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TECHNICAL MANUSCRIPT 371

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MARCH 1967

DEPARTMENT OF THE ARMY
Fort Detrick
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ABSTRACT

The conditions under which Venezuelan equine encephalomyelitis (VEE) virus attached to host cells markedly influenced the assay of virus by the fluorescent cell-counting technique. When virus inoculum was centrifuged onto McCoy cell monolayers, approximately 97% of virus was attached to cells within 10 minutes in contrast to 34% after stationary incubation at 35 C for 2 hours. Maximum binding of virus occurred only in the presence of 0.1 to 0.15 M NaCl. This salt requirement, added to evidence of pH dependence and temperature independence of VEE virus attachment to cells, indicated that the initial union involved electrostatic forces. Virus penetration, measured by the insensitivity of virus-cell complexes to viral antiserum, was complete in 30 minutes at 35 C. The process was temperature-dependent and unaffected by the ionic content of medium. For assay of VEE virus by the fluorescent cell-counting technique, infected cells may be enumerated as early as 12 hours after infection of cell monolayers. The relationship between virus concentration and cell-infecting units was linear; the distribution of fluorescent cells was random. Virus assay by the fluorescent technique was equivalent in sensitivity but more precise and rapid than that by intracerebral inoculation of mice.

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I. INTRODUCTION

Recently, rapid and quantitative assays were developed for a number of viruses[1-3] and a rickettsia[4] that are based on immunofluorescent staining of infected cell monolayers and enumeration of cells containing fluorescent viral or rickettsial antigens. The feasibility of extending this technique to the assay of Venezuelan equine encephalomyelitis (VEE) virus was investigated, because it was previously demonstrated that cells infected with the virus are amenable to immunofluorescent staining.[5] In developing the assay, certain specific requirements were encountered for the attachment of VEE virus to cells that, in some aspects, may be unique to this arbovirus. The availability of a quantitative assay of the virus and of a technique that promotes efficient, rapid, and almost simultaneous virus attachment to cells facilitated kinetic studies on the early reactions between this animal virus and host cells.

This report describes an investigation of initial virus-cell interactions in the course of developing a rapid assay of VEE virus by the fluorescent cell-counting technique. The relevance of the findings to other virus-cell host systems is discussed.

II. MATERIALS AND METHODS

A. VIRUS

The Trinidad strain of VEE virus was used throughout this study; its history has been recorded elsewhere.[6] A working suspension of virus was prepared by inoculating monolayers of McCoy cells grown in milk dilution bottles with 10^{-1} dilution of 20% chick embryo suspension of virus. After adsorption of inoculum at 35 C for 2 hours, cell monolayers were washed, overlaid with 5 ml of maintenance medium, and incubated at 35 C. Widespread destruction of cell monolayers occurred within 40 hours. The cell cultures were then frozen and thawed, and the culture fluid was distributed in 1-ml portions into glass vials and stored at -60 C. The suspension contained $10^{9.1}$ mouse intracerebral (MIC) LD_{50} units of virus per ml. Prior adaptation of VEE virus to the cell line was not a requisite because mouse brain or embryonated egg suspensions of virus readily infected cell monolayers.

B. CELL LINE AND CULTIVATION

The established cell line, McCoy, derived from human synovial tissue[7] was used in the assay of virus. Preliminary tests indicated that more than 99% of the cells were susceptible to the virus. Nutrient medium for the cell line consisted of medium 199 containing 0.5% lactalbumin hydrolyzate, 10% heat-inactivated calf serum, and 50 μg per ml of streptomycin and 75 μg per ml of kanamycin. Cells were cultivated on circular cover slips (15-mm diameter) inserted in flat-bottomed glass vials (19 by 65 mm). A 1-ml amount of cell suspension, containing 1 x 10^5 to 3 x 10^5 cells, was introduced onto cover slips that were then incubated at 35 C for 24 hours, or until a complete cell monolayer was formed. Cell monolayers were washed with an appropriate diluent before the addition of virus inoculum.

C. VIRUS ASSAY

Determinations were usually carried out in triplicate. Virus dilutions were prepared in phosphate-buffered saline (PBS), pH 7.1, free of calcium or magnesium ions. This consisted of 8.5 g NaCl, 1.07 g Na_2HPO_4 (anhydrous), and 0.39 g $NaH_2PO_4 \cdot 2H_2O$ per liter of distilled water. Inoculum in 0.2-ml volume was introduced directly onto cover slip cell monolayers following their transfer from glass vials to rotor chamber inserts.[2] The latter were employed because they withstand the high centrifugal force required to sediment VEE virus. To adsorb the inoculum with the aid of centrifugal force, rotor chamber inserts placed in a swinging bucket-type SW 25.1 rotor were centrifuged in a model L Preparative Ultracentrifuge* at 19,642 to 29,432 x g for 15 minutes at 30 C. The residual inoculum was removed after centrifugation, the cover slip cell monolayers were replaced into glass vials, and 1 ml of maintenance medium was then added to each vial. After incubation at 35 C for 1 hour, the maintenance medium was replaced with 1 ml of 1:40 dilution of virus antiserum in medium 199 (serum-neutralization index, 4.8 $\log_{10}$ for undiluted serum). The rationale for this procedure is presented in Section III. After further incubation at 35 C from 20 to 24 hours, cover slip cell monolayers were rinsed twice with cold PBS, fixed with cold (-60 C) acetone, and either prepared immediately for immunofluorescent staining and cell counting or stored at -60 C. Fluorescence of viral antigens in fixed cell cultures was not diminished when they were stored under these conditions for 8 weeks.

The method of intracerebral inoculation of mice for assay of virus has been described previously.[6]

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* Beckman Instruments, Inc., Spinco Division, Palo Alto, California.

D. VEE ANTISERUM CONJUGATE AND IMMUNOFLUORESCENT STAINING

Aerogenic vaccination of rhesus monkeys was employed to make VEE antiserum. One month after vaccination, the animals were bled. The antiserum was conjugated with fluorescein isothiocyanate by the method of Riggs et al.[8] To remove unbound dye, the conjugated antiserum was passed through a column of Sephadex G-25.

