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TECHNICAL MANUSCRIPT 321

VENEZUELAN EQUINE ENCEPHALITIS VIRUS: AN APPARENT DOUBLY BLOCKED CONDITIONAL LETHAL VIRUS

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DEPARTMENT OF THE ARMY
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VENEZUELAN EQUINE ENCEPHALITIS VIRUS: AN APPARENT DOUBLY BLOCKED CONDITIONAL LETHAL VIRUS

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ABSTRACT

The replication of Venezuelan equine encephalitis (VEE) virus is completely inhibited at 44 C. The inhibited steps were analyzed by changing the incubating temperatures and determining the rate and extent of infectious RNA synthesis, intact virus synthesis, and complement-fixing antigen formation during the changes. The inhibition appears to be due to the presence of two temperature-sensitive steps involved in the synthesis of VEE virus in chick embryo cells. At 44 C the first step occurs early in virus replication and can be completely reversed by returning cultures to 37 C. The site of inhibition appears to be located at some point beyond the time when the virus enters the cell and is uncoated and the beginning of viral RNA synthesis. The efficiency of reversing the inhibition after 24 hours' incubation at 44 C is approximately 100%, and suggests that the viral genome exists in a stabilized form in the cell. The second temperature-sensitive step is irreversible and occurs late in the virus synthesis process, probably between synthesis of viral RNA and formation of virus protein as measured by complement-fixing antigen, or as antigen that would be detectable by the fluorescent antibody technique. The question of whether VEE virus is fundamentally a singly or doubly blocked conditional lethal virus is discussed.

I. INTRODUCTION

Conditional lethal viruses are those that can infect cells, reproduce, and form complete, infectious virus under one set of (permissive) conditions, but under another set of (restrictive) conditions are blocked in one or more steps in virus reproduction. The genetic lesions responsible for the abortive infections observed under restrictive conditions have been found to be widely distributed over the viral genome.[1-3] Thus, one may study gene function and the biosynthesis and morphological development of the virus based on principles similar to those established by Beadle and Tatum,[4] who used differentially blocked biochemical mutants of neurospora for elucidating biosynthetic pathways.[5] The potential of conditional lethal viruses for studying animal viral genetics and physiology has been recently pointed out by Fenner and Sambrook.[6]

Like their bacterial viral counterparts, two subclasses of conditional lethal animal viruses have been isolated and studied: (i) temperature-sensitive viruses[7-11] and (ii) host-dependent viruses.[12-14] Following the observation that some of our group A arboviruses were temperature-sensitive in chick embryo cells,[15] it was of interest to locate and define the temperature-sensitive steps in their biosynthesis. Of the two viruses examined thus far, eastern equine encephalomyelitis and Venezuelan equine encephalomyelitis, an apparent double block was found in the latter.

II. MATERIALS AND METHODS

A. VIRUSES

Two group A arboviruses were used - the Trinidad strain of Venezuelan equine encephalitis (VEE) virus and the Louisiana strain of eastern equine encephalitis (EEE) virus. The origin, passage histories, properties of the strains, their preparation as seeds, and the suspended plaque method for their assays have been described by Brown.[15]

B. CELL CULTURES AND MEDIA

Chick embryo (CE) cell monolayers were prepared from minced, trypsinized 10-day-old chick embryos. Twenty to 30 million cells contained in 5 ml growth medium were added to 60-mm plastic petri dishes. The growth medium consisted of 0.5% lactalbumin hydrolyzate, 0.1% yeast extract, Hanks balanced salts solution, 10% calf serum, and 0.14% sodium bicarbonate. Confluent CE monolayers were obtained after 24 hours' incubation at 37 C in an incubator supplied with a 5% carbon dioxide - 95% air mixture. All experiments were performed on CE monolayers 24 hours old.

C. GROWTH STUDIES

For studying the growth response of the viruses at different temperatures, the monolayers were infected at an input multiplicity of about 100 plaque-forming units (pfu) per cell. The virus was allowed to adsorb to the CE monolayers for 15 minutes at room temperature. The infected monolayers were washed twice with phosphate-buffered saline (PBS), pH 7.4, in order to reduce the residual virus titer in the supernatant medium, and then were overlaid with lactalbumin hydrolyzate medium containing 10% calf serum. The cultures were then placed at the appropriate incubation temperature and the viral growth was followed at 2-hour intervals.

