in the cells of infectious virus-antibody complexes. The greater sensitivity of the 60 and 61 cells could possibly extend the usefulness of this technique.

#### 4.6. Susceptibility of the cell lines to viral infection.

The 20A cells have been shown to support the replication of TAH virus (Marhoul, 1971), WN virus (Pudney, 1973; Johnson, 1973) and YF virus (Johnson, 1973). Viruses that failed to multiply in these cells are CVO and UUK (Marhoul, 1971), ANA, CVO, ONN and BWA (Varma and Pudney, 1971) and DEN-2 (Johnson,

1973).

In my experiments similar results were obtained with WN, YF, DEN-2 and ONN viruses, but evidence of low virus output was found with ANA virus. BWA virus also multiplied in the cells, but this may be explained by the production of a small plaque type showing reduced mouse virulence. In the earlier studies mice were used throughout for virus assay. CVO, TAH and UUK viruses were not used in my studies.

Earlier studies with the 43 cells have shown that they support the replication of ANA, CVO, ONN and BWA viruses (Varma and Pudney, 1971), CVO and TAH viruses (Marhoul, 1971) and WN virus (Johnson, 1973). The cells failed to support the growth of YF and DEN-2 viruses (Johnson, 1973) as well as the tick-borne UUK virus (Marhoul, 1971).

All these findings were confirmed in my experiments except for the results with CVO, TAH and UUK viruses which were not used.

Prior to my experiments the 55 cell line has been infected with only 4 viruses, supporting the replication of TAH virus but not CVO, TBE or Yaba-1 viruses (Marhoul, 1973). None of these viruses were used in my experiments.

Johnson (1973), obtained replication of WN virus in the 60 cell line, and CPE indicating virus growth was observed in the 60 cells with both WN and JE viruses (Varma *et al*, 1974). CPE has also been observed in the 61 cells with WN, JE, DEN-2 and YF viruses (Varma *et al*, 1974, 1976). In my experiments these cell lines were susceptible to all these viruses, the only difference being that the vaccine strain of YF virus that was used failed to produce CPE in the 61 cells, although the virus multiplied readily.

Studies on the susceptibility of the XTC-2 cell line have recently been published (Leake *et al*, 1977).

For comparison the results of the susceptibility of the cell lines to infection with the 23 viruses tested are summarised in Table 14. The most susceptible cell line was the XTC-2 cell line supporting the growth

TABLE 14.  
 SUSCEPTIBILITY OF CELL LINES TO INFECTION  
 WITH 23 ARBOVIRUSES.

VIRUS	CELL LINES					
	20A	43	55	60	61	XTC-2
SIN	-	-	+	+	+	+
NDU	+	-	-	+	+	+
SF	+	-	+	+	+	+
CHIK	+	-	+	+	+	+
ONN	-	+	+	+	+	+
MAY	+	+	+	+	+	+
MID	+	-	-	+	+	+
LGT	-	-	-	-	-	+
LI	-	-	-	-	-	+
JE	+	+	+	+	+	+
WN	+	+	+	+	+	+
ZIKA	+	+	+	+	+	-
DEN-2	-	-	-	+	+	-
YF	+	-	-	+	+	-
BUN	+	+	+	+	+	+
GER	+	+	+	+	+	+
BWA	+	+	-	+	+	+
CE	+	+	+	+	+	+
ANA	+	+	+	+	+	+
CHP	+	+	+	+	+	+
SFS	-	-	-	-	-	+
ZIR	-	-	-	-	-	+
QRF	-	-	-	-	-	+

+ Growth.

- No Growth.

of 20 of the 23 viruses tested. The three viruses failing to multiply were all flaviviruses namely DEN-2, YF and ZIKA.

The most broadly susceptible mosquito cell lines were the highly sensitive 60 and 61 cell lines, both supporting replication of 18 of the 23 viruses tested. The viruses that failed to multiply in both cell lines were the four tick-borne viruses LGT, LI, QRF and ZIR, and the phlebotomus-borne bunyavirus SFS. Varma *et al* (1974) suggested that the 60 and 61 cell lines may show affinities with Singh's Ae.albopictus cell line, and this is emphasised by a comparison of the susceptibility of the three cell lines to 13 arboviruses that have been tested in all three lines (Table 15).

In comparison with the 60 and 61 cell lines, the other aedine cell line, 20A, supported the replication of 16 of the 23 viruses tested. The tick-borne and phlebotomus-borne viruses again failed to multiply, and in addition the alphavirus ONN failed to grow and low titres were obtained with the flavivirus DEN-2, which could be explained on the basis of virus elution from the cells as suggested by Johnson (1973).