The direct fluorescent antibody method was used to demonstrate immunofluorescence of viral antigens in infected cells. Fixed cell cultures were washed three times with PBS and stained with conjugated antiserum for 30 minutes at room temperature. Cover slip cell monolayers were rinsed in two changes of PBS to remove excess conjugate and then mounted in a semipermanent medium.[9]

E. FLUORESCENCE MICROSCOPY AND CELL COUNTING

Cover slip cell cultures were examined with an American Optical microscope equipped with a Fluorolume illuminator (model 645), Corning no. 5840 and Schott BG-12 exciter filters, and an E.K. no. 2A barrier filter. With this optical system at magnification 430X, the number of microscopic fields in the area of a 15-mm cover slip was 1,064. For each cover slip cell monolayer, 50 microscopic fields were examined for fluorescent cells. To calculate the number of cell-infecting units of virus per ml, the average number of fluorescent cells per field was multiplied by the number of fields per cover slip, the reciprocal of the dilution of virus inoculum, and a volume factor (for conversion to milliliters).

F. DETERMINATION OF VIRUS ATTACHMENT

Attachment was measured by following the disappearance of virus from inoculum after its addition onto cell monolayers. Cover slip monolayers were inoculated with virus suspension, multiplicity of infection 0.1, in 0.2-ml volumes. After designated intervals of incubation or centrifugation, residual inoculum was removed and the cell cultures were immediately washed twice with PBS. Residual inoculum was then introduced onto fresh cell monolayers to measure unattached virus. For this, the residual inoculum was adsorbed onto cell cultures by centrifugation at 19,642 to 29,432 x g for 30 minutes. Cover slip cell monolayers exposed to initial or residual inocula were then treated in the manner described earlier for virus assay. The amount of virus that was attached to cells at a given time was expressed as a percentage of the virus input. The virus input was the sum of the amounts of attached and free virus.

G. DETERMINATION OF VIRUS PENETRATION

Virus penetration into cells was measured by the insensitivity of attached virus to antiserum. Inoculum was attached to cell monolayers as described above. Cell cultures were washed twice with PBS, overlaid at designated intervals with 1 ml of 1:40 dilution of virus antiserum, and then incubated at 35 C for 20 hours. The quantity of virus that penetrated cells at a given time was expressed as a percentage of the input virus.

H. CALCULATION OF ATTACHMENT AND PENETRATION CONSTANTS

The attachment and penetration rate constants (k) were calculated from the relationship $\ln (V_0/V_t)nt$, where V_0 = the input virus concentration, V_t = unattached or unpenetrated virus at time t, and n = the number of cells per cm^3 determined by resuspension of cover slip cell cultures.

III. RESULTS

A. VIRUS ATTACHMENT*

Previous findings indicated that centrifugal force was highly efficient in promoting the attachment of virus to cells.[1-3] An experiment was performed to determine the rate of VEE virus attachment onto cell monolayers during stationary incubation at 35 C and during centrifugation (19,642 to 29,432 x g at 30 C). The concentration of cells per cover slip culture was 5.3×10^5 per ml. Inocula employed for stationary and centrifugation experiments were 10,853 and 5,171 cell-infecting units, respectively. Additional experimental details are described in Section II.

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* In this study, the terms employed to denote the early stages of virus-cell interactions are defined as follows: Adsorption is a general term referring to the various interactions and processes involved in the initial binding of virus to cells leading to the loss of identity of the virus. Its implications of physicochemical nonspecificity, however, limit its descriptive usefulness.[10,11] Attachment is the initial, specific union between virus and cells, which may or may not be reversible. Penetration refers to the progressive insensitivity of virus-cell complexes to viral antiserum.

The rate of virus attachment with each treatment is shown in Figure 1. Within 10 minutes, 97% of virus inoculum was attached when aided by centrifugal force. After stationary incubation at 35 C for 2 hours, only 34% of virus inoculum was attached. The attachment rate constant (k) was 3.3 x 10^{-7} cm^3 min^{-1} with centrifugation and 7.3 x 10^{-9} cm^3 min^{-1} with stationary incubation. The latter is consistent with findings reported for Newcastle disease virus and poliovirus under similar circumstances.[12,13] Because centrifugation was highly efficient and rapid for virus attachment to cells, it was adopted as the standard procedure.

In preliminary experiments, erratic virus assay values resulted when either McCoy cell maintenance medium or PBS containing $CaCl_2$ and $MgCl_2$ were used for dilution of virus inoculum. In addition, the values were often as much as 1.0 log unit less than those obtained by intracerebral inoculation of mice. To test the hypothesis that these events may be related to the attachment of virus to cells, different diluents were employed to prepare virus inocula. The molarity of $CaCl_2$ and $MgCl_2$ in diluents was similar to that usually found in cell cultivation reagents. Immediately after centrifugation of virus inoculum onto cells, monolayers were rinsed twice with PBS and treated in the prescribed manner. Results in Table 1 show that virus attachment and, consequently, virus assay values were significantly affected by the composition of the diluent. The largest quantity of virus that attached to cell monolayers occurred with PBS containing a monovalent cation; lesser amounts of virus attached in the presence of divalent cations. Magnesium ions were more marked in impeding virus attachment than were calcium ions. In the presence of calf serum, virus attachment decreased as the concentration of serum increased. In view of these findings, PBS free of calcium and magnesium ions was employed routinely as the virus diluent.

The possibility that the low assay values obtained with diluent containing divalent cations may be the result of virus elution from cells after initial attachment was investigated. Virus inoculum was prepared in PBS containing calcium and magnesium ions and in PBS free of these cations. Immediately after centrifugation of inoculum, cell monolayers were rinsed twice, and 0.2-ml volume of the corresponding diluent was added to cell monolayers. At 30-minute intervals, the diluent was removed and assayed for virus in the usual manner. Although virus appeared to elute more rapidly in PBS containing $CaCl_2$ and $MgCl_2$ than in PBS free of the divalent salts, the amount that eluted in the presence of either diluent was less than 0.1% within 2 hours (Table 2). Comparable results were noted with medium 199 containing 5% calf serum. Virus elution from cells was not a significant factor contributing to low virus assay values. An additional factor considered was that of virus aggregation. Virus suspended in PBS containing divalent salts was treated for 5-, 10-, and 15-second periods with an ultrasonic probe.* Assay values were comparable between sonic-treated virus suspensions and untreated controls. There was no indication from the results that virus aggregation occurred in the presence of divalent cations.

