A PLAQUE TECHNIQUE FOR THE ENUMERATION OF INFECTIOUS PARTICLES OF COLORADO TICK FEVER VIRUS IN L-929 CELL MONOLAYERS

by

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INTRODUCTION

A. Statement of the problem

In order to study any virus adequately, a sensitive and reproducible assay procedure to determine the number of infectious particles in a given sample is valuable.

In the past, investigations of Colorado tick fever virus (CTFV) have been performed using the tube dilution method of assay in mammalian cell cultures. This procedure consisted of inoculating culture tubes containing monolayers of susceptible cells with serial tenfold dilutions of virus. After several days of incubation, cytopathic changes or effects (CPE) in the cells and pH changes of the medium were noted. The titer, in terms of the dilution of virus infecting 50% of the cell cultures inoculated ($CCID_{50}$), was then calculated. The CPE, in the tube dilution method of assaying virus, is qualitative and can be used quantitatively only when several dilutions and multiple cell cultures at each dilution are used. Another method of analysis, called plaque assay system, is more practical and perhaps more sensitive for use quantitatively. For example, plaque counts made from 15 to 30 petri dishes or plates would be as significant as the results obtained from 1200 to 2400 culture tubes (Dulbecco and Vogt, 1954a).

In the plaque assay procedure cell monolayers are infected with dilutions of virus suspension. These infected monolayers are then overlaid with a semisolid nutrient solution which helps to limit

the spread of virus from an infected cell to neighboring cells. As a result local macroscopic areas of CPE, or plaques, develop. If the monolayer of cells is then stained with a vital stain such as neutral red, the plaques become visible as clear areas in a faint red background.

According to the one particle theory, each focus of infection is initated by a single virus particle or aggregation of particles not divisible by dilution. Thus, the plaque assay method enables one to determine the number of infectious particles, or plaque-forming units (PFU's), per unit volume. The value of such a system is evident. For example, during purification procedures the loss or gain of infectious virus per unit volume following each physical or chemical manipulation of the virus suspension can be followed with accuracy. Also, any work related to the stability of a virus to certain chemical and physical effects would give more significant data. Furthermore, studies related to attachment or adsorption kinetics, single-step growth curves, virus mutants, general characterization of viruses, etc. are possible and more meaningful with this system.

A plaque assay method for enumerating infectious CTFV particles was described in which the A-1 embryonic hamster fibroblast strain was used as the host cell (Deig and Watkins, 1964). Early attempts to reproduce these experiments were unsuccessful.[1] Furthermore, personal efforts to acquire the A-1 cell strain were unfruitful. Thus in order to study this virus adequately, it became necessary to develop another technique in which a different indicator cell was employed.

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[1] Gardner, C. E., "Personal Communication." 1964

The need for a plaque assay procedure using a stable cell line became more acute when it was noted that the A-1 cell strain had undergone various changes following numerous subcultures. These changes apparently affected both the susceptibility of the cells to CTFV and their viability under an agar overlay medium.[1]

B. Review of the pertinent literature on Colorado tick fever virus

1. Early history

An account of clinical symptoms of Colorado tick fever was first given by Becker (1930a). His description and classification of this disease indicated that the causative agent was either an attenuated strain of Rickettsia rickettsii or some entirely different agent (Becker, 1930b). Prior to this time, the names "Mountain tick fever" and "American mountain fever" had been used synonymously to describe this disease. A decade later, Topping (1940) conducted a study in which certain epidemiological observations were made while attempting to isolate the causative agent from afflicted individuals. This investigator found that tick bites were always associated with the disease, and all evidence pointed to the tick as the transmitting agent. In this study, guinea pigs, monkeys, rats, mice, and chicken embryos were inoculated with various preparations of blood, serum, or spinal fluid from patients having Colorado tick fever, but all attempts to isolate or observe spirochetes, parasites, or rickettsiae from these experimental animals were unsucessful.

2. Isolation of CTFV

Florio, Stewart, and Mugrage (1944) were able to isolate and pass

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[1] Deig, F. E., "Personal Communication." 1966

the virus in human volunteers and hamsters by injecting blood or serum from a patient in the febrile phase of the disease. These same workers later determined that the etiological agent of Colorado tick fever was a virus, and that the agent was filterable through a membrane with an average pore size of 24 mμ (Florio, Stewart, and Mugrage, 1946).

Koprowski and Cox (1946) adapted CTFV to mice and developing chick embryos by serial blind passage. Virus was found in brains, spinal cords, hearts, spleens, lungs, and blood of infected mice. The highest concentrations were found in brains and spinal cords. These same authors (1947) found that after 14 days of age, mice began to develop a natural resistance to infection with increased age. For example, 50% mortality was obtained if 21-day-old mice were inoculated with a dose giving 100% mortality in newborn or suckling mice. After the mice were 28 days of age, they no longer died when given massive doses of CTFV. The higher susceptibility of the suckling mouse obviously made this animal the principal means of isolation of the virus from naturally infected ticks and humans (Oliphant and Tibbs, 1950). More recently the usefulness of tissue culture for such isolations has been recognized (Pickens and Luoto, 1958).

3. Transmission and classification of CTFV

Even though it had been known for several years that ticks transmitted the etiological agent of Colorado tick fever, it was not until 1950 that the virus was isolated from wood ticks (Dermacentor andersoni) found in nature (Florio, Miller, and Mugrage, 1950a). This established CTFV as the first known tick-borne virus in the Western Hemisphere. These investigators also isolated the virus from D. variabilis and re-

ported that both of the above-mentioned ticks were capable of transmitting CTFV transovarially (Florio et al, 1950a; Florio, Miller, and Mugrage, 1950b). Recently, Eklund, Kohls, and Kennedy (1962) reported that D. andersoni appeared to be the principal vector of this agent as far as transmission to human beings is concerned. Furthermore, they were unable to show that transovarial passage of this virus occurred.

CTFV is transmitted by an arthropod vector and therefore, is classified as an arthropod-borne virus (arbovirus). Neutralization studies (Koprowski and Cox, 1947) and complement-fixation tests (DeBoer et al, 1947) revealed that this virus was not antigenically related to any other arbovirus. It is, therefore, classified as an ungrouped arbovirus.

4. Tissue culture studies

Pickens and Luoto (1958) were the first investigators to describe the isolation and propagation of CTFV in tissue culture. These workers were able to isolate CTFV from infected mouse brains, ticks, and human and monkey blood samples in cultures of KB human epithelial carcinoma cells. In some cases, a blind passage was required before the virus would produce CPE in these cultures. In comcomitant attempts to propagate the virus, it was found that the titer of virus increased after three to four serial passages in tissue culture. Finally, Pickens and Luoto (1958) observed that the time required for CPE to develop was proportional to the amount of virus inoculated. High concentrations of CTFV gave complete CPE in three days while lower concentrations required up to eight days to produce similar changes.

A plaque assay procedure for CTFV, in which the A-1 embryonic hamster tissue cell strain was used as host, was described by Dieg and Watkins (1964). According to these investigators, infectivity of this virus for A-1 cells is affected by several conditions of the assay system. For example, the efficiency of virus attachment or adsorption to cells was increased by adding fetal calf serum to the media, allowing attachment to take place at 25 C, and by maintaining the pH of the attachment medium between 7.1 and 8.1. It was also reported that the incorporation of fetal calf serum in the overlay medium allowed maximum plaque production. Similarly, plaque production was maximal if the concentration of $NaHCO_3$ in the overlay medium was less than 0.22%. Under optimal conditions plaques measuring 4 mm in diameter were obtained after incubation at 37 C for 4 to 6 days.

Trent and Scott (1964) compared the sensitivity of several cultured cell lines to infection by CTFV. The virus was found to induce CPE in L-929 mouse fibroblasts, FL human amnion cells, and primary chicken embryo cells as well as KB cells. The virus underwent adaptation when it was passed serially in L-929 cells; there was a sudden 1000-fold increase in measureable virus yield after the fourth passage. This level of virus replication was then maintained in all subsequent passages. A similar phenomenon was also noted by Pickens and Luoto (1958) in infected KB cells.

The rate of attachment of the cell-adapted virus to L-929 cells and its growth in these cells were also investigated (Trent and Scott, 1964). Approximately 90% of the virus had attached in 15 min and 99% in 30 min. A ten-to twelve-hr eclipse period followed, and maximum rate of viral synthesis was attained within an additional 10 to 24 hr,

depending upon the multiplicity of infection. Virus production reached its peak 35 hrs after infection was initiated. The average yield of virus was 125 $CCID_{50}$ per cell.

5. Chemical and physical properties of CTFV

Florio et al, (1946) originally reported that the hamster-adapted strain of CTFV was approximately 10 mμ in diameter. A more complete study, using filtration methods for estimating the size of the mouse brain-adapted strain of the virus, indicated that it was between 35 mμ and 50 mμ in diameter (Koprowski and Cox, 1947).

Infectivity of mouse-adapted virus was lost after 30 min at 60 C or 3 to 4 days at 37 C. Virus-containing mouse brain preparations stored at room temperature remained infective for some time; however, in 25 days, the titer of this virus decreased from $10^{6.85}LD_{50}$ to $10^{4.0}LD_{50}$/g of tissue as measured in suckling mice (Koprowski and Cox, 1947). The L-929 cell adapted-virus appeared to be even more heat labile, since the half life of this CTFV preparation was 21 min at 37 C and 72 min at 25 C (Trent and Scott, 1966). However, these results may indeed be due to differences in experimental conditions. For example, the L-929 cell-adapted virus was suspended in tissue culture medium while the earlier observations of Koprowski and Cox were made using a virus-mouse brain preparation.

Early investigators found that addition of serum would adequately stabilize the virus during manipulation and storage (Koprowski and Cox, 1947). More recent studies have indicated that glucose, glycerol, bovine serum albumin, gelatin, and glutamine as well as serum are capable of stabilizing CTFV (Trent and Scott, 1966).

While 5-fluoro-2-deoxyuridine and 5-bromo-2-deoxyuridine are known to cause a marked inhibition of DNA-containing viruses, they do not greatly affect the replication of viruses containing RNA (Spalzman N. P., 1960; and Hamparian, Hilleman, and Ketler, 1963). Experimental findings indicated that these compounds did not appreciably change the rate of formation of CTFV in a tissue culture system. Additional experiments demonstrated that Actinomycin D (known to inhibit DNA-dependent RNA synthesis) had no direct effect on viral replication. Both of the above experiments indicate that CTFV is an RNA-containing virus.(Trent and Scott, 1966).

C. Review of the plaque assay technique and factors affecting its sensitivity

1. Introduction

Standard plaque assay procedures for enumerating bacterial viruses were first described by Ellis and Delbruck in 1939. It was more than a decade later before a similar method of assaying animal viruses was reported (Dulbecco, 1952). Many modifications of this procedure have been employed in titrating various viruses. These changes were made to obtain plaques in systems where known techniques failed, to increase the sensitivity of the assay, or just as a matter of convenience and ease of operation.

2. The indicator cell

The selection of a suitable indicator cell is an important factor in designing a plaque assay procedure. The cells must be susceptible to the virus and, preferably, undergo changes detectable by the differences in reaction to vital stains observed between infected and uninfected cells.

Usually, cells in which CPE is induced by a virus in a liquid medium are chosen as indicator cells for use in a plaque assay technique for that virus. However, in certain cases these cells show no CPE when incubated under an agar overlay system (Hsiung and Melnick, 1957). In other instances, the agar overlay would permit CPE to develop without the formation of plaques (Rapp et al, 1959).

In general there are two major reasons for differences in the susceptibility exhibited by lines of cells to a given virus. These differences may be due to variations in genetic or embryological origin of the cells. Alternatively, they may be variations in susceptibility due to physiological conditions. Cloned cell lines may be preferred for plaque assays because of uniform degree of susceptibility. Some workers (Vogt and Dulbecco, 1958) have found that uncloned He La cell cultures contained cells resistant to infection by poliovirus. It was later reported that the difference in susceptibility was due to a heritable cellular difference and not host range virus variation (Darnell and Sawyer, 1959).

Physiological conditions play a major role in cell susceptibility as measured by the plaque assay system. For example, it was found that if the temperature of incubation during the 24 hr previous to viral attachment was 14 C or if required nutrients were absent from growth medium, there was a decrease in susceptibility of cells to Rous sarcoma virus (RSV) (Temin and Rubin, 1958). Virus yield was substantially affected by the pH of the culturing medium, and this was found to be related to the decreased metabolic rate of the cells (Gifford, Robertson, and Syverton, 1956). Similar studies gave evidence that He La cells become less susceptible to certain strains of polioviruses

under acidic conditions (Hsiung and Melnick, 1958).

3. Conditions of attachment

A single washing of cell monolayers before and after infection has been reported to have little or no effect on the attachment rate (Kaplan, 1957). On the other hand, multiple washings after the adsorption period have been found to cause a deteriorating affect on the cell monolayer (Bachrach et al, 1957; Nagai and Hammon, 1964).

The solution used for attachment and dilution of stock virus should contain adequate concentrations of various cations and serum. Allison and Valentine (1960) have shown that virus attachment to cell monolayers is dependent on the concentration of cations and is not depressed by the presence of proteins in the medium. In addition, these investigators found that the presence of protein in this medium reduced the amount of virus adsorbed to glass or plastic surfaces.

The ability of adenovirus to adsorb to monolayers of He La cells has been reported to be dependent on the presence of serum. The efficiency of attachment could be promoted by the inclusion of chicken or rabbit serum in the attachment medium. If human or horse serum was used, the amount of virus adsorbed to the cells was greatly reduced. (Philipson, 1961).

In general, a pH between 5.5 and 7.6 will allow efficient viral attachment to monolayered cells (Allison and Valentine, 1960). However, CTFV appears to require a pH above 7 and below 8 for attachment to cells, (Dieg and Watkins, 1964).

The volume of the virus inoculum affects the efficiency of attachment. For example, Bachrach et al, (1957) found that whereas 0.1 ml of a suspension of foot-and-mouth disease virus (FMDV) yielded

62 PFU, a 50% reduction in the number of plaques was obtained when the same amount of virus was suspended in 0.4 ml. When the volume was increased to 6.4 ml, only 10 plaques developed. With RSV it was shown that as the adsorption volume was increased from 0.1 ml to 0.8 ml there was a 65 to 75% decrease in efficiency of attachment (Temin and Rubin, 1958). A similar result was also observed by Porterfield and Allison (1960) with poxviruses.