Comparison of these results in the 20A cells with results obtained in other Ae.aegypti cell lines (Table 16) again shows a high degree of correlation, particularly with Singh's Ae.aegypti cell line. The only difference appears to be that the 20A cells supported the growth of JE virus, whereas Singh's cells were refractory to infection. Singh and Paul (1968) have also reported erratic results with SIN virus in their cells, and this is similar to the 20A cells where SIN virus replication was only demonstrated once in four experiments.

In contrast to the three Aedes cell lines the two Anopheles cell lines were less susceptible, with the 43 cells supporting replication of only 10 viruses and the 55 cells only 13. Of the 14 togaviruses tested only 5 multiplied in the 43 cells and 8 in the 55 cells, but in contrast 5 out of 6 bunyaviruses multiplied in both cell lines. As with the other mosquito cell lines the tick-borne and phlebotomus-borne viruses failed to

TABLE 15.  
 COMPARISON OF THE SUSCEPTIBILITY OF THE 60 AND  
 61 CELL LINES AND SINGH'S Ae.albopictus CELL  
 CELL LINE TO INFECTION WITH SOME ARBOVIRUSES.

VIRUS	CELL LINES		
	60	61	A.al.*
SIN	+	+	+
SF	+	+	+
CHIK	+	+	+
ONN	+	+	+
JE	+	+	+
WN	+	+	+
DEN-2	+	+	+
YF	+	+	+
LGT	-	-	-
CE	+	+	+
CHP	+	+	+
SFS	-	-	-
QRF	-	-	-

+ Growth.

- No Growth.

\* Data from SINGH (Review 1972).

TABLE 15.  
 COMPARISON OF THE SUSCEPTIBILITY OF THE 60 AND  
 61 CELL LINES AND SINGH'S Ae.albopictus CELL  
 CELL LINE TO INFECTION WITH SOME ARBOVIRUSES.

VIRUS	CELL LINES		
	60	61	A.al.*
SIN	+	+	+
SF	+	+	+
CHIK	+	+	+
ONN	+	+	+
JE	+	+	+
WN	+	+	+
DEN-2	+	+	+
YF	+	+	+
LGT	-	-	-
CE	+	+	+
CHP	+	+	+
SFS	-	-	-
QRF	-	-	-

+ Growth.

- No Growth.

\* Data from SINGH (Review 1972).

TABLE 16.  
 COMPARISON OF THE SUSCEPTIBILITY OF THE 20A CELL  
 LINE AND OTHER Ae.aegypti CELL LINES TO INFECTION  
 WITH SOME ARBOVIRUSES.

VIRUS	CELL LINES			
	20A	SINGH'S	PELEG'S	GRACE'S
		A.ae.	A.ae.	A.ae.
SIN	+	+	+	-
CHIK	+	+	NT	NT
SF	+	+	+	-
ONN	-	-	NT	NT
JE	+	-	+	+
WN	+	+	+	+
DEN-2	-	-	+	NT
YF	+	+	NT	+
LGT	-	-	NT	NT
CE	+	+	+	NT
CHP	+	+	NT	NT
SFS	-	-	NT	NT
QRF	-	-	NT	NT

+ Growth. - No Growth. NT Not Tested.

Data from SINGH (1972), PELEG (1968,1972,1974)  
 and SWEET and UNTHANK (1971).

TABLE 16.  
 COMPARISON OF THE SUSCEPTIBILITY OF THE 20A CELL  
 LINE AND OTHER Ae. aegypti CELL LINES TO INFECTION  
 WITH SOME ARBOVIRUSES.

VIRUS	CELL LINES			
	20A	SINGH'S	PELEG'S	GRACE'S
		A. ae.	A. ae.	A. ae.
SIN	+	+	+	-
CHIK	+	+	NT	NT
SF	+	+	+	-
ONN	-	-	NT	NT
JE	+	-	+	+
WN	+	+	+	+
DEN-2	-	-	+	NT
YF	+	+	NT	+
LGT	-	-	NT	NT
CE	+	+	+	NT
CHP	+	+	NT	NT
SFS	-	-	NT	NT
QRF	-	-	NT	NT

+ Growth. - No Growth. NT Not Tested.

