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A STUDY OF SOME BIOLOGICAL AND PHYSICAL CHARACTERISTICS
OF COLORADO TICK FEVER VIRUS PROPAGATED
IN CELL CULTURE

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LIST OF ABBREVIATIONS

Following are the explanations of the abbreviations used throughout this writing:

BUdR	5-Bromo-2'-desoxyuridine
BSA	Bovine serum albumin
CAV	Cell associated virus
CE	Chick embryo
CPE	Cytopathic effect
CRK	Cotton rat kidney
CRV	Cell released virus
CTF	Colorado tick fever
DNA	Desoxyribonucleic acid
DOC	Sodium desoxycholate
EBSS	Earle's balanced salt solution
EEE	Eastern equine encephalitis
FL	Cell line, human amnion
FMD	Foot-and-mouth disease
FU	5-Fluorouracil
FUdR	5-Fluoro-2'-desoxyuridine
FUdRP	5-Fluoro-2'-desoxyuridine monophosphate
HBSS	Hanks' balanced salt solution
HeLa	Cell line, human carcinoma of cervix
IRNA	Infectious ribonucleic acid
JAP	Japanese B encephalitis
KB	Cell line, human carcinoma of nasopharynx

L-929	Cell line, mouse fibroblast
MVE	Murray valley encephalitis
NCS	Normal calf serum
OMP	Oklahoma mouse passage
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
RMSF	Rocky Mountain spotted fever
RSSE	Russian spring-summer encephalitis
SLE	Saint Louis encephalitis
TE	Tick-borne encephalitis
VEE	Venezuelan equine encephalitis
WEE	Western equine encephalitis
WN	West Nile encephalitis

A STUDY OF SOME BIOLOGICAL AND PHYSICAL CHARACTERISTICS OF COLORADO TICK FEVER VIRUS PROPAGATED IN CELL CULTURE

CHAPTER I

INTRODUCTION

One of the fevers of unknown origin experienced by pioneers to the Rocky Mountain region was an acute, benign, non-exanthemic disease with intermittent fever. During the eighteenth and early nineteenth centuries the disease was referred to as American mountain fever. Frontier and military physicians in the region had difficulty in differentiating it from malaria, typhoid fever, and a disease later recognized as tularemia. Some of the early observers of American mountain fever considered the etiological agent to be rarefied mountain air, miasma, or even snow water. Rarely was the disease associated with a history of tick bite. Rocky Mountain spotted fever (RMSF) was initially reported from the Bitteroot Valley of Western Montana in 1873. For the next several decades American mountain fever was often confused with RMSF and considered by many clinicians to be a mild form of the latter. However, some observers considered the

diseases to be separate entities. During the first two decades of the twentieth century reports and scientific studies on American mountain fever were overshadowed by the great interest in RMSF. Not until the investigations of Becker in 1926 and 1930 was evidence presented to indicate that the milder disease was not a form of RMSF. Becker proposed the name Colorado tick fever (CTF) for the disease.

Since CTF has been recognized as a distinct clinical entity, continued effort has been made to elucidate the ecology of the virus and its importance in human disease. It is now known that the wood tick, Dermacentor andersoni, is the most important vector in dissemination of the virus to rodents and man. Several rodents which serve as hosts for immature forms of the vector tick were suspected to be viral reservoirs. It now appears that the distribution of CTF virus is localized to areas which correlate with the geographic distribution of the golden mantled ground squirrel (Citellus lateralis), Columbian ground squirrel (Citellus columbians), chipmunk (Eutamias amoenus), pine squirrel (Tamiasciurus hudsonicus) and deer mouse (Peromyscus maniculatus).

The disease in man is most common among sportsmen, sheepherders, and residents of the Rocky Mountain states. Human infections may be debilitating for a month, although death as a result of CTF is rare. Hemorrhage and central nervous system involvement may be more frequent than it is

now generally thought. In the past decade visitors to the Rocky Mountain forests have greatly increased and, as a result, the number of cases of Colorado tick fever has continued to increase. For this reason several attempts have been made to prepare a vaccine from virus infected tissues. Since viruses propagated in cell culture have been used satisfactorily for the preparation of vaccines against certain other viral diseases it was reasoned that these methods could be adapted for the preparation of a CTF virus vaccine. Before a vaccine can be prepared, a thorough understanding of the multiplication of the virus in the cell system of choice is needed. At present, basic information on the replication of CTF virus in cell cultures is not available.

The purpose of this investigation was to examine critically some of the more important biological and physical characteristics of CTF virus and its relationship to the living cell. In the course of the study the following aspects of the virus have been examined: (1) the *in vitro* host cell susceptibility range; (2) characterization of the growth cycle of the virus in cell culture; (3) the resistance of the virus to some chemical and physical agents; and (4) the type of nucleic acid contained in the virus. It is hoped that the information gained from this study might lay a foundation for preparation of CTF virus antigens to be used for a vaccine or for serological surveys.

CHAPTER II

REVIEW OF LITERATURE

In order to understand the relationships of Colorado tick fever (CTF) virus to other viruses it may be appropriate first to consider information gained from the study of other members of the arbovirus group. Where the biological or physical characteristics of arboviruses are not well understood viruses from other groups shall be used to illustrate certain points. In a review of a subject as large as this it is not practical to refer to every arbovirus or tissue culture system which has been studied. Instead, an attempt shall be made to summarize results obtained with viruses and virus-host cell systems which have been studied most thoroughly, particularly those that have characteristics in common with the virus of CTF.

Etiology of Colorado Tick Fever

Using procedures that had been developed for isolation of rickettsiae, early attempts to isolate the etiologic agent of CTF were unsuccessful (Topping *et al.*, 1940; Becker 1930). Becker found that guinea pigs did not become sick when inoculated with blood from patients with American

Mountain fever and rickettsiae could not be demonstrated in their tissues. Fourteen years later, Becker's observations were verified by Florio _et al._ (1944) who showed that the disease could be transmitted experimentally to hamsters and human volunteers. Persons immunized against RMSF were found to be susceptible to CTF. These studies clearly demonstrated the etiologic agent of CTF to be antigenically distinct.

Florio and his associates (Florio and Stewart 1947; Florio _et al._, 1946) were the first to demonstrate that the infection could be serially transmitted to man and hamsters with blood serum from patients who had acquired the disease naturally. Examination of blood smears by light microscopy failed to reveal anything unusual except a marked leucopenia. On the basis of these observations and the previous findings of Becker (1930), Florio and his associates concluded that CTF must be of viral etiology. Gradacol membrane filtration studies of infectious hamster serum revealed the diameter of the viral particle to be less than 25 millimicrons. Therefore they estimated that the particle has a diameter of about ten millimicrons. From filtration studies with collodion membranes, Koprowski and Cox (1947a) estimated the mouse-adapted virus to have a particle diameter of 35 to 50 millimicrons. The latter figure differed somewhat from that obtained by Florio _et al._ (1946). The discrepancy in particle size has never been resolved, however it is possible that the physical association of the mouse-brain virus with tissue

debris prevented the passing of virus through the membrane pores.

CTF virus which had been modified by Florio _et al._ (1944) to multiply in the hamster was adapted by Koprowski and Cox (1946) to replicate in the mouse and developing chick embryo. Studies by Oliphant and Tibbs (1950) on the relationship of age to susceptibility indicated that the suckling Swiss mouse, three to five days of age, was the animal of choice for propagation and isolation of the virus. Propogation of CTF virus in cells cultivated _in vitro_ was first described by Pickens and Luoto (1958). Their studies revealed that the virus would replicate and produce cytopathic effects (CPE) in cultures of KB cells. They observed that virus isolated from infected ticks and human sera would occasionally require several passages in KB cells before development of the CPE became consistent. Recently, Deig and Watkins (1963) have described an assay technique for mouse-brain virus in cultures of A-1 hamster fibroblasts.

The antigenic relationship of CTF virus with other arboviruses has been investigated with several techniques. Cross immunity tests in human volunteers showed that the viruses of dengue and CTF are antigenically distinct (Florio _et al._, 1946; Pollard _et al._, 1946). Neutralization tests carried out in mice (Koprowski and Cox, 1946, 1947a) and complement fixation tests (DeBoer _et al._, 1947, Miller _et al._, 1961) suggested that CTF virus is antigenically different

from Russian spring-summer encephalitis (RSSE), louping ill, Venezuelan equine encephalitis (VEE), Western equine encephalitis (WEE), Eastern equine encephalitis (EEE), Japanese B encephalitis (JAP), St. Louis encephalitis (SLE), yellow fever, and dengue viruses. Strains of CTF virus isolated in Eastern and Western parts of the United States were shown to be antigenically indistinguishable (Florio et al., 1950). Casals (1961) systematically studied a large number of the arboviruses and found CTF virus to be immunologically unrelated to any of them.

Arbovirus Host Cell Spectrum

Members of the arbovirus group have been shown to be capable of replication in cells of widely different origins. Haung (1942) first demonstrated that the CPE of WEE virus on fragments of embryonic chick tissues could be used as an indicator for the presence of this virus. Monolayer cultures of chick embryo (CE) cells have since been found to be as sensitive as suckling mice for the isolation of WEE, VEE, EEE and Kyasanur Forest disease viruses (Fastier, 1954; Kissling, 1957; Medearis and Kilrick, 1958; Bhatt, 1960; Lennette et al., 1961). Liquid covered monolayers of CE cells supported replication of Middleberg (Henderson and Taylor, 1959) and JAP viruses (McCollum and Foley, 1957), however the production of CPE was sporadic. Porterfield (1960) demonstrated that five viruses in group-A, eight in group-B, and two in the Bunyamwera-group produce clear, distinct plaques on monolayers

of CE cells. The Tr-1751 strain of dengue virus was the only virus studied which did not produce CPE. Henderson and Taylor (1959, 1960) and Henderson (1961) investigated the response of 21 arthropod-borne viruses in primary cultures of avian origin. In general, they found that group-A viruses multiplied and produced plaques in cell cultures from chick embryo and Peking duck kidney or embryo. Viruses of group-B, except Ilheus, could be propagated in Peking duck embryo or kidney but not in cultures of CE cells. These investigators observed that viruses of group-C do not replicate in tissues of avian origin, however they do produce plaques on cultures of rhesus monkey kidney cells.

Some of the arboviruses may be isolated and grown in cultures of kidney epithelial cells from various mammalian sources. The viruses of WEE, EEE, Sinbis, Mayaro, SLE, Murray Valley encephalitis, West Nile (WN), Ilheus, and JAP grow very well in cultures of hamster kidney cells (Rosenberger and Shaw, 1961; Lennette et al., 1961; Kissling, 1957; Diercks and Hammon, 1958; Henderson 1961). Cultures of rhesus monkey kidney cells have been shown to support replication and plaque formation of most viruses belonging to the Japanese B-West Nile complex (Bhatt and Work, 1957). The viruses of dengue and WEE (Hotta and Evans, 1956; Banta, 1957), as well as the virus of EEE (Medearis and Kilrick, 1958), also replicate in primary cultures of monkey kidney epithelium. Monolayer cultures of pig kidney epithelial cells have been

utilized successfully for the propagation and assay of JAP (Kato and Inoue, 1962) and tick-borne encephalitis (TE)[1] virus (Malkova et al., 1960).

Stable cell lines derived from neoplastic tissues will support growth of a limited number of arboviruses. Scherer and Syverton (1954) reported that EEE, WEE, WN, SLE and JAP viruses multiplied in monolayers of HeLa Cells, however cytopathic effects are consistent only in cultures infected by EEE and WN viruses. Banta (1957) confirmed these findings and in addition reported that dengue virus did not replicate in cultures of HeLa cells. Arboviruses which have been classified antigenically in Casal's group-C grow well and produce CPE consistently in cultures of HeLa and Detroit-6 cells (Buckley and Shope, 1961). McCollum and Foley (1957) reported that HeLa and Detroit-6 cells supported replication of two strains of JAP and WN viruses, although these viruses produced CPE consistently only in cultures of Detroit-6 cells. HeLa cells have been reported to support replication of various members of the RSSE complex with the appearance of partial or irregular cytonecrosis (Libikova et al., 1960). The replication of these viruses was determined by the interference phenomenon with WN virus or by assay of the cell culture fluids in mice. The Asibi strain of yellow fever virus replicated in cultures of KB cells (Eagle et al., 1956) and HeLa

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[1]The Russian authors use the term tick-borne encephalitis virus to indicate an undesignated virus of the Russian spring-summer encephalitis complex.

cells (Hardy, 1963a). Of the various cell types examined by Hardy HeLa cells were one of the least sensitive to infection and only sporadically showed CPE. This cell type had the greatest capacity to support the replication of this strain of yellow fever virus. KB cells are susceptible to infection by dengue (Schulze and Schlesinger, 1963) and CTF viruses (Pickens and Luoto, 1958). Both of these viruses required several passages in cell cultures before the development of CPE became consistent.

Several of the arboviruses have been adapted to replicate in cultures of L cells. When initially passed in L cells, WEE virus multiplied with varying degrees of cell degeneration (Chambers, 1957). Large amounts of virus frequently caused fewer cytopathic changes than did small amounts of virus. Cells which survived the initial infection with virus multiplied and continued to produce virus over a long period of time without showing any morphologic changes. These cells were immune to destruction induced by superinfection as long as virus persisted in the cultures. Eiring and Scherer (1961) found that continued passage of WEE and EEE virus in cultures of L cells resulted in the development of a virus which was persistently cytopathic for this cell line. These authors reported that in five of their 16 attempts to adapt EEE virus to L cells the virus failed to replicate for reasons unknown to them. Lockart (1960) suggested that L cells exposed to large amounts of inactive WEE virus became refractory

to destruction by active virus. He demonstrated that cultures of L cells become persistently infected with active WEE virus and postulated that the chronically infected state may be due to the operation of an interference mechanism. Recently, Lockart (1963) demonstrated that interferon was produced by cultures of L cells infected with WEE virus. He suggested that the irregular development of cytopathic changes and virus production were due to the presence of this compound. Monolayer and spinner cultures of L cells sustained the replication of VEE virus with the production of specific cytopathic changes (Hardy, 1959). Titration of VEE virus by CPE or plaque formation in L cells was not as sensitive as assay in suckling mice. A TE virus has been adapted to replicate in L cells (Stancek, 1963). The degree and type of cytopathic changes induced by the virus depended upon the amount of virus in the inoculum. The CPE resulting after infection with a high titer inoculum was of a lytic character. The CPE which developed after infection with a low titer inoculum was partial and the infected cells become rounded and detached from the glass. The titer of TE virus in L cells, using CPE development as the index of infection, was 10^3 to 10^5 fold lower than that calculated by mouse inoculation studies.

Growth Cycles of Arboviruses

The growth cycles of arboviruses in cells cultivated *in vitro* have been studied. Dulbecco and Vogt (1954b) first

characterized the growth cycle of WEE virus in monolayer and suspension cultures of CE cells. They reported that the adsorption of virus onto cells occurred rapidly. From these data the adsorption of WEE virus to CE cells appeared to proceed at a linear rate during the first 60 minutes of incubation. At the end of this time 84 per cent of the input virus was adsorbed to the cells. Cells which were maintained in suspension culture adsorbed the virus at a rate approximately five times slower than the cells in monolayer culture. Levine (1958) observed that the rate of adsorption of WEE virus was dependent upon the volume of the inoculum. He reported that only 30 to 70 per cent of the virus in the inoculum was adsorbed, regardless of the length of time of exposure. Wagner's investigation (1961) established that the attachment of EEE virus to monolayer cultures of chick embryo cells was slower than that of WEE and was dependent upon the volume of the inoculum. He observed that after a 90 minute adsorption period 40 per cent of the inoculum was still unadsorbed to the cells. The adsorption of WEE and EEE virus to CE cells was slower and not as efficient as that of poliovirus, in which 95 to 100 per cent of the input virus was adsorbed to susceptible cells in 20 minutes (McLaren _et al._, 1959).

The kinetics of arbovirus adsorption to cultures of mammalian cells has been reported for some members of Casal's group-B. Whether JAP virus was in contact with monolayers of

human skin for one minute or four hours, essentially the same yields of virus were produced after 24 hours of incubation (Banta, 1957). Schulze and Schlesinger (1963) demonstrated that the adsorption rate of dengue virus to monolayers of KB cells proceeded in a linear manner during the first 30 minutes and the amount of virus adsorbed was maximal after two hours of incubation. The rate and efficiency of adsorption were shown to be dependent upon the temperature of incubation. Adsorption of virus to the cell surface was 40 per cent more efficient at 37 C than at 25 C. The attachment of VEE virus to L cells was described by Hardy and Brown (1961a). Fifty per cent of the cells inoculated at a multiplicity of 1 LD_{50} per cell were infected during the first 15 minutes of adsorption. Ninety to 100 per cent of the cells inoculated at multiplicities of 10 or 100 LD_{50} per cell were infected during the first 15 minutes of exposure to the virus. Lockart (1960) reported that L cells infected with WEE virus at a multiplicity of two and one-half plaque forming units per cell adsorbed 48 per cent of the input virus in one hour.

The lag periods of arboviruses cultivated _in vitro_ vary from two and one-half to eighteen hours. Viruses of Casal's group-A usually have a shorter lag period. WEE, EEE, and VEE viruses characteristically have a lag period of two to four hours regardless of the host-cell type (Dulbecco and Vogt, 1954b; Rubin _et al._, 1955; Wagner, 1961; Lockart, 1960, 1963; Hardy and Brown, 1961a,b). Arboviruses belonging

to the biologically more diverse, and antigenically more complicated group-B usually have a lag period of six to 18 hours. The growth cycles of viruses of the JAP-WN complex in monolayer cultures of hamster kidney cells (Diercks and Hammon, 1959), HeLa, human intestine, human conjunctiva, monkey kidney, and human skin (NCTC2546) have a lag period varying from four to ten hours (Banta, 1957). Yellow fever (Hardy, 1963b) and dengue viruses (Schulze, 1964) have a longer lag period which extends from 12 to 16 hours. The duration of the lag periods of viruses of the RSSE complex varies from six to nine hours (Mayer et al., 1960; Stancek, 1963). However, Danes and Benda (1960) reported a lag period of 18 hours for a strain of the TE virus.

The synthesis, maturation, and release of infectious WEE virus from cultures of CE cells have been described in detail by Dulbecco and Vogt (1954b) and Rubin et al. (1955). Data from these studies have been interpreted to indicate that complete virions were not present intracellularly during the first hour of the lag period. The first infectious virus was demonstrated intracellularly during the second and third hours after infection. Intracellular virus increased in an exponential manner for the next three hours, accompanied by the appearance of extracellular virus which began about the fourth hour after inoculation. Increases in extracellular and intracellular virus were logarithmic from the fourth to the eighteenth hour when maximal titers were

attained. The pattern of WEE virus replication in L cells was similar to that previously described in CE cells (Lockart and Horn, 1963). EEE virus replication in CE cells was at a logarithmic rate from the fourth to the tenth hour and maximal titers of virus were produced 12 hours after infection (Wagner, 1961). Hardy and Brown (1961a,b) characterized the growth cycle of VEE virus in monolayer and spinner cultures of L cells. The four hour eclipse phase was followed by an exponential growth phase of eight to 12 hours, culminating in a peak titer at about 25 hours post infection. These data, together with those of Rubin _et al_. (1955), have been interpreted to indicate that complete virions are released from the cell within one to two minutes after their development. These studies indicated that the production of cell-associated (CAV) and of cell-released virus (CRV) were parallel throughout the growth cycle. The titer of the CRV always remained 10 to 20 times higher than that of the CAV.

The replication of viruses belonging to Casal's group-B have not been studied in as great detail as those of group-A. After inoculation of hamster kidney cultures with JAP virus, Diercks and Hammon (1959) observed the virus to increase in a geometric manner after a ten-hour lag period. Virus increased rapidly for the next 12 to 14 hours and reached maximal titers approximately 72 hours after infection. The multiplication rates of type two dengue virus in KB cell cultures was described by Schulze (1964). Viral

titers increased at an exponential rate for 18 hours following a 12 hour lag period. Maximal yields of infectious virus were produced 35 to 40 hours after inoculation of the cultures. Monolayer cultures of HeLa cells infected with the Asibi strain of yellow fever virus produced virus at an exponential rate for the first 24 hours after the lag period if the unadsorbed virus was removed one hour after adsorption (Hardy, 1963b). If the residual unadsorbed virus was not removed the logarithmic growth phase did not begin for 24 hours and was much slower. In these retarded cultures maximal yields of virus were not produced until 72 hours after infection.

The multiplication of viruses of the RSSE group has been studied in cultures of pig kidney, human embryonic tissue, duck kidney, and Detroit-6 cells (Mayer, 1960). Virus replicated in all cell lines at a linear rate for 10 to 12 hours after the lag period. Maximal titers were not attained until four or five days after infection. Mayer et al. (1960) reported that the growth of TE virus was more rapid in HeLa cells than in the other cell types studied. After the lag period the logarithmic phase of multiplication started about the ninth hour and continued until the twenty-fourth hour, when maximum titers were produced. Stancek (1963) characterized the growth of a strain of TE virus in L cells. Virus adapted to this cell line replicated at a logarithmic rate from the seventh to the twenty-fourth hour. Viral titers

continued to increase for another 24 hour period to attain maximal yields of virus 48 hours after infection.

Stability of Arbovirus to Physical and Chemical Agents

Lipid Solvents

Many reports in the literature have shown that certain viruses, notably those of the adeno, papavo, pox and picorna virus groups are highly resistant to inactivation by lipid solvents (Andrewes *et al.*, 1961; Andrewes, 1962; Hamparian *et al.*, 1963). Most studies have indicated that arboviruses, in sharp contrast to other RNA-containing viruses such as polio and encephalomyocarditis, are easily inactivated by these solvents. Sulkin and Zarafonetis (1947) reported that WEE, EEE, and SLE viruses, in contrast to poliovirus, are highly susceptible to the action of ethyl ether. Andrewes and Horstmann (1949) examined the effect of ether on the infectivity of a variety of viruses. They concluded that, "on the whole the viruses which we have found to be ether-resistant are 'tough viruses,' viruses which can be left at room temperature without fear that they will quickly lose their activity. The ether-sensitive ones are, on the whole more delicate." To show whether a virus contained essential lipid, Feldman and Wang (1961) determined that chloroform could be substituted for diethyl ether. Their investigation of the comparative efficiency of these two lipid solvents indicated that chloroform was the more efficient. Hamparian *et al.*

(1963) confirmed this observation and reported that chloroform gave more consistent and reliable results than did ether when used to detect the presence of essential lipid.

An important and interesting property of the arboviruses was seen in studies on the effect of bile salts on infectivity. The early studies by Smith (1939) indicated that the following ether-sensitive viruses are inactivated by bile salts: Influenza A, louping ill, and Rous sarcoma; Polio, foot-and-mouth disease (FMD) vaccinia and ectromelia viruses, all of which are ether-resistant viruses, resist inactivation by bile salts. Sunaga _et al_. (1960) showed that a high degree of correlation exists between sensitivity of a virus to diethyl ether and sensitivity to sodium desoxycholate (DOC). All arboviruses tested have been found to be sensitive to inactivation by DOC (Theiler, 1957; Casals, 1961). Sensitivity to the chemical was not, however, a characteristic limited to this group of viruses (Hamparian _et al_., 1963). It was shown that incubation of WN (Anderson and Ada, 1959) and EEE (Richter and Wecker, 1963) viruses with DOC under appropriate conditions releases infectious RNA (IRNA from the virions. Sunaga _et al_. (1960) suggested that the inactivation of arboviruses by DOC was due to the release of IRNA from the viral particle after damage to surface lipid by the DOC. This was followed by enzymatic digestion of the IRNA by RNAse present in the crude viral preparation.

Hydrogen Ion Concentration

Investigations of several animal viruses have shown that for each virus there is a range of hydrogen ion concentration over which it is most stable. At pH values higher or lower than the optimal, infectivity of the virus is usually rapidly destroyed. It has been suggested by Hamparian and his associates (1963) that this characteristic could be utilized in the classification of the animal viruses. Lepine (1931) demonstrated that louping ill virus was most stable within the pH range of 7.5 to 8.5. The virus of EEE was also more stable within this pH range, however there was a second region of relative stability between pH 3.5 and 5.0. Midway between a pH of 5.0 and 6.5 inactivation is complete (Finkelstein _et al_., 1938, 1940). Research on the pH stability of TE virus by two different groups of Russian investigators (Shubaladaze and Ananina, 1943; and Gresikova-Kohutova, 1959a) showed these group-B viruses to be most stable within a pH range of 7.4 to 8.2. In contrast to most of the arboviruses, JAP and SLE viruses were shown by Duffy and Stanley (1945) and Duffy (1946) to be more stable in an alkaline range of pH 8.5 to 9.0. Hardy and Brown (1961a) investigated the stability of VEE virus over pH values ranging from 6.0 to 7.4. They reported the virus to be most stable in the slightly alkaline pH range of 7.2 to 7.4. It may be concluded from the literature that, in general, crude arbovirus preparations are usually stable in an alkaline pH

range, between 7.4 and 8.0, and are rapidly destroyed at extremely high or low hydrogen ion concentrations.