It is apparent from these results that the smallest possible volume of the diluent that protects the cells from drying results in the most efficient attachment of virus to cells. Generally, if the attachment period requires several hours, 0.1 ml of virus suspension does not adequately cover and prevent the drying of a cell monolayer in a 60 mm-diameter plate. The problem is resolved by gently rocking the dish periodically. Valentine and Allison (1959) reported that this rocking technique has no effect on the rates of viral attachment.

4. Components of the overlay medium

An overlay consisting of agar, nutrient medium, serum, and a buffering system is generally utilized in the plaque assay procedure. These constituents can affect the physiological condition of the cells and in turn, affect the sensitivity of the assay system.

A. Agars

A technique for washing agar-agar to remove certain factors which were apparently inhibitory to plaque formation was described by Dulbecco and Vogt (1954a). Plaques produced by mengovirus mutants were larger under overlays containing washed Noble agar than those containing either unwashed Noble or purified agars (Campbell and Colter, 1965).

Noble agar appeared to allow normal plaque production to occur in

the assay of FMDV (Bachrach et al, 1957), myxoma virus (Schwerdt and Schwerdt, 1962), and encepholomyocarditis virus (EMC)(Takemoto and Liebhaber, 1961). As mentioned above, Noble agar reduced the size of mengovirus plaques. However, if protamine was added to media containing this agar, the plaque size of one mutant strain could be increased from 0.2 mm to 6.0 mm in diameter (Campbell and Colter, 1965).

Purified agar has been used sucessfully in the overlay media employed to assay fibroma and myxoma viruses (Padgett, Moore, and Walker, 1962).

Ionagar is another commercially washed agar which may prove useful in plaque assay procedures (Cooper, 1961).

Dulbecco and Vogt (1954a) recommended that 1.3% agar be used in the plaque assay procedure. The lowest concentration giving a firm gel (0.9%) is now commonly used by many investigators (Dougherty, 1964). Because the neutral red staining technique is often unsatisfactory, other concentrations of agar, 0.6% or less, have been used with different staining procedures. If the cells are firmly attached to the container, the 0.6% agar and virus-killed cells can be decanted. This would leave only the uninfected cell sheet which can then be fixed and stained to allow the counting of plaques (Holland and McLaren, 1959).

Plaques were also found to develop much sooner under this concentration of agar. The use of similar (Schwerdt and Schwerdt, 1962) or even lower agar concentrations (Hotchin, Deibel, and Benson, 1960) for other assay systems have also been described.

Although agar can commonly be used in overlay media, it contains substances which may inhibit plaque or virus production. Takemori and Nomura (1960) found that extracts of agar would inhibit the replication

of small-plaque-producing mutant strains of poliovirus. The size of EMC virus plaques was also found to be suppressed by inhibitors found in agar (Takemoto and Liebhaber, 1961).

It was reported that these inhibitors did not inactivate poliovirus by directly combining with the virus particles. The indications were that viral replication was inhibited by alterations in the metabolism of susceptible host cells (Takemori and Nomura, 1960). In recent investigations with mengovirus, these inhibitors (sulphated polysaccharides) were found to block cell-virus interaction by directly immobilizing the virus particle. Plaque formation was not completely inhibited, but smaller plaques were observed. Protamine added to the agar overlay medium apparently neutralized this inhibitory affect and allowed development of larger plaques (Colter, Davies, and Campbell, 1964; Campbell and Colter, 1965).

In some instances agar was found to prevent CPE and demonstrable virus replication. This necessitated the utilization of other substances for localizing infection in the monolayers. Methylcellulose, for example, has been found satisfactory for use in assaying fowl plague virus (Hotchin, 1955), measles (Rapp et al, 1959), and several group B arboviruses (Schulze and Schlesinger, 1963).

B. Nutrients

Incorporation of the proper kinds and concentrations of nutrients in the overlay may be important for maintaining the physiological condition of the cells. If this is indeed the case, the nutritional content of the overlay medium may be a critical factor in determining the sensitivity of a plaque assay procedure. Evidence supporting this was

originally reported by Dulbecco and Vogt (1954b) who found that Earle's balanced salt solution (EBSS), with or without glucose, was incapable of allowing plaque production by Western equine encephalomyelitis (WEE) virus in monolayers of chicken embryo cells. If chicken serum was added to the EBSS, plaques were produced. Similar findings were obtained with RSV. In this system, cell monolayers starved of glucose or glutamine for 24 hr were found to be less susceptible to virus infection than monolayers in medium containing normal concentrations of these constituents (Temin and Rubin, 1958).

The ability of adenovirus to form plaques in KB cells was reported to be dependent on the source and amounts of sera present in the overlay. Results of experiments involving various concentrations and combinations of rabbit, calf, and chicken sera were compared to determine the most sensitive system. A combination of 2% chicken and 1% calf serum was found to give the optimal number and size of plaques (Philipson, 1961).

Weeks of incubation are required before plaques develop in monolayers infected with polyoma virus. Therefore, two additions of overlay medium are necessary to maintain the cells until plaques develop. A recent technique used with simian virus 40 in fetal rhesus kidney cells, in which a 16-day incubation period is required to obtain plaques, has desirable characteristics. Additional nutrients are added in liquid form. The expended medium can be easily removed before each new feeding. This allows several feedings without continuously adding to the depth of the overlay medium (Ushijima and Gardner, 1967).

C. Control of pH

A report discussing the yield of poliovirus from He La cells grown

at various pH ranges appeared in 1956. In these studies it was found that low virus yields at decreased (below 7.2) or increased (above 7.8) pH levels were related to a decrease in metabolic rate of the host cell cultures (Gifford et al, 1956). These findings may have stimulated interest in determining the significance of the role played by the buffer system in determining the sensitivity of the plaque assay system.

Mutants of polioviruses have been isolated that have a low plating efficiency in the presence of 0.11% $NaHCO_3$. These mutants produced only 6×10^3 PFU/ml under an agar overlay at pH of 6.6, and 3×10^4 PFU/ml at pH 6.8. However, if the pH was between 7.0 and 7.7, approximately 1.4×10^8 PFU/ml were produced under an agar overlay. These mutants, called d mutants because of delayed plaque production, exhibited dependency on bicarbonate concentrations. Results of experiments conducted with d mutants indicated that the delay in plaque production was not due to a decreased attachment rate. Rather, it appeared to be due to a gradual decrease in susceptibility of the cells in this system to the d mutants as a result of some influence exerted on the cells under the acid overlay. It was also noted that areas infected by the d mutants did not show signs of infection under an acidic agar overlay for long periods of time. If the pH of the agar was raised, the cells apparently became susceptible again and plaques rapidly formed (Vogt, Dulbecco, and Wenner, 1957).

Similar studies of the effects of $NaHCO_3$ concentrations on plaque formation were determined with virulent and attentuated strains of poliovirus. Four different concentrations of $NaHCO_3$ ranging from 0.11% to 0.9% were used in the overlay media. The plaques of virulent strains were smallest at the lowest concentration and became increasingly larger

as concentrations increased. Slightly higher plaque counts were observed in most instances as the concentration was increased from 0.11% to 0.45%. The attenuated strains, on the other hand, produced larger plaques at higher concentrations and no plaques at all at the lowest concentration. Attachment and replication of polioviruses were not greatly affected by varying the $NaHCO_3$ concentration in liquid medium. On the basis of these studies, it was determined that the bicarbonate concentration rather than the initial pH of the overlay exerted the affect on plaque development and size (Hsiung and Melnick, 1958).

The concentration of $NaHCO_3$ used in the plaque assay procedure for Japanese B encephalitis virus appeared to affect the number and often the size of the plaques obtained. Optimum sensitivity regarding number and size of plaques appeared greatest when 0.1% $NaHCO_3$ was used. If higher or lower concentrations were used, a decreased efficiency of this assay system was observed (Nagai and Hammon, 1964). Sodium bicarbonate has been eliminated in the plaque assay of several viruses and substituted with tris (hydroxymethyl) aminomethane buffer, which was found to allow for more efficient plaque production (Porterfield and Allison, 1960).

The volume of overlay medium has also been reported to exert influences on the development of plaques (Vogt et al, 1957). An increase in thickness of the overlay from 0.8 mm to 1.2 mm resulted in a decreased titer of the d mutant strain virus from 7.6×10^7 to 3.8×10^4 PFU/ml. In such a system, maintaining a constant volume of overlay and an even depth of agar over the entire monolayer would be necessary in order to obtain accuracy. Gifford and Syverton (1957) reported that plaques

developed slower as agar depth increased because a longer latent period was required for development of virus under conditions of low O_2 tension.

The depths of agar which will allow plaques to develop are different for several group A arboviruses. These differences were found using the same host cell and are ascribed to differences in oxygen requirements necessary for virus replication (Baron, Porterfield, and Isaacs, 1961).

MATERIALS AND METHODS

Accepted guidelines for washing and care of all glassware used in the cell culture studies were followed (Paul, 1961). The detergent used was 7-X (Linbro Chemical Company, New Haven, Conn.).

A. Solutions and media

Unless otherwise stated, all solutions and media were stored at 2 to 4 C.

1. Antibiotics

A stock solution containing a mixture of 20,000 units potassium penicillin G (Chas. Pfizer and Co., New York) and 20,000μg streptomycin sulfate (E. R. Squibb and Sons, New York)/ml in sterile deionized distilled water (DDW) was stored at -20 C. Five-tenths ml of this solution was added to each 100 ml of medium to give concentrations of 100 units penicillin and 100μg streptomycin/ml of medium.

Amphotericin B (Fungizone) (E. R. Squibb and Sons, New York) was dissolved in sterile DDW at a concentration of 12,000μg/ml. This stock solution was stored at -20 C. For use, 0.33 ml was added to 100 ml of medium to give a concentration of 40μg amphotericin B/ml.

Kanamycin sulfate (donated by Bristol Laboratories, Schenectady) was dissolved in sterile DDW at 20,000μg/ml and stored at -20 C. Five-tenths ml in 100 ml of medium gave a final concentration of 100μg of kanamycin/ml.

Unless otherwise stated, all cell culture media and salt solutions contained the penicillin and streptomycin mixture. Kanamycin and amphotericin B were only included in medium used for culturing stock cells

and were never added to the agar overlay media.

2. Sodium bicarbonate

A stock solution containing 7.5% $NaHCO_3$ was prepared monthly and sterilized by Millipore (Millipore Filter Corp., Bedford, Mass.) filtration.

3. Hanks' balanced salt solution (HBSS) (Hanks and Wallace, 1949)

Twenty-times concentrated stock solutions for making HBSS were prepared as follows:

Solution A

Components	Amount
NaCl	160.0 g
KCl	8.00 g
$MgSO_4 \cdot 7H_2O$	2.00 g
$MgCl_2 \cdot 6H_2O$	2.00 g
$CaCl_2$	2.80 g

The first four components were dissolved in about 800 ml DDW. A separate solution containing the $CaCl_2$ in 100 ml DDW was then added to the solution containing the other ingredients. Finally, the total volume was brought to 1,000 ml with DDW.

Solution B

Components	Amount
$Na_2HPO_4 \cdot 7H_2O$	1.80 g
KH_2PO_4	1.20 g
dextrose	20.00 g
0.5% phenol red solution[1]	80.0 ml
DDW, quantity sufficient to make	1 liter

[1] Phenol red solution was purchased from Microbiological Associates, Inc., Albany, California (MAI).

Each solution was dispensed into 100-ml bottles and then sterilized at 115 C for 10 min.

Single-strength HBSS was prepared by adding 5 ml of solution A, 5 ml of solution B, and 0.5 ml of 7.5% $NaHCO_3$ to 90 ml sterile DDW.

4. Earle's balanced salt solution (EBSS) (Earle, 1943)

The composition of EBSS is given in the appendix. This solution was purchased as a sterile tenfold concentrate, without $NaHCO_3$, from Baltimore Biological Laboratories, Baltimore (BBL).

For use as single-strength EBSS, 10 ml of the stock solution and 3 ml of 7.5% $NaHCO_3$ were aseptically added to 87 ml of sterile DDW.

When double-strength EBSS was required for plaque assay, it was made by adding 20 ml EBSS (10X) to 77 ml of sterile DDW.

5. Saline A (Marcus, Cieciura, and Puck, 1956)

A tenfold concentrate of this solution was made in the following manner:

Components	Amount
NaCl	80.00 g
KCl	4.00 g
dextrose	10.00 g
0.5% phenol red	40.00 ml
DDW, quantity sufficient to make	1 liter

This solution was sterilized by Millipore filtration and dispensed into 100-ml bottles for storage.

Saline A was prepared for use in cell culture by adding 10 ml of the above stock solution and 0.5 ml of 7.5% $NaHCO_3$ to 88 ml sterile DDW.

6. Phosphate buffered saline (PBS) (Dulbecco and Vogt, 1954a)

PBS was prepared as follows:

Solution A

Components	Amount
NaCl	16.00 g
KCl	0.40 g
Na_2HPO_4	3.30 g
KH_2PO_4	0.40 g
DDW	1,500 ml

Solution B

$MgCl_2$	0.20 g
DDW	200 ml

Solution C

$CaCl_2$	0.20 g
DDW	200 ml

These solutions were autoclaved for 20 min at 15 psi. After cooling to 5 C, the final solution was prepared by adding solution B and solution C in order to solution A, and then bringing the volume to 2 liters with sterile DDW. The resulting solution was dispensed into sterile 8-oz prescription bottles for storage.

7. Basal medium Eagle (BME) (Eagle 1955a; Eagle et al, 1956)

The chemical composition of BME is listed in the appendix.