* Model BP-1, Blackstone Ultrasonics, Inc., Sheffield, Pa.

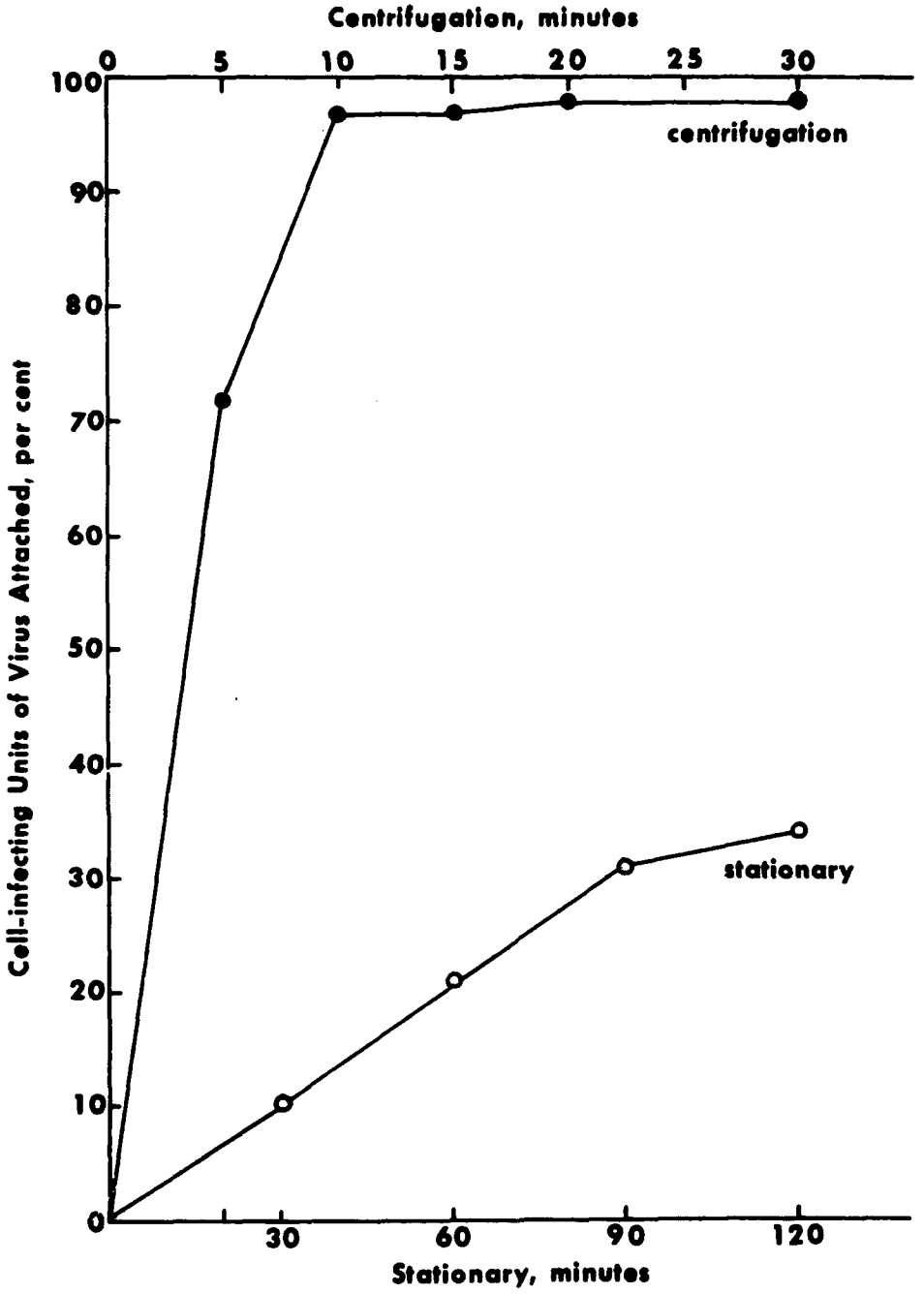

Figure 1. Attachment of VEE Virus onto Cover Slip Cultures of McCoy Cells by Centrifugation (19,642 to 29,432 x g, 30 C) and Stationary Incubation (35 C).

TABLE 1. EFFECT OF VIRUS DILUENT ON ATTACHMENT AND ASSAY OF VEE VIRUS

Diluent	Virus Attached, %	Virus Titer, 10^8 CIU[a] per ml
PBS[b]	99.0	16.7
PBS + 0.0009 M $CaCl_2$ and 0.0005 M $MgCl_2$	20.9	3.5
PBS + 0.0009 M $CaCl_2$	23.3	3.9
PBS + 0.0005 M $MgCl_2$	5.9	1.0
Medium 199 + 5% calf serum	31.7	5.3
Medium 199 + 10% calf serum	19.1	3.2
Medium 199 + 20% calf serum	8.9	1.5
Heart infusion broth	25.1	4.2

a. Cell-infecting units of virus. Virus input was 6,800 CIU with 0.2 ml inoculum.
b. 0.15 M NaCl buffered by 0.01 M phosphate buffer.

TABLE 2. ELUTION OF VEE VIRUS AT 35 C AFTER ATTACHMENT TO McCOY CELLS

Attachment Time, min	PBS[a] Plus Ca^{++} Mg^{++}		PBS Free of Ca^{++} Mg^{++}	
	CIU[b]	Virus Eluted, %	CIU	Virus Eluted, %
0	42	0.01	0	0
30	106	0.04	0	0
60	127	0.05	42	0.01
90	170	0.07	84	0.03
120	234	0.09	216	0.09

a. PBS consisted of 0.15 M NaCl buffered by 0.01 M phosphate solution plus 0.0009 M $CaCl_2$ and 0.0005 M $MgCl_2$.
b. Cell-infecting units of virus eluted. Virus input was 2.4 x 10^5 CIU with 0.2 ml inoculum.