Protective Proteins

Arboviruses are generally stable in a serum-saline diluent when maintained at 4 C for 30 days. They are stable up to three years when frozen at dry ice temperature (-65 C). The stability of yellow fever virus is increased by the use of a protein-containing diluent instead of saline (Bauer and Mahaffy, 1930). More recently it has been shown that thermal inactivation rates of WEE, EEE (Olitsky _et al._, 1950), Ilheus (Koprowski and Thomas, 1946), and TE virus (Gresikova-Kohutova, 1959) were greatly retarded when the virus-suspending diluent contained 10 per cent serum.

The resistance of CTF virus to freezing and lyophilization appears to be dependent upon the past history of the virus and the medium in which it is suspended. Florio _et al._ (1944) reported that CTF virus in human serum remains infectious for at least three and one-half years when frozen in the ice compartment of an electric refrigerator. Mouse brain-adapted virus diluted in media containing 10, 20, and 50 per cent horse or rabbit serum remained viable for three years when kept in a dry ice chest or in a lyophilized state (Koprowski and Cox, 1947a; Thomas and Eklund, 1960; Gerloff and Larson, 1959; Gerloff and Eklund, 1959). CTF virus which was adapted to replicate in cultures of KB cells was described by Pickens and Luoto (1958) as being unstable in cell culture

medium containing 10 per cent horse serum when stored in sealed ampules over dry ice. The virus was more stable when the diluent contained 50 per cent horse serum.

Thermal Inactivation

Most arboviruses are relatively sensitive to inactivation by heat. The viruses of WEE, EEE (Olitsky _et al._, 1950), Ilheus (Koprowski and Thomas, 1946), Murray Valley encephalitis (MVE) (Anderson and Ada, 1953), JAP (Kudo _et al._, 1937; Melnick, 1946), Semliki Forest disease (Cheng, 1961), louping ill (Lepine, 1931), and TE (Schubaladaze and Ananina, 1943) are readily inactivated by heating at 56 to 60 C for 10 to 30 minutes. The majority of these reports are difficult to compare and confine themselves to statements to the effect that a certain viral property is or is not destroyed by heating for a particular time at a designated temperature. Furthermore, the stability of the viruses is qualitatively difficult to compare because the basic techniques used have not been standardized. It is possible to generalize and conclude that, as a group, the arboviruses are very unstable when exposed to temperatures above 4 C. Banta (1957) reported that there were enough differences in the thermal decay curves of the viruses of dengue, WEE, JAP, and WN to differentiate among them.

Koprowski and Cox (1947a) studied the resistance of mouse-brain CTF virus to inactivation at temperatures of 37 C, 56 C, 60 C, and room temperature. Virus diluted in medium

containing 10 per cent rabbit serum remained infectious for mice after 30 minutes at 56 C. Infectivity of the virus was destroyed during exposure to a temperature of 60 C for 30 minutes. Virus suspensions incubated at 37 C were noninfectious after three days of incubation. Virus suspended in rabbit serum-saline remained infectious when kept at room temperature for as long as 64 days. When the virus was diluted in saline, infectivity was lost after five days of incubation at 37 C and after 40 days at room temperature.

Published reports on the kinetics of thermal inactivation of animal viruses present the picture of a complex process. There are, however, several well established characteristics of thermal inactivation curves which Woese (1960) summarized as follows: (1) first order kinetics are usually obeyed for a few log units only, as there exists in most virus populations a relatively thermal resistant fraction of about 10 per cent; (2) the Q_{10} for viral inactivation varies with the temperature; and (3) at certain temperatures virus characteristics such as infectivity and antigenic structure are lost simultaneously or independently according to the temperature.

Thermal inactivation of viruses is generally assumed to be exponential (Luria, 1953). Exponential inactivation, the expression of a first order reaction, indicates that a constant fraction of the virions undergo an inactivating change in each unit of time. The law of mass action states

that the rate of decrease in V_o, the input virus, at a given time, t, is proportional to V_t, the survivors at time t. This relationship is expressed mathematically as:

$$k = - \frac{2.303 \log_{10} {}^{V}t/V_o}{t}$$

where K is the specific reaction rate constant. This value is a characteristic of the thermal inactivation rate of a particular virus under the specified conditions under which the reaction is measured. The term V_t/V_o is a ratio indicating the initial virus concentration/prevailing virus concentration and is independent of the units of virus employed. A plot of the logarithm of this ratio, V_t/V_o against time gives a straight line, the slope of which is independent of V_o. This indicates that the rate of inactivation is dependent only upon the temperature of incubation.

In a recent review of viral inactivation Hiatt (1964) suggested that the kinetics of inactivation can be related through the reaction rate theory to the thermodynamic quantity E (heat of activation) by the Arrhenius equation. The integrated form of this equation is written as:

$$\ln K_2/K_1 = E/R\ (1/T_1 - 1/T_2)$$

where K_1 and K_2 are the velocity constants for the thermal inactivation at absolute temperatures, T_1 and T_2 respectively. In this equation, R represents the gas constant and E the energy of activation for the inactivation reaction. The real meaning of these thermodynamic expressions when applied to

the inactivation of a macromolecule such as a virion is a matter of question.

An excellent discussion by Pollard (1953) of viral thermal inactivation serves to illustrate the difficulties involved in attempting to interpret E in terms of changes in viral structure during inactivation by heat. Therefore, in the presentation of data in this review no attempt shall be made to evaluate these constants in terms of the number and types of bonds broken during inactivation. The values will be used only as emperical constants to characterize the inactivation of the particular virus in question. Data for the inactivation of representative members of several groups of animal viruses are presented in Table 1. The inactivation energies of vaccinia (Kaplan, 1958) and various myxoviruses (Woese, 1960) are even greater than those for the picornaviruses, indicating that more energy is required before chemical-structural changes in the virion are of sufficient magnitude to alter the biological function of the particle. Pollard (1953) suggested that the main difficulty in the study of viruses and other macromolecules lies in the large number of possible simultaneous reactions in such complicated systems. The temperature effect on inactivation rate is frequently measured by the Q_{10}, i.e., the effect of 10 C temperature increase on the reaction rate. Large Q_{10} values indicate that the reactions change from slow to rapid rates in a very restricted temperature range. Viruses which have a high

TABLE 1

THERMODYNAMIC PARAMETERS CHARACTERIZING THE THERMAL INACTIVATION OF SOME ANIMAL VIRUSES

Virus	E x 10^{3a}	Q_{10}	T½ in Minutes[c]	Reference
FMD	26	2.1	790	Bachrach *et al.* 1957a
Polio, 2	30	1.05	1224	Youngner, 1957
Coxsackie, A	20	---[d]	---[d]	Robinson, 1950
Vaccinia	90	1.8	---	Kaplan, 1958
Reovirus	48	1.6	153	Gomatos *et al.* 1962
Influenza, A	170	---	---	Woese, 1960
Influenza, B	340	---	---	Woese, 1960
Newcastle disease	129	---	---	Woese, 1960
Measles	188	2.5	120	Black, 1959

[a]Activation energy for thermal inactivation: calories $mole^{-1}$.

[b]Half-life at 37 C.

[c]Data are not provided by the authors.

E value usually have a low Q_{10} and a long half-life when exposed to temperatures of 37 C (Table 1).

The studies of Kaplan (1958) on vaccinia, of Bachrach and associates (1957a) on FMD and of Youngner (1957) on poliovirus revealed that typical thermal inactivation curves contained two components. The inactivation of each phase of the curves was described as obeying first order kinetics. Extrapolation of the slow inactivating phase back to the ordinate revealed that the estimated size of the heat resistant fraction was a function of the temperature of exposure. As the temperature of exposure was lowered, the extrapolated intercept of the slow inactivating component approached 100 per cent. Viral inactivation curves at temperatures below a certain value have only one component which is characterized by first order kinetics down to the last 10 per cent of the survivors. This was obvious with the data on FMD virus referred to previously. Thermal inactivation curves of WEE, EEE, WN, VEE, JAP, TE, and dengue viruses (Banta, 1957; Rosenberger and Shaw, 1961; Nir and Goldwasser, 1961b; Gresikova-Kohutova, 1959a) in the temperature range of 35 C to 37 C have only one component. Luria (1953) suggested that when inactivation curves are not linear the deviation is generally due to aggregation of the viral particles or to heterogenity of their heat sensitivity. Woodroffe (1960) observed that thermal decay curves of vaccinia virus suspensions prior to freezing were exponential and contained only one component.

Arbovirus Nucleic Acid

Two general methods are available for the determination of the type of nucleic acid present in a virus. These are, first, the isolation of the infectious nucleic acid and, secondly, the utilization of metabolic inhibitors which inhibit specifically the replication of viral nucleic acids.

The phenol extraction technique described by Gierer and Schramm (1956) for the isolation of infectious RNA (IRNA) from plant viruses gave great impetus to similar studies with animal viruses. RNA preparations from tissues infected with several RNA-containing animal viruses are capable of initiating infection in the susceptible host (Colter, 1958). Progeny virus from cells infected with viral IRNA is identical to the original virus from which the RNA extraction was made. In addition, it has been shown that poliovirus IRNA can infect and complete a single growth cycle in cells which are insusceptible to infection by complete virions (Holland *et al.*, 1959; McLaren *et al.*, 1960).

Infectious ribonucleic acid has been isolated from tissues infected with eight arboviruses: EEE (Wecker and Schaefer, 1957), WEE (Wecker, 1959), MVE (Anderson and Ada, 1953), WN (Colter *et al.*, 1957), Semliki Forest (Cheng, 1958), JAP (Nakamura and Ueno, 1963; Liu *et al.*, 1962), denque (Nakamura, 1961), and TE (Sokol *et al.*, 1959). The evidence that the infectivity of such preparations could be ascribed to viral RNA and not due to contamination by complete

virus is based on the following information from the above authors:

1. The infectivity of the IRNA preparation, but not that of the original virus is completely destroyed by RNAse.

2. The infectivity of the IRNA preparation is more stable to inactivation by heat than is the original virus preparation.

3. Intact virions sediment more rapidly during centrifugation than infectious particles in the IRNA preparation.

4. The infectivity of the viral IRNA can not be neutralized by homologous immune serum.

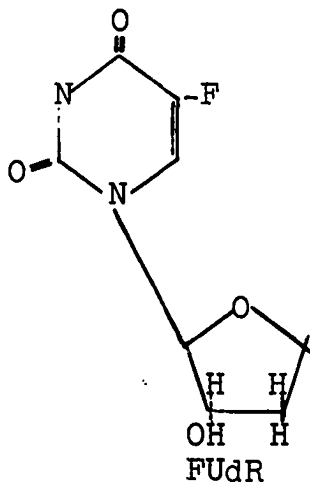
5. The infectivity of the IRNA can be recovered after precipitation by ethanol or one molar sodium cloride solution.

Of these criteria, the first three are necessary, but not sufficient to prove IRNA is infectious. Sokol _et al._ (1961) suggested that viral particles from which only part of the surface protein has been removed could have been inactivated by ribonuclease to give the impression that infectivity was due only to the IRNA. Virions in which the capsid was damaged migrated more slowly in a centrifugal field than the intact viral particles. They postulated that the lability of the partially damaged particles in such RNA preparations was due to contaminating host RNAse. The IRNA itself was relatively thermostable, since active preparations could be obtained from virus particles by heating at 90 to 100 C. This treatment ruptured the bonds between viral RNA and protein with subsequent denaturization of viral protein (Kaper and Steere, 1959).

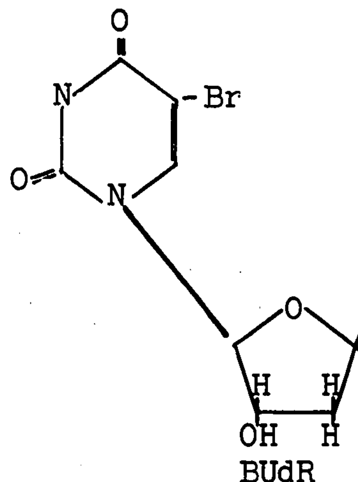
In summary, the main criteria for proving the isolation of IRNA from virus infected tissues are: (1) the recovery of infectivity after precipitation of the nucleic acids with reagents which completely inactivate the intact virion and (2) the lack of neutralization of IRNA by specific antibodies against the intact virus.

The results obtained in the investigations of IRNA isolated from crude arbovirus preparations were only preliminary (Wecker and Richter, 1962). Definite conclusions concerning the infectivity and other properties of arbovirus RNA will be possible only when highly purified preparations are studied more thoroughly.

Recently it has been suggested that the type of nucleic acid in viruses may be determined by treating infected cell cultures with metabolic inhibitors which prevent the synthesis of DNA-, but not RNA-containing viruses (Salzman, 1960; Salzman _et al_., 1962). Two halogenated pyrimidines, 5-fluoro-2'-deoxyuridine (FUdR) and 5-bromo-2'-deoxyuridine (BUdR) have been utilized for this purpose.



FUdR



BUdR

The primary effect of FUdR, after phosphorylation, is to inhibit the methylation of deoxyuridilic acid to thymidilic acid which, as a consequence blocks the synthesis of DNA (Cohen et al., 1958; Bosch et al., 1958; Habers et al., 1959). Thymidylate synthetase is specifically and competitively inhibited by 5-fluoro-2'-deoxyuridine-5'-monophosphate (FUdRP) but is not appreciably affected by 5'-fluorouridine-5'-monophosphate or thymidylate, the product of the reaction (Hartmann and Heidleberger, 1961). Mukerjee and Heidelberger (1962) reported that FUdR is a more efficient inhibitor of thymidylate synthesis in cell cultures than FUdRP. They reasoned that the fluoridated pyrimidine mononucleotide is unable to cross the cell membrane. To become active FUdRP must be dephoxphorylated, cross the cell membrane, and be rephosphorylated.

In addition to its effect on thymidylic acid synthesis, the deoxyribonucleotide may be hydrolyzed to the free base, fluorouracil (FU) which is converted to a ribonucleotide and incorporated into ribonucleic acid (Habers et al., 1959). By virtue of these interconversions, FUdR can inhibit the incorporation of exogenous uracil or orotic acid into RNA. The fluorouridylic acid which is produced by this reaction can be incorporated into viral RNA and result in the formation of a non-functional species of RNA. This reaction occurred at a rate directly proportional to the supply of nucleotides, uridylic and 5-fluorouridylic acid which is

available (Munyon and Salzman, 1962; Salzman and Sebring, 1962). A second factor which influences the incorporation of fluorouridylic acid into RNA is the efficiency of the cell in converting FUdR to FU. Two observed effects of fluorouracil analogues on nucleic acid synthesis are: (1) the inhibition of thymidylic acid synthesis and (2) the incorporation of fluorouridylic acid into RNA (Salzman _et al._, 1962).

It has been shown that HeLa (Rueckert and Mueller, 1960; Salzman and Sebring, 1962) and L cells (Paul and Hagiwara, 1962; Till _et al._, 1963) cease to divide in the presence of FUdR. This is a result of the inhibition of DNA synthesis induced by thymidylate starvation. When thymidine was added to the cultures in an amount 15 times greater than the concentration of the fluoridated pyrimidine, DNA synthesis began immediately and was followed by cell division (Stanners and Till, 1960; Till _et al._, 1963). At FUdR concentrations of 10^{-7} M or greater there appeared to be a complete block in DNA synthesis in cultures of HeLa and L cells. Under these conditions synthesis of DNA and protein continued at suboptimal rates to produce unbalanced growth and loss of cell viability.

The ability of viruses to replicate in cells during unbalanced growth indicates that the formation of functional RNA and protein proceeds in the absence of DNA synthesis. The same concentration of FUdR which prevents the formation of normal titers of vaccinia virus permits the synthesis of

normal yields of poliovirus (Salzman, 1960). FUdR does not have an inhibitory effect on RNA viruses such as reovirus (Gomatos et al., 1962), measles and vesicular stomatitis (Seymour and Olson, 1963), ECHO 28 (Hamparian et al., 1963), Newcastle disease (Simon, 1961), and Rous sarcoma (Rich et al., 1962). The synthesis of adenovirus (Flanagan and Ginsberg, 1961), herpes simplex (Hamparian et al., 1963), and pseudorabies (Kaplan and Ben-Porat, 1961) viruses is reduced in the presence of FUdR.

The desoxyribonucleoside 5-bromo-deoxyuridine (BUdR) is an analogue of thymidine. This molecule is an acceptable substrate for the phosphorylating and polymerizing enzymes that synthesize DNA (Bessman et al., 1958; Trautner et al., 1962; Littlefield and Gould, 1960; Kit et al., 1958; Dunn and Smith, 1957; Cheong et al., 1960a,b). The in vitro growth of mammalian cells after exposure to BUdR is retarded after the first cell division and approaches zero as the amount of BUdR incorporated into the DNA is increased (Littlefield and Gould, 1960).

When cultures of HEp (Cheong, et al., 1960a) and L cells (Hakala, 1959) are grown in BUdR-containing medium, up to 80 per cent of the DNA thymine is replaced by BUdR in one week. Shapiro and Chargaff (1960) and Hsu and Somers (1962) suggested that the cessation of growth in the presence of BUdR is the result of alterations in the genetic code which makes normal DNA replication impossible. Although DNA

replication in HeLa cells is arrested by BUdR, protein and RNA synthesis continue, in HeLa cells with the formation of giant cells which do not divide but gradually disintegrate (Hakala, 1959).

Infection of BUdR-treated HeLa or KB cells by vaccinia virus results in the incorporation of the analogue into viral DNA and the formation of large quantities of noninfectious, malformed viral particles (Easterbrook and Davern, 1963). The addition of excess thymidine to the BUdR medium reverses the inhibition of viral synthesis. These data indicate that the formation of competent, infectious, viral DNA is specifically supressed by this analogue. The synthesis of viral RNA in reovirus (Gomates *et al*., 1962) and vesicular stomatitis (Chamsy and Cooper, 1963) virus is not inhibited by this analogue. Salzman (1960) and Simon (1961) concluded that RNA viruses are replicated independent of DNA synthesis. On this basis Hamparian *et al*. (1963) suggested that BUdR could be employed to distinguish RNA-containing from DNA-containing viruses.

Additional evidence that a virus contains RNA may be provided by measuring the effect of the antibiotic, actinomycin, upon its replication. Actinomycins are composed of a phenooxazone chromophore to which two peptide chains are attached. The various actinomycins differ principally in the amino acid composition and sequence in the peptide chains.

Actinomycin D was reported by Goldberg and Rabinowitz

(1962) to inhibit the synthesis of RNA in cultures of HeLa cells. Similar observations were reported by Shatkin (1962). Treatment of cultures of L (Franklin and Baltimore, 1962) and chick embryo cells (Amos, 1964) with the antibiotic results in the immediate cessation of 99 per cent of cellular RNA synthesis. Hurwitz _et al_. (1962) studied the role of DNA in RNA synthesis in mammalian cells. They observed that although RNA synthesis is markedly inhibited under the influence of actinomycin D, the formation of DNA by DNA polymorase is not affected. It had been shown previously that actinomycins form stable complexes with DNA (Kernstan _et al_., 1960; Kawamata and Imanishi, 1960), however the exact mechanism of actinomycin inhibition was unknown. Further investigations by Goldberg _et al_. (1962) on the nature of the DNA-actinomycin complex have shown that the purine base guanidine is essential for the formation of the complex. The antibiotic is not specifically bound to guanidine because native, helical DNA forms a more stable complex with actinomycin than does a polymer of poly-guanidine. These investigators suggested that the guanidine residue, the base sequences in the immediate vicinity of guanidine, and the helical form of native DNA were all essential for formation of the actinomycin-DNA complex.

Studies of RNA synthesis by RNA polymerase preparations from HeLa cells indicate that actinomycin D is bound to DNA and competitively inhibits the DNA-dependent RNA

polymerase (Goldberg and Rabinowitz, 1962). Baltimore and Franklin (1962) showed that RNA polymerase preparations from L cells which required all four ribonucleoside triphosphates and primer DNA for the synthesis of RNA are completely inhibited by actinomycin. This inhibition could be reversed by the addition of DNA primer to the system. More recently, Franklin (1963) showed that the residual RNA synthesis in cells treated with actinomycin could be accounted for by the incorporation of nucleotides into the cytidine-phosphate-cytidine-phosphate adenine terminal bases of soluble RNA. From his data he concluded that all normal RNA synthesis in mammalian cells is directed by the DNA template.

Inhibition of cellular RNA synthesis by actinomycin does not suppress the replication of poliovirus (Shatkin, 1962). Reich *et al.* (1962) confirmed these observations with poliovirus and reported that DNA-containing vaccinia virus is inhibited by the antibiotic. The synthesis of RNA viruses such as Mengo (Franklin and Baltimore, 1962), reovirus (Gomatos *et al.*, 1962), and Chikongunya (Heller, 1963) is unaffected in cell cultures in which synthesis of cellular RNA is inhibited by actinomycin. These observations were interpreted to indicate that the biosynthesis of viral RNA is a fundamentally different process than the synthesis of cellular RNA. The formation of viral RNA and protein does not require the participation of DNA or DNA-dependent protein synthesis. The fact that actinomycin interferes with RNA

synthesis, which is dependent on either cellular or viral DNA, suggests that the synthesis of viral RNA requires the formation of a new, viral-specific RNA polymerase. Only recently, Baltimore (1964) reported the isolation of a viral-specific RNA polymerase from poliovirus-infected cells. This enzyme, which supplied with unprogrammed ribosomes and ribonucleotide triphosphates, synthesized viral RNA in a cell-free system.

CHAPTER III

MATERIALS AND METHODS

Viruses

The Florio strain of CTF virus used throughout these studies was obtained from Drs. Carl Eklund[1] and Leo Thomas[1] as the twenty-seventh mouse brain passage at the Rocky Mountain Laboratory. This represented the seventy-first passage of the Florio strain since it was adapted to multiply in mice from infectious hamster serum (Thomas, 1962).

The Dearing strain of reovirus, type 3, (Ramos-Alvarez and Sabin, 1958) was obtained from Dr. Peter Gomatos[2] as the twenty-eighth L cell passage.

HeLa cell-adapted herpes simplex virus, HF strain, (Flexner and Amoss, 1925) was received from the Communicable Disease Center, Atlanta, Georgia.

Animals

The Webster strain of white mice which has been maintained at the University of Oklahoma Medical School for four years was used for the production and titration of CTF virus

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[1]Rocky Mountain Laboratory, Hamilton, Montana.

[2]Rockefeller Institute, New York.

in vivo. A litter, as used in these studies, consisted of six suckling mice, three to five days old, or six weanling mice, 21 to 25 days old.

Young albino rabbits, weighing approximately two kg, were used for the production of antibodies to CTF virus.

Cultures of cotton rat kidney (CRK) cells were prepared from young adult cotton rats, *Sigmodon hispitis*. The HNGC strain of rats used in these studies was initially isolated from nature and colonized by Mr. Fred Hopper.[3]

Embryonated Honegger white Leghorn eggs, after 9 to 13 days of incubation, were used as the source of tissue for the preparation of chick embryo (CE) cell cultures.

Cell Cultures

Early phases of this study were designed to determine the comparative susceptibility of several cell types to infection with CTF virus. Six different cell cultures, four serially propagated and two primary, were screened before one of them was selected for intensive study. The different types of cell cultures used are listed in Table 2.

Seed cultures of KB, FL, and L-929 cells were obtained from Microbiological Associates, Bethesda, Maryland. The strain of HeLa cells employed was initially obtained through the courtesy of Dr. R. W. Brown.[4]

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[3]Department of Zoology, University of Oklahoma, Norman, Oklahoma.

[4]Carver Foundation, Tuskegee, Alabama.

TABLE 2

DESCRIPTION OF CELL CULTURES USED

Cell Type	References	Passage Information
CE, Chick embryo		Primary
CRK, Cotton rat kidney		Primary
L-929, Mouse fibroblast	Sanford *et al.* (1948)	over 100
FL, Human amnion	Fogh and Lund (1957)	28
HeLa, Carcinoma of human cervix	Gey *et al.* (1952)	over 100
KB, Carcinoma of human nasopharynx	Eagle (1955)	31

Reagents

The distilled water which was used for the preparation of solutions and for the final rinse of all glassware was first distilled in a copper distillation apparatus. It was then passed through a deionization column and was finally glass distilled.

Phosphate buffered saline (PBS) was prepared according to the formulation of Dulbecco and Vogt (1954a). Earle's balanced salt solution (EBSS) was prepared according to the technique of Earle _et al_. (1943) and Hanks' balanced salt solution according to the method of Hanks and Wallace (1949). Phenol red (10 mg per liter) was added as a pH indicator to all solutions before they were sterilized by positive pressure Seitz filtration.

Trypan blue at a final concentration of 0.1 per cent was used for making viable cell counts by the dye exclusion method (Siminovitch _et al_., 1957). The dye was added to cell suspensions from a 1.0 per cent stock solution prepared in distilled water.

PBS containing 10 per cent non-immune calf serum was used for making viral dilutions.

Trypsin[5] solution used for the preparation of primary cultures and for dispersal of cellular monolayers was prepared according to the method of Wallis _et al_. (1961).