The salt, vitamin and amino acid components of this medium were purchased as working solutions from MAI. Single-strength BME was prepared as follows:

Order	Component
1	83.0 ml sterile DDW
2	10.0 ml EBSS (10X)
3	1.00 ml BME amino acids (100X)
4	0.14 ml N NaOH
5	1.00 ml BME vitamins (100X)
6	1.00 ml 200 mM L-glutamine
7	0.50 ml penicillin-streptomycin mixture (200X)
8	0.5 ml kanamycin sulfate (200X)
9	3.0 ml 7.5% $NaHCO_3$
10	0.33 ml amphotericin B (300X)

The double-strength BME used for plaque assays contained the following constituents:

Order	Component
1	71.0 ml sterile DDW
2	20.0 ml EBSS (10X)
3	2.00 ml BME amino acids (100X)
4	0.28 ml N NaOH
5	2.00 ml BME vitamins (100X)
6	2.00 ml 200 mM L-glutamine
7	1.00 ml penicillin-streptomycin mixture (200X)

The 200 mM L-glutamine and BME vitamin concentrates were stored at -20 C.

8. Minimum essential medium (MEM) (Eagle, 1959)

The chemical composition of MEM is listed in the appendix.

Concentrated solutions of "essential" amino acids(EAA), "non-essential" amino acids (NEAA), vitamins and L-glutamine were purchased from MAI.

A single-strength medium was prepared in the following manner:

Order	Components
1	81.0 ml sterile DDW
2	10.00 ml EBSS (10X)
3	2.00 ml MEM-EAA (50X)
4	1.00 ml MEM-NEAA (100X)
5	neutralize to pH of 7 with N NaOH
6	1.00 ml MEM vitamins (100X)
7	1.00 ml 100 mM sodium pyruvate
8	1.00 ml 200 mM L-glutamine
9	0.50 ml penicillin-streptomycin (200X)
10	0.50 ml kanamycin sulfate (200X)
11	3.00 ml 7.5% $NaHCO_3$
12	0.33 ml amphotericin B (300X)

Double-strength MEM, when used in the overlay medium was prepared as shown on following page.

Double-strength MEM:

Order	Components
1	68.0 ml sterile DDW
2	20.0 ml EBSS (10X)
3	4.00 ml MEM-EAA (50X)
4	2.00 ml MEM-NEAA (100X)
5	neutralize to pH of 7.0 with N NaOH
6	2.00 ml MEM vitamins (100X)
7	2.00 ml 100 mM sodium pyruvate
8	2.00 ml 200 mM L-glutamine
9	1.00 ml penicillin-streptomycin (200X)

The MEM vitamin solution was stored at -20 C.

9. Medium 199 or M199 (Morgan, Morton and Parker, 1950; Salk, Youngner, and Ward, 1954.

A tenfold concentrate of M199 without $NaHCO_3$ was purchased from MAI. A single-strength solution of M199 was prepared by adding 10 ml of the M199 (10X) working solution, 2.67 ml $NaHCO_3$ and all antibiotics to 87 ml sterile DDW.

The double-strength M199 for use in preparing the overlay medium consisted of 20 ml M199 (10X) in 77 ml sterile DDW.

The components of M199 are listed in the appendix.

10. Lactalbumin hydrolysate media

a) Lactalbumin hydrolysate, 0.5% in HBSS (LaH).

This medium was prepared by dissolving 5 g of lactabumin hydrolysate [Nutritional Biochemicals Corp., Cleveland (NBC)] in approximately 800 ml DDW. After the volume was brought to 900 ml with DDW, 90-ml aliquots were dispensed into bottles and autoclaved at 10 psi for 10 min.

These bottles were then allowed to cool to room temperature before they were stored at 2 to 4 C.

To prepare this medium for use, 5 ml each of sterile HBSS twentyfold concentrated stock solutions A and B were added to each bottle of the lactalbumin hydrolysate solution. Finally, 0.47 ml of 7.5% $NaHCO_3$ was added.

b) Lactabumin hydrolysate, 0.5% in EBSS (LaE)

The procedure in preparing LaE was similar to that used in preparing LaH. However, the 5 g was dissolved in DDW brought to a final volume of 870 ml, and 87-ml aliquots were dispensed into each bottle before autoclaving.

Preparations of LaE required the addition of sterile 10 ml EBSS tenfold concentrate and 3 ml of 7.5% $NaHCO_3$ to the bottle containing the sterile 87 ml of lactabumin hydrolysate solution.

In order to prepare double-strength LaE for a plaque assay, the following procedure was employed:

Ten grams of lactabumin hydrolysate were dissolved in approximately 650 ml of DDW. The volume was then brought to 770 ml with DDW, and 77 ml aliquots were placed in 6-oz prescription bottles for autoclaving at 115 C for 10 min. This was then cooled and stored until needed. When double-strength LaE was required for plaque assays, 20 ml EBSS tenfold concentrate was added to 77 ml of the lactalbumin hydrolysate solution.

c) Lactalbumin hydrolysate (0.5%) and yeast-extract (0.1%) in EBSS (LaEYe)

This medium is similar to LaE except that 1 g of yeast extract p/liter (NBC) is included. All other procedures for preparing this medium are identical to the preparation of LaE.

11. Trypsin

Trypsin (1-300 grade) was obtained from NBC. A stock solution of 0.25% trypsin in Saline A was filter-sterilized and stored in 1-ml aliquots at -20 C. This solution was diluted tenfold in Saline A for use with cell cultures.

12. Serum

Lyophilized calf serum was purchased from BBL and stored at -20 C. It was then reconstituted with sterile DDW shortly before use.

Rabbit serum, used in preparing the virus stock suspensions, was purchased from MAI and stored at -20 C.

13. Neutral red

Neutral red was dissolved in 0.85% NaCl at a concentration of 0.0001% and sterilized by autoclaving at 10 psi for 10 min.

14. Crystal violet

A.1.4% solution of crystal violet was diluted 1:100 in tap water for use in staining the cell monolayers.

15. Immune serum

Reference hyper hyperimmune anti-CTFV serum was kindly supplied by Dr. Leo Thomas, United States Public Health Service Laboratories, Hamilton, Montana.

16. Agar

Bacto-agar, purified agar, and Noble agar were purchased from Difco Laboratories, Detroit. Oxoid Ionagar no. 2 was obtained from Consolidated Laboratories, Inc., Chicago Heights, Ill.

A modification of Dulbecco and Vogt's technique (1954a) was used for washing the Bacto-agar. One-half pound of this agar was washed 25 times in tap water, 10 times in DDW (using 5 liters/wash) and 3

times in acetone (2 liters/wash). The acetone was removed by filtering through a Buchner funnel. The agar was then dried under 3 desk lamps and collected when completely dry.

The agars were diluted in DDW to give solutions which were twice the concentration, of agar, desired in the final overlay mixture. They were autoclaved at 20 psi for 20 min and cooled to 42 C in a water-bath immediately prior to use.

B. Virus

The GS-20 strain of CTFV (Gerloff and Eklund, 1959) was used in all experiments. It was derived from the Florio- strain which had undergone 30 serial passages in hamsters and 57 intracerebral passages in suckling mice; Gerloff and Eklund passed the virus 7 times in embryonated eggs and 4 more times in suckling mice. It was then designated as the GS-20 strain. Gardner (1962) subsequently obtained the virus from Dr. Gerloff and passed it 6 times in suckling mice. Two additional suckling mouse passages were made by the present author, and the last of these was used in the experiments reported herein.

A modification of Gardner's (1962) technique was used in making these passages of the virus. An inoculum of 0.01 ml of 10^{-3} dilution of the virus in 0.85% NaCl containing 10% normal rabbit serum (NRSS) was injected intracerebrally into 2-to 4-day old Swiss albino mice. When the mice became moribund after 3 days, they were killed by freezing. The brains were harvested, weighed, and ground in a sterile mortar and pestle with alundum. A 10% suspension (weight/volume) of the tissue was made in whole NRS and centrifuged at 2000 rpm for 20 min at 4 C. The supernatant, which contained the virus, was removed and dispensed into ampoules. These were sealed and stored at -70 C.

C. Cell culture techniques

KB (Eagle, 1955b), L-929 (Sanford, Earle, and Likely, 1948), and embryonic human skin (EHS) cells (all obtained from MAI) were cultured in one-liter Roux bottles. The cells were grown in a medium consisting of 90 parts BME and 10 parts calf serum (BME-Ca10). Incubation was at 37.5 C in an atmosphere of 95% air and 5% CO_2. To prepare cell suspension for transfer, the monolayered cell cultures were first washed several times with 10 ml of Saline A, prewarmed at 37 C, and then trypsinized for 5 to 10 min at 37.5 C with 5 ml of prewarmed diluted trypsin solution. Following this, the cells were gently pipetted back and forth several times to disperse any clumps, and 5 ml of BME-Ca10 were added to the suspension. After thorough but gentle mixing, a cell count was performed using a hemocytometer. The suspension was then diluted with growth medium to the desired cell concentration.

D. Tube dilution titration of CTFV

A standard tube dilution method for assaying CTFV in cell cultures (Gerloff and Eklund, 1959; Gardner 1962; Trent and Scott, 1964) was used to estimate the amount of CTFV present in the stock suspensions. Trypsinized cells were diluted to a concentration of 2×10^4 cells/ml in BME-Ca10. This cell suspension was then dispensed into Wallis- Melnick culture tubes (Demuth Glassware, Parkersburg, West Virginia) in 1-ml aliquots. The cultures were incubated at 37.5 C for 18 to 24 hr in the CO_2-air mixture. At the end of this time, the cells had attached to the tubes and were ready for use.

Serial tenfold dilutions of the stock virus preparation were made in LaH containing 5% calf serum (LaH-Ca5). The medium was removed from

the culture tubes, and each of 4 or 6 tubes then received an inoculum of 1 ml of the virus dilution. Control tubes received an inoculum of medium alone. All tubes were then sealed with silicone-rubber stoppers and incubated at 35 C. The medium was replaced every three days with fresh LaH-Ca5 in which the concentration of $NaHCO_3$ had been doubled. The cultures were held for 10 days and examined at two-day intervals for CPE. Virus titers in terms of $CCID_{50}$ were calculated by the method of Reed and Muench (1938).

E. Plaque assay procedure

A modification of the technique described by Dulbecco and Vogt (1954a) was employed. The general procedure is stated in the following section.

One million cells in a volume of 5 ml of BME-Ca10 were placed into 60-mm plastic petri dishes (Falcon Plastics, Los Angeles). The dishes were incubated at 37 C for approximately 24 hr until a nearly confluent monolayer developed. The growth medium was removed, and each monolayer washed with 3 ml of Saline A. After removing the wash fluid, 0.1 ml of a known dilution of the stock virus was added to each of the cell monolayers. The virus had been diluted in M199 containing 10% calf serum (M199-Ca10), and all dilutions had been made at 4 C. Virus adsorption was allowed to proceed at room temperature for 90 min. The agar overlay medium was added to the monolayers after removal of the inoculum with another washing using 3 ml Saline A. In all experiments control cultures were treated identically except that the adsorption medium contained only the diluent or normal mouse brain suspensions diluted in a manner similar to the virus-containing mouse brain suspensions. To prepare overlay medium, equal volumes of washed Bacto-agar

CalO, both held at 42 C, were mixed. Finally, 7.5% $NaHCO_3$, prewarmed to 37 C, was added to the mixture to give a final concentration of 0.15%. Four ml of the resultant solution were added to each culture dish and allowed to cool and gel.

The plates were incubated at 37.5 C in the air-CO_2 mixture for 3 days, after which the cells were stained. If the agar concentration in the overlay was 0.4% or less, the overlay was gently poured off, and the monolayers were stained using a modification of the technique of Holland and McLaren (1959). Five ml of 75% ethanol were added to the monolayer to fix the cells. After 1.5 to 2 min, the alcohol was decanted and the cells were stained with crystal violet for approximately 20 seconds. The stain was decanted, and the cell layer was rinsed with tap water. The plates were inverted and allowed to dry. Plaques appeared as transparent circles in a dark purple background.

In experiments where the agar concentration of the medium was 0.5% or greater, the overlay could not be decanted. Consequently, a different staining procedure was employed. The technique used was that of Dulbecco and Vogt (1954a). One ml of neutral red solution was added to the agar overlay after 3 days of incubation. After an additional 5 hr of incubation, the stain was removed by suction. Plaques, which appeared as translucent areas on a faint brownish-red background, were counted immediately.

RESULTS

A. Tube dilution titrations of CTFV

Results of two tube dilution titrations of CTFV in L-929 cells are given in Table 1. The titer of virus as calculated by the method of Reed and Muench (1938) was $10^{9.67}$ $CCID_{50}$/g of mouse brain in the first test and $10^{9.6}$ in the second.

TABLE 1

TITRATION OF CTFV BY THE TUBE DILUTION METHOD IN L-929 CELL CULTURES

Virus dilution [a]	Results	
	Expt. 1	Expt. 2
10^{-3}	4/4[b]	6/6[b]
10^{-4}	4/4	6/6
10^{-5}	4/4	6/6
10^{-6}	4/4	6/6
10^{-7}	4/4	6/6
10^{-8}	4/4	6/6
10^{-9}	4/4	6/6
10^{-10}	1/4	1/6
10^{-11}	0/4	0/6
Controls	0/4	0/6

[a] Prepared from a gram of CTFV-infected mouse brain tissue diluted 1:10 (weight/volume) in whole normal rabbit serum, then by serial 10-fold dilutions in LaH-Ca5.

[b] Numerator = number of tubes exhibiting CPE; denominator = total number of tubes per dilution.

Similar titrations of the virus performed in EHS cells gave a calculated titer of $10^{8.33}$ $CCID_{50}$ in the first experiment and $10^{8.5}$ in the second. In KB cells the titers were $10^{6.75}$ and $10^{6.5}$ $CCID_{50}$ respectively.

TABLE 2

TITRATION OF CTFV BY THE TUBE DILUTION METHOD IN EHS CELL CULTURES

Virus dilution[a]	Results	
	Expt. 1	Expt. 2
10^{-3}	4/4[b]	4/4[b]
10^{-4}	4/4	4/4
10^{-5}	4/4	4/4
10^{-6}	4/4	4/4
10^{-7}	4/4	4/4
10^{-8}	3/4	4/4
10^{-9}	0/4	0/4
10^{-10}	0/4	0/4
10^{-11}	0/4	0/4
Control	0/4	0/4

a Prepared from a gram of CTFV infected mouse brain tissue diluted 1:10 (weight/volume) in whole rabbit serum, then by serial 10-fold dilutions in LaHCa5.

b Numerator = number of tubes exhibiting CPE: denominator = total number of tubes per dilution.