To ascertain if the electrolyte requirement for maximum attachment of VEE virus to cells is dependent upon the presence of a specific cation, other monovalent cations were substituted for sodium in 0.01 M phosphate buffer. The results in Table 3 indicate that maximum attachment of virus was attained only with NaCl. Less effective in promoting virus attachment were the monovalent cations potassium and ammonium. The finding that a small quantity of virus is attached to cells in the presence of sucrose solution is similar to previous observations with herpes virus and poliovirus.[13-15]

TABLE 3. VEE VIRUS ATTACHMENT TO McCOY CELLS IN THE PRESENCE OF MONOVALENT CATIONS

Diluent	Virus Attached, %	Virus Titer, 10^8 CIU[a] per ml
PB[b] + 0.1 M NaCl	99.00	18.00
PB + 0.1 M KCl	5.20	0.95
PB + 0.1 M K_2SO_4	0.55	0.10
PB + 0.1 M NH_4Cl	0.83	0.15
PB + 0.25 M sucrose	4.11	0.74

a. Cell-infecting units of virus. Virus input was 7,565 CIU in 0.2 ml inoculum.
b. Phosphate buffer, 0.01 M.

The quantity of virus attached to cells as a function of NaCl concentration was investigated. Virus dilutions were prepared in 0.01 M phosphate buffer containing concentrations of NaCl ranging from 0.01 to 0.5 M. The range of molarity for testing was limited because cell monolayers exhibited plasmolysis or plasmoptysis beyond the designated range. Virus attachment to cells was carried out and determined in the usual manner. Results in Figure 2 show that concentrations of NaCl from 0.1 to 0.15 M were optimal for achieving maximal binding of virus to cells. Greater or lesser salt concentrations markedly inhibited attachment. These findings are compatible with the proposed role of salt concentration in attaining an electrostatic complementary configuration between virus and cells for maximal attachment.[16]

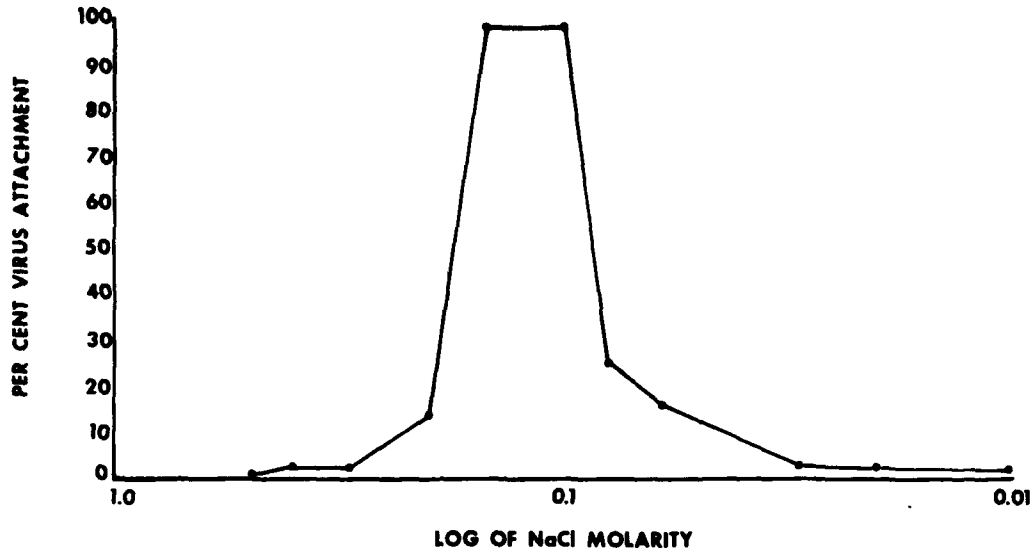

Figure 2. Attachment of VEE Virus to McCoy Cells in the Presence of Different NaCl Concentrations.

Previous studies demonstrated that the attachment response of bacteriophage to its bacterial host[17,18] and Newcastle disease virus to host cells[13] was affected by variations in the pH of the suspending medium. This suggested that chemical groups participated in the binding of virus to cells. To determine whether pH of the suspending medium was important in the virus-host cell system currently under study, Sorensen's phosphate buffer containing 0.15 M NaCl, adjusted to give pH values from 4.3 to 8.7, was used as the attachment medium. That chemical groups (carboxyl and amino) are also involved in the attachment of VEE virus to cells is suggested by the pH dependence of the reaction (Table 4). Maximal binding of virus occurred near neutrality. Under either acid or basic conditions, when ionization of carboxyl and amino groups was depressed, virus attachment decreased markedly.

TABLE 4. EFFECT OF pH ON ATTACHMENT OF VEE VIRUS TO McCOY CELLS

Attachment Medium[a] pH	Virus Attached, %	Virus Titer, 10^8 CIU[b] per ml
4.3	2.8	0.3
5.1	5.7	0.6
6.1	8.5	0.9
7.2	98.0	11.0
8.2	40.9	4.5
8.7	18.0	2.0

a. Sorensen's phosphate buffer with 0.15 M NaCl.
b. Cell-infecting units of virus. Virus input was 2,234 CIU in 0.2 ml inoculum.

The effect of temperature on the attachment of VEE virus to cells during centrifugation was studied by sedimenting inoculum onto cell monolayers at temperatures from 30 to 4 C. With the exception of this variable, the procedure for virus attachment and assay was similar to that described earlier. The finding that initial binding of virus to cells was independent of temperature is further evidence of the electrostatic nature of the reaction.[11] Similar results were obtained by others with bacteriophage,[19] Newcastle disease virus,[13] poliovirus,[12] herpes virus,[14] and fowl plague and vaccinia viruses.[20]

B. VIRUS PENETRATION

It was necessary to determine the rate of virus penetration into cells when it was discovered that enumeration of individual fluorescent cells was precluded by the appearance of microplaques between 12 and 16 hours after virus infection of cell monolayers. To provide a more convenient time for counting individual infected cells, virus antiserum was employed to prevent a second cycle of infection of cell monolayers by extracellular virus. The earliest time at which antiserum may be added without neutralizing attached virus was determined by following the rate of virus penetration into cells. The experiment was carried out at 35, 28, and 4 C. Results in Figure 3 indicate that virus penetration into cells is temperature-dependent. At 35 C, there was a short, initial period of approximately 10 minutes when virus attached to the cell surface was highly sensitive to antiserum. Thereafter, virus penetration proceeded at a linear rate with a velocity constant (k) of 6.9×10^{-8} cm^3 min^{-1} and was complete in approximately