Data from SINGH (1972), PELEG (1968,1972,1974)  
 and SWEET and UNTHANK (1971).

multiply . However, low levels were detected in the 43 cells infected with the phlebotomus-borne SFS virus, but these results could also be explained on the basis of elution of adsorbed virus from the cells.

Schneider's An.stephensi cell line is the only other anopheline cell line that has been examined. Buckley (1971, et al , 1975) found that both CHIK and ONN viruses multiplied in this cell line, but in 1973, Singh et al found that these cells were refractory to infection with CHIK virus as well as DEN-2 and the tick-borne KFD and GAN viruses. They found that JE and CHP viruses multiplied in these cells. My results in the 43 cells generally support these findings with multiplication of ONN, JE and CHP but not DEN-2 or the tick-botne viruses. CHIK virus failed to multiply in the 43 cells.

Buckley (1969 b, 1972) has suggested that the lipid solvent sensitive arboviruses from ticks and phlebotomines fail to multiply in Singh's Aedes cell lines, and my results support and extend this observation as all the tick-borne and phlebotomus-borne viruses I tested failed to grow in any of the Aedes or Anopheles cell lines. Buckley's suggestion that the relatively solvent resistant arboviruses (orbiviruses) would multiply in one or both of Singh's cell lines was not tested in my experiments.

From my results it is interesting to draw parallels with the susceptibility of cell lines in vitro and the in vivo virus-vector relationship. Thus one might speculate that the growth of several bunyaviruses in the 43 cell line reflects certain affinities of the bunyaviruses for anophelines, as suggested by Chamberlain et al (1969). Such suggestions must, however, be carefully examined. Varma (1972) has pointed out that the arthropod host spectrum of arboviruses may extend to particular genera, families, orders or classes of arthropods, and that most instances of host-virus specificity are examples of ecological specificity. It is, perhaps, not surprising therefore, that my results in vitro show that these cell lines exhibit a wide

spectrum of susceptibility. For example, the Aedes cell lines 60 and 61 support the growth of viruses such as SF virus, isolated from numerous Aedes species mosquitoes in Africa, ONN virus, isolated from Anopheles species mosquitoes in Uganda and Kenya, MAY virus, isolated from Mansonia species in Trinidad and from Haemagogus and Culex species in S.E.Asia, and CHP virus that has been associated with phlebotomines in India.

Even so, several examples of specificity at the generic level appear in the results. The two alphaviruses NDU and MID, both isolated from Mansonia and Aedes mosquitoes, and the two flaviviruses YF and DEN-2, isolated from Aedes mosquitoes ( and Haemagogus for YF), all fail to grow in the cell lines derived from Anopheles mosquitoes whilst growing in the Aedes cell lines (with the exception of DEN-2 virus in the 20A cells). None of these viruses have been isolated from anopheline mosquitoes. Specificity at the level of family can be seen with the failure of the phlebotomus-borne SFS virus to multiply in the mosquito cells, and specificity at the level of order can be seen with the failure of four tick-borne viruses, LGT, LI, QRF and ZIR, to multiply in the mosquito cell lines. All these tick-borne viruses were solvent sensitive viruses, and it would be interesting to test viruses such as GJM, a bunyavirus isolated from both ticks and mosquitoes, and the orbiviruses such as CTF or KEM in the mosquito cell lines.

Of the viruses I used, 16 are proven mosquito-borne arboviruses, with three CHP, BWA and NDU classified as probable arboviruses. The fact that all these viruses multiplied in the 60 and 61 cells could be taken as in vitro evidence that these viruses are mosquito-borne in vivo.

#### 4.7. Viral growth patterns.

If we consider the results obtained for the growth of individual viruses the variability of the growth patterns is immediately apparent. Singh (1972), whilst reviewing the literature on the growth of arboviruses in Aedes cell lines, suggested that there appeared to be two types of growth patterns, the first being where the virus titre never exceeded the inoculum by more than 10-fold, and the second where an increase in virus titre from  $10^2$ - $10^6$  times the inoculum can be seen. This rise in virus titre was seen from 3-6 days after infection, and was then followed by a steady decline in titre.

The subsequent literature, and my results, show that this is an over-simplification. From my results, for example, the increase in virus titre ranged from the level of the inoculum, as with BWA virus, to as much as a  $10^7$  increase, as with URU virus in the 61 cells. Peak virus titres were reached as quickly as 1 day after infection with several of the alphaviruses in the 61 cells, to as late as 21 days with JE virus in the 20A cells. After the peak titres were reached titres can decline rapidly, as with SF virus in the 61 cells, be maintained at a steady level as with several flaviviruses and bunyaviruses in the 61 cells, or decline slowly. However, if we consider the growth patterns of the viruses in individual cell lines, some useful generalisations can be made.