Chick embryo extract was prepared according to the

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[5]Difco Certified, Difco Laboratories, Detroit, Michigan.

technique of Merchant et al. (1960) except that the tissue suspension was frozen at -25 C for three hours, placed at 4 C for 18 hours, and then allowed to thaw. The homogenate was subsequently centrifuged at 3,000 x g for one hour.

Calf serum was prepared from whole blood obtained from a local abattoir. The blood was collected in gallon jars, allowed to clot and placed at 4 C overnight for clot retraction. The serum was collected and centrifuged at 1000 x g at 4 C, and the clear, supernatant fluid was removed and stored in 35 ml volumes at -20 C. Before the serum was added to growth medium or viral diluent it was thawed and sterilized by Seitz filtration.

All cell culture media and balanced salt solutions contained penicillin[6] (100 units per ml) and streptomycin[7] (50 mcg per ml). To insure that the cell cultures remained free of pleuropneumonia-like organisms, kanamycin[8] (100 mcg per ml) was added to the growth medium for a 10 day period after every 60 days of cell growth (Pollock et al., 1960). All antibiotics were prepared in sterile distilled water and stored at -20 C in 1.0 ml volumes until they were added to reagents or media.

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[6]Potassium penicillin-G, Parke, Davis and Co., Detroit, Michigan.

[7]Streptomycin sulfate, E. R. Squibb and Sons, New York, New York.

[8]Kanamycin sulfate, Bristol Laboratories, Syracuse, New York.

Lyophilized mouse antiserum used for the identification of L cell-adapted CTF virus was obtained from Dr. Leo Thomas. It was rehydrated with 1.0 ml of sterile glass distilled water.

Tissue Culture Media

Serially propagated cells were grown in Eagle's minimum essential medium (growth medium) (Eagle, 1959). Stock concentrates of amino acids and vitamins for this medium were obtained commercially[9] and added to EBSS supplemented with 2 mM glutamine[10] and 10 per cent calf serum. After monolayers had become confluent the cultures were maintained in a maintenance medium which consisted of 0.5 per cent lactalbumin hydrolysate,[11] 0.1 per cent yeast extract[12] and five per cent calf serum dissolved in EBSS (LaEC5).

CE cell cultures were grown in a medium described by Lennette *et al*. (1961) except that calf serum was substituted for lamb serum. During virus propagation, the CE cell cultures were maintained with Eagle's minimum essential medium containing 1 per cent chick embryo extract and 2 per cent calf serum.

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[9]Microbiological Associates, Bethesda, Maryland.

[10]Mann Research Laboratories, Inc., New York, New York.

[11]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[12]Difco Laboratories, Detroit, Michigan.

CRK cell cultures were initiated and maintained in a lactalbumin medium as suggested by Lennette et al. (1961).

Metabolic Inhibitors and Nucleotides

A thymidine analogue, 5-bromo-2'-deoxyuridine (BUdR) was commercially obtained.[13] Hoffman and LaRoche, Inc., Nutley, New Jersey prepared the 5-fluoro-2'-deoxyuridine (FUdR) which was obtained through the courtesy of the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Maryland. Actinomycin D was obtained as a gift from Merck, Sharp and Dohme Research Laboratories, West Point, Pennsylvania. Thymidine and uridine were commercially obtained.[14]

Propagation of Cell Cultures

Primary Cell Cultures

Chick embryo cells. Cultures of CE cells were prepared according to the method of Lennette et al. (1961) except that the tissue mince was trypsinized during repeated, 15 minute intervals at room temperature. The cell-containing trypsin solution was then decanted into a 250 ml centrifuge bottle in an ice bath. After sufficient numbers of cells had been obtained the cell-trypsin suspension was centrifuged

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[13]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[14]Nutritional Biochemicals Corporation, Cleveland, Ohio.

horizontally at 400 x g for 15 minutes at 4 C and the supernatant fluid was replaced with approximately 20 ml of warm growth medium. Samples of the cell suspension were then removed and counted in a crystal violet counting reagent (Sanford *et al.*, 1951). The cells were enumerated in a hemocytometer under direct microscopic observation (10X). Cultures were initiated in 250 ml Kimax glass bottles and in 16 x 150 mm culture tubes with 1.2 x 10^6 and 5 X 10^5 cells, respectively. Bottles were incubated in a horizontal position and culture tubes were incubated in stationary racks[15] at an angle of approximately 10 degrees from the horizontal. All cultures were incubated at 36.5 C. Monolayers of CE cells were usually confluent within 48 hours after seeding.

Cotton rat kidney cells. Kidneys were removed aseptically from exsanguinated, young adult cotton rats. After the capsule and cortex were removed, the kidneys were washed twice in warm HBSS and minced into small fragments with scissors. The tissue fragments were then washed twice in HBSS and suspended in 50 ml of warm trypsin solution. The tissue mince was incubated for one hour at 37 C without stirring. The supernatant fluid was discarded and replaced with cold trypsin solution. The trypsin-tissue mixture was then transferred to a fluted trypsinization flask, placed in the refrigerator and stirred with a magnetic flea for 16 to 18 hours. The resulting cell suspension was removed and

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[15]Seelye Craftmen, Minneapolis, Minnesota.

centrifuged at 400 x g for 15 minutes at 4 C. The supernatant fluid was aspirated and discarded and the sediment was washed twice with HBSS and resuspended in 15 ml of kidney growth medium. Samples of the cell suspension were removed and the number of cells was counted by the crystal violet technique of Sanford _et al_. (1951). CRK cell cultures were initiated with a suspension containing 5 X 10^5 cells ml^{-1}. Twelve ml of this suspension were placed in bottles and 1.0 ml volumes were distributed into roller tubes. Monolayers were confluent seven to ten days of incubation.

Serially Propagated Cell Lines

Stock cultures of HeLa, KB, FL, and L cells were grown in bottles of 36.5 C. When monolayers became confluent the medium was aspirated and replaced with 10 ml of trypsin solution and the cultures were returned to the incubator for 10 minutes. The cells were detached from the glass by drawing the trypsin-cell suspension up and down several times with a pipette. They were then packed by centrifugation at 500 x g for five minutes at room temperature and suspended in 5 ml of growth medium. Samples of the cell suspension were removed and diluted 1:10 in growth medium. Trypan blue was then added to the mixture and it was incubated for three to four minutes. The dye-cell suspension mixture was then stirred with a pipette and the number of viable cells counted in a hemocytometer. Stock

cultures of cells were initiated in bottles with approximately 3×10^6 viable cells per 12 ml of growth medium. Monolayers were usually confluent after seven days of incubation after seeding, and contained approximately 1.6×10^7 cells. Roller and stationary tube cultures, used for viral growth and adsorption experiments, were initiated with 5×10^5 viable cells in a 1.0 ml volume of growth medium. Tube cultures were maintained in stationary racks for 18 to 24 hours after seeding and then transferred to a roller drum for the remainder of the growth period. The resulting monolayers contained approximately 9×10^5 cells at 48 hours and 1.4×10^6 cells at the end of 48 hours and 72 hours, respectively. Monolayers 72 hours old were used routinely for viral studies. Monolayer tube cultures of L cells, used for viral titrations, were initiated with 2.0×10^5 cells in 0.5 ml of growth medium. The cellular monolayers were confluent after 48 hours of incubation and contained approximately 7.0×10^5 cells. Growth medium in all monolayer bottle and tube cultures was changed every third day.

Suspension cultures of mouse fibroblasts were grown in Eagle's minimum essential medium supplemented with 10 per cent calf serum as modified for suspension culture.[16] The cultures were maintained in water-jacketed spinner flasks[17] at 37 C. Spinner cultures were initiated with 7.2×10^4 to

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[16]Microbiological Associates, Bethesda, Maryland.

[17]Belco Glass, Inc., Vineland, New Jersey.

1.0×10^5 viable monolayer-cultivated cells ml^{-1} of suspension medium (Siminovitch et al., 1957; McLimans et al., 1957). Growth was usually started in 50 to 100 ml volumes of cell suspension. At 48 hour intervals the cells were collected by centrifugation at 500 x g for five minutes at room temperature and resuspended in fresh medium. Each time the medium was changed, the cultures were gassed for 10 minutes with air containing 5 per cent carbon dioxide. Viable cell counts were made with trypan blue every 18 to 24 hours.

Preparation of Seed Virus

The twenty-seventh Rockly Mountain Laboratory mouse passage of the Florio strain of CTF virus was used to initiate viral propagation in this laboratory. Suckling mice were inoculated intraperitoneally with 10^4 LD_{50} of virus and were sacrificed when they became moribund, usually on the fourth day after inoculation. A 10 per cent suspension of pooled infected brain material was prepared in HBSS which contained 50 per cent normal calf serum. After the suspension was centrifuged at 1,000 x g for 15 minutes at 4 C the supernatant fluid was removed and distributed in vials which were subsequently sealed and stored at -65 C. This is mouse passage one. Virus from Oklahoma mouse passage three (OMP-3) which was used had an average titer of $10^{7.2}$ LD_{50} ml^{-1}.

Cell-adapted CTF virus was prepared in monolayer roller tube cultures of L or FL cells. Confluent monolayers which contained 1.6×10^6 viable cells were inoculated with

0.2 ml of virus suspension which contained 1000 $TCID_{50}$. The inoculum was allowed to adsorb for two hours at room temperature. The unadsorbed virus was then removed and the monolayers were washed twice with HBSS. Viral growth medium, i.e., Eagle's minimum essential medium containing double concentrations of amino acids and vitamins, was added to each culture and they were incubated for 50 hours in a roller drum. At the end of the viral growth cycle, heat inactivated calf serum was added to give a final concentration of 50 per cent and the cell suspension was subjected to one cycle of freezing and thawing. The suspensions from all cultures were then pooled and sealed in vials which were stored at -65 C. Colorado tick fever virus, L cell passage number eight, was used for most of the studies. Repeated titration of this virus suspension in L cells gave an average $TCID_{50}$ of $10^{6.2}$0.2 ml^{-1}.

Seed cultures of herpes simplex virus were prepared in L cell monolayers. Cultures were infected with 10^4 $TCID_{50}$ of L cell-adapted virus in a 1 ml volume. Adsorption was allowed to take place at room temperature for two hours after which growth medium was then added. Thirty hours later, the cells and supernatant fluids were collected and frozen and thawed three times. Cellular debris was removed by centrifugation at 1,000 x g for 30 minutes at 4 C and the supernatant fluids were stored at -65 C. The virus used in these studies was L cell passage number seven. The average titer of this

pool was $10^{5.2}$ $TCID_{50}$ 0.1 ml^{-1}.

Reovirus type 3, Dearing strain, was propagated in L cells as described previously for herpes simplex virus. The seed virus suspension had a titer of $10^{8.2}$ $TCID_{50}$ 0.2 ml^{-1}.

Quantitation of Virus

Titration of Virus in Mice

Colorado tick fever virus-infected cell culture fluids and mouse brain suspensions were assayed in suckling and weanling mice. Tenfold dilutions of the viral suspensions were prepared in virus diluent and samples and each dilution were inoculated into six mice. Suckling mice were inoculated with 0.05 ml volumes by the intraperitoneal route and weanling mice were inoculated with 0.03 ml volumes by the intracerebral route. Mortality was used as the index of infection and 50 per cent endpoints were calculated according to the method of Reed and Muench (1938).

Titration of Virus in Cell Culture

Herpes simplex, CTF, and reoviruses were assayed on monolayer cultures of L cells. Serial, tenfold dilutions of the virus suspensions were prepared in virus diluent which was maintained in an ice bath. Five monolayers were inoculated with 0.2 ml volumes of each viral dilution and the virus was adsorbed to the cells for two hours at room temperature. Lactalbumin medium was then added to the cultures and they were placed on a roller drum in the 36.5 C incubator.

Cellular monolayers were observed microscopically for CPE on the third and fifth days after inoculation. The $TCID_{50}$ was calculated according to the method of Reed and Muench (1938) and expressed as the positive log to the base 10 of that dilution of the virus suspension which would infect 50 per cent of the cultures.

Production of CTF Virus Antibodies in Rabbits

Antibodies against CTF virus were produced in rabbits which were inoculated with a vaccine composed of equal volumes of Freund's complete adjuvant[18] and a 10 per cent suspension of CTF virus-infected mouse brain which had a titer of $10^{7.2}$ LD_{50} ml^{-1}. Each animal was given 1.0 ml volumes of the vaccine intramuscularily on day 0, 10, 21, and 50. Seven days following the last injection the animals were exsanguinated by cardiac puncture. The blood was collected in sterile, 250 ml centrifuge bottles and allowed to clot at room temperature. The clots were then ringed and the bottles were stored overnight in the refrigerator. On the following day the blood was centrifuged at 1,000 x g for 30 minutes and the serum was collected, pooled, and stored at -20 C.

Neutralization Tests

Neutralization tests used for the identification of L cell-adapted virus and for the determination of the antibody

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[18]Difco Laboratories, Detroit, Michigan.

content of immune serum were performed according to the method of Gerloff and Eklund (1959), except that complete monolayers of L cells were used in place of KB cells. Pooled rabbit antiserum or rehydrated mouse antiserum obtained from Dr. Thomas was mixed with equal volumes of tenfold dilutions of either mouse-or L cell-adapted virus. The virus-serum mixtures were incubated for one hour at room temperature. Six suckling mice or five L cell cultures were then inoculated with samples of each dilution of the virus-serum mixture. The titers of the mouse-adapted and L cell-adapted viruses in the presence of non-immune serum were determined by the same technique.

Viral Adsorption

In a typical experiment confluent monolayers of L cells in roller tubes were inoculated with 0.2 ml of viral suspension which contained 20 $TCID_{50}$ of virus per cell (adsorption mixtures). Tubes without cells (control mixtures) were inoculated with a similar amount of virus to serve as a control for inactivation of the virus. Adsorption and control mixtures were incubated at 25 C in stationary racks. At 15 minute intervals five tubes or cultures were removed from each group and the free virus was collected by washing the monolayers or glass surfaces three times with HBSS containing 50 per cent calf serum. The difference between the amount of free virus recovered from the mixtures with and without monolayers was considered as the amount of adsorbed virus.

Virus Growth Curve Experiments

Confluent monolayers of L cells containing approximately 1.6 X 10^6 cells were inoculated with 0.2 ml of L cell-adapted virus which contained 0.2 to 40.0 $TCID_{50}$ ml^{-1}. After a two hour adsorption period at 25 C, the cultures were washed three times with HBSS. Virus growth medium was then added and the cultures were placed on either a roller drum or a stationary rack, according to the design of the experiment. At four hour intervals, the supernatant fluids from three to five tubes were collected, pooled, and centrifuged at 1,000 x g for 15 minutes at 4 C. The supernatants, which contained the cell-released virus (CRV), were fortified with calf serum to give a final concentration of 50 per cent and were stored at -65 C. The sediments were added to the cell samples which had been removed from the glass by two freeze-thaw cycles in PBS containing 50 per cent serum. These samples were then centrifuged at 1,000 x g for 15 minutes at 4 C. The cellular debris was discarded and the supernatants, designated as cell associated virus (CAV), were stored at -65 C. When samples were prepared for the determination of their total viral content, calf serum was added to the cultures to give a final concentration of 50 per cent. The cultures were then frozen and thawed twice and centrifuged at 1,000 x g for 15 minutes at 4 C. The cellular debris was discarded and the supernatant fluids, which contained the total virus (CAV plus CRV), were stored at -65 C.

In experiments where spinner cultures were used cell growth was initiated with 5 X 10^5 cells ml^{-1} in 50 ml volumes and maintained until the cultures contained 1.9 X 10^6 cells ml^{-1}. Twelve hours prior to infection the cells in suspension culture were diluted in fresh medium to contain 1.2 X 10^6 cells ml^{-1}. Cell adapted CTF virus was then added to the cell suspension to give the desired multiplicity of infection. No attempt was made to remove the unadsorbed virus from the cultures. At four hour intervals 2.0 ml of the cell suspension were removed from the culture, supplemented with serum to give a final concentration of 50 per cent and samples were prepared to be assayed for total viral content.

In order to determine the duration of the eclipse phase of the growth cycle, confluent monolayers of L cells were inoculated with 0.2 ml of virus suspension which contained 40 $TCID_{50}$ of virus $cell^{-1}$. After two hours of adsorption the cultures were washed three times with HBSS to remove the unadsorbed virus and 1.0 ml volumes of undiluted rabbit, anti-CTF virus serum were added to each half of the cultures and 1.0 ml volumes of undiluted, normal calf serum were added to the remainder. After one hour at 36.5 C the serum was removed and replaced with growth medium. During the first 18 hours of infection samples were taken at two hour intervals and assayed for total viral content.

Stability of CTF Virus to Physical and Chemical Agents

Freeze-Thaw Treatment

Since stock CTF virus was stored at -65 C and virus was usually liberated from infected cells by repeated freeze-thaw cycles it was important to determine the stability of the virus to freezing and thawing. Accordingly, stock CTF virus was diluted in HBSS (pH 7.5) which contained:

(1) 10 per cent normal calf serum (NCS),
(2) 25 per cent NCS,
(3) 50 per cent NCS,
(4) 20 per cent glucose,
(5) 20 mM glutamine,
(6) 20 per cent glycerol,
(7) 20 per cent bovine serum albumin (BSA),
(8) 10 per cent BSA, or
(9) 2 per cent gelatin.

The virus suspensions were frozen rapidly in an alcohol bath at -65 C and then thawed quickly in a water bath at 37 C. This procedure was repeated three times and samples were removed for viral assay following each freeze-thaw cycle. All samples were placed in an ice bath and assayed immediately for viral content.

Sonic Oscillation

Since ultrasonic oscillation can be used as an alternate method for liberating virus from infected cells, studies

were undertaken to measure its effect on the infectivity of CTF virus. Stock suspensions of virus were diluted 1:10 in HBSS which contained 10, 25, or 50 per cent NCS. The suspensions were then exposed to sonic oscillation for one minute in a 250 watt, 10 kilocycle sonic oscillator. Samples were removed immediately and assayed for infectivity.

Hydrogen Ion Concentration

Studies were done to determine the stability of CTF virus infectivity over a wide range of pH values. Acetate-veronal buffer (Michaelis, 1931) and phosphate buffer containing 10 per cent NCS were prepared at pH values ranging from 3.9 to 8.5. Stock virus was diluted 1:10 in the various buffers and after one hour at 4 C samples were then removed from each of the buffer solutions and the pH was adjusted to 7.2 before they were assayed for virus in cell culture. The pH of each virus-containing solution was determined with a pH meter[19] immediately after the viral dilution was prepared and again at the end of a one hour period at 4 C.

Lipid Solvents

The stability of cell-adapted CTF virus in lipid solvents was investigated. The initial experiments were performed with chloroform as described by Feldman and Wang (1961). One ml of a stock virus suspension which contained $10^{5.8}$ $TCID_{50}$ of virus was mixed with 0.05 ml of chloroform

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[19]Beckman Instruments, Inc., Palo Alto, California.

and shaken continuously for 10 minutes at 4 C. The suspension was then centrifuged at 300 x g for 15 minutes at 4 C. A sample was removed from the clear supernatant above the chloroform layer and assayed immediately for infectivity. A control sample, unexposed to chloroform, was handled in a similar manner.

Experiments were also performed to determine the effect of sodium desoxycholate (DOC) on the infectivity of the virus. For this purpose virus was diluted 1:10 in HBSS (pH 7.2) containing:

(1) 1.0 per cent DOC with 1.0 per cent BSA,
(2) 1.0 per cent DOC with 10 per cent BSA,
(3) 0.2 per cent DOC with 1 per cent BSA,
(4) 0.2 per cent DOC with 10 per cent BSA,
(5) 10 per cent BSA, or
(6) 1 per cent BSA.

HBSS was the diluent. The virus suspensions were maintained at 4 C for one hour and assayed immediately for viral content.

Thermal Inactivation

Experiments were designed to determine the thermal inactivation rate constants of cell-adapted CTF virus. From this information the heat of activation for the loss of infectivity, half-life of the virus at the various temperatures and Q_{10} for the reaction could be calculated. Sterile rubber stoppered tubes containing a solution of HBSS with 5 per cent BSA were prewarmed in a water bath within ± 1.0 C of

the desired temperatures of 25, 37, 45, and 56 C. Cell-adapted virus was diluted in each of the prewarmed diluents and at 30 minute intervals samples were removed, fortified with BSA to give a final concentration of 10 per cent frozen rapidly in an alcohol bath at -65 C. All samples were assayed for infectivity one week later.

Effect of Metabolic Inhibitors on L Cell Multiplication

Replicate tube cultures were initiated with 2.0 X 10^5 viable L cells in 1.0 ml volumes of growth medium. After 24 hours of incubation at 36.5 C the cultures were divided randomly into groups and the growth medium was replaced with one of the following experimental media:

(1) Growth medium with no inhibitor,

(2) Growth medium with 0.3 mcg FUdR ml^{-1},

(3) Same as (2), but with 2.8 mcg thymidine ml^{-1},

(4) Same as (2), but with 2.6 mcg uridine ml^{-1}.

The cultures were then incubated at 36.5 C on the roller drum. At the time the experimental media were first applied, cell counts were made on three to five replicate cultures. Growth medium was replaced with 0.9 ml of trypsin solution and the culture was incubated at 36.5 C for 10 minutes to produce a cell suspension. One-tenth ml of trypan blue solution was added to 0.9 ml of cell suspension, mixed by pipetting and the number of viable cells was counted in a hemocytometer. Counts from three to five monolayers were averaged and this figure was used as a base count for determining cell growth

on subsequent days of the experiment. Counts of viable cells were made at 24 hour intervals for each experimental medium. The rate of cellular growth was calculated as the ratio of the number of viable cells in the culture on the day of the count (N) to the number of cells in the culture when the experimental medium was first applied.

Experiments designed to determine the effect of BUdR on the multiplication of L cells were performed in a similar manner. In these experiments the multiplication of L cells was studied in: (1) Growth medium with no analogue; (2) Growth medium with 100 mcg of BUdR ml^{-1}, and (3) Same as (2), but with 100 mcg of thymidine ml^{-1}.

Investigations on the effect of actinomycin D on L cell division were also performed using these techniques. Cell multiplication in growth medium with no antibiotic and in medium containing 1.0 mcg ml^{-1} of actinomycin D were compared.

CHAPTER IV

EXPERIMENTAL RESULTS

Cytopathogenicity and Multiplication of Virus

In Figure 1 are shown the morphologic changes which develop in L cells after infection with either mouse-or cell-adapted CTF virus. Photomicrograph number 1 represents a typical monolayer of noninfected L cells. Cytonecrotic changes first became evident approximately 40 hours after infection (micrograph 2) at which time the cells were rounded, with vacuolated and granular cytoplasms. At 48 hours after infection the monolayer lesion appeared to be composed of aggregates of rounded, opaque and anucleated cells (micrograph 3). Seventy-two hours after inoculation, the infected cells had undergone further necrosis and had become detached to produce a plaque as shown in micrograph 4. Similar cytopathic changes occurred in FL cells.

During the first three passages of virus in L cells, titers of $10^{2.7}$ to $10^{3.4}$ $TCID_{50}$ were detectable (Table 3). The yield of virus after the fourth passage was $10^{2.5}$ $TCID_{50}$ greater than after the first, second, and third passages. Subsequent propagations of this adapted CTF virus in L cells repeatedly yielded titers of 10^{5} $TCID_{50}$ or greater.