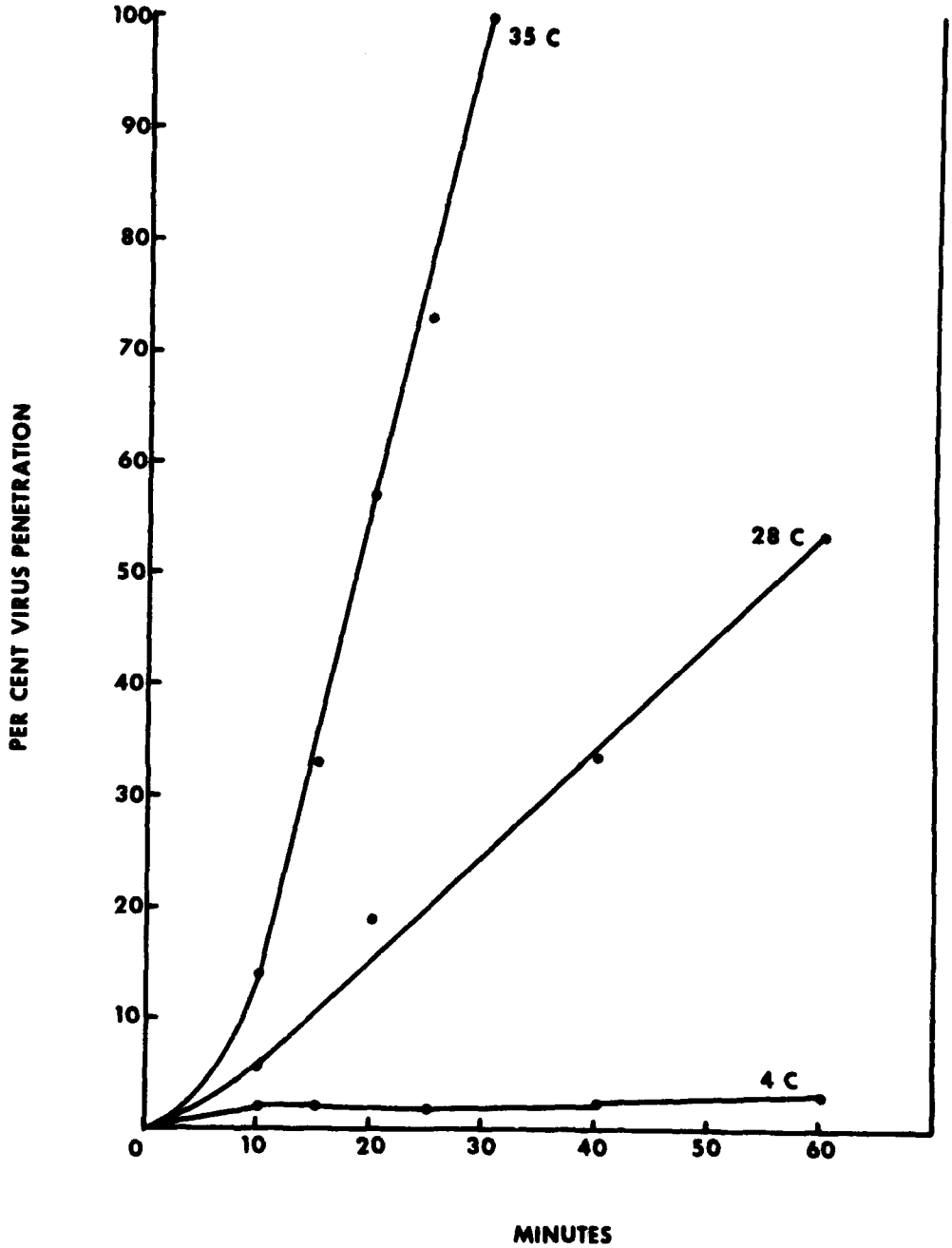

Figure 3. Effect of Temperature on the Penetration of VEE Virus into McCoy Cells as Measured by Insensitivity to Viral Antiserum.

30 minutes. At 28 C, virus penetration also appeared to be linear but only 50% of attached virus penetrated into cells in 56 minutes. The quantity of virus that penetrated into cells at 4 C was less than 5% in 60 minutes. For routine assay of virus, cell cultures were incubated at 35 C for 1 hour after virus attachment. Antiserum was then added to prevent subsequent infection of cell monolayers by newly released virus.

Earlier, it was demonstrated that a specific monovalent cation was required to attain maximal attachment of VEE virus to cells. The presence of divalent cations appeared to affect the process. To ascertain if specific cations were needed to affect virus penetration into cells, cell monolayers were incubated at 35 C for 1 hour with medium of different cationic content immediately after virus attachment. The test media for virus penetration were then replaced with 1 ml of an appropriate dilution of virus antiserum and the cell cultures were incubated for 20 hours. From the comparable assay values that were obtained with the different media (Table 5), it is evident that the presence of electrolytes was not a prerequisite for virus penetration. Because cell monolayers were routinely overlaid with medium 199 containing 5% calf serum after virus attachment, this practice was continued.

TABLE 5. ROLE OF CATIONS IN THE PENETRATION OF VEE VIRUS INTO McCOY CELLS

Medium for Virus Penetration	Virus Titer, 10^{10} CIU[a] per ml
Deionized H_2O + 0.25 M sucrose	1.0
PB[b] + 0.0009 M $CaCl_2$, 0.0005 M $MgCl_2$	1.0
PB + 0.0009 M $CaCl_2$, 0.0005 M $MgCl_2$, 0.02 M EDTA[c]	1.0
PB + 0.15 M NaCl	1.1
Medium 199 + 5% calf serum	1.2

a. Cell-infecting units of virus. An alternative virus preparation was used in this experiment.
b. Phosphate buffer, 0.01 M.
c. Disodium (ethylenedinitrilo)tetraacetate.

C. INCUBATION PERIOD

The rapid rate of VEE virus multiplication in cell cultures[21] was indirectly substantiated by observations on the initial fluorescence of infected cells. The earliest visual signs of cellular infection by virus, in the form of faint, cytoplasmic fluorescence, appeared 8 hours after adsorption of inoculum. By 12 hours, the amount and intensity of fluorescence had markedly increased so that infected cells could be easily discerned (Fig. 4). Fluorescent viral antigen was diffuse, granular, and confined to cell cytoplasm. Between 12 and 16 hours, fluorescent microplaques were noted in cell monolayers that were indicative of a second cycle of virus infection. Microplaques usually contained from 5 to 20 fluorescent cells. The number doubled or tripled at 20 hours (Fig. 5). With extended incubation periods, microplaques coalesced to form a fluorescent cell monolayer.