In the 20A cells, the initial rise in virus titre was variable, reaching a peak from 2-3 days after infection with the alphaviruses to a more steady rise with SF, CE and CHP viruses. With the two flaviviruses JE and YF peak titres were not reached until late in the experiment (21 days for JE and 14 days for YF). The highest titre observed in the 20A cells was 6.3 dex pfu per ml with the alphavirus URU (but see section 3.3.1.1 for results with SIN virus).

Compared to the 20A cells a consistent growth

pattern in the 43 cell line was only seen with the bunyaviruses, where the initial rise in titre was reached from 2-5 days after infection, after which there was a steady or marked fall. The rhabdovirus CHP also multiplied well in these cells, and the highest titre of 7.5 dex pfu per ml was seen with this virus 6 days after infection.

In contrast to the 43 cells, the bunyaviruses reached low titres in the other anopheline cell line, 55, although the growth patterns of the togaviruses tested were more consistent, with an increase in titre from between 2-5 days after infection followed by a steady decline. The highest titre observed in the 55 cells was 6.5 dex pfu per ml with the alphavirus URU and the rhabdovirus CHP.

Virus growth patterns in the 60, 61 and XTC-2 cells usually showed quite close agreement between the 60 and 61 cells, with the XTC-2 cells showing titres generally 1-2 dex lower. The alphaviruses showed a consistent rapid rise in titre reaching peak levels 1-2 days after infection. Virus titres were then maintained at a high level, as with SIN virus, or fell rapidly, as with SF virus. Initial peak virus titres as high as 9.2 dex pfu per ml were seen with URU virus in the 61 cells, and titres greater than 7.0 dex pfu per ml were usually seen in both the 60 and 61 cells. The lowest alphavirus titre was 6.0-6.5 dex pfu per ml with the alphavirus ONN.

In contrast to the rapid rise in titre with the alphaviruses, the initial rise in titre of the flaviviruses tested was slower, ranging from 3 days with JE virus to as long as 10-14 days with YF virus. Virus titres never approached the levels seen with the alphaviruses with peak titres of up to 6.0 dex pfu per ml being seen.

A similar pattern was seen with the bunyaviruses with a gradual rise in virus titre reaching levels similar to the flaviviruses.

Close agreement can be seen in the viral growth

patterns obtained in the 61 cell line and in Singh's Ae.albopictus cell line with SIN virus (Singh and Paul, 1968; Stevens, 1970), CHIK virus (Singh and Paul, 1968), SF virus (Davey and Dalgarno, 1974), JE virus (Singh and Paul, 1968; Igarashi et al, 1973), WN virus (Singh and Paul, 1968), DEN-2 virus (Singh and Paul, 1968; Sinarachatanant and Olson, 1973) and CE virus (Whitney and Deibel, 1971).

It is tempting to speculate that individual viruses may have characteristic growth patterns in arthropod or lower vertebrate cell lines. Thus the similarity of the growth curves seen with the alphaviruses could reflect their antigenic relatedness. One important exception is the marked difference in the growth curves obtained with ONN and CHIK viruses. Although these two viruses are closely related, they can be readily distinguished on the basis of their growth in vitro. This biological difference, expressed by differing growth patterns in culture, could be used to rapidly distinguish closely related virus strains.

#### 4.8. CPE and plaque formation.

In most cases the mosquito cell lines did not show any CPE on infection with the arboviruses used. This cannot be attributed to the low incubation temperature (28°C) at which the mosquito cells are grown, as a high proportion of the viruses produced CPE in the XTC-2 cells grown at the same temperature. CPE in the mosquito cell lines was restricted to the 60 and 61 cell lines, and was only seen with the flaviviruses WN, JE and DEN-2. The response of the two cell lines was different, in that in the 60 cells a focal cell death was seen whereas in the 61 cells syncytia formation was observed.

Attempts to produce plaques in the mosquito cells, grown in plastic trays under CMC overlay, proved negative, even with WN virus which produces CPE in the 60 and 61 cells. These results were disappointing, but not entirely unexpected as problems have been found

with commercially available plastic tissue culture trays. Pudney (personal communication) has recently demonstrated that newly available trays vary in their capacity to support good growth of mosquito cells, although I could find no apparent differences in the growth of XTC-2 or mammalian cells in these trays. This sensitivity of mosquito cells to different plastic surfaces complicates experimental techniques, but, in spite of this, Pudney et al (1976) have successfully obtained plaques with YF virus in the 61 cells, and promising results are now being obtained with other viruses.