The early studies at several different temperatures were performed in the temperature gradient plate described by Brown.[15] Later studies, employing only 37 or 44 C temperatures, were carried out in water-jacketed incubators. All cultures were incubated in the presence of a 5% carbon dioxide - 95% air mixture.

D. EXTRACTION AND ASSAY OF INFECTIOUS RNA

Infected CE monolayers were removed with a rubber policeman and suspended in 0.02 M phosphate - 0.001 M versene buffer, pH 7.4. These suspensions were extracted twice with cold phenol and viral RNA was precipitated from the aqueous phase with ethanol containing 2% potassium acetate. The precipitate was dissolved in PBS and then assayed by the method described by Colon and Idoine[16] on CE monolayers treated with 1 M NaCl.

E. COMPLEMENT FIXATION TEST FOR ANTIGEN

The complement fixation test was performed according to the standardized diagnostic complement fixation method of the U.S. Public Health Service.[17] The antiserum was obtained from guinea pigs immunized with living virus, purified through a sucrose gradient. This antiserum did not react with the host cell components, and specifically detected the presence of virus antigen in infected cell extracts.

III. RESULTS

Chick embryo cells have unusual heat tolerance and can survive temperatures up to 46.5 C for at least 48 hours. The growth of EEE virus in CE monolayer cultures incubated at various temperatures is shown in Figure 1. Maximal growth rate was obtained up to 44 C; the rate of growth and yield decreased thereafter. However, even after 30 hours at 50 C, virus was detectable. This suggested that some virus multiplication had occurred, especially if one considers the rapid inactivation rate of EEE virus in cell-free media (Mika et al.[18] and unpublished results). During the same experiment, in marked contrast to EEE virus, VEE virus in the same cell cultures showed a significant reduction in growth rate and yield at 42 C; it multiplied very little at 44 C (Fig. 2). VEE virus was apparently inactivated rapidly at 46 C. The data in Figures 1 and 2 show that the difference in growth response to temperatures between the two viruses can be ascribed to the viruses and not to the host cells. These differences are apparently not due to differences in heat stability, because VEE virus held at 50 C in a cell-free medium seems to be significantly more heat-stable than EEE virus (Mika et al.[18] and unpublished results). The data suggest that a virus-induced enzyme(s) or some other virus-specific process(es) involved in the synthesis of VEE virus is sensitive to temperatures above 40 C.

The remainder of the experiments were designed to locate and describe the temperature-sensitive steps in the synthesis of VEE virus.

A. TEMPERATURE-SHIFT EXPERIMENTS

A large number of CE monolayers infected with VEE virus were prepared and treated as follows. A control group of plates was placed in a 44 C incubator and the remainder were incubated at 37 C. At hourly intervals one set of plates was transferred from 37 to 44 C, and at the same time one plate from each temperature group was assayed for virus titer. With this system it was possible to determine the effect on the growth cycle of transferring cultures from 37 to 44 C at different but sequential stages after infection. The results of a typical experiment are shown in Figure 3.

As cultures were transferred from 37 to 44 C, most of the virus replication was halted almost immediately. If the plates were transferred before 2 hours, the growth curve followed that of the 44 C control culture. Transferring the cultures after 2 hours resulted in an apparent inhibition of viral replication so that the virus titer remained at approximately the same level that the cultures had at time of transfer. If infection of cultures was carried out with IRNA instead of intact virus and incubated immediately at 44 C again, no virus replication could be detected. This suggests that sensitivity to high temperature occurred after uncoating of virus.

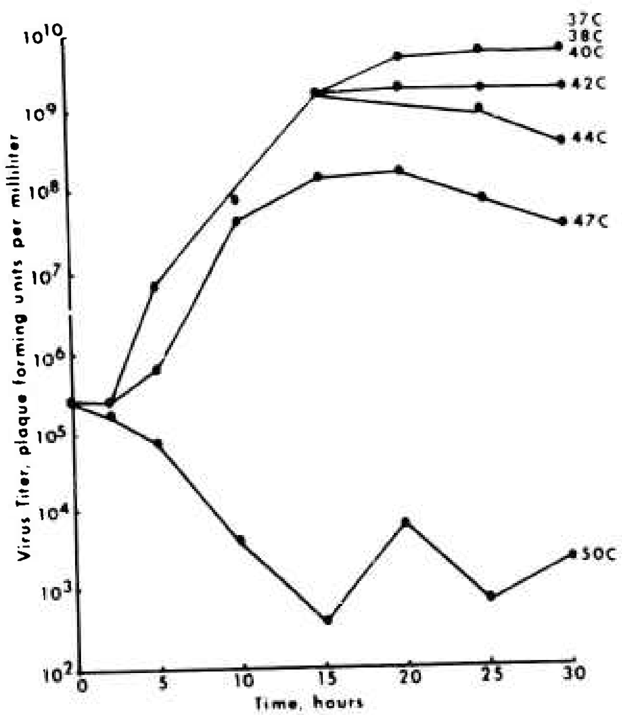

Figure 1. Growth Response of EEE Virus at Different Temperatures.