Counts of infected cells were compared between cell cultures incubated with and without antiserum for periods of 8, 10, 12, 16, 20, and 24 hours. At either condition, the number of infected cells was doubled between 10 and 12 hours. Counts of individual infected cells were precluded with the appearance of microplaques in cell cultures incubated for 16 hours without the antiserum overlay. In the presence of antiserum, counts of infected cells were comparable between 12 and 24 hours. From observations on the development of fluorescent viral antigen and infected cell counts, individual infected cells may be enumerated as early as 12 hours after adsorption of virus inoculum. Antiserum must be added to inoculated cell cultures, however, when the incubation time is extended beyond 12 hours. In this study, an incubation period of 20 hours was employed for virus assay.

D. QUANTITATIVE EVALUATION OF ASSAY

Results in Figure 6 reveal a linear relationship between twofold dilutions of virus over a range of 1.5 log units and the number of cell-infecting units of virus. These data suggest that each fluorescent cell resulted from infection by a single infective virus particle or aggregates not divisible by dilution.

In a single experiment, 11 determinations were performed to estimate the precision of the assay of VEE virus. Cover slip cell monolayers were infected by a standard quantity of virus inoculum and then treated in the prescribed manner. The number of cell-infecting units of virus per ml of inoculum ranged from 1.0×10^9 to 1.7×10^9 with a mean of 1.4×10^9 and standard deviation (SD) of ± 0.19. Expressed as a percentage, the SD was 13% of the mean.

Figure 4. Fluorescent VEE Viral Antigen in McCoy Cells 12 Hours after Infection. 126X.

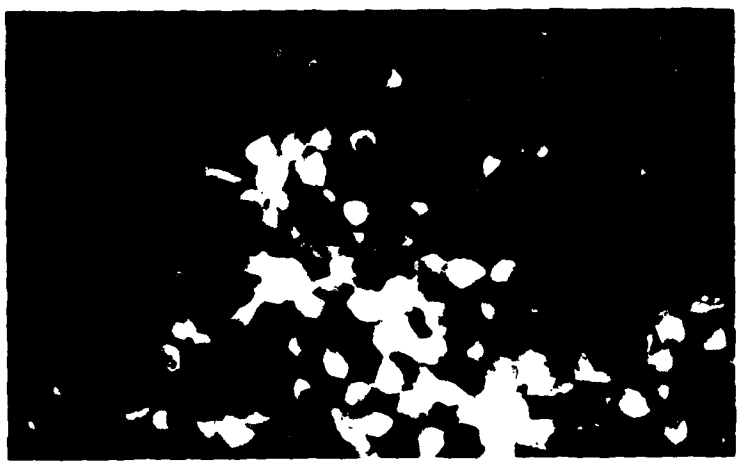

Figure 5. McCoy Cell Monolayer with Fluorescent Microplaque after Infection with VEE Virus 20 Hours Earlier. 126X.

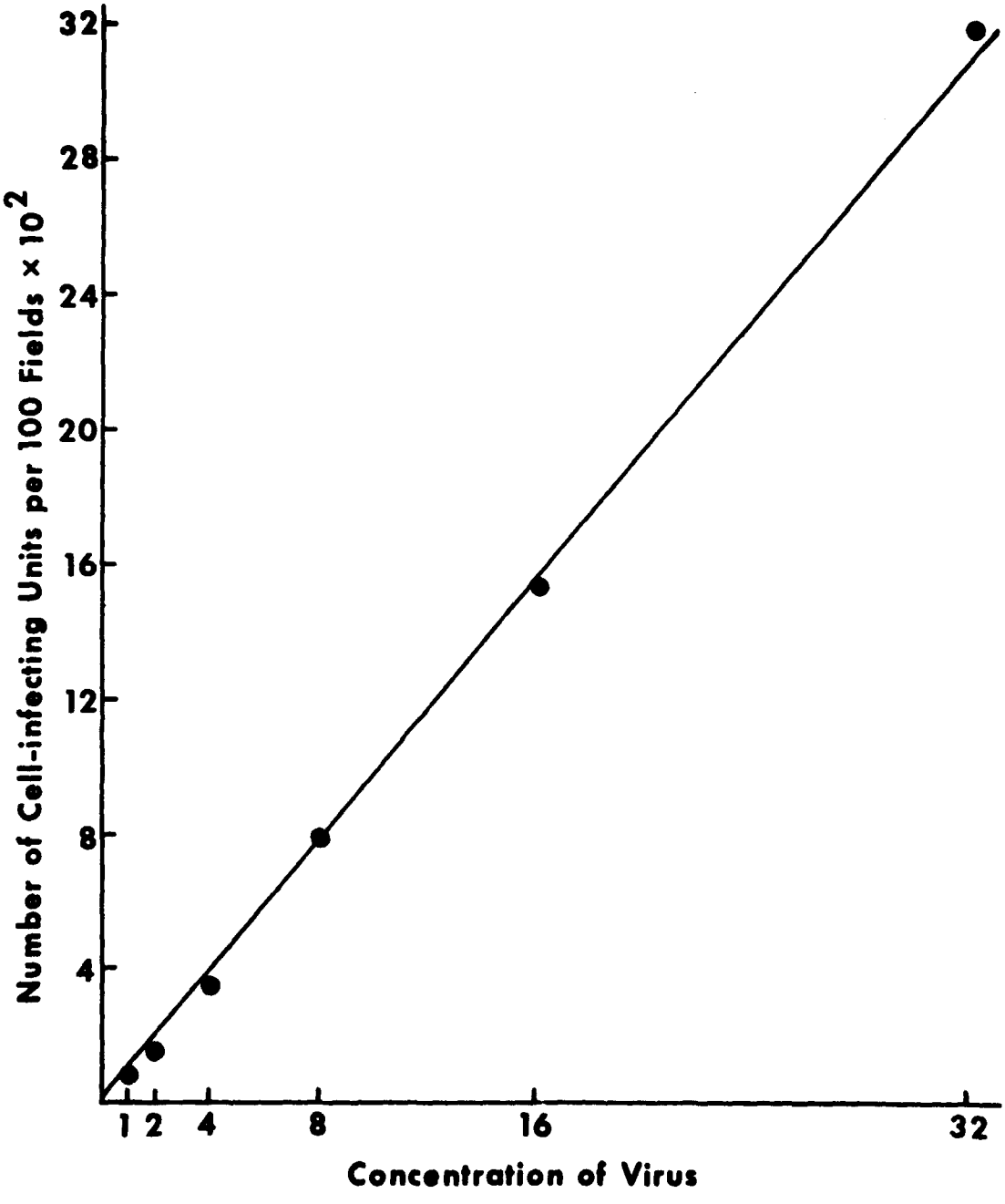

Figure 6. Linear Function between the Number of Cell-Infecting Units and Concentration of VEE Virus.