#### 4.9. Persistent infections.

The general lack of CPE in mosquito cells is probably a reflection of the usually harmless and persistent infection in whole mosquitoes, and favours the argument that the arboviruses may have their evolutionary origins in arthropods (Schlesinger, 1971).

Persistent infections were readily established in the 60, 61 and XTC-2 cells and could, almost certainly, have been studied in the other mosquito cell lines. In studies with cultures persistently infected with WN virus, total homologous interference was seen with superinfecting WN virus in the mosquito cell lines, whereas heterologous SF virus multiplied normally in these cultures suggesting the lack of an interferon-like system. These results are in agreement with Kascsak and Lyons (1974) results using Ae. albopictus carrier cultures.

In contrast to the results in the 60 and 61 cells, both homologous and heterologous interference was observed in the experiments with the XTC-2 cells, and experiments to further characterise this interferon-like activity are planned. Interferon-like activity has been demonstrated in tortoise cell cultures (Galabov et al, 1973), and in fish cells (Falcoff and Fauconnier, 1965; Beasley et al, 1966), and the confirmation of an interferon system in the XTC-2 cells would extend

the range of in vitro studies to include amphibian cells. This would support the hypothesis that 'the capability of vertebrate cells to produce antiviral substances appeared early in the phylogenesis of the vertebrates' (Galabov et al, 1973), and that 'interferon-like substances might represent one of the more ancient defence mechanisms against viral parasitism (Falcoff and Fauconnier, 1965).

#### 4.10. Alterations in virus progeny from persistently infected cells.

Persistently infected cells could be subcultured over long periods of time, and the virus produced studied in both Vero cells at 37°C and XTC-2 cells at 28°C.

WN virus from persistently infected 60 cells continued to resemble the stock virus for a considerable time (more than 300 days), but after more than two years in culture a small plaque type had appeared, and plaque formation in both Vero and XTC-2 cells was slower. The fact that the plaque type was restricted in the XTC-2 cells at 28°C suggests that the strain was not temperature sensitive.

Studies with the alphaviruses SF, SIN and CHIK, showed that a rapid production of mixed plaque types can take place, compared to the slow change with WN virus.

A small plaque variant of BWA virus was also produced by infected 20A cells (see section 3.3.3.3.) and to my knowledge this is the first time that a small plaque variant of a bunyavirus has been observed in mosquito cells.

Buckley (1971 b) has found that a small plaque variant of CHIK virus produced in Ae.albopictus carrier cultures shows reduced virulence for mice whilst retaining efficiency as an immunogen, and as my results show that small plaque variants are produced not only with alphaviruses but flaviviruses and bunyaviruses as well, these cell lines may be of particular use for the

comparative study of a broad spectrum of viruses in persistently infected cells. This may have practical applications in the production of attenuated virus strains as a first step in the production of vaccines.

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## APPENDIX.

Mitsuhashi and Maramarosch's Medium.

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	40 mg
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	20 mg
KCl	40 mg
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	40 mg
NaCl	24 mg
D-Glucose	800 mg
Lactalbumin hydrolysate	1300 mg
Yeastolate	1000 mg
Distilled water	160 ml

Adjust pH to 7.0 with 2% KOH. Sterilise by membrane filtration and store at 4°C. Prior to use heat inactivated foetal bovine serum to 20% and antibiotics are added.

Modified Kitamura's Medium.

NaCl	1300 mg
KCl	100 mg
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	20 mg
$\text{KH}_2\text{PO}_4$	20 mg
$\text{NaHCO}_3$	20 mg
D-Glucose	800 mg
Lactalbumin hydrolysate	1300 mg
Yeastolate	1000 mg
Distilled water	160 ml

Adjust pH to 7.0 with 2% KOH. Sterilise by membrane filtration and store at 4°C. Prior to use heat inactivated foetal bovine serum to 10% and antibiotics are added.