TABLE 3

TITRATION OF CTFV BY THE TUBE DILUTION METHOD IN KB CELL CULTURES

Virus dilution[a]	Experiment 1	Experiment 2
10^{-3}	6/6[b]	4/4[b]
10^{-4}	6/6	4/4
10^{-5}	6/6	4/4
10^{-6}	6/6	4/4
10^{-7}	2/6	0/4
10^{-8}	0/6	0/4
10^{-9}	0/6	0/4
10^{-10}	0/6	0/4
10^{-11}	0/6	0/4
Control	0/6	0/4

[a] Prepared from a gram of CTFV infected mouse brain tissue diluted 1:10 (weight/volume) in whole rabbit serum, then by serial 10-fold dilutions in LaHCa5.

[b] Numerator = number of tubes exhibiting CPE; denominator = total number of tubes per dilution.

The results of the foregoing assays indicated that the L-929 cell was the most susceptible to CTFV of the cells tested. To confirm this, the susceptibilities of KB, L-929, and EHS cells to CTFV were then compared. The results of two experiments are presented in Table 4. The data indicates that under the conditions of the experiments, L-929 cells are more susceptible than either EHS or KB cells. Based on these results, L-929 cells were selected as the indicator cells to be used in establishing a plaque assay procedure for this virus.

TABLE 4

COMPARISON OF SUSCEPTIBILITIES[a] OF VARIOUS CULTURED CELLS TO CTFV

Cell Cultures	$CCID_{50}$/g CTFV-Infected Mouse Brain Tissue	
	Expt 1	Expt. 2
KB	$10^{6.5}$	$10^{6.5}$
EHS	$10^{8.67}$	$10^{8.67}$
L-929	$10^{9.67}$	$10^{9.5}$

[a] Based on assays performed using the tube dilution method.

B. Neutralization by tube dilution assay

To acertain if the stock CTFV suspensions used in these studies was contaminated with other viruses, neutralization tests were carried out in L-929 cell cultures.

Since a gram of virus-infected mouse brain tissue was found to contain approximately $10^{9.6} CCID_{50}$ of CTFV, the homogenate was subsequently

diluted in LaH-Ca5 to a final viral concentration of 200 $CCID_{50}$/ml. Anti-CTFV and normal mouse sera were inactivated at 56 C for 30 min, then diluted in LaH-Ca5.

Two and five-tenths ml each of the viral suspension were added to equal volumes of 1:10 or 1:20 dilutions of the inactivated sera. The controls included diluent without virus for each of the sera to ensure the absence of any nonspecific toxic material for the cells. Additional cultures challenged only with diluent or virus served as medium and virus controls.

The mixtures were incubated at 37 C for 1 hr. Culture tubes were then inoculated with 1 ml each of the mixtures and incubated at 35 C. Microscopic observations and medium changes with LaHCa5 were made at 3-day intervals. By the 6th day, CPE was apparent in cultures infected with the virus-normal mouse serum and virus control mixtures. Observations on the 9th day revealed that the cells in these cultures were completely destroyed. The cultures containing antiserum-virus mixtures showed no apparent changes indicative of virus infection. Similarly, the antiserum, normal serum, and medium control cultures remained unchanged. The results of this experiment, shown in Table 5, signified that the stock suspension did not contain virus other than CTFV.

C. CPE production in monolayers of L-929 cells infected with CTFV

These experiments were undertaken to determine whether specific nutritional requirements were necessary for CPE production in L-929 cells. This experiment was carried out in plastic petri dishes since monolayers of cells were desired. A nearly confluent monolayer of

TABLE 5

NEUTRALIZATION OF CTFV WITH MOUSE SERUM IN TUBE CULTURES OF L-929 CELLS

Serum	Final Serum Dilution	$CCID_{50}$ of CTFV/Culture Tube	Results
Normal	1:20	no virus	0/4[a]
	1:40	no virus	0/4
	1:20	100	4/4
	1:40	100	4/4
Immune	1:20	no virus	0/4
	1:40	no virus	0/4
	1:20	100	0/4
	1:40	100	0/4
None	0	no virus	0/4
	0	100	4/4

[a] Numeration = number of tubes exhibiting CPE; denomination = total number of tubes used.

L-929 cells is shown in Figure 1. Although some of these cells appeared to be fibroblastic, there is an apparent change in morphology when the monolayer is completely confluent. At this stage of growth, foci of cells several layers thick were observed which could affect the assay. Consequently, the monolayers in the near-confluent stage were used. When this stage of growth was obtained, the growth medium was removed and 0.1 ml of 10^{-7} dilution of virus was added to each dish. Control cultures received 0.1 ml of diluent alone. After allowing 90 min for the virus to attach to the cells, 5 ml of M199, MEM, BME, LaH, LaE, or LaEYe, supplemented with 2% or 10% calf serum were added to each plate.

The cultures were observed daily with an inverted microscope. The appearance of CPE in virus-infected cultures was interpreted as an indication that the medium was perhaps suitable for experiments under an agar overlay. The results in Table 6 show that M199, MEM, BME, LaE, or LaH supplemented with either 2% or 10% calf serum might be acceptable for use in further studies. The toxicity of yeast extract was apparent since degeneration of both control and infected L-929 cells occured within two days under LaEYe medium.

Close observation during this experiment revealed that micro-plaques had formed in L-929 cell monolayers maintained in all of the liquid media except LaEYe. One such micro-plaque observed in a culture incubated with M199 and supplemented with 2% calf serum (M199-Ca2), on the 2nd day of infection is shown in figure 2.

M199-Ca10 was selected as the experimental medium for plaque assay studies because of the greater variety of nutrients contained in this medium. Further experiments revealed that M199-Ca10 would indeed allow CTFV to form plaques in L-929 cells under an agar overlay system.

FIGURE 1. A monolayer of L-929 cells at the stage used in plaque assay.

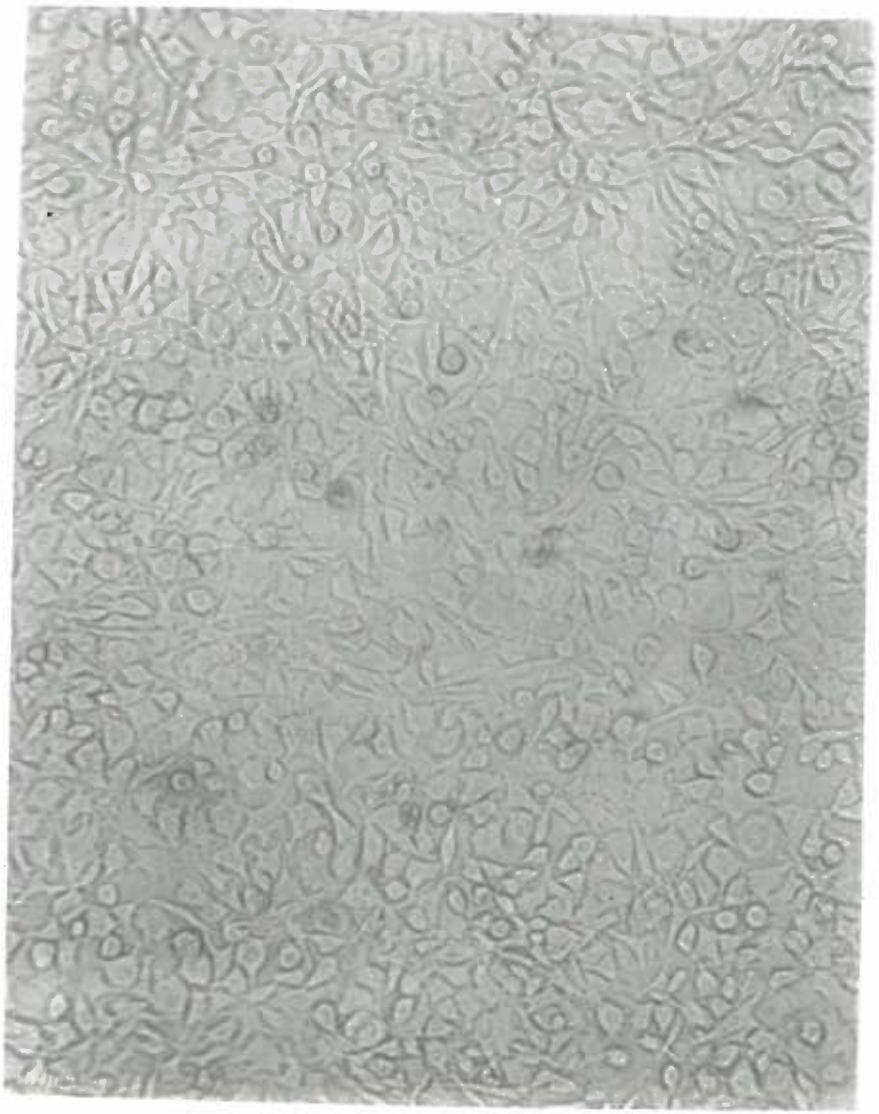


TABLE 6

EFFECTS OF NUTRIENT MEDIA AND SERUM CONCENTRATIONS ON SUSCEPTIBILITY OF L-929 CELLS TO CTFV

Concentration of Calf Serum	Media	Infected Plates	Control Plates
2%	LaH	CPE	normal
	LaE	CPE	normal
	M199	CPE	normal
	MEM	CPE	normal
	BME	CPE	normal
	LaEYe	degeneration[a]	degeneration[a]
10%	LaH	CPE	normal
	LaE	CPE	normal
	M199	CPE	normal
	MEM	CPE	normal
	BME	CPE	normal
	LaEYe	degeneration	degeneration

[a] Degeneration of cell monolayer was complete in two days and apparently due to toxic factors in the yeast extract.

FIGURE 2. A microplaque induced in a L-929 cell monolayer by CTFV, 48 hr after infection.

Before the effects of several other variables were investigated, certain procedures were temporarily established based on the experimental findings of other investigators. For example, the agar overlay medium contained 0.15% $NaHCO_3$ (Dieg and Watkins, 1964); the adsorption volume was 0.1 ml (Dougherty, 1964); the attachment period was 90 min (Trent and Scott, 1964).

D. The effect of agar concentration on plating efficiency

In most assays, the concentration of agar in the overlay is based on that used in the procedure first reported by Dulbecco and Vogt (1954a). To determine what effect agar concentration might have on plaque titer and size, the following experiments were performed.

The plaque assay procedure described earlier was followed; the only variable was that double-strength agar solutions which would give final concentrations of agar ranging from 0.1% to 0.9% were used. The data for these experiments were obtained from 6 petri dishes at each agar concentration unless otherwise stated. The results of these experiments are shown in Tables 7 and 8. There is no meaningful difference between the number of plaques obtained under the varying agar concentrations, particularly since the minute plaques were difficult to enumerate at the higher concentrations. However, it is apparent that at the highest concentrations of 0.8% and particularly 0.9%, plaque sizes were reduced. The lower agar concentrations appeared to favor the development of larger-sized plaques.

TABLE 7

EFFECT OF CONCENTRATION OF WASHED AGAR IN THE OVERLAY MEDIUM ON THE AVERAGE NUMBER OF PLAQUES PER DISH

Final Concentration of Agar in the Overlay (percent)	Average Number of Plaques per Dish[a]				
	Expt. 1[a]	Expt. 2[b]	Expt. 3	Expt. 4	Expt. 5
0.1	51 (47-57)	*	*	*	*
0.2	49 (38-58)	*	*	*	*
0.3[c]	55 (30-74)	32 (29-35)	41 (35-44)	38 (37-41)	48 (42-53)
0.4	62 (56-70)	31 (27-36)	*	*	*
0.5	70 (68-72)	37 (32-41)	41 (37-42)	40 (39-42)	52 (47-59)
0.6	66 (56-71)	37 (28-48)	*	*	*
0.7	62 (56-71)	40 (37-44)	42 (39-44)	42 (38-45)	50 (46-57)
0.8	61 (56-64)	37 (35-39)	*	*	*
0.9	41 (34-50)	19 (16-21)	21 (18-22)	24 (20-28)	34 (30-37)

[a] The range of the counts is enclosed in parenthesis.

[b] Values obtained from average of three plates at each concentration.

[c] 0.3% agar concentration selected as standard for further experiments.

* Not done.

TABLE 8

EFFECT OF CONCENTRATION OF WASHED AGAR IN OVERLAY MEDIUM ON THE SIZE OF PLAQUES PRODUCED BY CTFV-INFECTED L-929 CELLS

Final Concentration of Agar in the Overlay (percent)	Plaque Diameter in mm[a] (±0.25 mm)				
	Expt. 1[b]	Expt. 2[b]	Expt. 3	Expt. 4	Expt. 5
0.1	3.5	*	*	*	*
0.2	3.0	*	*	*	*
0.3	3.0	2.5	2.5	3.0	3.0
0.4	2.5	2.5	*	*	*
0.5	2.0	2.0	2.0	2.0	2.0
0.6	2.0	2.0	*	*	*
0.7	2.0	1.5	1.5	1.5	1.5
0.8	1.5	1.0	*	*	*
0.9	0.5	0.5	0.5	0.5	0.5

[a] There were two distinct plaque sizes on each dish. Only the large plaques, which constituted about 70% of the total number of plaques, were measured.

[b] The values for these experiments were obtained from 3 plates with each agar concentration.

* Not done

E. A comparison of staining methods

Neutral red and crystal violet staining procedures were compared to determine which resulted in the most clearly defined plaques. Individual plaques stained by each of these methods are shown in figures 3 and 4. These pictures indicate that the resolution of plaques obtained with crystal violet were superior to those obtained with neutral red. In view of these findings, crystal violet was adopted as the standard stain.

Crystal violet was unsuitable with overlay containing an agar concentration of 0.5% or greater, since these overlay media could not be adequately removed without disturbing the cell monolayer. Furthermore, if crystal violet was added to firm agar overlays in the same manner as neutral red, the entire overlay stains dark purple. Under this condition plaques cannot be seen. On the other hand, concentrations of 0.2% or lower tended to allow the development of comet-shaped plaques. Such plaques interfered with counting, particularly when two plaques were in close proximity.