The mode of distribution of fluorescent cells on a cover slip monolayer was determined by examining 200 microscopic fields at random. The frequencies of fields containing infected cells correspond closely to the theoretical frequencies (Fig. 7). The χ^2 test of goodness of fit of the observed data to the theoretical Poisson distribution showed no significant deviation (probability of 0.83 with 6 degrees of freedom). Fluorescent cells were randomly distributed in cell monolayers.

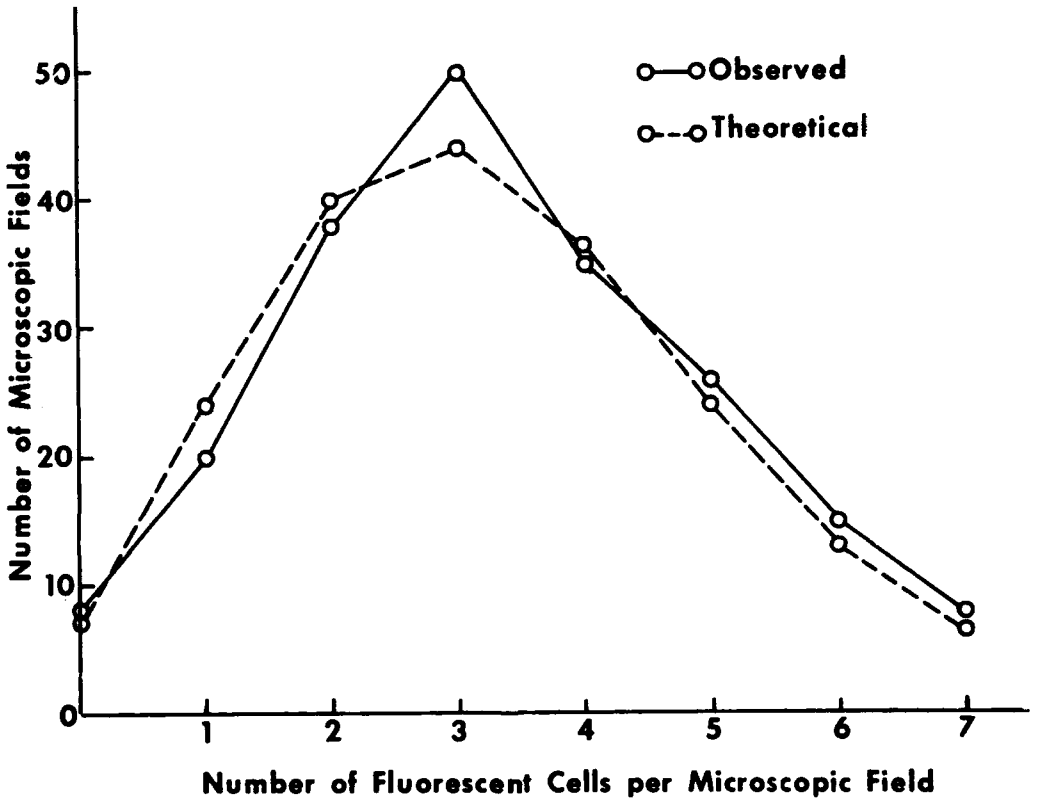

Figure 7. Frequency Distribution of Fluorescent Cells on McCoy Cell Monolayer Infected with VEE Virus.

The sensitivity of the fluorescent cell-counting assay was compared with that of intracerebral inoculation of mice. Results in Table 6 show that assay values were comparable by either method. The fluorescent cell-counting assay, however, exhibited less variability than the mouse assay. By the fluorescent method, results were attained within 20 hours; by the mouse assay, an observation time of 10 days was needed before values could be estimated.

TABLE 6. COMPARISON OF TWO PROCEDURES FOR ASSAY OF VEE VIRUS

Assay Procedure	Virus Titer	Standard Deviation	Standard Error
Fluorescent cell-counting	9.1[a/]	±0.05	±0.09
Intracerebral inoculation of mice	9.1[b/]	±0.18	±0.18

a. Reciprocal of cell-infecting units of virus ($\log_{10}$) per ml, based on six titrations, determined in 20 hours.
b. Reciprocal of LD_{50} ($\log_{10}$) mouse intracerebral units of virus per ml based on six titrations, 10-day observation period.

IV. DISCUSSION

The mechanism of VEE virus attachment to host cells appeared to be similar in many aspects to that of other animal virus and bacteriophage systems.[11] Studies on the primary interactions between virus and cells indicated, however, that maximal initial binding of virus to cells was achieved only under specific circumstances. The conditions under which virus attached to host cells profoundly influenced the assay of VEE virus by the fluorescent cell-counting technique. Centrifugation of virus inoculum onto cell monolayers promoted efficient and rapid attachment of virus. Approximately 97% of virus inoculum was bound to cells within 10 minutes in contrast to 34% after stationary incubation at 35 C for 2 hours. These results were comparable to those obtained with yellow fever virus.[2] Virus attachment mediated by centrifugal force attains added importance in view of the theory of Brownian motion as it relates to the arrival rate of virus particles in suspension at the surface of a cell system. Virus in Brownian motion may take an average of 4 hours to diffuse 0.1 mm, and several hours may elapse before half the virus particles reach the cell surface.[22] The effects of thermal inactivation on virus particles during extended incubation for attachment are minimized by the rapidity of virus binding to cells when augmented by centrifugal force. Furthermore, the short time required to achieve complete and almost simultaneous attachment of virus by this procedure helps to delineate that reaction from virus penetration and, thereby, makes it possible to measure accurately the rate of the penetration.