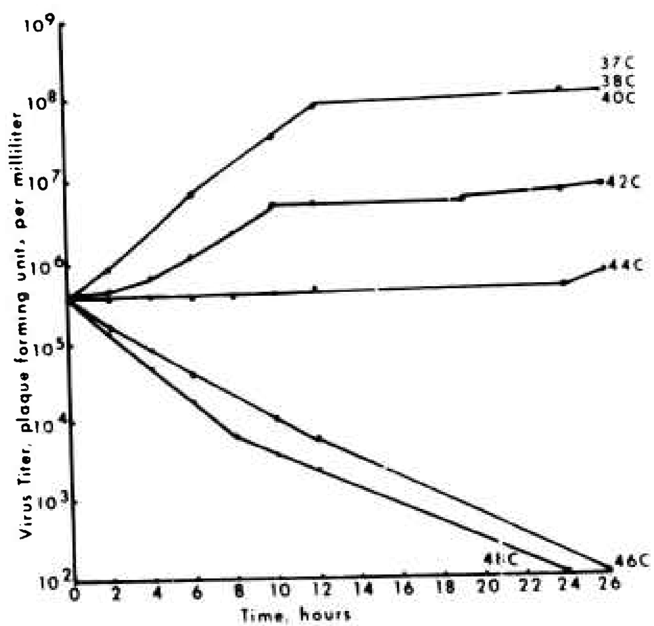

Figure 2. Growth Response of VEE Virus at Different Temperatures.

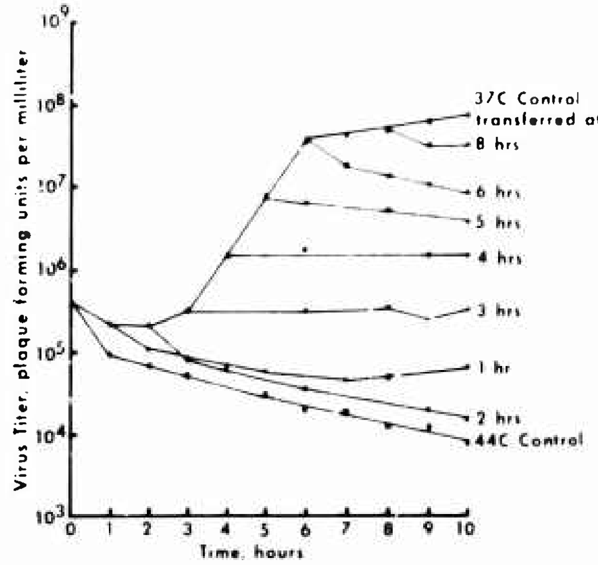

Figure 3. Effect of Transferring Plates from 37 to 44 C Upon Growth Response of VEE Virus.

The effect of transferring VEE-infected CE cultures in a shift-downward experiment from 44 to 37 C is shown in Figure 4. The procedure used for sampling and transferring cultures was the same as that just described except that the infected monolayers were placed initially at 44 C. Virus growth was initiated within 4 hours after transferring the cultures to the 37 C incubator. If the transferred cultures were incubated at 37 C long enough, the virus titer would eventually reach that of the 37 C control culture. A similar experiment was performed in which the cultures were transferred from 44 to 37 C at 28 hours; virus growth was initiated as usual and attained the usual peak titer.