Tick Medium VP<sub>12</sub>

NaCl	390 mg
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	55 mg
MgCl <sub>2</sub> ·6H <sub>2</sub> O	110 mg
MgSO <sub>4</sub> ·7H <sub>2</sub> O	120 mg
KCl	55 mg
CaCl <sub>2</sub> ·2H <sub>2</sub> O	40 mg
D-Glucose	200 mg
Choline chloride	25 mg
Inositol	40 mg
NaHCO <sub>3</sub>	50 mg
Lactalbumin hydrolysate	500 mg
Bovine albumin fraction 5	100 mg
5% Glutamine	0.6 ml
BME Vitamin mixture 100x	2.0 ml
Distilled water	97.4 ml

Adjust pH to 7.0 with 2% KOH. Sterilise by membrane filtration and store at 4°C. Prior to use heat inactivated foetal bovine serum to 10% and antibiotics are added.

EDTA (Versene) Solution.

Ethylenediaminetetra-acetic acid	200 mg
NaCl	8000 mg
KCl	200 mg
NaH <sub>2</sub> PO <sub>4</sub>	1150 mg
KH <sub>2</sub> PO <sub>4</sub>	200 mg
D-Glucose	200 mg
Distilled water	1000 ml

Membrane filter sterilised and stored at 4°C.

Trypsin Solution.

NaCl	2400 mg
KCl	120 mg
Na <sub>2</sub> HPO <sub>4</sub>	18 mg
KH <sub>2</sub> PO <sub>4</sub>	18 mg
Bacto-trypsin	3000 mg
Distilled water	300 ml

Stirred on a magnetic stirrer for several hours and then clarified by centrifugation. Sterilise by membrane filtration and store at -20°C.

Carboxymethylcellulose Overlay Medium.

A 3% solution of carboxymethylcellulose (Sodium salt) is prepared in distilled water, and the solution is sterilised by autoclaving at 10lbs per sq in for 10 minutes. The stock solution may be stored at room temperature.

Double strength L-15 medium is prepared from Flow powdered L-15 and membrane filter sterilised.

Immediately after filtration 2x L-15 and CMC solution are mixed on a 1:1 basis and the mixture is stored at 4°C. Prior to use 10% tryptose phosphate broth, the required amount of foetal bovine serum and antibiotics are added.

Napthalene-Black Stain.

Napthalene-Black	1.0 g
Glacial Acetic Acid	60 ml
Sodium Acetate	13.6 g
Distilled water	to 1000 ml

Trypsin Solution.

NaCl	2400 mg
KCl	120 mg
Na <sub>2</sub> HPO <sub>4</sub>	18 mg
KH <sub>2</sub> PO <sub>4</sub>	18 mg
Bacto-trypsin	3000 mg
Distilled water	300 ml

Stirred on a magnetic stirrer for several hours and then clarified by centrifugation. Sterilise by membrane filtration and store at -20°C.

Carboxymethylcellulose Overlay Medium.

A 3% solution of carboxymethylcellulose (Sodium salt) is prepared in distilled water, and the solution is sterilised by autoclaving at 10lbs per sq in for 10 minutes. The stock solution may be stored at room temperature.

Double strength L-15 medium is prepared from Flow powdered L-15 and membrane filter sterilised.

Immediately after filtration 2x L-15 and CMC solution are mixed on a 1:1 basis and the mixture is stored at 4°C. Prior to use 10% tryptose phosphate broth, the required amount of foetal bovine serum and antibiotics are added.

Napthalene-Black Stain.

Napthalene-Black	1.0 g
Glacial Acetic Acid	60 ml
Sodium Acetate	13.6 g
Distilled water	to 1000 ml

Trypsin Solution.

NaCl	2400 mg
KCl	120 mg
Na <sub>2</sub> HPO <sub>4</sub>	18 mg
KH <sub>2</sub> PO <sub>4</sub>	18 mg
Bacto-trypsin	3000 mg
Distilled water	300 ml

Stirred on a magnetic stirrer for several hours and then clarified by centrifugation. Sterilise by membrane filtration and store at -20°C.

Carboxymethylcellulose Overlay Medium.

A 3% solution of carboxymethylcellulose (Sodium salt) is prepared in distilled water, and the solution is sterilised by autoclaving at 10lbs per sq in for 10 minutes. The stock solution may be stored at room temperature.

Double strength L-15 medium is prepared from Flow powdered L-15 and membrane filter sterilised.

Immediately after filtration 2x L-15 and CMC solution are mixed on a 1:1 basis and the mixture is stored at 4°C. Prior to use 10% tryptose phosphate broth, the required amount of foetal bovine serum and antibiotics are added.

Napthalene-Black Stain.

Napthalene-Black	1.0 g
Glacial Acetic Acid	60 ml
Sodium Acetate	13.6 g
Distilled water	to 1000 ml