Since the agar concentration in the overlay did not affect the numbers of plaques produced, 0.3% agar was selected as the standard for all other experiments. This concentration was removed easily without disturbing the monolayer and was of sufficient firmness to prevent formation of comet-shaped plaques. Furthermore, plaques of optimum size were produced under this concentration of agar.

Figure 5 illustrates normal and CTFV-infected monolayers of L-929 cells stained with crystal violet. The plaques are well defined and easily recognized. In addition, at least two distinct sizes of plaques can be seen.

FIGURE 3. Microscopic observation of a CTFV-induced plaque in a L-929 cell monolayer stained with neutral red.

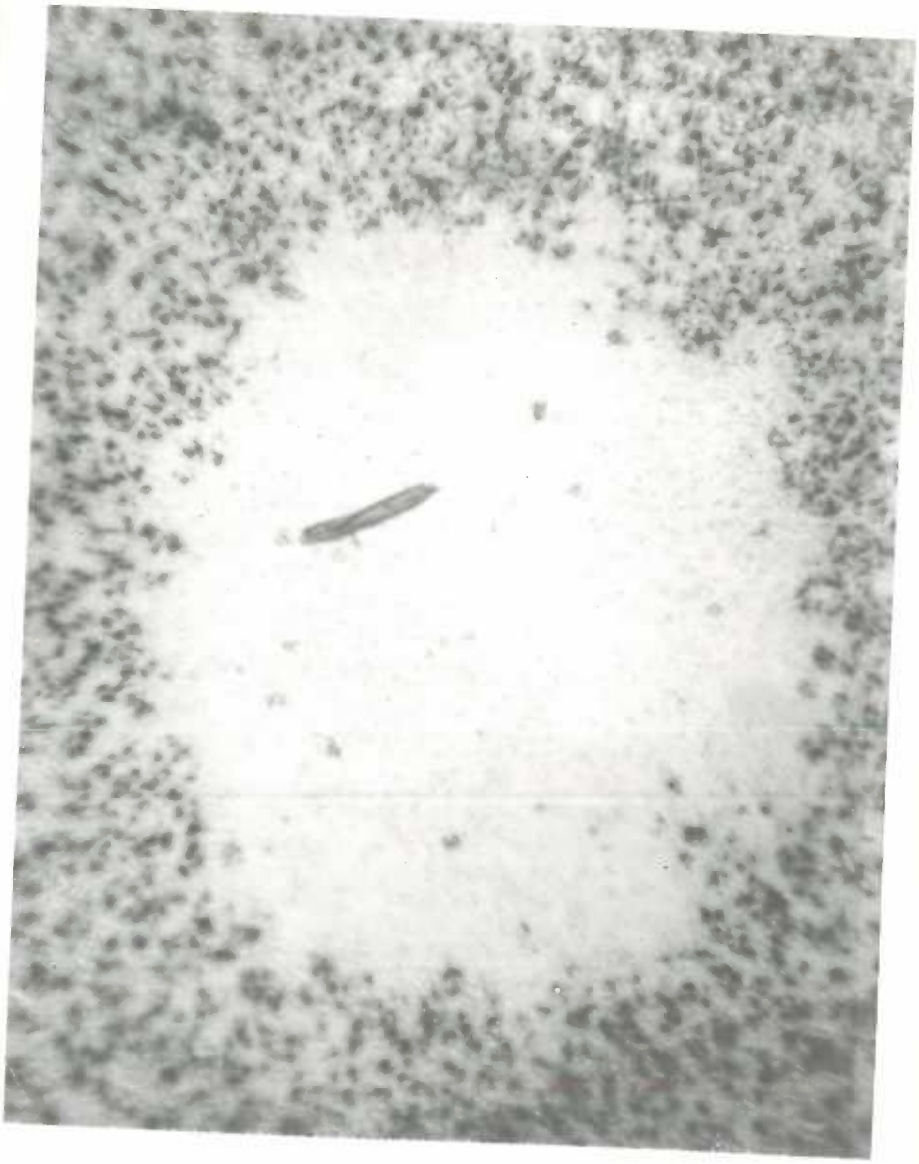


FIGURE 4. A microscopic observation of a CTFV-induced plaque in a L-929 cell monolayer stained with crystal violet.

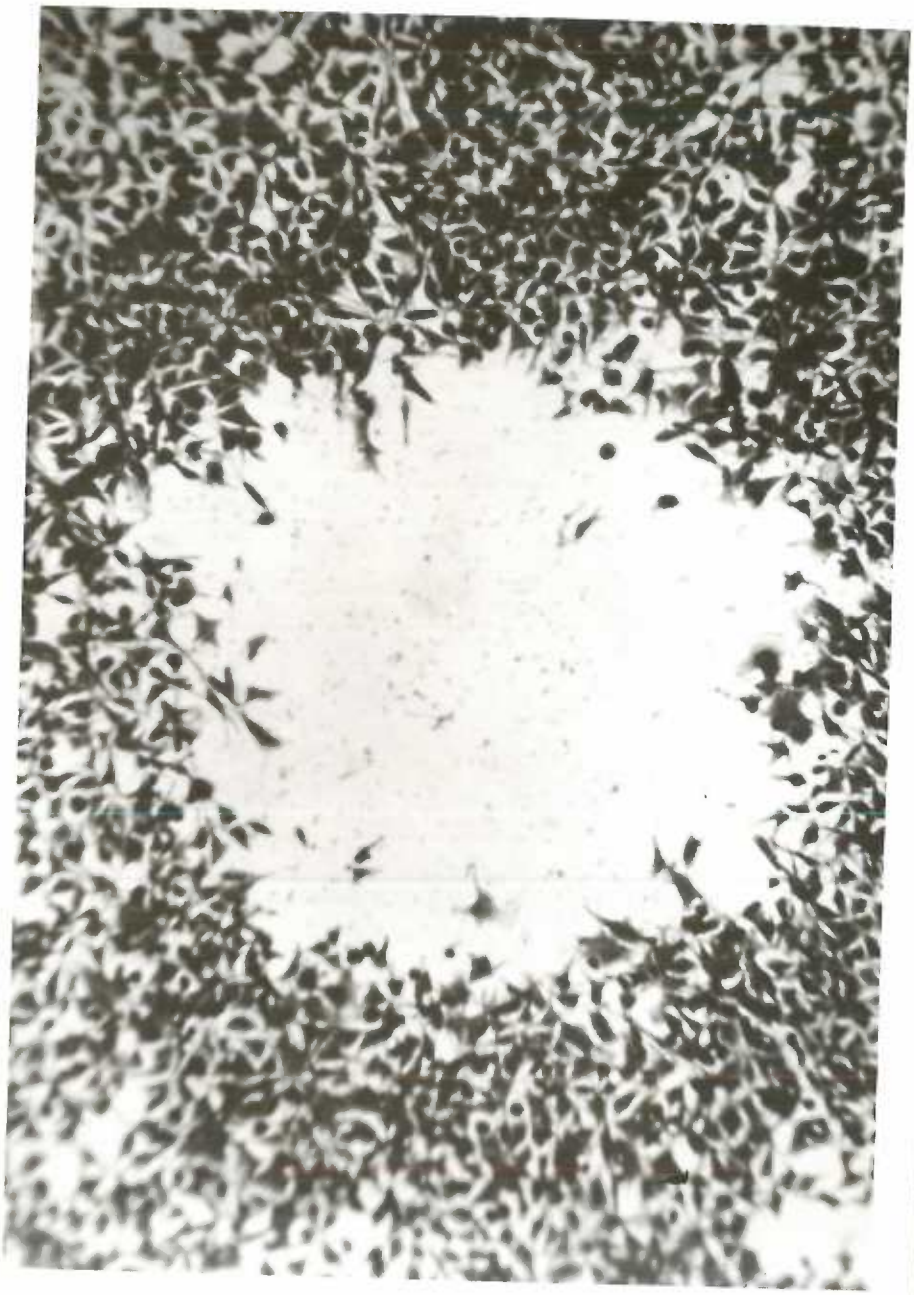
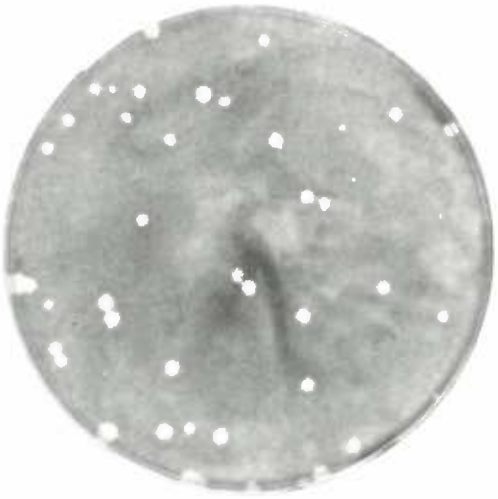


FIGURE 5. A. Uninfected L-929 cell monolayer stained with crystal violet.

B. CTFV-infected L-929 cell monolayer stained with crystal violet.

A.



B.

F. Effects of different types of agar on plaque production

Five different agar preparations were used in an effort to determine which would be most suitable for the assay procedure. Ionagar No. 2, Purified, Noble, Bacto-agar or washed Bacto-agar was incorporated into the overlay medium at a final concentration of 0.3%. The results of these experiments are shown in Tables 9 and 10.

Little difference in size and number of plaques were observed in cultures overlaid with media containing washed, Ionagar No. 2, Bacto-agar, or purified agar. Plaques formed under overlay media prepared with these agars were 2 to 3 mm in diameter while those formed under Noble agar were 0.5 mm. In addition, the numbers of plaques under Noble agar were reduced by as much as 50%.

TABLE 9

EFFECT OF DIFFERENT TYPES OF AGAR ON THE NUMBER OF PLAQUES PRODUCED IN CTFV-INFECTED L-929 CELLS

Type of Agar	Average Number of PFU/Plate[a]		
	Expt. 1	Expt. 2	Expt. 3
Washed agar[b]	39 (35-44)	50 (46-57)	51 (47-59)
Oxoid Ionagar 2	37 (31-42)	51 (47-53)	45 (38-51)
Bacto-agar	37 (35-39)	46 (43-50)	54 (51-56)
Noble agar	14 (10-21)	24 (13-43)	20 (15-26)
Purified agar	36 (33-43)	46 (43-47)	49 (42-53)

[a] The range of the counts is enclosed in parenthesis.

[b] Washed agar was selected as standard for use in future experiments.

TABLE 10

EFFECT OF DIFFERENT TYPES OF AGAR ON THE SIZE OF PLAQUES PRODUCED BY CTFV INFECTED L-929 CELLS

Type of Agar	Plaque Diameter[a] in mm (± 0.25 mm)		
	Expt. 1	Expt. 2	Expt. 3
Washed agar	3.0	3.0	3.0
Ionagar 2	2.5	2.0	2.0
Bacto-agar	3.0	2.5	3.0
Noble agar	pin point	0.5	0.5
Purified agar	3.0	2.5	2.5

[a] There were two distinct plaque sizes, and on each dish only the large plaques, which constituted about 70% of the total number of plaques, were measured.

G. Efficiency of plaque production by different media with varying serum concentrations

In an earlier study, several nutrient media with varying concentrations of calf serum were tested to determine the suitability of an appropriate medium supporting CTFV replication in L-929 cells. This study was inconclusive since actual virus titers were not determined nor were the experiments done under plaque assay conditions. Therefore, M199, MEM, LaE, BME, and EBSS each containing 2, 5, or 10% calf serum were incorporated into the overlay medium with 0.3% agar. All other conditions of the plaque assay procedure were kept constant in an effort to determine which medium-serum mixture allowed the maximum number of plaques to form in these L-929 cell monolayers. Three dilutions of virus with 3 to 5 plates per dilution were utilized. The results of a typical experiment are shown in Table 11.

The concentration of calf serum in the overlay was found to be a contributing factor in increasing the sensitivity of this assay system. Serum diluted in a balanced salt solution did not provide the necessary conditions to allow maximal number of plaques to develop. MEM, BME, and LaE were comparable to M199 in providing suitable conditions for CTFV replication in L-929 cells.

H. The effect of overlay volume on plaque size and numbers

The depth of the overlay medium has been shown to have a contributing effect on the number of plaques produced (Vogt et al, 1957) as well as the rate of plaque formation (Gifford and Syverton, 1957). Therefore,

TABLE 11

EFFECT OF NUTRIENT MEDIA AND OF CALF SERUM CONCENTRATIONS IN THE OVERLAY ON PLAQUE TITER OF CTFV IN L-929 CELLS

Percent Calf Serum	Nutrient Media	PFU of CTFV/g of Infected Mouse Brain Homogenate X 10^9	(Range)
2%	M199	17	(164-183)[b]
	MEM	17	(162-181)[b]
	BME	5	(46-57)[b]
	LaE	10	(90-105)[b]
	EBSS	1	(10-16)[b]
5%	M199	34	(23-53)[b]
	MEM	21	(9-30)
	BME	22	(9-36)
	LaE	40	(35-47)
	EBSS	2	(18-25)[b]
10%	M199[c]	62	(57-68)
	MEM	55	(45-66)
	BME	44	(33-53)
	LaE	42	(35-53)
	EBSS	3	(21-27)

[a] Three dilutions were utilized and the determinations of PFU/g are based on the dilutions that gave between 20 and 200 plaques per petri dish.

[b] Count made at 10^8 dilution.

[c] M199 supplemented with 10% calf serum selected as standard overlay medium for further experiments.

experiments were performed to determine whether the volume of overlay medium (which determined the depth) had any effect on the production of plaques by CTFV in L-929 cells.

Volumes of overlay varying from 3.5 ml to 10 ml were added to CTFV-infected monolayers. The results of these experiments are shown in Tables 12 and 13.

After a 3-day incubation period, no significant decrease in plaque numbers were observed, but a decrease in the sizes of plaques by as much as 66% was found when volumes of 8 or 10 ml were used.