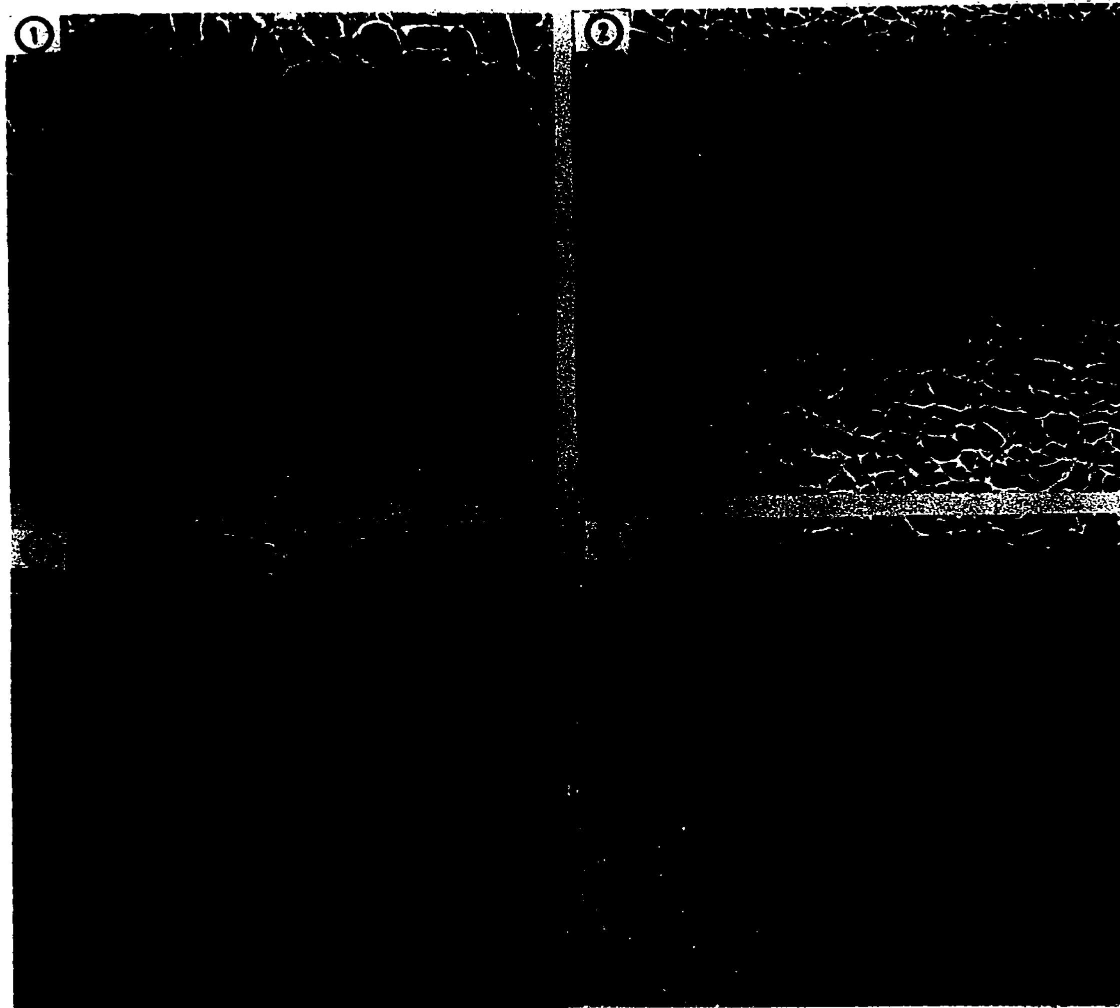

Figure 1. Cytonecrotic changes in L cells after infection with CTF virus. Micrograph 1 is an uninfected culture, 200 X; micrographs 2, 3 and 4 are infected cultures at 40, 48 and 72 hours respectively, 100 X.

TABLE 3

MULTIPLICATION OF CTF VIRUS IN L-929 CELL CULTURES

Passage Number	Cumulative Dilution of the Original Inoculum	$TCID_{50}$/0.2 ml in L Cells	LD_{50}/0.05 ml in Suckling Mice
OMP-3[a]		6.4[b]	6.8[b]
L-1[c]	10^{-3}	2.7	2.2
L-2	10^{-6}	3.2	--[d]
L-3	10^{-9}	3.4	2.6
L-4	10^{-14}	5.9	4.8
L-5	10^{-17}	6.1	5.6
L-6	10^{-20}	6.3	--
L-7	10^{-24}	6.4	5.5
L-8	10^{-26}	6.2	5.3

[a]Oklahoma mouse passage 3.10 per cent suspension (Total passage number 74).

[b]Titer expressed as positive $\log_{10}$ of virus dilution which would infect 50 per cent of cultures or mice.

[c]L cell passage number 1.

[d]Data not available.

Monolayers of FL cells supported viral replication and produced titers of $10^{6.5}$ to $10^{7.8}$ $TCID_{50}$ during the initial and subsequent passages (Table 4). Cytonecrosis regularly appeared 40 to 48 hours after infection with cell-adapted virus. The amount of virus produced and the time required for the appearance of cytopathic changes were essentially the same during eight serial propagations. Virus which had replicated in FL cells was almost as virulent for mice as the mouse-adapted virus from which it was derived.

Identification of Virus

Table 5 contains the results of neutralization tests which were done to identify the virus propagated in L cell cultures. The antiserum prepared against the mouse-brain virus neutralized 99.9 per cent of the homologous virus and 99.7 and 98.5 per cent of virus from L cell passages eight and twelve respectively.

Sensitivity of Different Cell Types to Infection

The sensitivity to CTF virus infection of six different *in vitro* cultivated cell types is shown in Table 6. Parallel titrations of mouse-brain (passage 74)-, L cell (passage eight)-, and FL cell (passage six)-adapted viruses were done on replicate monolayers of FL, L, HeLa, KB, CE and CRK cells. These same viral suspensions were also assayed in weanling and suckling mice.

Cultures of FL, L and chick embryo cells were as

TABLE 4

MULTIPLICATION OF CTF VIRUS IN FL CELL CULTURES

Passage Number	Cumulative Dilution of the Original Inoculum	$TCID_{50}$/0.2 ml in FL Cells	LD_{50}/0.05 ml in Suckling Mice
OMP-3[a]		7.5[b]	6.8[b]
FL-1[c]	10^{-3}	6.5	5.2
FL-2	10^{-6}	7.2	--[d]
FL-3	10^{-9}	6.7	--
FL-4	10^{-12}	6.8	5.7
FL-5	10^{-15}	6.5	5.2
FL-6	10^{-19}	7.6	5.2
FL-7	10^{-21}	7.5	5.8
FL-8	10^{-23}	7.8	5.6

[a]Oklahoma mouse passage 3, 10 per cent suspension (Total passage number 74).

[b]Titer expressed as positive $\log_{10}$ of virus dilution which would infect 50 per cent of the cultures or mice.

[c]FL cell passage 1.

[d]Data not available.

TABLE 5

IDENTIFICATION OF L CELL-ADAPTED CTF VIRUS WITH SPECIFIC MOUSE ANTISERA

Experiment Number	MBV[a] Plus		LCV_8[b] Plus		LCV_{12}[c] Plus	
	Normal Serum	Anti-serum	Normal Serum	Anti-serum	Normal Serum	Anti-serum
1	7.5[d]	4.3	5.2	2.6	4.8	1.5
2	6.8	3.8	5.6	3.2	---	---
3	7.2	4.0	---[e]	---	5.3	2.7

[a]Mouse brain-adapted virus, Oklahoma passage 5.

[b]L cell-adapted virus passage 8.

[c]L cell-adapted virus passage 12.

[d]Titer expressed as positive $\log_{10}$ per 0.03 ml of virus dilution which would infect 50 per cent of mice, LD_{50}.

[e]Data not available.

TABLE 6

TITERS OF CTF VIRUS IN CELL CULTURES AND MICE

Experimental Cell or Animal	Source of Virus: Mouse-brain adapted[a]	Cell culture-adapted: FL[b]	Cell culture-adapted: L[c]	CPE[d]
Chick embryo cells	7.1[e]	6.1[e]	6.4[e]	+
Cotton rat kidney cells	0	0	0	0
L-929	6.4	6.7	6.5	+
FL	7.5	7.8	7.2	+
HeLa	0	0	0	0
KB	4.2	3.8	3.6	+
Mouse, suckling (IP)	6.8	6.5	5.3	
Mouse, weanling (IC)	6.9	6.8	5.7	

[a]Oklahoma mouse passage 3, 10 per cent brain suspension (Total passage number 74).

[b]FL cell-adapted virus, passage 6.

[c]L-929 cell-adapted virus, passage 8.

[d]Cytopathic effect in cell cultures: indicates CPE production; - indicates no CPE production.

[e]Titers expressed as the positive $\log_{10}$ of $TCID_{50}$ or LD_{50}.

sensitive to infection as suckling or weanling mice. Of the cell types studied, FL cells were the most susceptible and KB cells the least sensitive to infection. Cytopathic changes, as described previously, developed in FL, L, and chick embryo cells within 48 hours after infection by either cell culture or mouse-adapted virus. The morphologic response of KB cells to infection appeared 12 to 18 hours later. Neither mouse-brain nor cell-adapted viruses produced cytopathic changes in cultures of HeLa or cotton rat kidney cells.

Replication of Virus in Cell Cultures

Confluent monolayer cultures of FL, L, CE, KB, HeLa, and cotton rat kidney cells were prepared and inoculated with L cell-adapted CTF virus at a multiplicity of 1.4 $TCID_{50}$. The results of one experiment, which are plotted in Figure 2 showed that cultures of HeLa or CRK cells did not support the replication of L cell-adapted virus. The amount of virus present in these cultures decreased during the 72 hour period of observation after infection. Cultures of KB cells supported growth of the virus at a slower rate and produced less infectious virus than did the cultures of L, FL and CE cells. The replication of virus in the latter three cell types was rapid after the initial 24 hours of incubation and maximum titers of virus were produced 72 hours after infection.

At this point in the investigations the L-929 cell line was selected to be used to study in detail the inter-

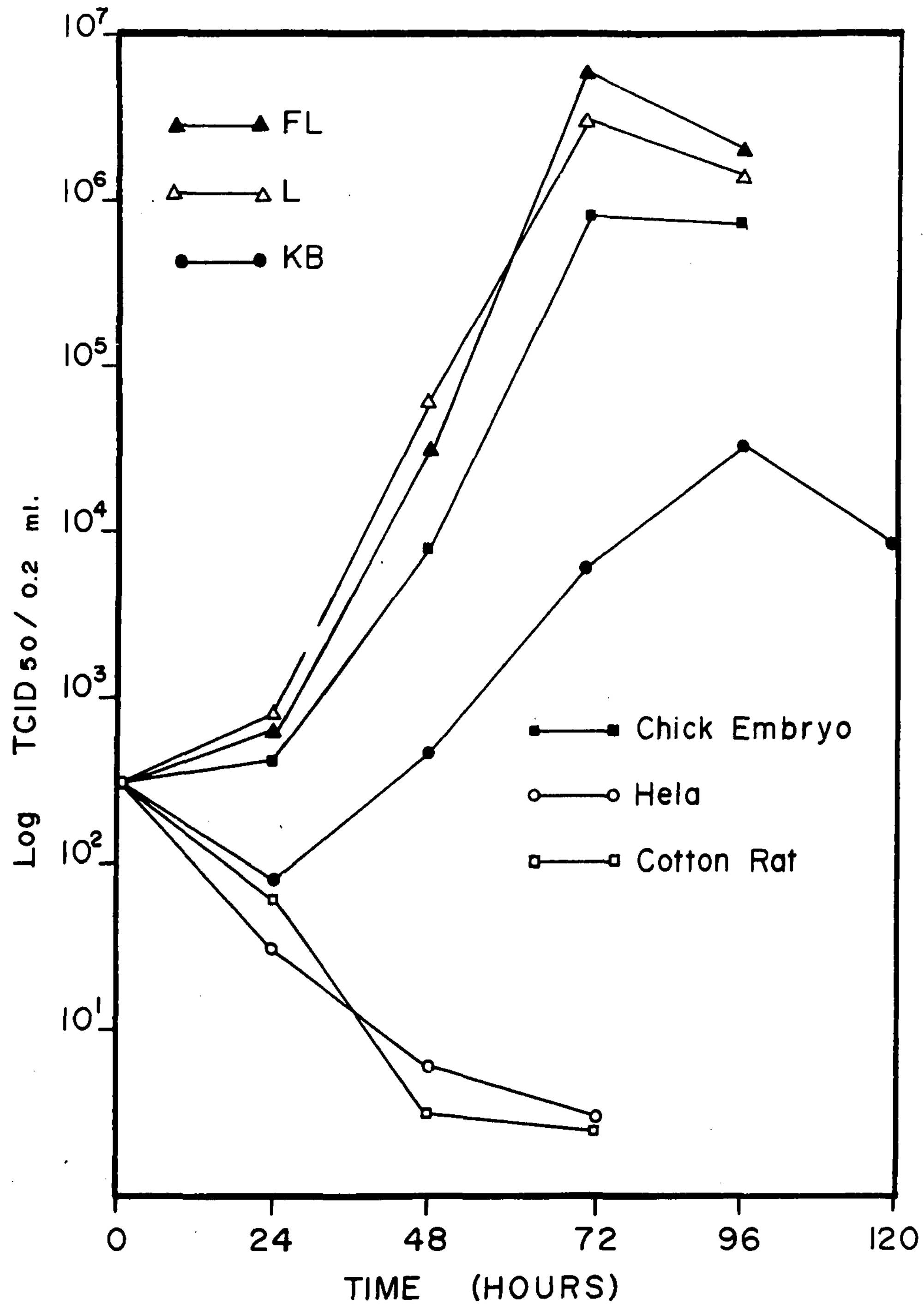

Figure 2. Replication of L cell-adapted CTF virus in cell cultures. Multiplicity of infection 1.4 $TCID_{50}$. Total virus is plotted as a function of time in hours.

action of CTF virus and cells which support its synthesis. These cells are metabolically hardy and have been studied extensively.

Viral Adsorption

The data plotted in Figure 3 are the combined results of three adsorption experiments. The most rapid adsorption of the virus occurred during the first 30 minutes of incubation. Subsequently, the rate declined markedly. It can be seen that over 90 per cent of the virus was adsorbed during the first 15 minutes of incubation. After two hours of incubation less than 1.0 per cent of the input virus remained unadsorbed to the cells.

CTF Virus Growth Curve

Eclipse Period

To establish the time interval required for the appearance of intra-cellular virus, experiments were designed to determine the length of the eclipse period. Results of a typical experiment are shown in Figure 4. When the cultures were treated with antiserum no infectious virus was found to be associated with the cells two hours after adsorption. The first progeny viruses in these cells were detected eight hours after adsorption and the amount of virus increased between the eighth and tenth hours. Infectious virus was found to be associated with the cells treated with normal serum throughout the lag phase. The rate of increase in CAV was exponential

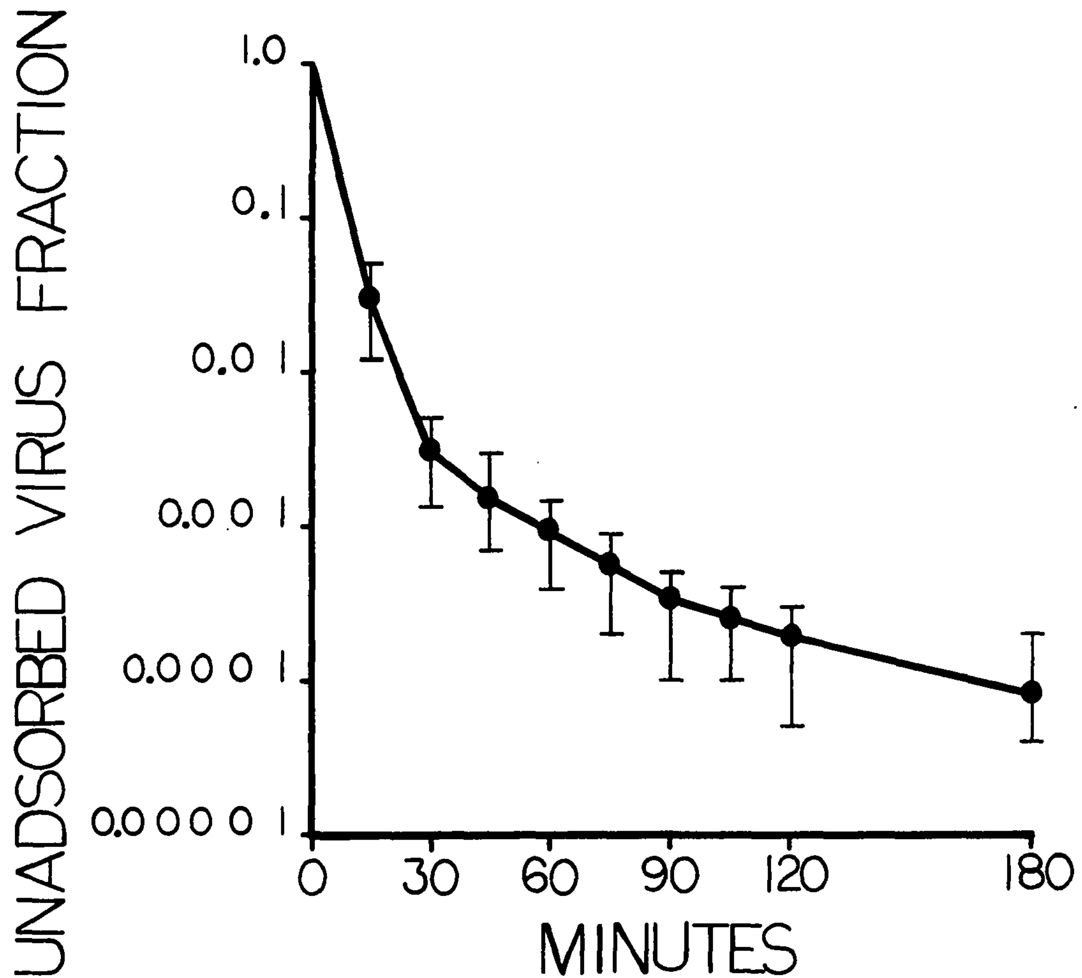

Figure 3. Adsorption of CTF virus onto L cells at 25 C. Viral input multiplicity (V_o) = 10 to 20 $TCID_{50}$. Unadsorbed virus at time t = (V_t). Unadsorbed virus fraction = (V_t/V_o). Data are composite results of three experiments. Vertical bars indicate standard deviation from mean values.

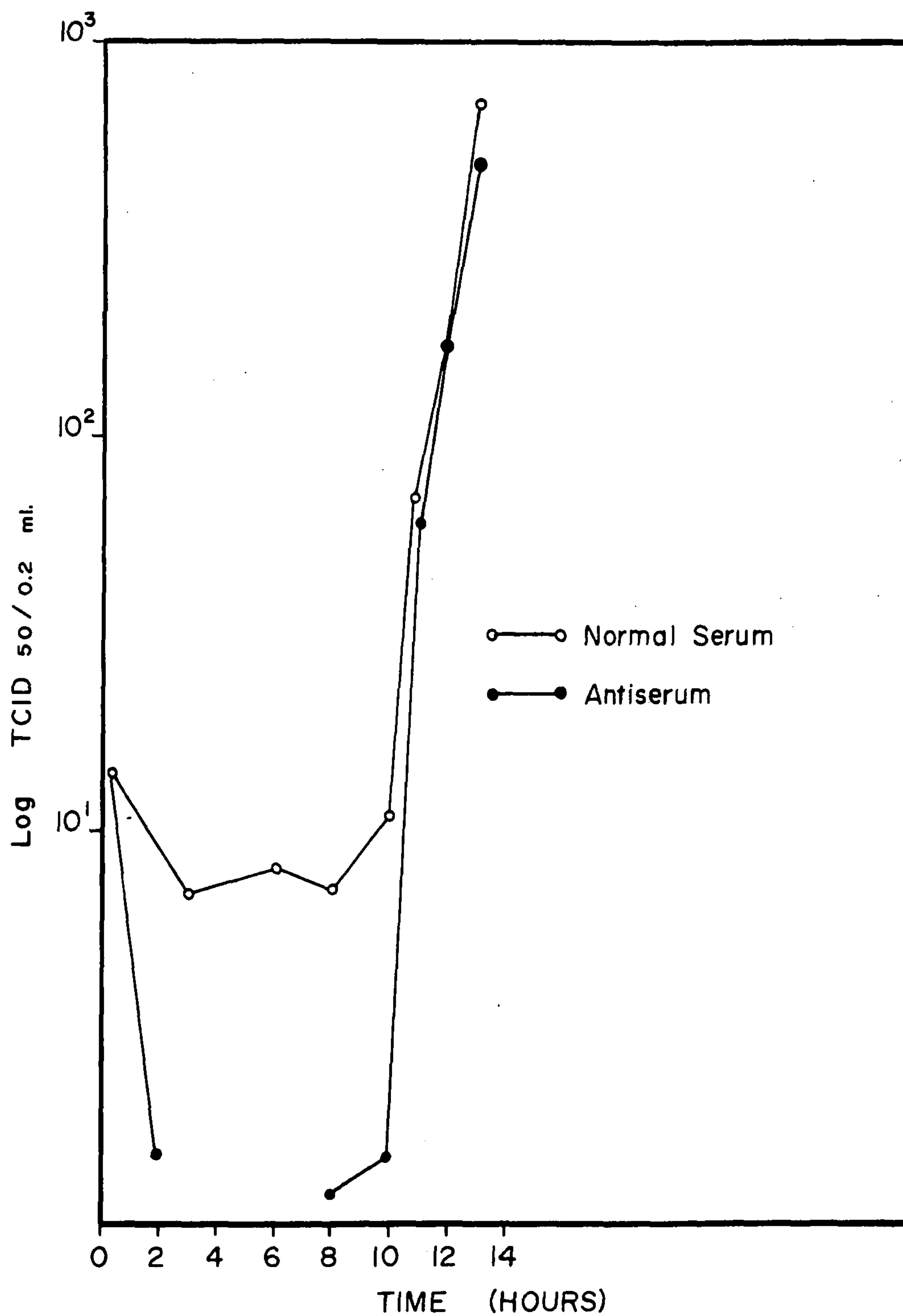

Figure 4. Eclipse period of CTF virus in L cells. Cell-associated virus is plotted as a function of time in hours.

in the antiserum and normal serum treated cells between the tenth and fourteenth hours after adsorption.

Viral Replication

Complete monolayers of L cells, 72 hours old, were inoculated with L cell-adapted virus at a low input multiplicity of 0.2 $TCID_{50}$. At four hour intervals after the adsorption period, sample to be assayed for CAV, CRV, and total virus were prepared. The data plotted in Figure 5 show that the lag phase appeared to extend through the twelfth hour and was followed by an exponential phase of viral growth. Viral replication was logarithmic in rate from the twelfth to the thirty-sixth hour at which time the total virus titer was $10^{5.4}$ $TCID_{50}$ 0.2 ml^{-1}. This represented an average of 10 $TCID_{50}$ of infectious virus per cell present in the culture at the time of infection. In the succeeding 60 hours of incubation the total viral titer increased to $10^{6.8}$ $TCID_{50}$ 0.2 ml^{-1} and represented approximately 125 $TCID_{50}$ $cell^{-1}$. The amount of cell associated virus increased in a linear manner for the initial ten hours after the lag period and continued to increase gradually from the eighteenth to the sixtieth hour when a maximum titer of $10^{5.5}$ $TCID_{50}$ 0.2 ml^{-1} were produced. The increase in CRV occurred very rapidly from the fourteenth to the thirty-fourth hour after infection. For the next 40 hours the titer of free virus remained relatively constant. Approximately 80 hours after infection the titer of virus released from the cells increased rapidly

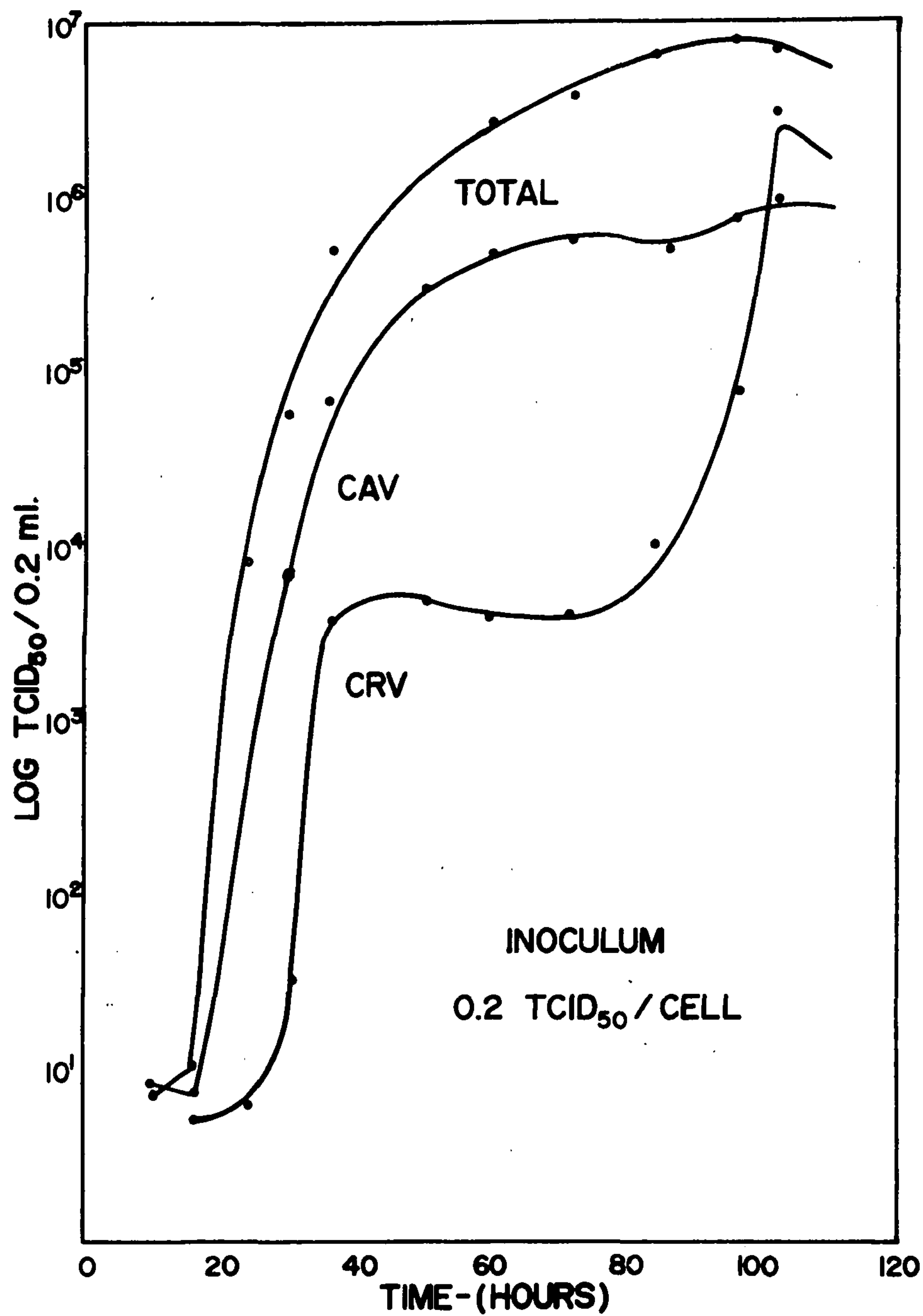

Figure 5. Growth curves of CTF virus in L cells infected with an input multiplicity of 0.2 $TCID_{50}$. Cell-associated virus (CAV), cell-released virus (CRV) and total virus are plotted as a function of time in hours.

to reach the maximum of $10^{6.2}$ $TCID_{50}$ of infectious virus $0.2\ ml^{-1}$ at 102 hours. Throughout the entire growth cycle the titer of CAV remained higher than the titer of CRV. This indicated that even after maximal titers of CRV were produced large quantities of virus remained with the cellular debris.