The attachment of VEE virus to cells was highly specific in its salt requirement in that maximal binding occurred only in the presence of a buffer solution containing defined concentrations of the monovalent salt NaCl. No effective substitute for the sodium cation was found in the limited number of monovalent salts that were tested. Divalent cations in the medium appeared to inhibit virus attachment. That divalent cations do not function as bridges between virus and cells is substantiated by these findings. The influence of cations on attachment of viruses to host cells may be interpreted in terms of interactions among particles that carry net negative charges.[23] Cations may function by altering the electrostatic configuration of the attachment sites so that complementation occurs between sites on the virus and on the cell surface. The salt requirement, added to evidence of pH dependence and temperature independence of the attachment of VEE virus to cells, supports the concept that the initial union involves electrostatic forces. Similar findings were reported with bacteriophages[11,16] and with other animal viruses.[12,13]

Although divalent cations, usually calcium and magnesium, are generally necessary for the electrostatic attachment of bacteriophages[24] and animal viruses to host cells,[10,12,25] there are numerous exceptions. The requirements for maximal binding of different T phages vary with the valency of cations and with the salt concentration.[26] Preliminary experiments carried

out in this study with another arbovirus, yellow fever (Group B member), indicated that its medium requirement for maximal attachment differed from that of VEE virus (Group A member). Centrifugation of yellow fever virus inoculum onto McCoy cells revealed that maximal virus attachment occurred in the presence of medium 199 containing 5% calf serum, and in decreasing amounts in PBS containing either $MgCl_2$ and $CaCl_2$ or NaCl alone. In contrast, the presence of calf serum in the attachment menstruum, while not affecting the viability of VEE virus, inhibited its attachment to cells. Virus attachment efficiency decreased as the serum concentration increased. In general, these data support the conclusions based on experiments with the bacteriophage system,[23] that the requirements for virus attachment are virus-specific and not cell-specific. It is possible, therefore, that the different requirements for maximal attachment of these two arboviruses may be a group characteristic, but additional tests are needed with several representative arboviruses before any generalization is warranted.

The attachment of virus to cells in vivo is not necessarily followed by elution, and, if it does occur, the amount eluted is small.[27] Our limited observations with VEE virus tend to uphold this viewpoint. Elution of VEE virus from host cells was minimal (less than 0.1% within 2 hours) and was not affected by the presence of either mono- or divalent cations in the medium. The small quantity of virus that eluted retained its infectivity. Similarly, Newcastle disease, fowl plague, or vaccinia viruses did not elute from host cells under conditions in which they readily elute from red cells.[13,28] No significant elution could be demonstrated of rabbitpox virus from HeLa cells.[10] These findings, however, may not have general applicability in view of seemingly contradictory observations. Smith and Sharp[29] reported that part of the attached vaccinia virus subsequently eluted from L cells, and Joklik and Darnell[30] demonstrated that more than half of the poliovirus particles eluted from HeLa cells. The eluted poliovirus was altered so that it could not initiate infectivity. Poliovirus eluted from HeLa cell plasma membrane preparations incubated at 37 C but not at 0 C.[31] It appears that the virus-cell system and the conditions employed to demonstrate virus elution markedly influence the phenomenon.

The finding that VEE virus was initially held at the cell surface for several minutes, where it was susceptible to antiserum, and that virus penetration then proceeded as a first-order reaction was similar to reported observations with poliovirus.[15,32,33] Virus penetration, measured by antiserum insensitivity, was complete in approximately 30 minutes at 35 C. The variable penetration times reported for other animal viruses, from 20 minutes to several hours,[15,34-38] may be a reflection of diverse experimental conditions and the difficulty in isolating the penetration stage from the rest of the infective cycle.[27] In contrast to the temperature independency of the attachment reaction, the penetration of VEE virus was temperature-dependent. Virus penetration was more rapid at 35 than at 28 C. Penetration at 4 C was minimal, less than 5%. These results, together with evidence obtained with other animal viruses,[14,15,36,39-41] suggest that the penetration process may involve cellular enzymes. Although penetration of a variety of

bacteriophages is dependent upon the environmental supply of divalent cations [24,42] penetration of VEE virus was independent of the ionic content of the medium.

The rapidity of the fluorescent cell-counting assay of VEE virus described in this report is an outstanding feature. Individual infected cells may be enumerated as early as 12 hours after infection. Because virus multiplication is rapid,[21] the early release of virus into the extracellular environment results in a second cycle of infection and the subsequent appearance of microplaques between 12 and 16 hours after initial infection. Microplaque formation may be prevented by overlaying cell cultures with viral antiserum 1 hour after virus penetration. The incubation period may then be extended beyond 12 hours and still permit counting of individual infected cells. Paralleling the findings of previously described fluorescent cell-counting virus assays,[1-3,43] the relationship between virus concentration and cell-infecting units was linear and the distribution of infected cells was random. The fluorescent method of assay was equivalent in sensitivity but less variable than the highly sensitive assay of VEE virus by intracerebral inoculation of mice.[44]

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13. ABSTRACT

The conditions under which Venezuelan equine encephalomyelitis (VEE) virus attached to host cells markedly influenced the assay of virus by the fluorescent cell-counting technique. When virus inoculum was centrifuged onto McCoy cell monolayers, approximately 97% of virus was attached to cells within 10 minutes in contrast to 34% after stationary incubation at 35 C for 2 hours. Maximum binding of virus occurred only in the presence of 0.1 to 0.15 M NaCl. This salt requirement, added to evidence of pH dependence and temperature independence of VEE virus attachment to cells, indicated that the initial union involved electrostatic forces. Virus penetration, measured by the insensitivity of virus-cell complexes to viral antiserum, was complete in 30 minutes at 35 C. The process was temperature-dependent and unaffected by the ionic content of medium. For assay of VEE virus by the fluorescent cell-counting technique, infected cells may be enumerated as early as 12 hours after infection of cell monolayers. The relationship between virus concentration and cell-infecting units was linear; the distribution of fluorescent cells was random. Virus assay by the fluorescent technique was equivalent in sensitivity but more precise and rapid than that by intracerebral inoculation of mice.

14. Key Words

*Venezuelan equine encephalomyelitis	Adsorption	Electrostatics
*Fast	Viruses	Temperature
Binding	Cells	Ions
Penetration	Sodium chloride	

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