TABLE 12

THE EFFECT OF OVERLAY VOLUME ON THE NUMBER OF PLAQUES PRODUCED IN L-929 CELLS INFECTED WITH CTFV

Volume of Overlay (in ml)	Average PFU/Plate[a]					
	Expt. 1		Expt. 2		Expt. 3	
3.5	55[b]	(51-59)	*		*	
4.0	55	(46-65)	50[c]	(46-57)	50[c]	(45-53)
6.0	53	(42-61)	51	(39-59)	48	(46-51)
7.0	47	(36-58)	*		*	
8.0	40	(37-43)	45	(43-47)	40	(39-41)
10.0	39	(38-42)	45	(42-50)	37	(30-41)

[a] The range of the counts is enclosed in parenthesis.

[b] Average No. of PFU from three plates.

[c] Average No. of PFU from six plates.

* Not done.

TABLE 13

THE EFFECT OF THE VOLUME OF OVERLAY ON THE SIZE OF PLAQUES PRODUCED IN L-929 CELLS INFECTED WITH CTFV

Volume of Overlay (in ml)	Plaque Diameter in mm[a]		
	Expt. 1	Expt. 2	Expt. 3
3.5	3.0	*	*
4.0	3.0	3.0	2.5
6.0	3.0	3.0	2.0
7.0	2.5	*	*
8.0	2.0	2.0	1.5
10.0	1.0	1.0	1.0

[a] There were at least two distinct plaque sizes on each dish. Only the large plaques, which constituted about 70% of the total number of plaques, were measured.

* Not done.

I. Effect of varying the concentration of $NaHCO_3$ incorporated in the overlay medium

The pH of overlay medium could influence the development of plaques by modifying the physiological conditions of the indicator cells. This could, in turn, affect the infectious processes of CTFV in L-929 cells. Since $NaHCO_3$ is incorporated in the overlay medium, its influence on plaque titer and size was investigated.

Several preparations of the overlay medium containing 0.3% agar and M199-Cal0 were prepared. The concentration of the $NaHCO_3$ in the medium were varied between 0.03% and 0.3%. The monolayers were infected, overlaid, incubated, and stained with crystal violet as described earlier. The results of this experiment are shown in Figure 6.

The average number and size of plaques per plate were proportional at the different concentrations of $NaHCO_3$. Controls containing no $NaHCO_3$ were void of plaques. The maximum number and size of plaques were attained in $NaHCO_3$ concentrations ranging from 0.15% to 0.24%. At higher or lower concentrations, plaque size and numbers were correspondingly reduced.

J. Washing of cell monolayers

Experiments were conducted to ascertain whether washing of cell monolayers before and after virus adsorption had any effect on the number of plaques produced. L-929 monolayers were washed 1, 2, or 3 times before and after the 90 min adsorption period. Each washing was performed using 4-ml volumes of Saline A, which were permitted to remain in contact with the cells for only a few seconds. The results shown in Table 14 indicate that washing of the monolayers before viral adsorption

FIGURE 6. The effect of $NaHCO_3$ concentration on numbers and size of plaques produced in L-929 cell monolayers.

a) Average number of plaques/dish produced when the concentration of $NaHCO_3$ found at the appropriate point on the abscissa was incorporated into the overlay medium.

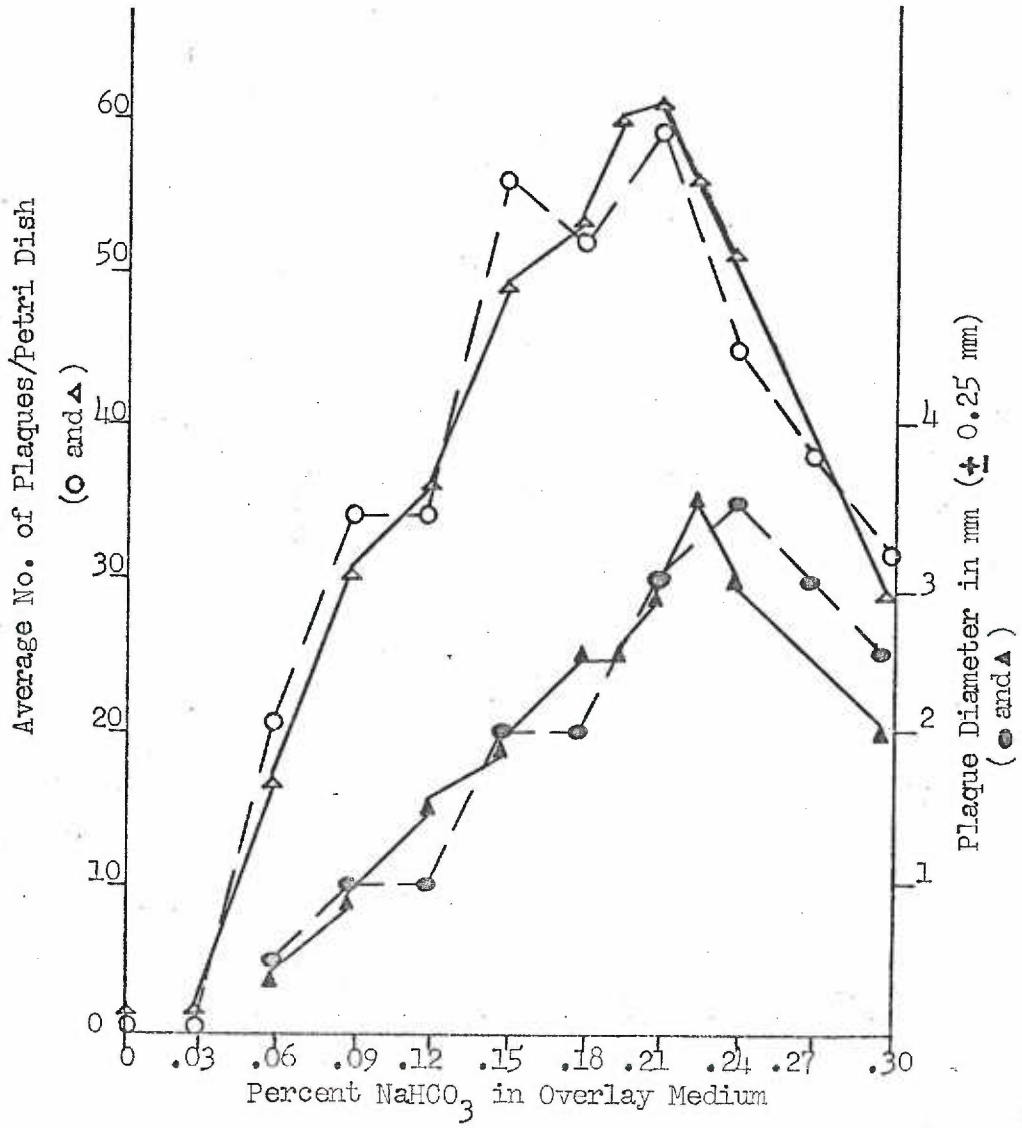
(o) Expt. 1

(Δ) Expt. 2

b) There were two distinct plaque sizes on each dish. Only the large plaques which constituted about 70% of the total number of plaques, are measured. The plaque size as above was the one produced when the concentration of $NaHCO_3$ found at the appropriate point on the abscissa was incorporated into the overlay medium.

(●) Expt. 1

(▲) Expt. 2


Average No. of Plaques/Petri Dish
(○ and △)
Plaque Diameter in mm (± 0.25 mm)
(● and ▲)
Percent NaHCO3 in Overlay Medium
0
10
20
30
40
50
60
1
2
3
4
.03
.06
.09
.12
.15
.18
.21
.24
.27
.30

slightly increased the efficiency of plating. However, further washing did not increase the number of plaques produced. Little effect was noted when monolayers were washed after the designated period of virus adsorption.

TABLE 14

THE EFFECT OF WASHING L-929 MONOLAYERS PRE- and POSTINFECTION WITH CTFV

Number of Washings		Average No. of PFU per Plate	
Before Adsorption	After Adsorption	Expt. 1	Expt. 2
0	0	35 (29-40)[b]	21 (20-23)
0	1	34 (31-36)	17 (15-19)
0	2	*	16 (14-20)
0	3	*	11 (8-14)
1	0	55 (52-58)	33 (27-42)
2	0	*	35 (20-44)
1	1[a]	54 (51-56)	44 (34-49)
2	2	45 (40-49)	37 (35-39)
3	3	33 (30-36)	32 (29-34)

[a] The range of the counts is enclosed in parenthesis.

[b] One pre- and one post-wash selected as standard.

* Not done.

K. Certain conditions affecting adsorption of CTFV to monolayers of of L-929 cells.

In view of the unstable nature of CTFV it was appropriate at this time to determine the effect of volume of adsorption medium on CTFV-induced plaque titer. One-tenth to 1.0 ml of diluent containing a known

number of PFU was allowed to adsorb for 90 min. The results of a typical experiment are shown in Figure 7. Optimum adsorption of CTFV to L-929 cells occured when a volume of 0.1 ml was employed. Only 39% of the infectious virus present adsorbed when 0.5 ml was used. The efficiency of adsorption decreased further with only 20% of the infectious CTFV present adsorbed if the volume was increased to 1.0 ml.

The time required for maximum virus attachment was also investigated. Based on the adsorption volume studies, 0.1 ml of a suspension containing 100 PFU of CTFV was added to monolayers of L-929 cells. After a prescribed period of attachment, the cell monolayers were washed with Saline A to remove any unadsorbed virus before overlay medium was added. The adsorption period was varied from 5 to 180 min, and each determination was based on counts from 10 dishes. The results of a typical experiment are shown in Figure 8. It was found that 93% of the infectious virus present adsorbed to the L-929 cells within 90 min. No increase in titer was obtained after 120 min of incubation.

L. Neutralization of CTFV in the plaque assay system

It has already been determined that the CTFV suspension was not contaminated with other viruses. To confirm that the plaques formed in L-929 cells resulted from CTFV infection, neutralization tests were repeated in the agar overlay system.

The stock suspension of mouse brain containing 4×10^9 PFU/ml of CTFV was diluted to a titer of 1000 PFU/0.5 ml of the virus, which were added to equal volumes of the serially diluted inactivated sera. Normal mouse brain suspension was diluted to give a concentration of tissue material identical to the CTFV-infected suspension. After in-

FIGURE 7. The effect of varying the volume of medium on efficiency of CTFV attachment to L-929 cell monolayers.

The volume of medium used to allow virus attachment to the cell monolayer was varied in an effort to determine which volume allowed the most efficient adsorption of virus to L-929 cell monolayers. The number of plaques produced in each sample was compared with the known number of PFU in the sample. This value was then expressed as a per cent and plotted on the graph.

Because of these results, the adsorption volume used in further experiments was 0.1 ml.

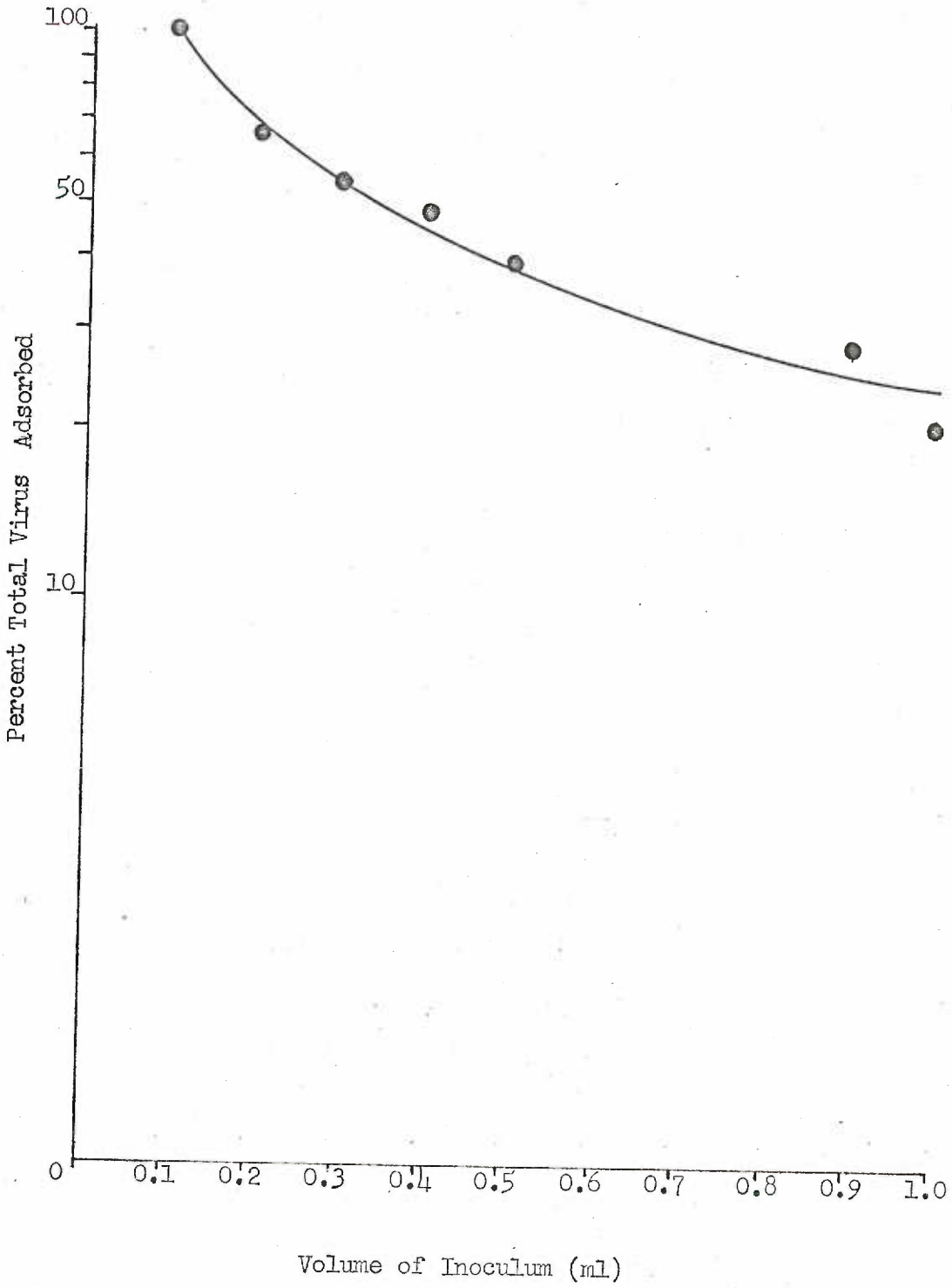
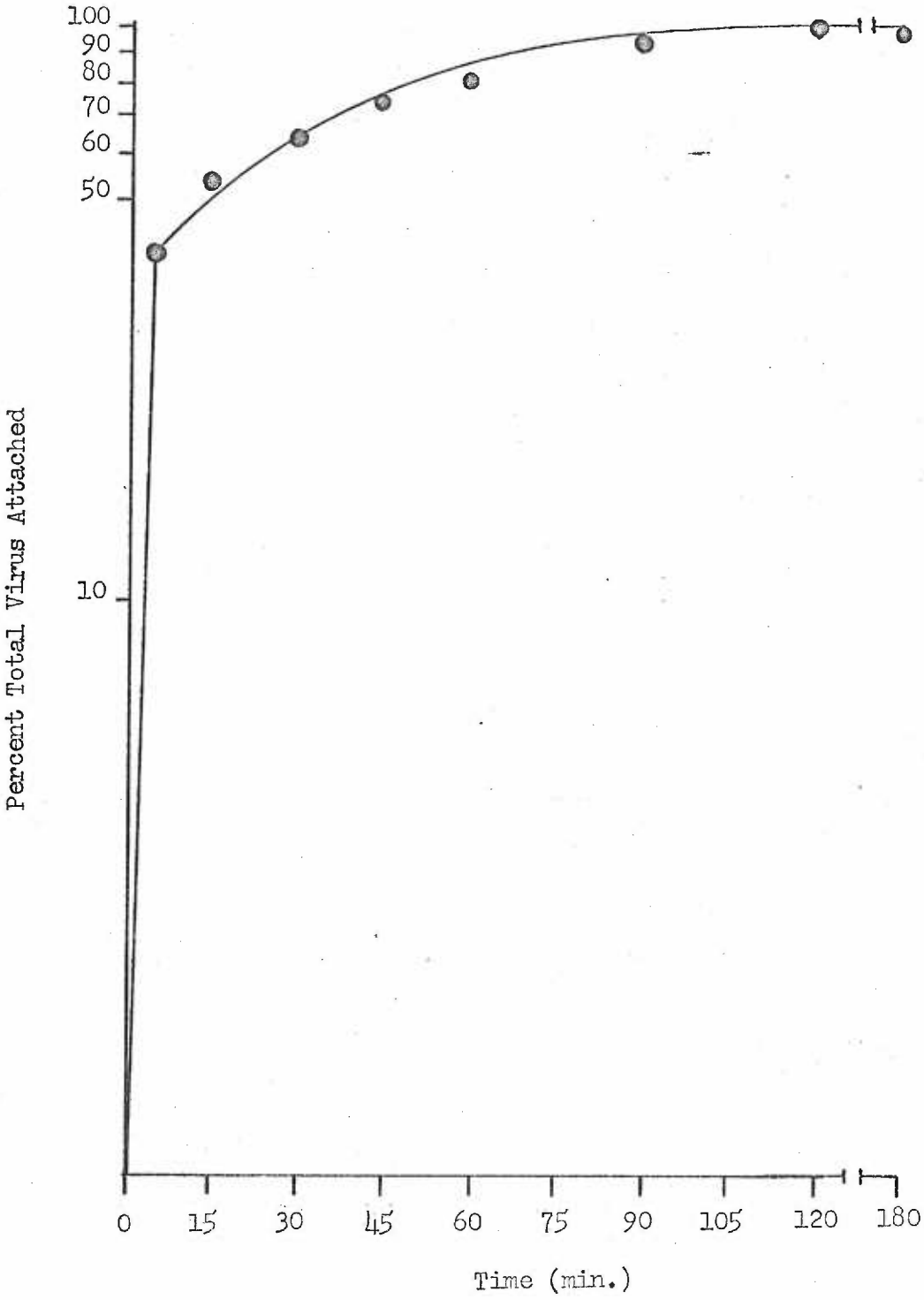