Data from the three previous experiments seemed to indicate that the lag phase of the growth cycle may vary in length from 8 to 12 hours; the length being inversely proportional to the titer of virus in the inoculum. Experiments were therefore done to determine the effect of different multiplicities of infection on the production of virus in L cells. Monolayer cultures prepared as described previously were divided into three groups and infected at a multiplicity of 0.4, 4.0 or 40 $TCID_{50}$. At four hour intervals after adsorption, samples from each group were prepared for the determination of total viral content and stored at -65 C until assayed. From the results presented in Figure 6 it can be observed that the duration of the lag phase of the growth cycle was nine to ten hours and was independent of the viral titer of the inoculum. The most obvious difference in the growth patterns was the slope of the curves during the logarithmic synthesis phase. Viral replication in cultures infected with input multiplicities of 4.0 and 40 $TCID_{50}$ was quite similar. A ten hour exponential phase of growth is observed and essentially the same amounts of virus were

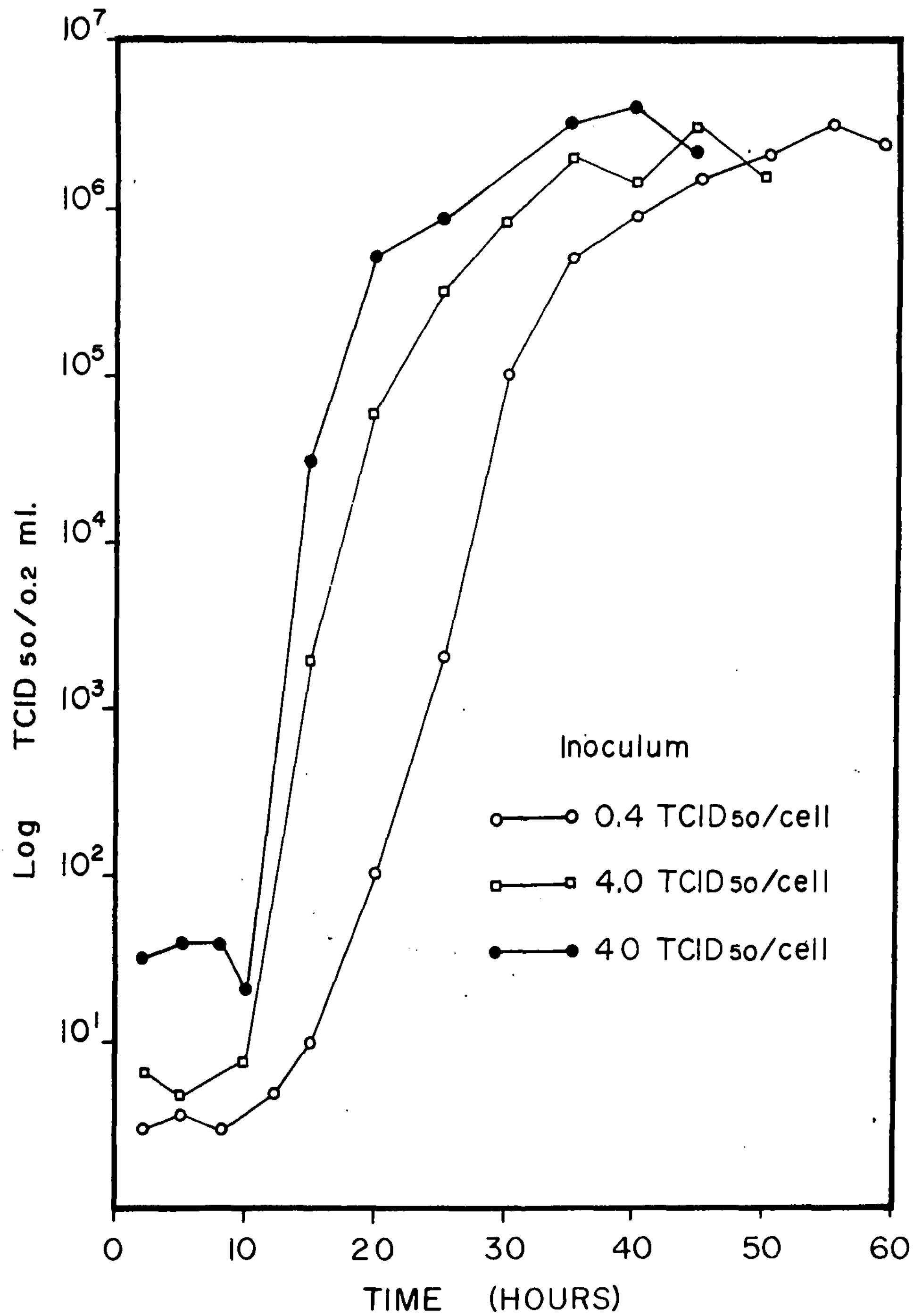

Figure 6. Growth curves of CTF virus in L cells infected with input multiplicities of 0.4, 4.0 and 40 $TCID_{50}$. Total virus is plotted as a function of time in hours.

produced within 35 to 40 hours after infection. The multiplication of virus in cultures infected at a lower multiplicity of infection (0.4 $TCID_{50}$) was obviously slower than that in cultures infected at high multiplicities. Peak viral titers resulting from an inoculation of 0.4 $TCID_{50}$ $cell^{-1}$ were not reached until 55 hours after infection. There were no apparent differences in the total virus produced in cultures infected at low, medium, and high multiplicities of infection.

Experiments were then designed to determine the effect of a high multiplicity of infection on the production of CAV, CRV, and total virus. Replicate L cell cultures were initiated and maintained in a routine manner for the first 72 hours and were then infected at a multiplicity of 40 $TCID_{50}$. The viral growth curve plot of a typical experiment can be seen in Figure 7. Total virus increased in an exponential manner after the tenth hour and reached $10^{6.8}$ $TCID_{50}$ 0.2 ml^{-1} by 36 hours after infection. The linear rate of increase in CAV continued for 12 hours after the lag period and peak titers of virus were seen 45 hours after inoculation. The rate of virus release from the cells paralleled the vote of formation of intracellular virus through most of the growth cycle and maximum titers of CRV were attained 45 hours after infection.

Experiments thus far had been arbitrarily done in monolayer cultures maintained on a roller drum. To determine

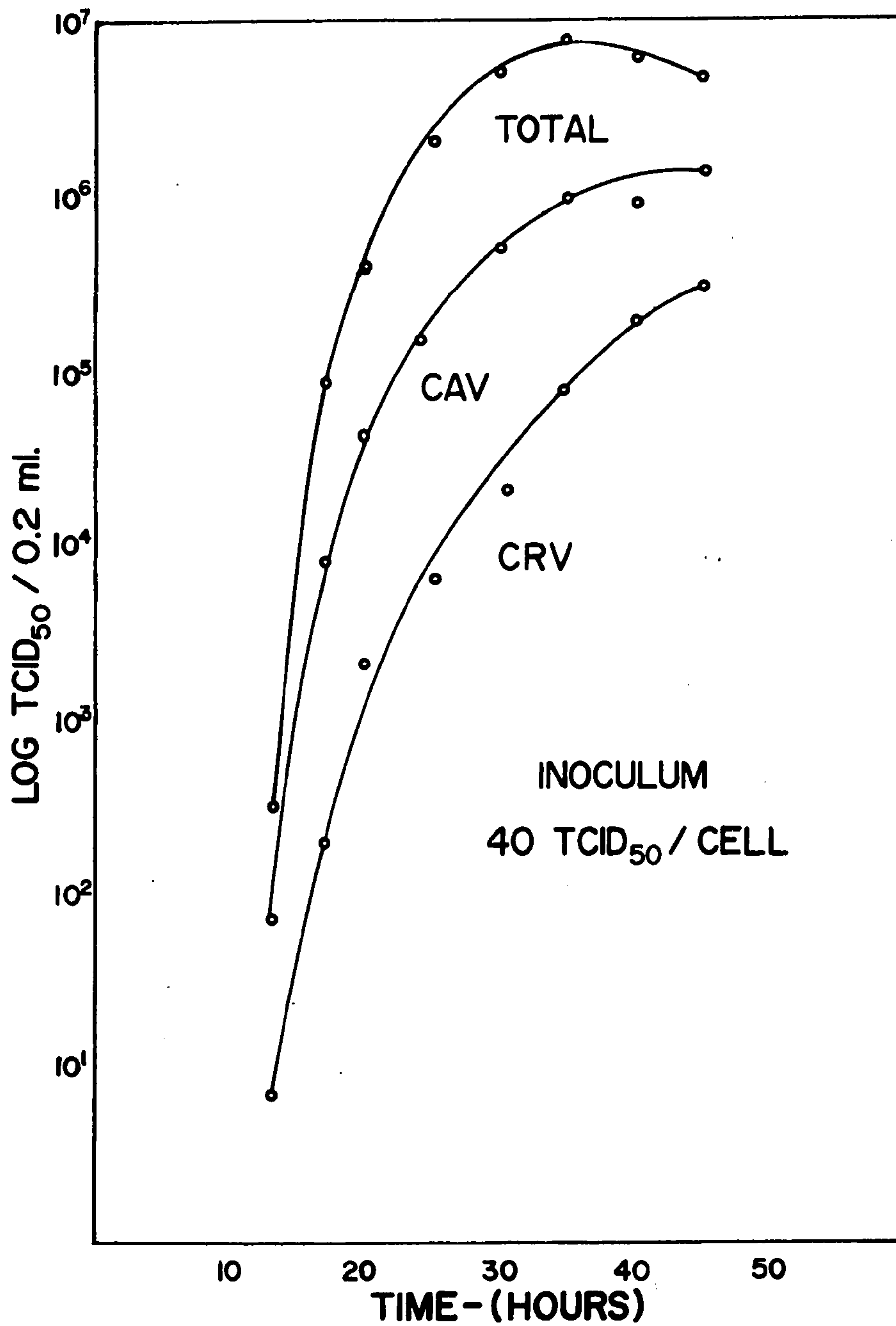

Figure 7. Growth curves of CTF virus in L cells infected with an input multiplicity of 40 $TCID_{50}$. Cell-associated virus (CAV), cell-released virus (CRV) and total virus are plotted as a function of time in hours.

the effect of other cell culture methods on the viral growth cycle, experiments were conducted in monolayer cultures maintained in stationary and roller tubes and in suspension cultures. All cultures were infected with virus from L cell passage eight at a multiplicity of 4.0 $TCID_{50}$. Samples were removed at four hour intervals from each of the culture types and assayed for total viral content. The data from a typical experiment, graphically presented in Figure 8, clearly indicate that no real differences exist in the rate of viral synthesis and final yields of virus in stationary as compared with roller monolayer cultures. In contrast to the situation in monolayer cultures, the production of virus in suspension cultures was slower and extended over a longer period of time. The maximum titer of infectious virus produced in the suspension cultures was about one $\log_{10}$ lower than that produced in the monolayer cultures.

A comparison between the production of CPE in roller and stationary cultures was investigated next. Parallel, duplicate titrations of L cell-adapted virus were performed in roller and stationary monolayer cultures of L cells. All cultures were observed at 12 to 16 hour intervals for five days to determine the time when cytopathic changes first appeared. The extent of these changes was graded and recorded after 120 hours of incubation. Data from an experiment of this type are presented in Table 7. Cytonecrotic changes became apparent 8 to 14 hours sooner in the roller cultures

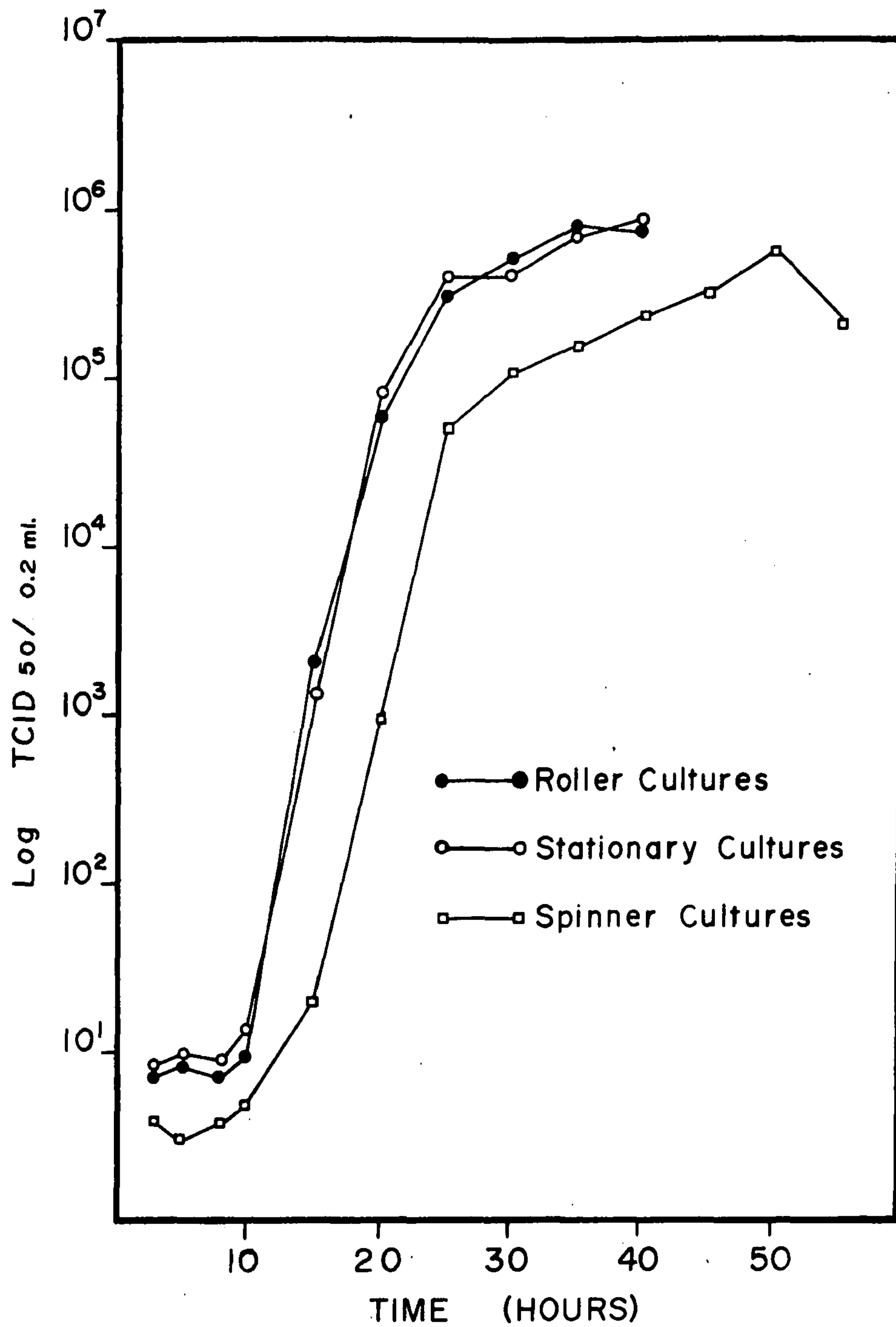

Figure 8. Replication of CTF virus in stationary and roller tube monolayer cultures and in spinner cultures of L cells. Multiplicity of infection 4.0 $TCID_{50}$. Total virus is plotted as a function of time in hours.

TABLE 7

COMPARATIVE DEVELOPMENT OF CYTOPATHIC CHANGES IN STATIONARY AND ROLLER CULTURES OF L CELLS INFECTED WITH CTF VIRUS

Dilution of Seed Virus	Times of CPE Development in Hours		Extent of CPE Development in Five Days Incubation	
	Roller Culture	Stationary Culture	Roller Culture	Stationary Culture
10^{-1}	35-40	48-54	4+[a]	4 +
10^{-2}	35-40	54	4 +	4 +
10^{-3}	40	54	4 +	4 +
10^{-4}	48	72	4 +	3 +
10^{-5}	48	96	4 +	2 +
10^{-6}	72		3 +	
10^{-7}				

[a]For recording the CPE a scale of 1+, 2+, 3+, 4+ was used representing 25, 50, 75, and 100 per cent cell destruction, respectively.

than in stationary cultures infected with the same dilution of virus. Destruction of the cellular monolayer as a result of viral infection was always more pronounced and widespread in roller cultures than in the stationary monolayers. Using the development of CPE in cultures maintained on the roller drum as an index of infection, the titer of the stock virus suspension was calculated to be $10^{4.8}$ $TCID_{50}$ 0.2 ml^{-1}. The titer of this same viral preparation calculated from the CPE in stationary cultures was $10^{3.5}$ $TCID_{50}$ 0.2 ml^{-1}, or approximately 90 per cent lower than that in the roller cultures.

Stability of CTF Virus to Physical and Chemical Agents

Freeze-Thaw and Sonic Oscillation Treatment

The results of studies on the effect of freezing and thawing on cell-adapted CTF virions in various concentrations of calf serum are given in Table 8. When suspended in a buffer containing 10 per cent serum, that serum concentration used in cellular growth medium, the virus was rapidly inactivated by repeated freezing and thawing. The diluent which contained 25 per cent serum stabilized viral infectivity after a loss of approximately 0.7 $TCID_{50}$ in the first freeze-thaw cycle. Virus suspended in a medium containing equal volumes of serum and HBSS was stable through three, repeated freeze-thaw cycles.

Although high concentrations of serum prevented inactivation of the virus during freezing, it was thought

TABLE 8

EFFECT OF FREEZE-THAW AND SONIC OSCILLATION ON INFECTIVITY OF CTF VIRUS

Per Cent Serum	Freeze-Thaw Cycle			Sonic Oscillation
	1x[b]	2x	3x	
10	4.5[a]	2.1	1.4	6.5
25	5.4	4.7	4.8	6.2
50	6.7	6.8	6.5	6.5

[a]Titer expressed as positive $\log_{10}$ of virus dilution which would infect 50 per cent of cultures, $TCID_{50}$.

[b]Number of freeze-thaw cycles.

desirable to find a chemically defined additive which would be as effective. Since inactivation of virus is probably due primarily to dehydration several compounds which minimize the crystalization of water during freezing were studied to determine whether they could stabilize CTF virions. The data from a typical experiment, presented in Table 9, indicate that the virus can be stabilized during repeated freezing and thawing by the addition of glúcose, glycerol or bovine serum albumin (BSA) to a final concentration of 20 per cent and also by 20 mM glutamine, 10 per cent BSA, and 2 per cent gelatin. All of these compounds were as efficient as 50 per cent serum in stabilizing the infectivity of CTF virus.

The results of studies in which CTF virus was diluted in HBSS containing various concentrations of calf serum and exposed to sonic vibration for one minute are presented in Table 8. It can be seen that with none of the suspending solutions did ultrasonic treatment have any injurious effects on the biological activity of CTF virions.

Hydrogen Ion Concentration

Changes in hydrogen ion concentration can seriously effect the infectivity of viruses. Studies were therefore undertaken to examine the stability of CTF virus over a wide range of pH values. The results of one of these experiments are presented in Table 10. It is clearly evident that CTF virus was most stable at pH 7.5 to 8.0 and that the stability was independent of the buffer system used. Exposure of virus

TABLE 9

STABILIZATION OF CTF VIRUS DURING FREEZE-THAW MANIPULATIONS

Freeze-Thaw Cycle	Glucose 20 per cent	Glutamine 20 mM	Glycerol 20 per cent	BSA[b] 20 per cent	BSA 10 per cent	Gelatin 20 per cent	Serum 50 per cent
0	3.5[a]	3.5	3.5	3.5	3.5	3.5	3.4
1	3.2	3.3	3.5	3.6	3.2	3.7	3.7
2	3.5	3.5	3.8	3.5	3.2	3.8	3.8
3	3.5	3.8	3.8	3.8	3.0	3.4	3.5

[a]Titer expressed as positive $\log_{10}$ of virus dilution which would infect 50 per cent of cultures, $TCID_{50}$.

[b]Bovine serum albumin.

TABLE 10

EFFECT OF HYDROGEN ION CONCENTRATION
ON INFECTIVITY OF CTF VIRUS

pH	Acetate-Veronal Buffer		Phosphate Buffer
	Experiment A	Experiment B	Experiment C
8.5	1.5[a]	1.2	1.6
8.3	--[b]	2.5	2.2
8.1	--	3.3	3.2
8.0	--	3.6	3.5
7.8	--	3.8	3.7
7.7	3.6	3.8	3.3
7.5	--	3.4	3.2
7.1	--	3.5	3.4
6.7	--	2.3	2.6
6.4	2.8	2.5	2.0
5.5	1.6	1.5	2.6
4.7	0.83	--	--
3.9	0.50	--	--

[a]Titer expressed as positive $\log_{10}$ per 0.2 ml of virus dilution which would infect 50 per cent of cultures, $TCID_{50}$.

[b]Data not available.

to hydrogen ion concentrations higher or lower than optimal resulted in a rapid loss of infectivity.

Lipid Solvents

Table 11 shows the results of studies in which CTF virus was in contact with chloroform for ten minutes. In all three experiments more than 99 per cent of the infectivity of the virus preparation was destroyed by the organic solvent.

The results of experiments on the effect of sodium desoxycholate (DOC) on the infectivity of CTF virus are presented in Table 12. Infectivity of the virus was stable in HBSS which contained 1.0 and 10 percent BSA, however the virus was rapidly inactivated by the bile salt. The degree of inactivation was directly proportional to the concentration of DOC in the buffer.

Thermal Inactivation

Results of a typical experiment on thermal stability of cell-adapted CTF virus are presented in Table 13. Virus exposed to 56 C was almost totally inactivated during 60 minutes of incubation. The infectivity of virus suspensions incubated at 25, 37, and 45 C decreased gradually over a four hour period. A semilog plot of the uninactivated virus fraction at each temperature, V_t/V_o, against time is shown in Figure 9. At each temperature of inactivation the decay of 90 per cent or more of the input virus was exponential. The

TABLE 11

EFFECT OF CHLOROFORM ON THE INFECTIVITY OF CTF VIRUS

Experiment	Control	Chloroform Treated	Per Cent of Control
1	5.8[a]	1.3[a]	0.0031
2	5.2	1.6	0.024
3	5.6	1.8	0.017

[a]Titer expressed as positive log_{10} per 0.2 ml of virus dilution which would infect 50 per cent of cultures, $TCID_{50}$.

TABLE 12

EFFECT OF SODIUM DESOXYCHOLATE ON THE INFECTIVITY OF CTF VIRUS

Experiment	BSA[a] Control Per cent 1	BSA[a] Control Per cent 10	1 Per cent DOC[b] + 1 Per cent BSA	1 Per cent DOC + 10 Per cent BSA	0.2 Per cent DOC + 1 Per cent BSA	0.2 Per cent DOC + 10 Per cent BSA
1	5.5[c]	5.4	2.8	3.5	4.5	4.5
2	5.3	5.5	3.2	3.2	4.6	4.8

[a]Bovine Serum Albumin.

[b]Sodium desoxycholate.

[c]Titer expressed as positive $\log_{10}$ per 0.2 ml of virus dilution which would infect 50 per cent of the cultures, $TCID_{50}$.

TABLE 13

HEAT INACTIVATION OF CTF VIRUS

Time in Minutes	Temperature of Incubation			
	25 C	37 C	45 C	56 C
0	6.2[a]	6.2	6.2	6.2
30	6.1	5.7	5.0	3.9
60	6.0	5.5	4.5	0.9
90	5.8	5.0	3.8	0.50
120	5.7	4.7	2.8	--[b]
150	5.7	4.2	1.5	--
180	5.6	3.8	0.8	--
210	5.4	3.7	0.50	--
240	5.1	3.5	---	--

[a]Titer expressed as positive $\log_{10}$ per 0.2 ml of virus dilution which would infect 50 per cent of cultures, $TCID_{50}$.

[b]Data not available.

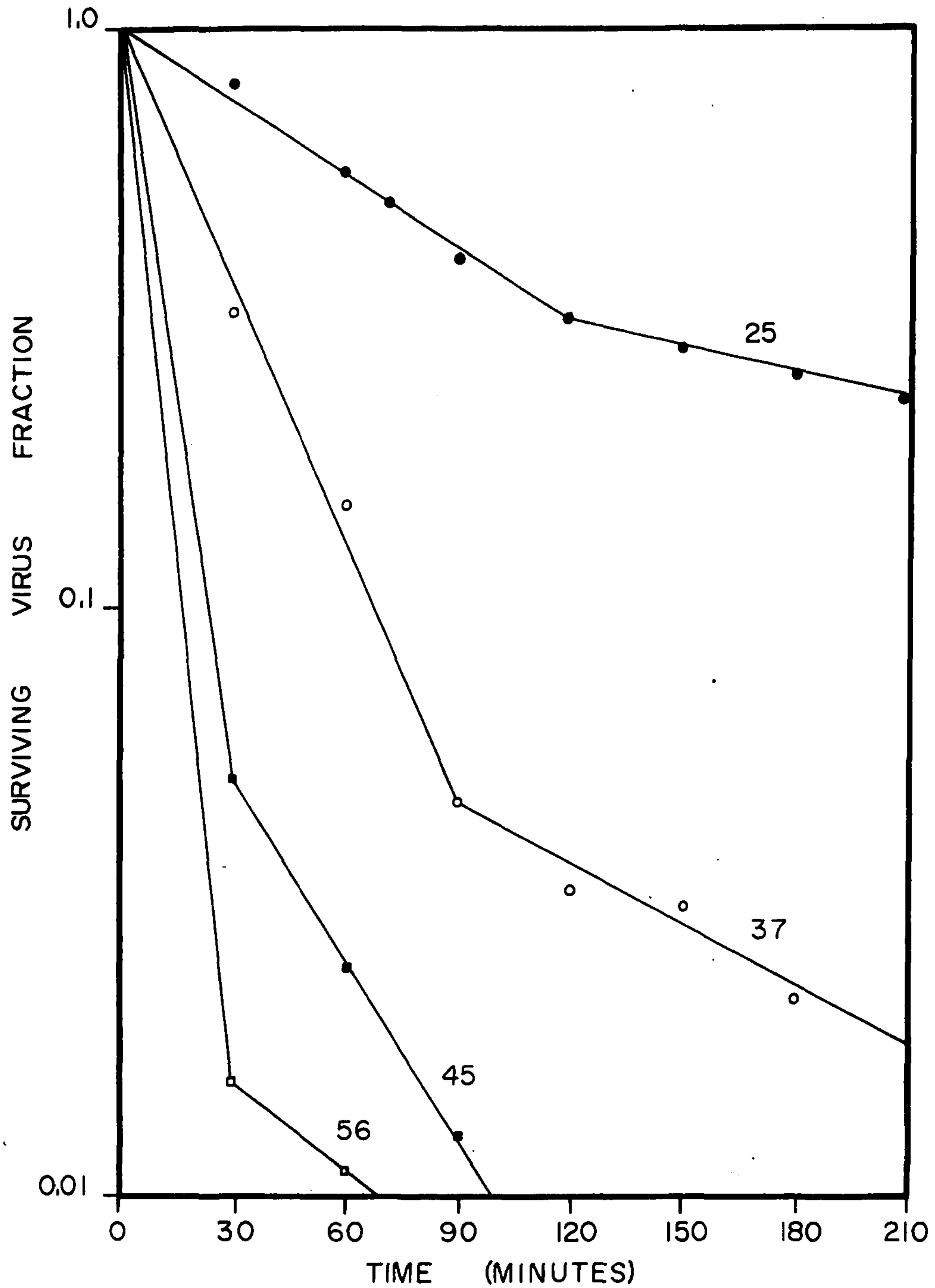

Figure 9. Heat inactivation of CTF virus at four incubation temperatures. Input virus (V_o) = $10^{5.2}$ $TCID_{50}$. Uninactivated virus at time t = (V_t). Surviving virus fraction = (V_t/V_o).

decay curves at all temperatures were biphasic and the inactivation of each phase of the curves appeared to show first order kinetics. If the slope of the slow inactivating phase of each curve is extrapolated back to the ordinant, it will be seen that the slope is independent of the temperature of inactivation. As the temperature was lowered, the intercept approached 50 per cent. This can be seen by comparing the extrapolations of the second phases of the 25 C and 37 C curves. The inactivation constant, K, for the initial 90 per cent of viral inactivation was calculated for each temperature by the first order reaction equation and these values are presented in Table 14.

An Arrhenius plot of these inactivation constants is presented in Figure 10. From this plot it may be observed that a linear relationship exists between the logarithm of the inactivation constant and the reciprocal of the absolute temperature of inactivation. The energy of activation for the thermal inactivation of cell-culture-adapted and propagated CTF virus was calculated by the Arrhenius equation and found to be 17,829 calories per mole. The half-lives at 25, 37, 45, and 56 C were determined to be 71, 21, 12, and 9 minutes, respectively. The temperature coefficient, Q_{10}, for the rate of inactivation of CTF virus by heat was computed from the ratio of the inactivation constants at $T^{o} + 27$ and T^{o} and found to be 2.63.

TABLE 14

REACTION RATE CONSTANTS FOR THERMAL INACTIVATION OF CTF VIRUS INFECTIVITY

Temperature C	$10^3/T^a$	$K \times 10^3$ minutes^{-1} [b]
56	0.3039	216.0
45	0.3144	99.7
37	0.3225	35.0
25	0.3350	9.74

[a]Degrees absolute temperature.

[b]Specific rate constant.

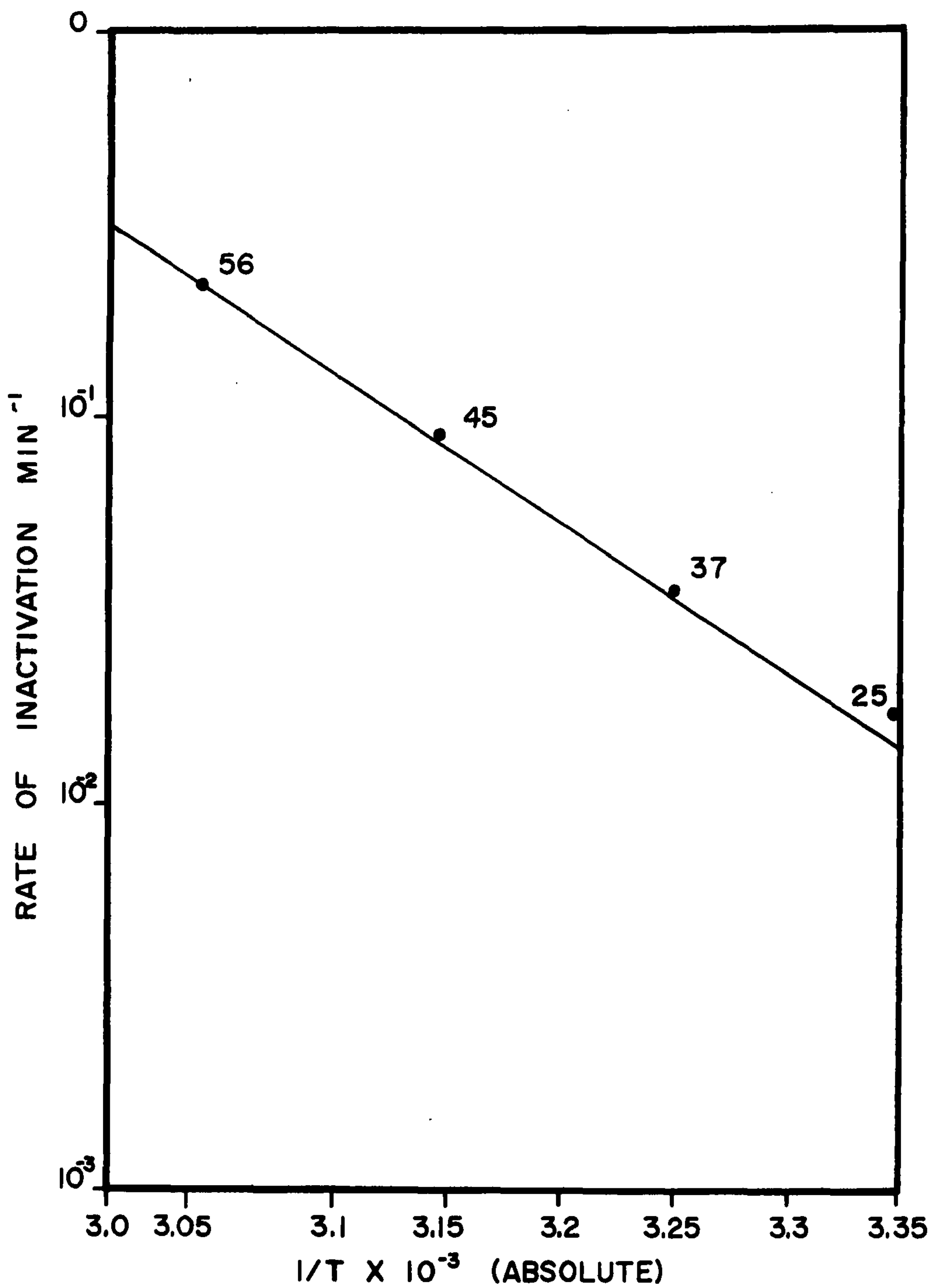

Figure 10. The dependence of the specific rate constants of CTF virus thermal inactivation on the absolute temperature of incubation, Arrhenius plot.

Effects of Metabolic Inhibitors on CTF Virus Synthesis and L Cell Multiplication

Effect of FUdR

Preliminary studies were done to determine the effect of FUdR on the multiplication of L cells. Data from a typical experiment are presented in graphic form in Figure 11. Unless indicated otherwise the cells were maintained in the presence of the designated media throughout the experiment. Cells nourished in growth medium containing no inhibitor continued to multiply until they had increased in number by a factor of 7.5 at the end of five days (line C). The addition of FUdR-containing medium to the cultures on day one (line F) or on day two (line $C_{24}F$) immediately inhibited cell division. The cells treated with FUdR maintained their normal morphology for 48 to 72 hours after the experimental medium was added. After 96 hours of exposure to the inhibitor the number of viable cells decreased rapidly and those cells still viable became rounded and detached from the glass during the next 48 hours. The combination of FUdR and uridine (line FU) failed to support cell division, although the cells maintained their normal morphology for a longer period of time than they did in medium which contained only FUdR. Cultures in which DNA synthesis was arrested for 18 hours (line $F_{18}C$) resumed normal growth 30 hours after the inhibitor-containing medium was replaced with growth medium. It will be seen that thymidine prevented inhibition of cell

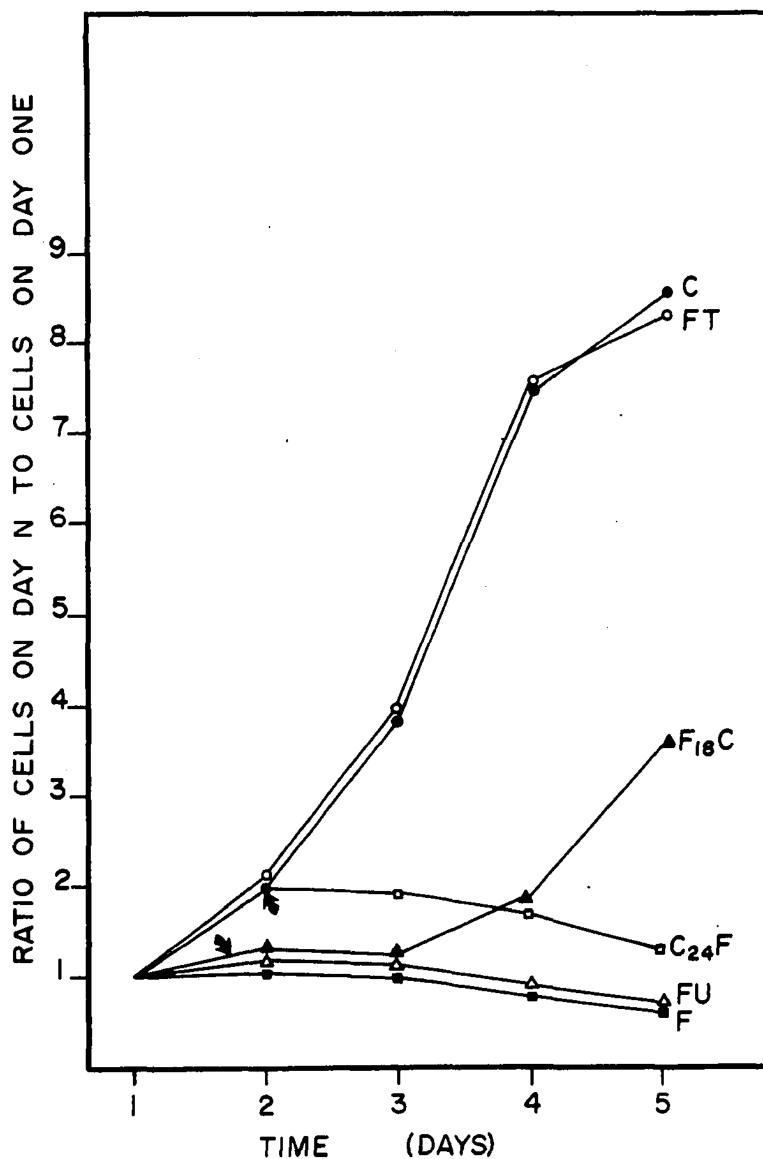

Figure 11. Effect of FUdR on multiplication of L cells. Each point on the graph represents the ratio of the average number of cells per tnree to five cultures on day of count (N) to the number of cells on the first day after seeding. The curves represent multiplication of cells in growth medium which contained:

C: No FUdR.
FT: 0.3μg FUdR ml^{-1}, 2.8μg thymidine ml^{-1}.
$F_{18}C$: 0.3μg FUdR ml^{-1} for 18 hours then growth medium added (arrow).
$C_{24}F$: Growth medium for 24 hours then 0.3μg FUdR ml^{-1} added (arrow).
FU: 0.3μg FUdR ml^{-1}, 2.6μg uridine ml^{-1}.
F: 0.3μg FUdR ml^{-1}.

growth by FUdR when it was added in an amount tenfold greater than that of the inhibitor (line FT).

Experiments were designed to compare the ability of FUdR treated cells to support replication of a DNA-containing virus (herpes simplex) and CTF virus. Confluent monolayers of L cells were maintained in growth medium or in medium containing 0.3 mcg FUdR ml^{-1} for one hour prior to infection. These cultures were then infected at a multiplicity of 4.0 $TCID_{50}$. As seen in Figure 12 the production of CTF virus was affected very little when DNA synthesis and host cell multiplication were inhibited by FUdR. Final yields of virus were only about tenfold lower in the presence of FUdR than in the corresponding untreated cultures. In contrast, the replication of herpesvirus was reduced about 1,000 fold in the presence of FUdR.

There is a general tendency to interpret the effect of FUdR on viral synthesis as an indication of the type of nucleic acid present in a given virus (Salzman *et al.*, 1962; Hamparian *et al.*, 1963). It was therefore considered essential to compare the effect of FUdR on a known RNA virus, a known DNA virus and on CTF virus. Experiments were therefore done to compare the effect of FUdR on the sensitivity of L cells to infection with CTF, herpes simplex virus (DNA-containing) and reovirus (RNA-containing). The viruses were titrated simultaneously in untreated cells and those treated with various concentrations of FUdR and FUdR plus thymidine

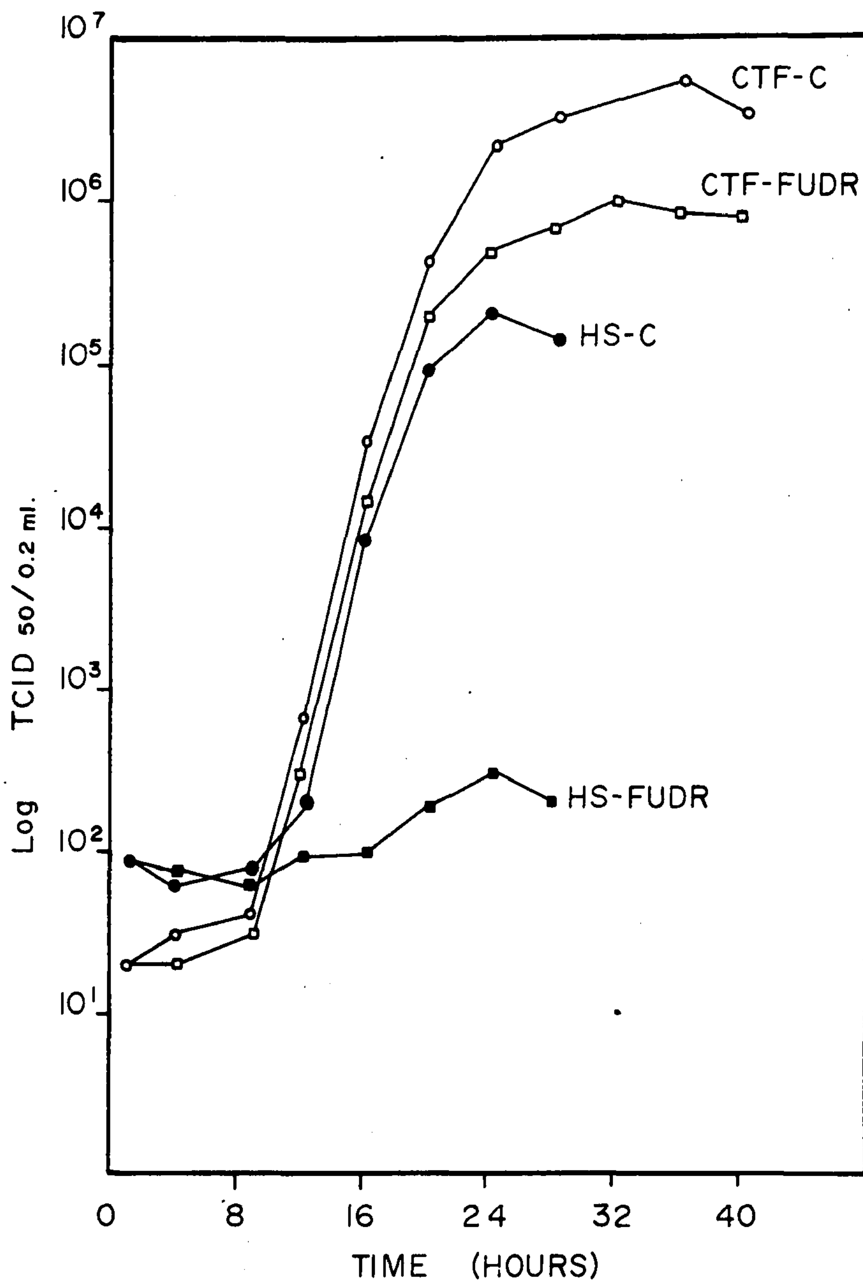

Figure 12. Effect on FUdR on replication of CTF and herpes simplex viruses in L cells.

CTF-C: CTF virus production in growth medium.

CTF-FUdR: CTF virus production in medium containing 0.3μg FUdR ml^{-1} added 1 hour before infection.

HS-C: Herpes virus production in growth medium.

HS-FUdR: Herpes virus production in medium containing 0.3μg FUdR ml^{-1} added 1 hour before infection.

for one hour prior to assay. The results of one of these experiments are tabulated in Table 15. It can be seen that the titer of herpes simplex virus was reduced by an average of 10^3 $TCID_{50}$ in the presence of 3.2 and 0.326 mcg FUdR ml^{-1}. The addition of 2.76 mcg thymidine ml^{-1} to FUdR-containing medium prevented the inhibition of the DNA-containing herpes-virus. The sensitivity of cells to infection by reovirus, an RNA virus, was not appreciably altered by FUdR. The titer of CTF virus was decreased approximately 0.7 log_{10} by FUdR, however cultures to which thymidine was also added were inhibited to the same degree.

Effect of BUdR

The previous experiments had produced results which could be interpreted to indicate that CTF virus contained RNA. This hypothesis was investigated further through the use of BUdR which antagonizes the formation of functional DNA. Preliminary experiments were done to determine the effect of the analogue on the multiplication of L cells. The results of one of these experiments are presented in Figure 13. Cells maintained in growth medium with no inhibitor, continued to divide until they had increased in number by a factor of eight (line C). Cultures in which BUdR-containing medium was introduced on day one (line B) and on day two (line $C_{24}B$) completed about one cell division in the presence of the analogue. The number of viable cells remained constant and the morphology of the cells appeared normal for 72 to 96 hours

TABLE 15

CTF VIRAL NUCLEIC ACID AS DETERMINED BY FUdR INHIBITION

Virus	Control	FUdR 3.2 mcg ml^{-1}	FUdR .326 mcg ml^{-1}	FUdR .326 mcg ml^{-1} and Thymidine 2.76 mcg ml^{-1}	Nucleic Acid Type
Herpes simplex	5.2[a]	2.6	1.8	5.2	DNA
Reovirus	8.6	8.8	8.4	8.8	RNA
CTF	5.4	4.8	4.6	4.8	Probably RNA

[a]Titer expressed as positive $\log_{10}$ per 0.2 ml of virus dilution which would infect 50 per cent of cultures, $TCID_{50}$.

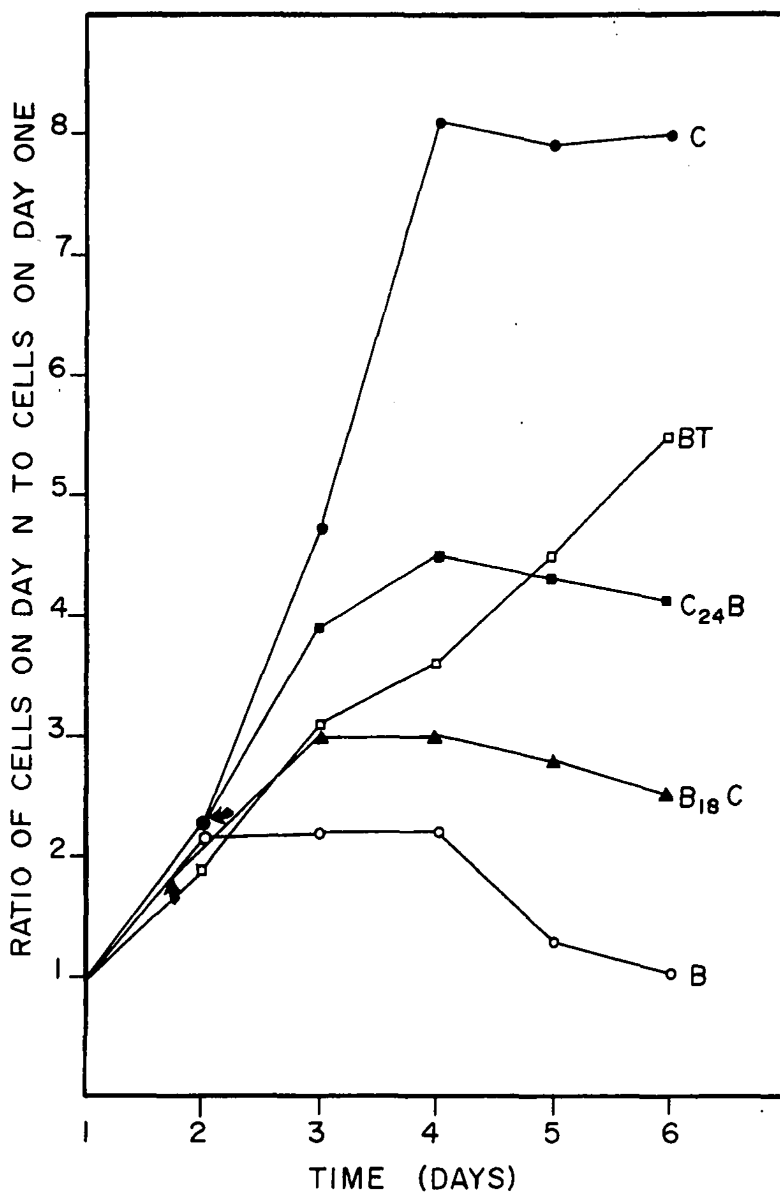

Figure 13. Effect of BUdR on multiplication of L cells. Each point on the graph represents the ratio of the average number of cells per three to five cultures on day of count (N) to the number of cells on the first day after seeding. The curves represent multiplication of cells in growth medium which contained:

C: No BUdR.

BT: 100μg BUdR ml^{-1}, 100μg thymidine ml^{-1}.

$C_{24}B$: Growth medium for 24 hours then 100μg BUdR ml^{-1} added (arrow).

$B_{18}C$: 100μg BUdR ml^{-1} for 18 hours then growth medium added (arrow).

B: 100μg BUdR ml^{-1}.

after the analogue was added. After five days of incubation in BUdR-containing medium the cells were greatly increased in size and the cell count began to decrease. In medium which contained 100 mcg of BUdR and 100 mcg of thymidine ml^{-1} (line BT), cell division continued at a reduced rate in comparison to the untreated cultures. When BUdR was present for 18 hours, followed by removal of the analogue and addition of growth medium, the cells ceased to divide after they had completed the first cell division in the presence of BUdR (line $B_{18}C$).