100
50
10
0
Percent Total Virus Adsorbed
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Volume of Inoculum (ml)

FIGURE 8. Attachment of CTFV to L-929 cell monolayers as a function of time. Approximately 100 PFU of CTFV were allowed to attach to L-929 cell monolayers for varying lengths of time before further adsorption was stopped by washing and an addition of overlay medium to the cell monolayer. The average number of plaques present was expressed as a per cent of the maximum virus found to adsorb. In all further experiments, an attachment period of 120 min was used.


100
90
80
70
60
50
10
Percent Total Virus Attached
0
15
30
45
60
75
90
105
120
180
Time (min.)

cubation at 37 C for 1 hr, 0.1 ml of each mixture was added to monolayers of L-929 cells. At the end of the adsorption period, the overlay medium was added, and the plates were incubated for 3 days before staining and counting.

The result of one of these experiments is shown in Table 15. Monolayers treated with normal mouse brain suspension or sera without virus did not develop any plaques. These findings, coupled with the failure of normal mouse serum to prevent plaque formation, indicated that the virus inducing plaques in the L-929 cell monolayers was only present in the CTFV-infected mouse brain suspension. Since the immune serum is known to specifically neutralize CTFV, it is apparent that the production of plaques was the result of infection with CTFV.

M. Comparative susceptibilities of L-929 and KB cells to infection with CTFV under plaque assay conditions.

Thus far, the plaque assay procedure devised has been used to titrate CTFV with L-929 cells. This procedure may also allow plaques to develop with anohter cell line, such as KB cells, demonstrating degenerative changes when infected with CTFV. Of particular interest with KB cells was the fact that in the tube dilution assay the final titer of CTFV was $10^{6.5}$ $CCID_{50}$/g of mouse brain tissue as compared to $10^{9.5}$ in L-929 cells. To acertain whether this difference in titer would be demonstrable under agar overlay, CTFV was again titrated in KB cells. The entire procedure of infection, adsorption, overlay, incubation and staining was as described in materials and methods. The results are shown in Table 16.

TABLE 15

NEUTRALIZATION OF CTFV WITH MOUSE SERUM IN MONOLAYERS OF L-929 CELLS

Serum	Final Serum Concentration	Expected Number of PFU/Plate	Average Number of PFU/Plate	(Range)
Normal	1:20	No virus	0	-
	1:40	No virus	0	-
	1:20	100	107	(102-110)
	1:40	100	101	(99-104)
Immune	1:20	No virus	0	-
	1:40	No virus	0	-
	1:20	NMB[b]	0	-
	1:40	NMB	0	-
	1:20	100	0	-
	1:40	100	0	-
	1:80	100	0	-
	1:160	100	0	-
	1:320	100	0	(0-1)
	1:640	100	15	(9-20)
	1:1280	100	60	(51-66)
	1:2560	100	97	(90-101)
	1:5120	100	106	(104-108)
None	0	No virus	0	-
	0	100	103	(100-106)

[a] Average of 3 plates per serum dilution.

[b] Normal mouse brain.

The titer of the stock CTFV suspension was found to be similar under this system in both L-929 and KB cells. A notable difference was observed in regards to plaque size. As shown in Figure 9, the plaques produced in KB cells were considerably smaller than those produced in L-929 cells. Under the same conditions that gave plaques 3 mm in diameter in L-929 cells, plaques obtained in KB cells were no larger than 1 mm in diameter.

TABLE 16

THE COMPARATIVE TITER OF CTFV IN MONOLAYERS OF L-929 AND KB CELLS UNDER PLAQUE ASSAY CONDITIONS

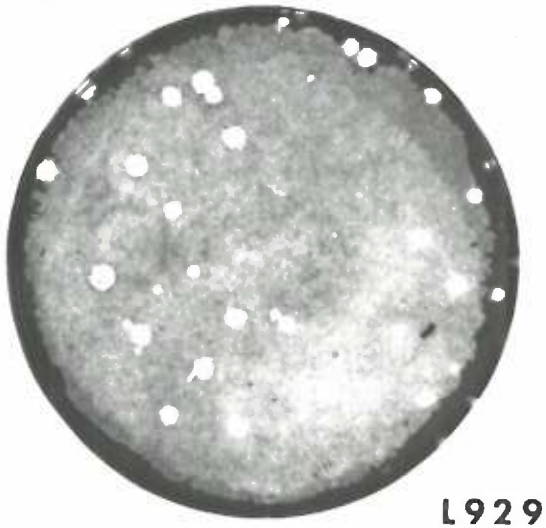
Cell Line	Final Virus Dilution	Average[a] No. of PFU/Plate			
		Expt. 1		Expt. 2	
L-929	10^{-6}	TNTC[b]	-	TNTC	-
	10^{-7}	TNTC	-	TNTC	-
	10^{-8}	45	(44-46)[c]	36	(32-41)
	10^{-9}	5	(2-7)	5	(3-8)
	10^{-10}	0	-	0	-
	Control	0	-	0	-
KB	10^{-6}	TNTC	-	TNTC	-
	10^{-7}	TNTC	-	TNTC	-
	10^{-8}	30	(26-34)	26	(22-29)
	10^{-9}	0	-	1	(0-2)
	10^{-10}	0	-	0	-
	Control	0	-	0	-

[a] Average of four plates at each dilution.

[b] TNTC: plaques too numerous to count.

[c] Ranges of counts are enclosed in parenthesis.

FIGURE 9. A comparison of CTFV-induced plaques in L-929 cell and KB cell monolayers. Plaques of CTFV in a L-929 cell monolayer after 72 hr of infection. Plaques of CTFV in a KB cell monolayer after 72 hr of infection.



L929

KB

DISCUSSION

A procedure for assaying infectious CTFV units by enumeration of plaques in monolayers of L-929 cells has been developed in this study. The initial requirement in any plaque assay procedure is the sensitivity of a cell line to the lytic activity of a given virus. Although KB and embryonic human skin cells were susceptible to infection with CTFV, L-929 cells were selected for further studies in view of the higher titers of virus obtained from the tube culture assays. It was not determined if true intrinsic differences in cell sensitivities were present or if the differences in apparent sensitivity were due to the physiological conditions of the experiments.

Several different media are commercially available. Certain cell lines can be cultured in only specific medium whereas others appear to have no preference. L-929 cells could be cultured in M199, MEM, BME, and LaE without difficulty provided calf serum was added as a supplement. There were no observable differences in plaque titers in L-929 cells cultured in overlays containing any of these media. However, the concentration of the serum supplement did affect the titer and size of the plaques with 10% offering the most favorable results. Among the media tested, M199 was adopted for future assays, since it contained the largest variety of defined ingredients.

The standard stain used by many investigators is neutral red, which does not stain foci of degenerated cells. This in turn could be visualized as plaques induced initially by the infection of one cell

and presumably by one infectious particle. When this stain was added to the overlay medium of CTFV-infected L-929 cells, the plaques were not clearly defined. Crystal violet applied in the same manner was unsuitable, since the entire overlay stained darkly obliterating the plaques. To attempt the removal of overlay prepared from 0.9% agar was also unsatisfactory, since part of the cell monolayer was removed along with the agar overlay. However, if the agar concentration could be reduced to the consistency where infection was still confined to a given focus and yet permit removal of the overlay without disturbing the monolayer, the cells could be fixed, then stained with crystal violet.

The concentration of agar in the overlay had no effect on the plaque titer, but the normal concentrations used in plaque assays of 0.8 to 0.9% did reduce the plaque diameter. A nutrient overlay containing 0.3% agar was found to possess sufficient firmness to localize the plaques. In addition, this facilitated the removal of the overlay at the termination of the incubation period without being detrimental to the monolayer. Finally, plaques visualized under crystal violet staining were easily observed and well defined. This procedure of staining also had the desireable advantage in that the plates could be kept indefinitely for possible future reference.

As was the case with media, several different types of agar are available. Ionagar No. 2, washed and normal Bacto-agar, and purified agar each allowed comparable numbers and size of plaques when added to the overlay. However, Noble agar inhibited the production and size of plaques. Similar results have been reported with mengovirus (Campbell and Colter, 1965). Apparently, Noble agar interfered with the infectious

process of CTFV-infected L-929 cells. The use of washed agar was continued in this laboratory because of its availability.

Another variable investigated was the volume of the overlay medium. Although volume was not noticed to be a critical factor, some differences in the number and size of plaques produced under greater depths of the larger overlay volumes may have been due to slower metabolic rates of cells under low oxygen tension. Since a 4-ml overlay volume was the most economical volume which afforded proper protection of the monolayer from dehydration, it was chosen as the standard volume to be used in the plaque assay system described.

The pH of the medium could be a contributing factor since the higher serum concentration was found to be conducive to optimal plaque production. In this regard, the concentration of $NaHCO_3$ in the overlay could maintain an optimum or favorable pH condition during the 3-day incubation period. Dieg and Watkins (1964) reported that any concentration less than 0.22% allowed optimal production of plaques by CTFV in A-1 cells, but this was not found to be the case with CTFV-infected L-929 cells. In concentrations less than 0.12% or greater than 0.24%, the plaque size and titer were reduced. Although a wider range in concentrations could suffice, the optimal condition for plaque titer and size was found to be approximately 0.21% $NaHCO_3$ in the overlay medium.

Considering only the results obtained in CTFV-infected L-929 cells under varied $NaHCO_3$ concentrations, we might be able to explain the decrease in plaque production at certain concentrations on the basis of stability of CTFV. For example, this virus is rapidly inactivated at any pH below 6.4 and above 8.3 (Trent and Scott, 1966). Therefore, the

virus particles produced by an infected cell under such conditions may be inactivated before adsorption to the surrounding cells can take place. Also, as the normal cellular metabolism is continued the pH of the overlay medium correspondingly decreases. This added pH change may increase the rate of inactivation of CTFV and concurrently alter the physiological condition of the cells. This alteration could affect the susceptibility of L-929 cells to infection with CTFV. On the other hand, even if infection is successful, a decrease in metabolic activity of the cell may persist and interfere with the efficient replication of CTFV. Similar results were observed with poliovirus in primate cells by Gifford and Syverton (1957).

The apparent differences in ability of L-929 and A-1 cells to allow optimum plaque production under an overlay medium containing less than 0.12% $NaHCO_3$ may be due to differences in the rates of cellular metabolism. For example, L-929 cells are tumorigenic (Sanford et al, 1948) while the A-1 cell line was derived from normal cells. A comparison of cellular metabolism under varying pH conditions should provide further information in this regard.