The replication of CTF and herpes simplex viruses in BUdR treated cultures was next characterized. Monolayers of L cells were prepared and when growth was confluent one half of the cultures were treated with 100 mcg BUdR ml^{-1} for four hours prior to infection. Treated and untreated cultures were then infected with either CTF or herpesvirus at a multiplicity of 4.0 $TCID_{50}$. The results of a typical experiment are plotted in Figure 14. The rate of synthesis of infectious herpesvirus was retarded and the final titers of virus produced were about 1,000 times less in the presence of BUdR as compared with the untreated cultures. In contrast, BUdR had no significant effect on the production of CTF virus.

Effect of Actinomycin D

Since results of the previous experiments with FUdR and BUdR indicated that the nucleic acid of CTF virus is RNA, the next studies were designed to determine the relationship

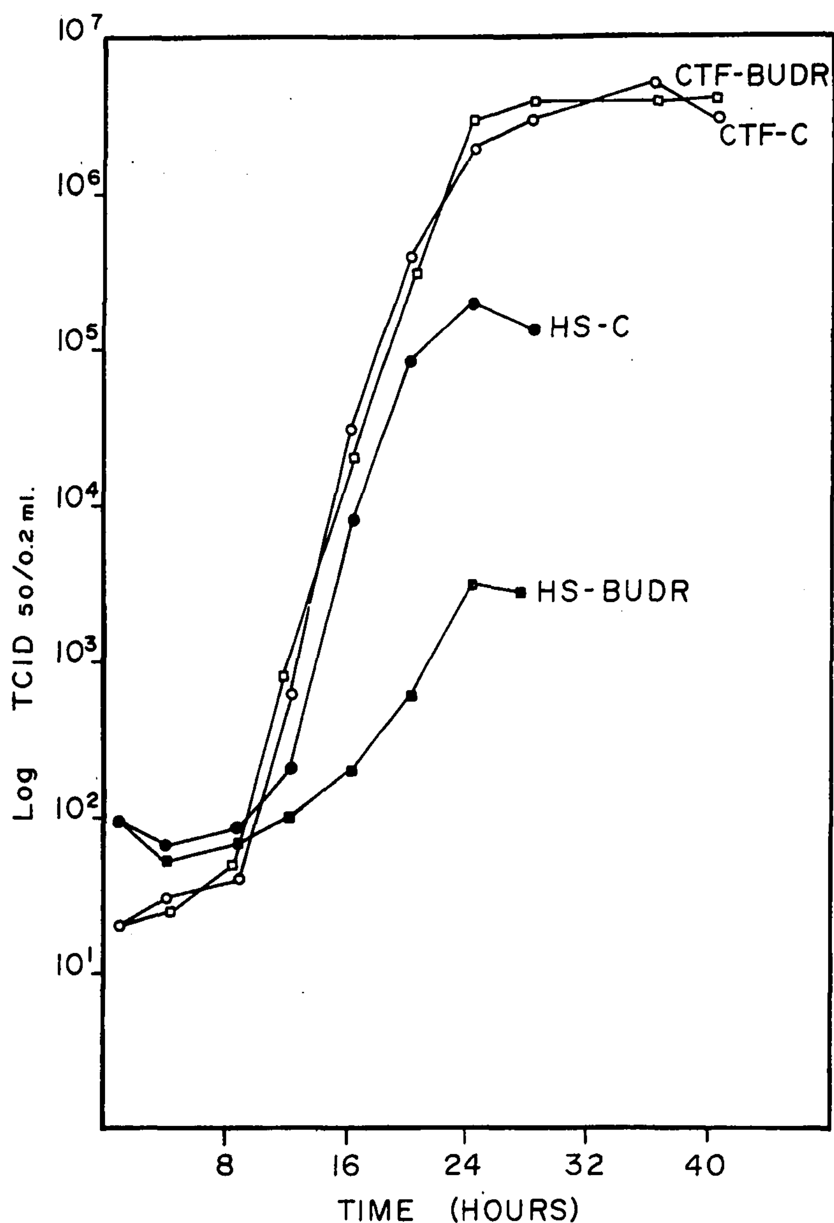

Figure 14. Effect of BUdR on replication of CTF and herpes simplex viruses in L cells.

CTF-C: CTF virus production in growth medium.

CTF-B: CTF virus production in medium containing 100μg BUdR ml^{-1} added 4 hours before infection.

HS-C: Herpes virus production in growth medium.

HS-B: Herpes virus production in medium containing 100μg BUdR ml^{-1} added 4 hours before infection.

of cellular RNA synthesis to CTF viral replication. In the first experiments, the effect of actinomycin D on the multiplication of L cells was examined. Replicate tube cultures were prepared and medium containing 1.0 mcg actinomycin D ml^{-1} was added to the cultures on day one or at the end of periods 12 or 36 hours. The results of a typical experiment are presented in Figure 15. The addition of actinomycin to the cultures on day one (line A_o) inhibited cell division completely after the first 24 hours. If the antibiotic was not added to the cultures until 12 (line $C_{12}A$) or 36 (line $C_{36}A$) hours later, cell division continued very slowly and ceased within 24 hours after the drug was added. Microscopic examination of the cultures revealed that the cells treated with actinomycin were rounded and very granular after 24 hours exposure to the drug. After the cells had been maintained in the antibiotic for 72 hours the cell count began to decrease rapidly and by 96 hours most of the cells had detached from the glass.

Finally, experiments were performed to determine if CTF virus could replicate in cells in which RNA synthesis was arrested by actinomycin D for various periods of time prior to infection. Monolayer cultures of L cells were prepared and infected at a multiplicity of 4.0 $TCID_{50}$. The results of one of these experiments are shown in Figure 16. When 1.0 mcg actinomycin D ml^{-1} was added to the cells at the time of infection (line A_o) and maintained throughout

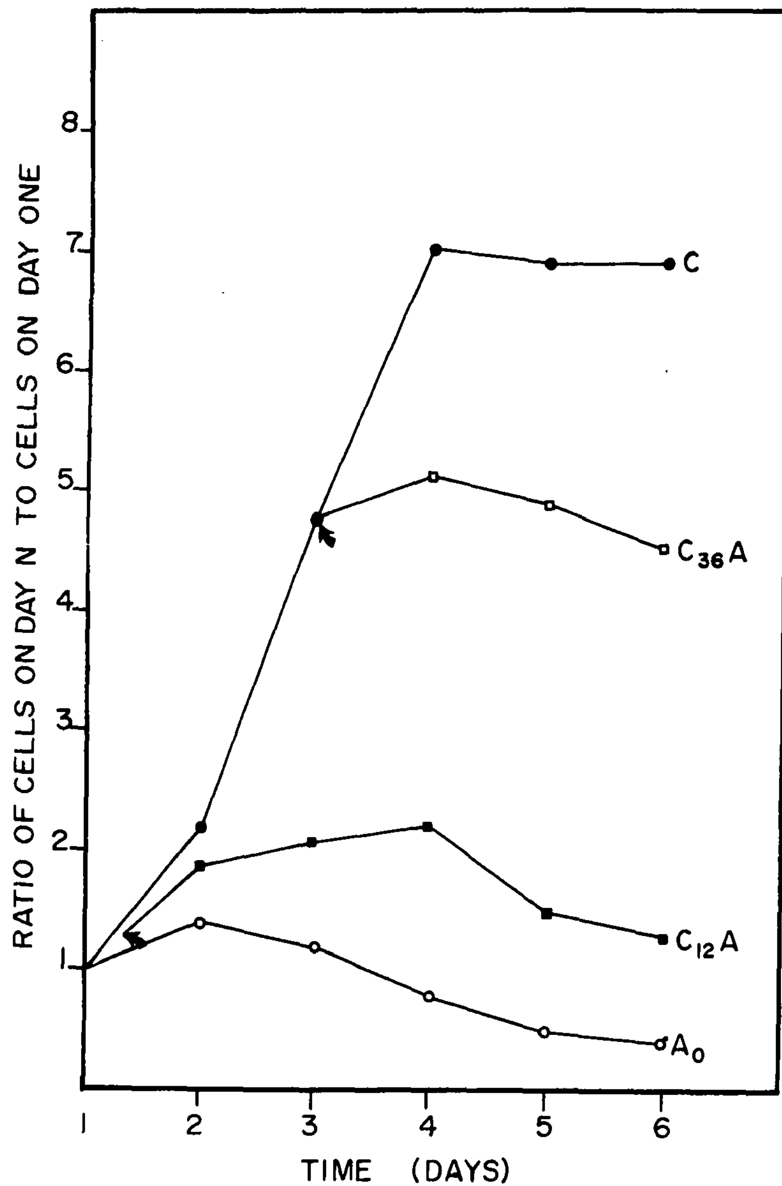

Figure 15. Effect of actinomycin D on multiplication of L cells. Each point on the graph represents the ratio of the average number of cells per three to five cultures on day of count (N) to the number of cells on the first day after seeding. The curves represent multiplication of cells in medium which contained:

C: No actinomycin D.

$C_{36}A$: Growth medium for 36 hours then 1.0μg actinomycin D ml^{-1} added (arrow).

$C_{12}A$: Growth medium for 12 hours then 1.0μg actinomycin D ml^{-1} added (arrow).

A_0: 1.0μg actinomycin D ml^{-1} added on day one.

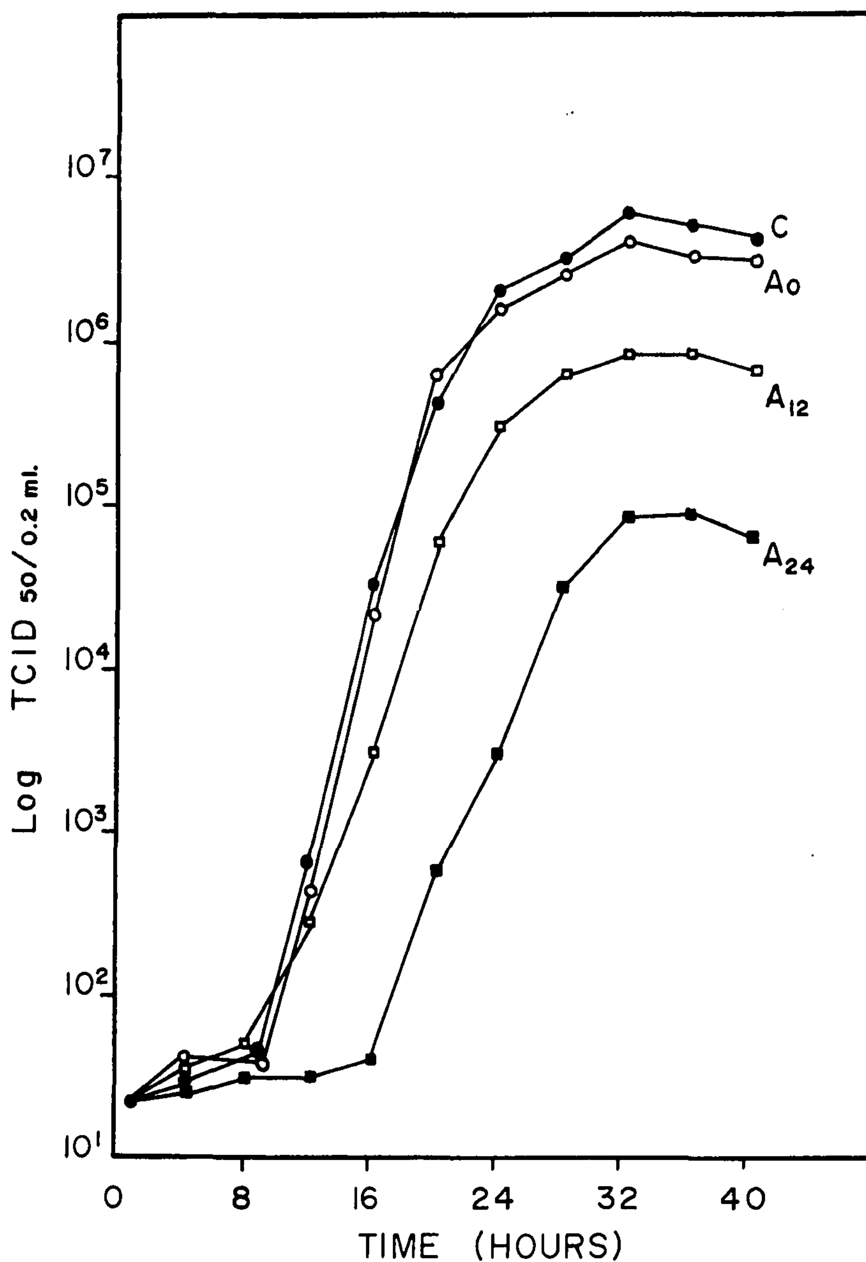

Figure 16. Effect of actinomycin D on replication of CTF virus in L cells. The curves represent production of virus in cells maintained in growth medium with:

C: No actinomycin D.

A_0: 1.0μg actinomycin D ml^{-1} added at time of infection.

A_{12}: 1.0μg actinomycin D ml^{-1} added 12 hours before infection.

A_{24}: 1.0μg actinomycin D ml^{-1} added 24 hours before infection.

the virus growth cycle, viral titers and the growth patterns were similar to those observed in the untreated cultures (line C). The rate of viral synthesis in cells pretreated with the antibiotic for 12 hours before infection (line A_{12}) was also similar to that in untreated cultures, although the total yield of virus from these cells was about 10-fold lower. When the cultures were pretreated for 24 hours (line A_{24}) before infection the lag phase of the growth cycle was extended to near the sixteenth hour and the total titers of virus produced were decreased by approximately 100-fold.

CHAPTER V

DISCUSSION

The cell culture technique described by Weller *et al.* (1949) gave great impetus to studies on the characterization of animal viruses and their relationships with the living cell. Since that time numerous investigators have described the replication of various arboviruses in cells cultivated *in vitro*. Until the present study, however, little was known about the replication of CTF virus in cell culture. Data from this investigation clearly indicate that CTF virus will grow in fibroblast and epithelial type cells. When mouse-brain virus was inoculated into monolayer cultures of FL cells high titers of virus were produced and cytopathic changes developed during the initial and succeeding passages of the virus in this cell system. Infection of L cells with mouse-brain virus resulted in destruction of the cells and the production of small amounts of virus during the first three passages. Beginning with the fourth, and continuing through all consecutive passages of the virus in L cells, high titers of virus were produced. The necessity for repeated passage of a virus in a cell system before viral replication and cytopathic changes become consistent has been

noted with WEE, EEE (Eiring and Scherer, 1961), Coxsackie 7 (Habel and Loomis, 1957), equine abortion (Randall, 1957) and yellow fever viruses (Hardy, 1963a). Pickens and Luoto (1958) reported that mouse-adapted CTF virus required several passages in KB cells before cytopathic changes were produced consistently.

The exact mechanism involved in the adaption of a virus to multiply in a new host is not thoroughly understood. It is known that mouse brain suspensions of some viruses are heterogeneous with respect to cytopathogenicity and virulence. Infection of a new type of host cell with a heterogeneous virus suspension may result in the selection of preexisting mutant from the inoculum which has an increased virulence for the new cell type. An alternate genetic explanation of this phenomenon could be the induction and selection of a mutant arising during maturation of the virus in cell culture. The possibility exists that interfering substances were present in the mouse brain suspension which were diluted on serial passage so that they could no longer suppress viral multiplication. Also, noninfectious interfering virus (Chambers, 1957) or interferon (Lockart and Horn, 1962) might have been produced during early passages of the virus in L cells. If so, cessation of the production of interfering virus and interferon after several passages is difficult to explain. Finally, it seems probable that spontaneous changes in the susceptibility of L cells to CTF virus

would not account for the increase in virus production. Cellular alterations could hardly have occurred with such regularity in experiments performed over a three year period. Moreover, L cells used for all experiments were from the L-929 cloned cell line which has maintained uniform morphology and growth characteristics since it was acquired in this laboratory in 1961.

Viruses which are adapted to multiply in cell culture often change their virulence for the original host. In the present study Colorado tick fever virus which multiplied in FL and L cells was not significantly less virulent for mice than the virus from which it had been derived. The virulence of yellow fever (Theiler and Smith, 1937; Smith and Theiler, 1937) and VEE viruses (Hearn _et al._, 1961) was decreased after continued cultivation _in vitro_. Koprowski and Cox (1947b) observed that CTF virus which had been adapted to replicate in the developing chick embryo was changed in pathogenicity for hamsters. The virus, however, was still virulent for human volunteers (Koprowski _et al._, 1951). In the studies reported herein continued passage of CTF virus in cell culture did not augment any decrease in the virulence of the virus for mice.

The antigenic properties of mouse-brain and L cell-adapted CTF virus were compared by neutralization studies. Antiserum prepared against mouse-brain virus neutralized 99.9 per cent of the homologous virus during one hour of

incubation. This same serum neutralized 99.7 per cent of the virus from L cell passage eight and 98.5 per cent of the virus from passage 12. The input virus in these experiments varied over a $10^{2.5}$ fold range and for this reason the rate of neutralization and fraction of surviving virus after any given period of incubation in different experiments can not be compared (Andrewes and Elford, 1933). These findings were interpreted to indicate that the virus which adapted to replicate in cultures of L cells was antigenically similar, if not identical to the mouse-brain virus from which it was derived. It is interesting to note that yellow fever virus (Hardy, 1963b) which was "adapted" to replicate in HeLa cells closely resembled mouse-brain virus in antigenic structure.

The capacity of various cell types to support the replication of CTF virus is similar to that seen with many other arboviruses. Monolayer cultures of CE cells are probably the cell culture system of choice for the isolation and propagation of arboviruses in Casal's group A (Fastier, 1954; Kissling, 1957; Medearis and Kilrick, 1958; Porterfield, 1960; Henderson and Taylor, 1960). The capacity of CE cells to support the multiplication of most of the viruses in group B is less than that reported in the present study for CTF virus (McCollum and Foley, 1957; Henderson and Taylor, 1959; Libikova _et al_., 1960; Diercks and Hammon, 1958). In contrast to the other viruses of the RSSE complex, the virus of Kyasanur Forest disease (Bhatt, 1960) replicates and

produces cytopathic changes in chick cells comparable to that observed here with CTF virus.

Nir and Goldwasser (1961b) reported that WN virus replicates in cotton rats and in cultures of cotton rat kidney cells. Koprowski and Cox (1947a) had previously shown that when these animals are inoculated with CTF virus they maintain a viremia for several days. The mouse-brain virus did not kill the cotton rats and they developed high titers of antibodies to the virus. Data from the studies reported herein indicated that CTF virus does not replicate in cultures of cotton rat kidney cells. It is of interest to note that Pickens and Luoto (1958) have reported that CTF does not replicate or produce cytopathic changes in cultures of monkey kidney cells.

Serially propagated cell types of tumor origin were less sensitive to infection by CTF virus than were cells derived from normal tissues. Monolayers of HeLa cells did not support viral synthesis and the strain of KB cells maintained in this laboratory was less sensitive to infection and produced smaller amounts of infectious virus than did any other cell type studied. These observations are quite similar to those of Banta (1957), McCollum and Foley (1957), and Scherer and Syverton (1954) showing that, in general, arboviruses do not replicate or produce consistent CPE in cell cultures derived from malignant tissues. The viruses of yellow fever (Hardy, 1963a) and dengue (Schulze and Schlesinger, 1963) do

replicate in HeLa and KB cells respectively, although these cells are not as sensitive to infection as other cell types. Pickens and Luoto (1958) reported that KB cells could be used for the isolation and propagation of CTF virus. The present studies confirm this observation but indicate further that KB cells are not the cell culture of choice for the isolation and propagation of CTF virus. In contrast, arboviruses of group-C replicate to high titer and produce extensive cytopathic effects in cultures of HeLa and Detroit-6 cells (Buckley and Shope, 1961).

Cultures of L cells (mouse fibroblast) have been used widely for the study of vaccinia (Kit and Dobbs, 1962a), Mengo (Franklin and Baltimore, 1962), and reoviruses (Gomatos _et al_., 1962) but have not been used extensively for the study of arboviruses. The viruses of VEE (Hardy and Brown, 1961a,b), WEE and EEE (Eiring and Scherer, 1961) and TE (Stancek, 1963) replicate and produce CPE in monolayer cultures of L cells. In the present studies cultures of L cells were as sensitive to CTF virus infection and had the same capacity to produce virus as did FL or chick embryo cells. Thus, a critical evaluation of the capacity of cells to support viral replication and their physiological hardiness indicated that the L cell system is ideal for continuation studies with CTF virus, as reported herein.

Monolayer cultures of FL and L cells infected with CTF virus developed a well defined and characteristic CPE.

The first signs of morphologic changes are the vacuolation and granulation of the cytoplasm. Within the next 24 hours the cells start to round and clump together. This process starts in focal areas and then spreads throughout the monolayer. Eventually the entire culture is affected and the cells undergo lysis and detach from the glass to form a plaque. There is no indication of syncytial formation. The cytonecrotic changes induced by VEE (Hardy and Brown, 1961a) and TE virus in L cells (Stancek, 1963) resemble those described here for CTF virus. The cytonecrotic changes induced by different arboviruses are not distinctive. The cellular response is always lytic in nature and is essentially the same throughout the entire group of viruses (Pereira, 1961).

Invasion of a cell by a virus is a process which may be divided into three phases: (1) adsorption, (2) viriopexis, and (3) uncoating of the viral nucleic acid. The rate at which each of these phases occurs in the mobilization of the cell for the production of virus is usually characteristic of each virus. The first of these phenomena, adsorption, was characterized for CTF virus in the present study. Critical examination of the data from three experiments indicates that adsorption was 99 per cent complete during the first 30 minutes after inoculation. Dulbecco and Vogt (1954b) and Levine (1958) reported that 70 to 84 per cent of WEE virus is adsorbed to chick embryo cells in one hour. Wagner (1961) reported that 60 per cent of the input

EEE virus adsorbes to chick embryo cells in one and one-half hours. The adsorption of dengue (Schulze and Schlesinger, 1963), JAP (Banta, 1957), and VEE (Hardy and Brown, 1961a) viruses to mammalian cells is maximal after one hour. The rate of adsorption of arboviruses to mammalian cells has heretofor been determined indirectly by the development of plaques on the adsorption plate after various periods of incubation. The method used in the present investigation was a direct method which quantitated the free virus after a given period of incubation. Because the methods used in the present investigation and in the other studies with mammalian cells were different it may not be valid to compare the results. It does appear, however, that the adsorption rate of CTF virus is equal to or greater than the adsorption rate of other arboviruses. A plaque assay technique for CTF virus in L cells could give more information on the adsorption phase.

It is noteworthy that the lag period of CTF virus growth was eight hours and that maximal yields of virus were reached at 35 hours. Isaacs (1959) has pointed out that, in general, RNA-containing viruses have a shorter lag period and a more rapid rate of increase than do DNA-containing viruses. Several references may be cited to support this statement (Bachrach *et al*., 1957b; Isaacs, 1959; Franklin and Henry, 1960; Eggers and Tamm, 1961; Franklin, 1962; Ginsberg, 1958). If, as the evidence suggests, CTF virus is

a RNA-containing virus, it is unusual that its multiplication is relatively slow as compared with that of many RNA-containing viruses.

Among the arboviruses, lag period, which includes the eclipse phase, varies from a minimum of two hours to a maximum of 18 hours. Critical studies by Rubin et al. (1955) on growth of WEE virus in chick embryo cells indicated that synthesis of intracellular virus begins after a one hour eclipse phase. The lag period persisted for two to two and one-half hours. This description of WEE virus is closely similar to that reported for EEE virus (Wagner, 1961) and VEE virus (Hardy and Brown, 1961a,b). Arboviruses which belong to group B characteristically have a longer lag period than do most group A viruses. For example, the growth cycle of viruses of the JAP-WN complex (Banta, 1957), yellow fever (Hardy, 1963b), and dengue (Schulze, 1964) have a six to ten hour lag period in mammalian cells. Although CTF virus is not antigenically related to the viruses of the RSSE complex the lag periods of TE virus (Stancek, 1963) and CTF virus are of the same duration in L cells. An explanation for these differences in the duration of the lag phases of group A and group B viruses could be that: (1) longer periods of time are required for uncoating of the virion, or (2) viruses of group B and CTF virus require the formation of many new enzymes before the synthesis of progeny virus can begin. The answer to this problem might be obtained by studying the

inhibition of cellular synthesis after infection.

The rapid phase of viral replication which follows the eclipse period is usually exponential for the first few hours. The slope and duration of the linear portion of the growth curve are dependent upon several factors. The primary factor which determines the slope of the growth curve is the biological nature of the virus. It may be influenced by many factors among which are the host cell type, medium used, multiplicity of infection, physiological state of the cell, and method of cell cultivation. The logarithmic phase of CTF replication at a low multiplicity of 0.2 $TCID_{50}$ $cell^{-1}$ was found to be 24 hours in the present study. The exponential phase after infection at multiplication of 4.0 and 40 $TCID_{50}$ $cell^{-1}$ was approximately 10 hours in duration. There were no detectable differences in the total amount of virus produced when cultures were infected at high and at low multiplicities. The observations of Banta (1957) and Diercks *et al.* (1961) on the effects of different multiplicities of infection on the growth cycle of JAP virus resemble those seen in this study. Viral growth in cultures inoculated at multiplicities of less than one $TCID_{50}$ $cell^{-1}$ lasted 18 to 24 hours longer than in cultures infected at higher multiplicities. These data have been interpreted to indicate that the number of cells which produce virus during the first growth cycle is related to the multiplicity of infection. Increasing the multiplicity greater than that sufficient to

infect all susceptible cells in the culture does not alter the growth cycle appreciably. It might be assumed that the growth curves resulting from infection of cultures at multiplicities greater than one are one-step growth curves.

Studies on the effect the method of cell cultivation on the replication of CTF virus revealed that the development of cytopathic changes is more rapid and complete in roller tube cultures. The rate of virus production was essentially the same in both the stationary and roller tube monolayers if the conditions were such that all susceptible cells were infected at the beginning of the experiment. Fronthingham (1959) reported that type II poliovirus produced more pronounced CPE and replicated faster in rolled cultures than in stationary cultures. He also observed that rotation of the cultures had no effect on the multiplication or the development of cytopathic changes by Sindbis virus. The replication and cytopathogenicity of reovirus was greatly enhanced in roller cultures of monkey kidney cells (Lerner and Cherry, 1961). The reason for this superiority of roller cultures might be that: (1) the continual mixing of the medium and alternate bathing of the cultures in the liquid and air phases provides better physiological conditions for viral development; (2) physical agitation of the medium over the infected cells accentuates the development of morphological changes which in stationary cultures would not be apparent; or (3) the spread of extracellular virus from infected to

non-infected cells is enhanced. The last of these possibilities seems to be the more plausible. Although definitive data on the route of spread of arbovirus from cell to cell are not available, the type of cellular response seen and the fact that an infected host always has a viremia would suggest that the virus spreads to uninfected cells in the liquid phase. The continual washing and mixing of the medium over the cells in roller cultures would facilitate the spread of virus to new host cells.

Comparisons of viral replication in spinner cultures and monolayer cultures revealed that the kinetics of CTF replication in spinner cultures is slower. The lag phase in spinner cultures appeared to be approximately two hours longer than that in monolayer cultures and maximum titers of virus produced were one log lower. These differences may be more apparent than real for two reasons. First, viral adsorption in suspension cultures is less efficient than that in monolayer cultures (Dulbecco and Vogt, 1954b) and second, CTF virus is more readily inactivated at 37 C, the adsorption temperature in the spinner cultures, than at 25 C, the temperature at which the virus was adsorbed to the monolayer cultures. Under conditions of reduced adsorption efficiency and higher adsorption temperature the multiplicity of infection was undoubtedly greatly reduced. Comparison of the viral growth curve in suspension cultures infected at a multiplicity of 4.0 $TCID_{50}$ with that of the monolayer culture

infected with a multiplicity of 0.4 $TCID_{50}$ reveals that the rates of viral multiplication are similar. Therefore, the kinetics of CTF virus replication in suspension culture appears to be essentially the same as that in monolayer cultures.

Arboviruses are more labile, in general, than most other animal viruses. The lability of tissue culture-propagated CTF virus under slightly acid conditions is of particular interest. The rapid inactivation of the virus at pH values lower than seven is in marked contrast to the situation with poliovirus, which is completely stable at a pH as low as 1.5 for 24 hours at 4 C (Bachrach and Schwerdt, 1952). Such extreme differences in resistance of two animal viruses must reflect certain differences in their physical state and/or chemical composition. The minute fraction of the infectious CTF virus that resists inactivation at pH 5.5 is destroyed when the pH is lowered to 4.7. The data also indicated that specific ions were not involved in the inactivation process since controls (pH 7.7) in veronal acetate and phosphate buffers did not lose their infectivity. These results are in agreement with those reported for EEE virus (Finkelstein *et al.*, 1938, 1940). Hardy and Brown (1961a) found that VEE virus is most stable at pH values of 7.2 to 7.8. In contrast, Duffy and Stanley (1945) and Duffy (1946) reported that the viruses of JAP and SLE are most stable in the pH range of 8.4 to 8.8.

The necessity of maintaining CTF virus in a protein containing medium had been observed previously by Koprowski and Cox (1947a). It seemed desirable to determine the optimal conditions for (1) preserving viral infectivity during storage at -65 C and (2) releasing CAV by repeated freezing and thawing. High concentrations of protein or other chemicals which minimize crystallization of water during freezing, were required to stabilize viral infectivity during storage at -65 C. These data may be interpreted to indicate that water plays an important role in the maintenance of infectivity and structure of the CTF virion. It is generally conceded in a living organism that water is physiologically entrapped by a definite cell membrane. The water of hydration is primarily considered to be associated with the cell contents. The problem becomes more complex when a particle in an aqueous solution is considered. In this case water may be entrapped within the particle to cause it to swell, adsorbed onto the surface of a molecule, or chemically bound to chemical groups on the surface of the virion. On a weight basis virus particles usually contain approximately equal quantities of water and nucleoprotein. Lauffer and Bendet (1954) determined that influenza A, influenza B, papilloma, and vaccinia viruses bind 3.78, 3.04, 1.69 and 0.42 grams of water per gram of virus respectively. In addition, the stability of a particular virus was found to be inversely proportional to the amount of bound water associated with the

virion. Labile viruses, such as CTF, may bind large quantities of water which crystallize during freezing to rupture the virion or destroy infectivity by dehydration. This possibility was not examined experimentally in the present investigation.

For many years it has been recognized that some viruses are more heat stable than others under certain environmental conditions. In fact, attempts have been made to characterize viruses by an apparent constant known as the temperature of inactivation or thermal death point. In more recent years, the inactivation reaction of viruses has been recognized as a process which takes place according to the laws of chemical kinetics. A stable virus is one which becomes inactivated at a slow or negligible rate, and an unstable virus is one which becomes inactivated at a more rapid rate. A virus with a high thermal death point and energy of activation is one for which a high temperature is required to obtain a rapid rate of inactivation and conversely, a virus with a low thermal death point and activation energy becomes inactivated rapidly at a lower temperature. The activation energy of 17,829 calories $mole^{-1}$ for the loss of infectivity of CTF virus is smaller than that observed with most RNA containing animal viruses. It is similar, however, to that reported for measles virus (18,000 calories $mole^{-1}$) (Black, 1959) and tobacco mosaic virus (Ginoza, 1958). These low values are incompatible with an inactivation mechanism

due to the rupture of hydrogen bonds. Ginoza (1958) postulated that inactivation energies of this order are due to the formation of phosphotriester linkages and subsequent hydrolysis and rupture of the RNA chain at the linkage point. Thermal agitation could result in the collapse of the tertiary structure of the RNA or lead to a simple chemical reaction in some of the bases of the nucleic acid chain (Woese, 1960). Pollard (1953) suggested the inactivation of viruses to result from the opening of the chains of the nucleic acid or protein to permit pendulum-like oscillations to take place and subsequent release of bound water. The denaturation of such a macromolecule as a virion can be thought of as a change in configuration toward greater stability but toward a form unsuited for biological functions.

Heat inactivation studies on the L cell-propagated CTF virus indicate that it is extremely sensitive to heat. Thermal decay of the virus appears to proceed at a rate similar to that characteristic of the other arboviruses which have been studied (Olitsky _et al_., 1950; Koprowski and Thomas, 1946; Anderson and Ada, 1953; Kudo _et al_., 1937; Cheng, 1961; Lepine, 1931). Thermal death of cell-propagated CTF virus was reached in 30 minutes at 60 C. It had previously been reported by Koprowski and Cox (1947a) that the thermal death point of mouse-brain CTF virus was reached in 30 minutes at 60 C. Their results indicated that the infectivity of the mouse-brain virus is relatively stable in serum-saline for

several days at 37 C. If the rate of inactivation of their preparations can be assumed to be linear through the first few logs, half-lives of 20.6 hours at 37.5 C and 29.5 hours at 25 C can be calculated from their data, for comparative purposes. These figures are quite different from the half-lives of 72 minutes at 25 C and 21 minutes at 37 C computed for the L cell-adapted virus in the present study. The differences in heat sensitivity may be due to (1) the selection of a more heat sensitive virus during cell culture adaption, or (2) the presence of some components of the mouse brain which may have a protective effect on the virus prepared *in vivo*.

Elementary concepts of kinetics can be used to interpret at least some of the phenomena observed relative to the thermal inactivation of viruses. Many viral inactivations seem to be first order reactions, that is reactions in which the number of particles undergoing change per unit time is directly proportional to the number of unchanged particles remaining at that time. In such reactions a straight line is obtained when the logarithm of the residual, unchanged material, is plotted against time. A striking feature of the heat inactivation curve of CTF virus is its apparently consistent biphasic nature. Deviations from first order kinetics are generally due to either aggregation of virions or to heterogeneity of their heat sensitivity. As pointed out by Woodroofe (1960) freezing of vaccinia virus in a

liquid state induces some physical or chemical changes in a small portion of the virus which serve to increase its heat stability. The virus used in the present investigation had been frozen prior to thermal inactivation and therefore, the biphasic nature of the inactivation curve might have been less pronounced if freshly prepared material had been used. On the other hand the resistance of a small fraction of the cell propagated virus to rapid inactivation could raise questions concerning the homogenity of the virus population. If the slope of the slow inactivating phase is extrapolated back to the origin the estimated size of the heat resistant fraction in the stock virus suspension varies from 0.2 to 50 per cent, depending upon the temperature of used (Figure 9). It would seem reasonable to believe that if the heat resistant fraction is determined by genetic characteristics the size of the fraction would be constant and unaffected by the rate of inactivation. Because the virus preparation was crude, a virus inactivating factor might have been liberated from the tissue debris during heating. An alternate explanation could be that heat inactivated CTF virus induces interferon to be produced in the assay system, and thereby making the true titer of the unchanged virus. The presence of a slow inactivating fraction in the virus suspension might also be due to clumps of virus which protect active virus inside the clumps.

Animal viruses may be divided into two groups

according to the presence or absence of peripheral structural lipids. Removal of such lipid from the virion results in the degradation of the particle and the loss of infectivity. Viruses which contain structural lipids are assembled at a cellular membrane. The capsomeres may be held together by lipid and nucleic acid located in the nucleoid, or the lipid may form an envelope around the virion and be separated from the capsid. Detailed information concerning the maturation of arboviruses are presently available only for WEE virus. Dulbecco and Vogt (1954b) followed the release of virus in single cell experiments and observed that new virus is released continuously and gradually rather than in a burst-like manner. These data were interpreted to indicate that the virus matures near the cell periphery or that complete virions are rapidly extruded from the interior of the cell. Morphologic studies of WEE-infected CE cells show clearly that maturation of the virus occurs at a cellular membrane (Morgan _et al_., 1961). Complete virus is formed in vesicles which move to the periphery of the cell and extrude mature virus from the cell. These workers suggested that the limiting viral membrane is a contribution from the cellular membrane. More recently, Pfefferkron and Hunter (1963) have shown by radioisotope techniques that the phospholipid of Sindbis virus is derived from the cellular membrane. It is reasonable to assume that lipid solvents, such as chloroform and DOC, inactivate arboviruses by degrading the membrane

surrounding the particle. Anderson and Ada (1953, 1959) demonstrated that IRNA preparations extracted from WN virus by phenol and DOC are similar. More recently, Richter and Wecker (1963) have shown that IRNA released from EEE virus-infected cells by DOC appears to be viral RNA prior to its incorporation into mature viral particles. They also observed that IRNA can be extracted from mature EEE virions by warm DOC solutions.

Under temperature and pH conditions optimal for the preservation of CTF viral infectivity, a solution of sodium desoxycholate or chloroform was found to rapidly inactivate the virus. Essentially the same findings were reported by Theiler (1957) and Sunaga _et al_. (1960) for other arboviruses. These workers suggested that the action of lipid solvents on the virion was to strip off the protein and release the IRNA. The infectivity of the IRNA was destroyed by contaminating ribonuclease present in the crude viral preparations. Since DOC has been shown to liberate IRNA from other arboviruses, it seems reasonable to assume that the inactivation of CTF virus by this chemical is due to a similar process. The inactivation of CTF virus by lipid solvents cannot be regarded as indirect evidence that lipids form part of the virus structure since these reagents can also denature the protein and thus render the virus incapable of being adsorbed onto the host cell. Clarification of the mechanisms involved in the inactivation of CTF virus by lipid solvents must await

electron microscopy studies on the virus and its maturation process and experimentation on the source and nature of the viral lipids.

Studies with metabolic inhibitors revealed evidence that CTF virus nucleic acid is probably not of the DNA type. *The rate of replication of virus was not inhibited by FUdR* or BUdR. The fluoro-derivative is an inhibitor of thymidylate synthesis (Cohen et al., 1958; Rich et al., 1960), whereas the bromo-derivative is known to be incorporated into DNA (Hakala, 1959). Both of these compounds cause marked inhibition of DNA-containing vaccinia virus (Salzman, 1960; Simon, 1961). FUdR is also an active inhibitor of adenovirus (Flanagan and Ginsberg, 1961) and herpes simplex virus multiplication (Kit and Dubbs, 1962b). On the other hand, the compound does not inhibit the replication of polio (Salzman, 1960), influenza, or Newcastle disease viruses (Hamparian et al., 1963) which are known to contain RNA. BUdR inhibits the multiplication of herpes simplex virus (Dubbs and Kit, 1964) but not that of Newcastle disease virus and poliovirus (Simon, 1961). The available evidence is fully in accord with the view that FUdR and BUdR inhibit DNA-, but not RNA-, containing viruses.

The synthesis of DNA-containing viruses is dependent upon the maintenance of accurate replication of DNA and its capacity to direct the formation of viral specific nucleic acid and protein. Compounds which inhibit or distort the

production of native DNA prevent the replication of viral DNA. Short nucleotide sequences of DNA can be produced *in vitro* in the absence of RNA, but concrete evidence is lacking to indicate that non-viral RNA can reproduce itself independently of DNA. It is now generally agreed that most RNA-containing viruses replicate independently of DNA synthesis. The investigations of Franklin and Baltimore (1962) and Baltimore (1964) have shown that Mengo and polio viruses induce a cytoplasmic, RNA-dependent RNA synthesis which is responsible for the formation of viral RNA. Because of these differences in the mechanism of replication of viral DNA and RNA, inhibitors have been useful in illucidating the mechanisms and rate of viral DNA replication. The use of these compounds may also provide insight into the native of the viral nucleic acid moiety.

The effects of BUdR on the replication of mammalian cells are well known. The material is readily incorporated into HeLa and L cell DNA during the first replication of the DNA (Hakala, 1959). Further cell division is greatly reduced because BUdR replaces thymidine in the DNA. The data from the present study confirm these observations. Thymidine is not very effective in protecting L cells against BUdR inhibition in an amethopterin medium. In general, the results indicate that thymidine and BUdR are used to approximately the same degree and an excess of one is necessary to overcome the effect of the other. In spite of the close similarity

between BUdR and thymidine with respect to spatial configuration and size, once the analogue has taken the place of thymidine in the DNA or L cells it creates an abnormality which prevents further synthesis of DNA. After only 24 hours, the effects of BUdR can not be reversed. Hakala (1959) suggested that the composition of the protein, which the non-dividing cells continue to synthesize might differ from those normally present in the cells. It could be speculated that a change of this sort is the immediate cause for the inability of BUdR-treated L cells to synthesize new DNA.

In the present investigations the addition of 0.3 mcg FUdR ml^{-1} to the culture medium immediately inhibited the growth of L cells. This inhibition was completely reversed by the addition of excess thymidine. A combination of FUdR and uridine did not support cell division. This indicated that the cessation in cell division induced by FUdR in the L cell monolayers was due specifically to thymidine starvation. These observations agree closely with those of Salzman and Sebring (1962). Till _et al_. (1962) postulated the following probable distribution of cells in the growth cycle after 16 hours of unbalanced growth in the presence of FUdR. Cells that were in the S-phase at the time the FUdR was added were randomly distributed through S and addition of the inhibitor prevented further progress of the cells through S as soon as the intracellular pools of thymidine are depleted. These cells, upon release from the thymidine block, proceed out of

S and through G_2, to mitosis. On the other hand, the cells not in S at the time FUdR was added proceed around the cycle until they reached the beginning of S, but they are prevented from entering S. These cells, though prevented from synthesizing DNA, continue to produce new RNA and protein.

Certain interesting observations were made in the studies on the synthesis of CTF virus in the presence of FUdR and BUdR. BUdR did not inhibit the rate of replication of CTF virus nor decrease the final titer of virus produced. Under conditions where DNA synthesis was prevented by FUdR, CTF virus was produced at a rate comparable to that in the control cultures, however the final yield of virus produced was about ten-fold lower. This indicated that the formation of CTF viral nucleic acid and protein is only 10 per cent as efficient in the presence of thymidylate starvation as in normal conditions. The addition of excess thymidine to the FUdR-containing medium did not reverse this inhibition. One is tempted to conclude that CTF virus contains RNA, and that the inhibition observed reflects the effects of several metabolic alterations induced by FUdR on viral replication. This may indicate that CTF virus is metabolically more fastidious than another RNA-containing virus, e.g., reovirus (Gomatos _et al_., 1962). Another possibility, which has not been explored in the present investigation, is that fluorouracil is incorporated into the RNA of CTF virus to make it nonfunctional. Salzman _et al_. (1962) observed that poliovirus

RNA synthesized in cells treated with FUdR contained FU. However, reovirus synthesized in the presence of the fluoridated pyrimidine did not contain FU (Gomatos *et al.*, 1962). These findings may well reflect distinct physiological differences in the mechanisms of the replication of different RNA-containing viruses. Sufficient information is not available at present, however, to draw any meaningful conclusions in this regard.

In experiments by Reich *et al.* (1962) and Reich *et al.* (1961) it was shown that actinomycin D completely inhibits the synthesis of both cellular RNA and DNA-containing vaccinia virus. The replication of poliovirus was unaffected by the antibiotic. More recently, Franklin and Baltimore (1962) reported that the replication of Mengovirus in cells treated with actinomycin D follows a normal pattern when the antibiotic is added at the time of infection. When L cells are pretreated with actinomycin their ability to support multiplication of reovirus and vaccinia virus is impaired to a similar degree (Gomatos *et al.*, 1962). If the antibiotic was present during the postinoculation period only, growth of vaccinia virus was considerably more suppressed than was that of reovirus. In contrast to this, the synthesis of Chikungunya virus in monolayers of CE cells is enhanced by pretreatment of the cells with actinomycin (Heller, 1963). Heller suggested that this enhancement is due to the suppression of interferon formation when the cells are pre-

treated with the antibiotic. The experiments with actinomycin described herein indicate that CTF virus replicates in the absence of DNA-directed RNA synthesis. The ability of pretreated cells to produce CTF virus was impaired to a degree directly proportional to the time of pretreatment. It is likely that under conditions of prolonged pretreatment, i.e., 24 hours, the metabolism of the cells was altered in several respects by the time the virus was introduced, thus obscuring the selective effects of the drug. It is indeed remarkable that cells so physiologically embarrassed can support the replication of virus. The interruption of cellular RNA synthesis induced by 1.0 mcg ml^{-1} of actinomycin resulted in a decrease in protein synthesis within four hours (Reich *et al.*, 1961). The ability of a virus to replicate in cells where both RNA and protein synthesis have been halted would indicate that the "machinery" involved in its replication must be sufficiently supplied from preexisting pools of amino acids and nucleotides, or alternately have a very efficient salvage mechanism. The inhibition of CTF viral replication in cells in which RNA synthesis has been arrested for 12 to 24 hours may reflect a need for protein during the early stages of viral synthesis. CTF virus may be metabolically more fastidious than some of the other RNA-containing viruses and require the presence of more protein and nucleic acid precursors. The present data indicate that CTF virus contains RNA and that the mechanism for its replication is independent of DNA and DNA-dependent RNA synthesis.

CHAPTER VI

SUMMARY

The Florio strain of CTF virus was adapted to proliferate in monolayers of L and FL cells. During the fourth to sixth passage of mouse-brain virus in L cells there was a 10^3-fold increase in the amount of infectious virus produced as compared with that in earlier cultures. All subsequent passages of the virus in L cells produced consistently high titers of virus. Cultures of FL cells infected with mouse-brain virus developed typical cytopathic changes and produced high titers of virus during the first and all subsequent passages. The virus which was produced in both of these *in vitro* systems was essentially as virulent for mice as the parent mouse-adapted virus.

Identity of the virus from the eighth and twelfth cell passages in L cells was confirmed by neutralization tests with specific immune serum. The cell culture-propagated virus was antigenically similar to, if not identical, with the original mouse-brain virus.

Parallel, comparative titrations of suckling mouse-, L cell-, and FL cell-propagated virus were performed in mice and in monolayers of L, FL, KB, HeLa, chick embryo, and cotton

rat kidney cells. Cytopathic effects and viral replication were seen in all cell cultures except HeLa and cotton rat kidney. Mice and L, FL, and chick embryo cell cultures were almost equally sensitive to infection. KB cells were intermediate in sensitivity. Growth curves of the L cell-adapted virus in monolayer cultures of L, FL, and CE cells were essentially the same. The titer of virus produced in CE cells was about ten-fold lower than that produced in L or FL cells. Virus multiplication in KB cells was slower and the titers of infectious virus which were produced were about 100 times lower than in the other cell types studied.

CTF virus was found to adsorb to monolayer cultures of L cells rapidly. Ninety per cent of the input virus was adsorbed to the cells during the first 30 minutes of incubation. After two hours of incubation only about one per cent of the input virus remained unadsorbed to the cells.

The eclipse period of CTF virus growth in L cells lasted six hours. The duration of the lag phase was found to be 10 hours and to be independent of the multiplicity of infection. Following infection at a low multiplicity, 0.2 and 0.4 $TCID_{50}$ $cell^{-1}$, the logarithmic rate of increase in virus lasted about 24 hours. Maximal titers of virus were produced 96 hours after infection. Infection of cells at multiplicities of 4.0 and 40.0 $TCID_{50}$ $cell^{-1}$ resulted in a single cycle of virus replication. The "one-step" growth curve of CTF virus had an exponential replication phase of

10 hours and maximal titers of virus were produced 35 hours after infection. Curves which describe increases in cell-associated and cell-released virus were separated by four hours. Furthermore, at the time the maximal yields of virus were produced, 80 to 90 per cent of the virus was released from the cells. The patterns of viral multiplication in monolayer cultures maintained in stationary racks and on the roller drum were essentially the same. Cytopathic changes developed sooner, and were more extensive, in the roller cultures than in the stationary monolayers. Synthesis of virus in spinner cultures of L cells followed approximately the same pattern as that in monolayer cultures.

CTF virus was stabilized during repeated freezing and thawing by the addition of either 50 per cent calf serum, 20 per cent glucose, 20 per cent glycerol, 10 per cent BSA, 2 per cent gelatin, or 20 mM glutamine to the diluent.

The optimal pH maintainance of CTF viral infectivity during storage in veronal-acetate and phosphate buffer was found to be 7.5 to 7.8.

Chloroform and sodium desoxycholate readily inactivated CTF virus.

The infectivity of cell culture-propagated CTF virus was rapidly destroyed by heat. Heat inactivation curves calculated at temperatures between 25 C and 56 C indicate that the viral population may be heterogeneous in its heat stability. Inactivation of the heat sensitive component of

the viral preparation seems to be a first order reaction, while the heat resistant fraction is inactivated at a constant, slow rate unrelated to the temperature of exposure. The half-life of CTF virus at 25, 37, 45, and 56 C was calculated to be 72, 21, 12 and 9 minutes, respectively. The Arrhenius constant for this system was approximately 17,800 calories $mole^{-1}$. This corresponds to a Q_{10} of 2.6.

Under conditions where cell division and RNA synthesis were inhibited by FUdR or BUdR, CTF virus replicated at a rate comparable with that in untreated cultures. However, final titers of virus produced in medium which contained FUdR were decreased about 10-fold. Monolayers of L cells which had been pretreated with FUdR were not quite as sensitive to infection with CTF virus as untreated cultures. The addition of excess thymidine to the cultures did not reverse this inhibition, suggesting that the effect was not specific for thymidylate starvation. FUdR-treated cultures were as sensitive to reovirus infection as were normal cells. Addition of either FUdR or BUdR to cultures of cells markedly suppressed the replication of herpes simplex virus. These data suggest that the nucleic acid of CTF virus is of the RNA type.

The replication of CTF virus in cells in which DNA-dependent RNA synthesis was suppressed by actinomycin D, was essentially the same as that in untreated cultures when the antibiotic was added at the time of infection. If cells were

pretreated for 12 hours prior to infection the rate of viral replication was similar to that observed in untreated cells. If, however, the cells were pretreated for 24 hours prior to infection, the lag period was extended six hours, total viral yields were reduced, and the rate of replication was decreased. These results are in agreement with the hypothesis that CTF viral RNA replicates independently of DNA-directed RNA synthesis The components of the CTF virion are probably formed through the mediation of an RNA-dependent RNA polymerase.

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