An 0.1 ml adsorption volume was originally chosen since Allison and Valentine (1960b) reported that the rate of virus adsorption to a monolayer of cells was inversly proportional to the depth of the fluid covering the cells. Similarly, Lockhart and Groman (1958) found that the efficiency and rate of adsorption of the unstable Western equine encephalitis (WEE) virus were optimum when a minimal volume sufficient to protect the cells from drying was used. The volume used in the early studies with CTFV in L-929 cells was found to be an appropriate selection

since larger volumes, which were more suitable in regards to protection against drying, reduced the efficiency of viral attachment. For example, a 0.2 ml volume decreased the plaque titer by approximately 33%.

Although other investigators have reported that 99% of the CTFV had attached to L-929 cells within 30 min (Trent and Scott, 1964), a 90-min adsorption period was used in the initial experiments in an effort to ensure that most of the infectious CTFV was adsorbed to the cells. However, on the basis of the results obtained in this study, it appears that an attachment period of 2 hr was necessary for maximum viral adsorption.

The possible discrepancy could lie in the strain of CTFV used by Trent and Scott. Whereas experiments described in this thesis were conducted with mouse brain-propagated virus, Trent and Scott used the L-929-adapted virus. It is highly plausible that the cell-adapted virus posessed a higher efficiency of attachment to L-929 cells than the mouse brain-adapted virus.

Although the entire procedure for the plaque assay of CTFV was designed using L-929 cells, other lines of cells showing susceptibility to this virus in the tube culture assay should demonstrate plaques under an agar overlay system. As presented in this thesis, KB cells were found to be susceptible to CTFV, but when these cells were used as the indicator cells in the plaque assay system, differences in susceptibilities were noticed. The virus titers under the overlay were 1000-fold higher than in the fluid medium. The possible explanations for these results are as follows:

A volume of 1 ml was used in the initial infection of tube cultures in contrast to 0.1 ml for plaque assay. This increased volume could

have decreased the efficiency of attachment as was observed in the plaque assay procedure. However, differences in titer were not observed in the L-929 cells under both assay conditions. Possibly, the reversible phase of viral attachment in KB cells may be longer than in L-929 cells, and this prolonged period of "unsteady state" could very well facilitate inactivation.

Conceivably, the rates of viral replication may play a vital part. It was noted that the plaque size seen in L-929 cells was considerably larger than found in KB cells. Excluding the possibility of agar or serum inhibitors, the KB cells may release virus after a longer eclipse period and in considerably lower numbers than L-929 cells. This could account for the reduced plaque size.

All tube culture assays were terminated 10 days after infection. Consequently, if it was possible to retain cultures of the KB cells for perhaps 20 days, a comparable titer might have been obtained. Unfortunately the KB cells began to degenerate nonspecifically after 10 days of incubation under these conditions.

In conclusion, differences in plaque size were noted in all petri dishes. Although plaque purification was not attempted to ascertain genetic stability, it is possible that the virus suspension used contained a heterogeneous population of CTFV mutants. These mutants may have expressed their genotypic characteristics through plaque morphology. In view of such mutants having been reported for other viruses, including poliovirus (Takemori et al, 1956), EMC virus (Takemoto and Liebhaber, 1961), and WEE virus (Ushijima, 1961), further studies concerning these properties should be investigated.

SUMMARY AND CONCLUSION

A plaque assay procedure for Colorado tick fever virus (CTFV) in monolayers of L-929 cells was described, and several factors which might affect the sensitivity of the system were investigated. The experimental results are summarized as follows:

A. Susceptibilities of L-929, KB, and embryonic human skin (EHS) cells to infection with CTFV in tube cultures were compared. Because of the higher susceptibility under these conditions, L-929 cells were selected as the indicator cell in developing a plaque assay technique for this virus.

B. The concentration of agar in the overlay had little effect on the numbers of plaques produced. However, at concentrations greater than 0.8%, plaque size was markedly reduced. A 0.3% agar concentration was selected since this resulted in the production of optimum-sized plaques. In addition, this concentration facilitated the removal of the overlay without affecting the monolayers and enabled the use of crystal violet for staining. This stain permitted the visualization of clearly defined plaques in contrast to those observed when monolayers were stained with neutral red.

C. Ionagar No. 2, Bacto-agar, purified agar, and washed agar were all shown to give optimal plaque production. Noble agar reduced the plaque size as well as plaque numbers.

D. Several nutrient media supplemented with 2, 5, or 10% calf serum were compared. M199, MEM, BME, or LaE supplemented with 10% calf serum were comparable in providing optimal plaque production. M199 with 10% calf serum was found to allow consistently higher numbers

of plaques. However, there appeared to be a correlation between increased serum concentrations and plaque numbers in all media.

E. The volume of overlay had no effect on the numbers of plaques produced by CTFV in L-929 cells, but plaque size was suppressed under greater volumes.

F. For maximal numbers and size of plaques, a concentration between 0.15% and 0.24% $NaHCO_3$ in the overlay medium was optimal.

G. A volume of 0.1 ml was found to be most efficient for the adsorption of CTFV to L-929.

H. After a 120-min adsorption period there was no further increase in the number of plaques produced.

I. An experiment to determine the relative susceptibilities of KB and L-929 cells to CTFV infection under the plaque assay conditions showed both cells gave comparable numbers of plaques. However, the plaques formed in KB cell monolayers were considerably smaller and less clearly defined than CTFV induced plaques in L-929 cells.

J. Finally, it was observed that at least two different sizes of plaques were present in L-929 infected monolayers. The possible significance in terms of viral mutants was briefly discussed.

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APPENDIX

Earle's Balanced Salt Solution

Components	mg/liter
$CaCl_2 \cdot 2H_2O$	265.0
KCL	400.0
$MgSO_4 \cdot 7H_2O$	200.0
NaCl	6,800.0
$NaHCO_3$	2,200.0
$NaH_2PO_4 \cdot H_2O$	140.0
Dextrose	1,000.0
Phenol Red	10.0

Basal Medium Eagle

Components	mg/liter
Amino Acids	
L-Arginine HCl	21.1
L-Cystine	12.0
L-Glutamine	292.0
L-Histidine HCl·H_2O	10.5
L-Isoleucine	26.2
L-Leucine	26.2
L-Lysine HCl	36.5
L-Methionine	7.5
L-Phenylalanine	16.5
L-Threonine	23.8
L-Tryptophan	4.0
L-Tyrosine	18.1
L-Valine	23.4
Vitamins	
D-Biotin	1.0
D-Ca-Pantothenate	1.0
Choline Chloride	1.0
Folic Acid	1.0
i-Inositol	1.8
Nicotinamide	1.0
Pyridoxal HCl	1.0
Riboflavin	0.1
Thiamine HCl	1.0
Inorganic Salts and Other Components	
Earle's BSS	
$CaCl_2 \cdot 2H_2O$	265.0
KCl	400.0
$MgSO_4 \cdot 7H_2O$	200.0
NaCl	6,800.0
$NaHCO_3$	2,200.0
$NaH_2PO_4 \cdot H_2O$	140.0
Dextrose	1,000.0
Phenol Red	10.0

Minimum Essential Medium

Components	mg/liter
"Essential" Amino Acids	
L-Argine HCl	126.4
L-Cystine	24.0
L-Glutamine	292.0
L-Histidine HCl H_2O	41.9
L-Leucine	52.4
L-Lysine HCl	73.1
L-Methionine	14.9
L-Phenylaline	33.0
L-Threonine	47.6
L-Tryptophan	10.2
L-Tryosine	36.2
L-Valine	46.8
L-Isoleucine	52.5
"Non-Essential" Amino Acids	
L-Alanine	89.0
L-Asparagine	13.0
L-Aspartic acid	13.0
L-Glycine	7.5
L-Glutamic acid	15.0
L-Proline	12.0
L-Serine	11.0

Minimum Essential Medium

Components	mg/liter
Vitamins	
D-Ca-Panthothenate	1.0
Choline Chloride	1.0
Folic Acid	1.0
i-Inositol	2.0
Nicotinamide	1.0
Pyridoxal HCl	1.0
Riboflavin	0.1
Thiamine HCl	1.0
Inorganic Salts and Other Components	
Earle's BSS	
$CaCl_2 \cdot 2H_2O$	265.0
KCl	400.0
$MgSO_4 \cdot 7H_2O$	200.0
NaCl	6,800.0
$NaHCO_3$	2,200.0
$NaH_2PO_4 \cdot H_2O$	140.0
Dextrose	1,000.0
Phenol Red	10.0
Sodium Pyruvate	110.0

Medium 199

Components	mg/liter
Amino Acids	
L-Alanine	25.0
L-Arginine HCl	70.0
L-Aspartic Acid	30.0
L-Cysteine HCl	0.1
L-Cystine	20.0
L-Glutamic Acid	67.0
L-Glutamine	100.0
L-Glycine	50.0
L-Histidine HCl·H_2O	22.0
L-Hydroxyproline	10.0
L-Leucine	60.0
L-Lysine HCl	70.0
L-Methionine	15.0
L-Phenylalanine	25.0
L-Proline	40.0
L-Serine	25.0
L-Threonine	30.0
L-Tryptophan	10.0
L-Tyrosine	40.0
L-Valine	25.0
L-Isoleucine	20.0

Medium 199

Components	mg/liter
Vitamins	
P-Aminobenzoic Acid	0.050
Ascorbic Acid	0.050
D-Boitin	0.010
Calciferol	0.100
D-Ca-Pantothenate	0.010
Cholesterol	0.200
Choline Chloride	0.500
Folic Acid	0.010
i-Inositol	0.050
Menadione	0.010
Nicotinamide	0.025
Nicotinic Acid	0.025
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.010
Thiamine HCl	0.010
DL-α-Tocopherolphosphate (Na_2)	0.010
Tween 80*	5.000
Vitamin A	0.100

Medium 199

Components	mg/liter
Other Components	
Adenine $HCl \cdot 2H_2O$	12.10
Adenosine-5'-Monophosphoric acid, dihydrate (AMP) (Muscle Adenylic Acid)	0.20
Adenosine-5'-Triphosphate disodium, tetrahydrate (ATP)	1.00
Deoxyribose	0.50
Dextrose	1,000.00
L-Glutathione	0.05
Guanine $HCl \cdot H_2O$	0.33
Hypoxanthine	0.30
Phenol Red	20.00
Ribose	0.50
Sodium Acetate $\cdot 3H_2O$	83.00
Thymine	0.30
Uracil	0.30
Xanthine	0.30
Inorganic Salts	
$CaCl_2 \cdot 2H_2O$	186.0
$Fe(NO_3)_3 \cdot 9H_2O$	0.7
KCl	400.0
KH_2PO_4	60.0
$MgCl_2 \cdot 6H_2O$	100.0
$MgSO_4 \cdot 7H_2O$	100.0
NaCl	8,000.0
$NaHCO_3$	1,250.0
$Na_2HPO_4 \cdot 7H_2O$	94.0

AN ABSTRACT OF THE THESIS OF

Marvin Eugene Frazier for the Master of Science in Microbiology

A PLAQUE TECHNIQUE FOR THE ENUMERATION OF INFECTIOUS PARTICLES OF COLORADO TICK FEVER VIRUS IN L-929 CELL MONOLAYERS

Approved Charles E. Gordon
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ABSTRACT

In the past, investigations of Colorado tick fever virus (CTFV) have been performed using the tube dilution method of assay in mammalian cell cultures. However, in order to study this virus adequately, it has become necessary to develop a procedure for the enumeration of infectious CTFV particles.

In the plaque assay procedure cell monolayers are infected with dilutions of virus suspensions. These infected monolayers are then overlaid with a semisolid nutrient solution which limits the spread of virus from an infected cell to neighboring cells. As a result, local macroscopic areas of CPE, or plaques develop. If the monolayer is then stained with a vital stain such as neutral red, the plaques become visible as clear areas in a faint red background.

According to the one particle theory, each focus of infection is initiated by a single virus particle or aggregation of particles not divisible by dilution. Thus, the plaque assay method enables one to determine the number of infectious particles, or plaque-forming units, per unit volume.

Susceptibilities of L-929, KB, and embryonic human skin (EHS) cells to infection with CTFV in the tube cultures were compared. Because of the higher susceptibility under these conditions, L-929 cells were selected as the indicator cell in developing a plaque assay technique for this virus. Such a method of assay for CTFV in monolayers of L-929 cells subsequently described and several factors which might affect the sensitivity of the system were investigated.

The concentration of agar in the overlay had little effect on the

numbers of plaques produced. However, at concentrations greater than 0.8%, plaque size was markedly reduced. A 0.3% agar concentration was selected since this resulted in the production of optimum-sized plaques. In addition, this concentration facilitated the removal of the overlay without affecting the monolayers and enabled the use of crystal violet. This stain permitted the visualization of clearly defined plaques in contrast to those observed when monolayers were stained with neutral red.

Ionagar No. 2, Bacto-agar, purified agar, and washed agar were all shown to give optimal plaque production. Noble agar reduced the plaque size as well as plaque numbers.

Several nutrient media supplemented with 2, 5, or 10% calf serum were compared. M199, MEM, BME, or LaE supplemented with 10% calf serum were comparable in providing optimal plaque production. M199 with 10% calf serum was found to allow consistently higher numbers of plaques. However, there appeared to be a correlation between increased serum concentrations and plaque numbers in all media.

The volume of overlay had no effect on the numbers of plaques produced by CTFV in L-929 cells, but plaque size was suppressed under greater volumes.

For maximal numbers and size of plaques, a concentration between 0.15% and 0.24% $NaHCO_3$ in the overlay medium was optimal.

A volume of 0.1 ml was found to be most efficient for the adsorption of CTFV to L-929.

After a 120-min adsorption period there was no further increase in the number of plaques produced.

An experiment to determine the relative susceptibilities of KB

and L-929 cells to CTFV infection under the plaque assay conditions showed both cells gave comparable numbers of plaques. However, the plaques formed in KB cell monolayers were considerably smaller and less clearly defined than CTFV-induced plaques in L-929 cells.

Finally, it was observed that at least two different sizes of plaques were present in L-929 infected monolayers. The possible significance in terms of viral mutants was briefly discussed.