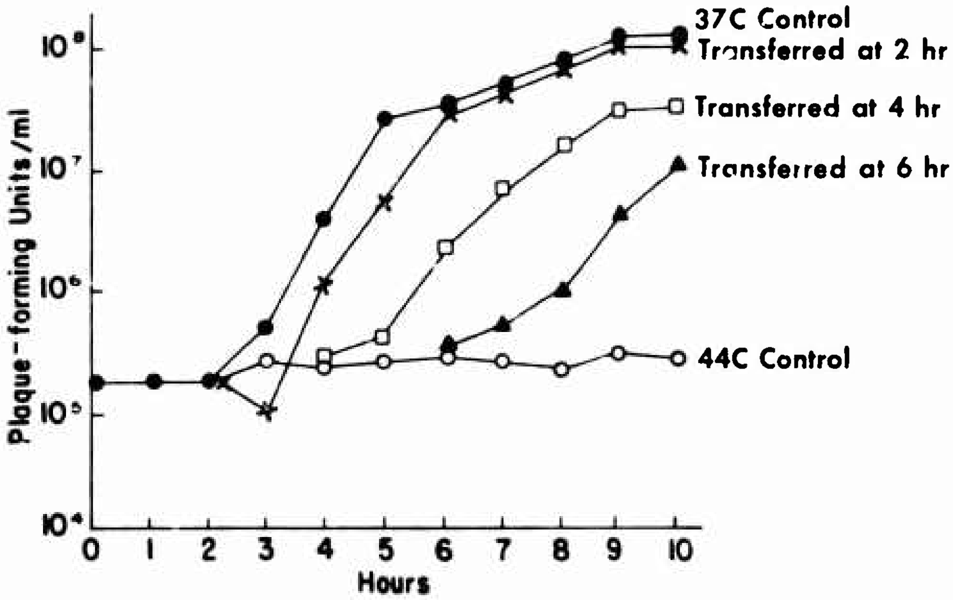

Figure 4. Effect of Transferring Infected Chick Embryo Cell Cultures from 44 to 37 C at Different Times Upon Growth Response of VEE Virus.

These data indicated that, although the virus-induced system appeared to be inhibited by temperatures of 44 C, it did not seem to be irreversibly damaged, because transferring the cultures to 37 C re-established viral synthesis after approximately the same latent period as controls. The latter result suggested that the inhibition was reversible with good efficiency.

To estimate more quantitatively the efficiency of reversibility of the inhibition at 44 C, the following experiment was carried out. CE monolayers were infected with approximately 70 pfu of VEE virus and allowed to adsorb the virus for 15 minutes. After washing, 5 ml of liquid medium were added to each plate. Five plates were placed at 44 C for 24 hours, then placed at 37 C for ½ hour, washed once, and overlaid with nutrient agar of the same composition as that used for the suspended cell plaque technique. Two sets of controls were included. The first was used to test whether pre-incubation of uninfected cells at 44 C in itself reduced the efficiency of plaque formation compared with the normal plaque technique. Thus, uninfected cultures incubated at 44 C for the same periods of time as the test cultures were brought back to 37 C for ½ hour. They were then inoculated with 70 pfu of VEE virus, allowed to adsorb virus for the same time as the test cultures, and overlaid with nutrient agar. The second set of controls was simply a typical plaque assay in which the monolayers inoculated with 70 pfu were overlaid immediately after adsorption and incubated at 37 C; these therefore served as controls for the first set of controls. The results of this experiment (Table 1) showed that inhibition of plaque formation by virus incubated at 44 C for 24 hours can be reversed with essentially 100% efficiency. These results and those of previous experiments indicated that after uncoating, the virus RNA is stabilized in a form that resists inactivation at 44 C for 24 hours.

TABLE 1. REVERSIBILITY OF INHIBITION OF VEE VIRUS PLAQUE FORMATION AT 44 C

Plaque Count (Average of Five Plates)[a]		
Control 1[b]	Control 2[b]	Test[b]
Normal Plaque Technique 37 C[c]	Cell cultures incubated at 44 C for 24 hours followed by normal plaque technique at 37 C	Infected cells overlaid with liquid medium, incubated at 44 C for 24 hr, then normal plaque technique at 37 C
64	70	73

a. Each plate was inoculated with 70 pfu.
b. See text for details.
c. All adsorptions at room temperature (about 22 C) 15 minutes.

B. PRELIMINARY EVIDENCE FOR A SECOND BLOCK

Figure 5 illustrates the effect of two sequential shifts in temperatures upon the growth response of VEE virus. One set of infected CE cell cultures was placed at 44 C, held for 24 hours, and then transferred to 37 C. A second set of cultures was placed at 37 C for 2 hours, transferred to 44 C for 22 hours, and then returned to 37 C. The virus growth response at 37 C was followed for an additional 9 hours. The curves in Figure 5 show that the virus growth of infected cultures held only at 44 C was not inhibited and rose to maximal titer when placed at 37 C. However, when similar cultures were incubated for 2 hours at 37 C prior to being transferred to 44 C, virus titers did not increase upon being returned to 37 C the next day.

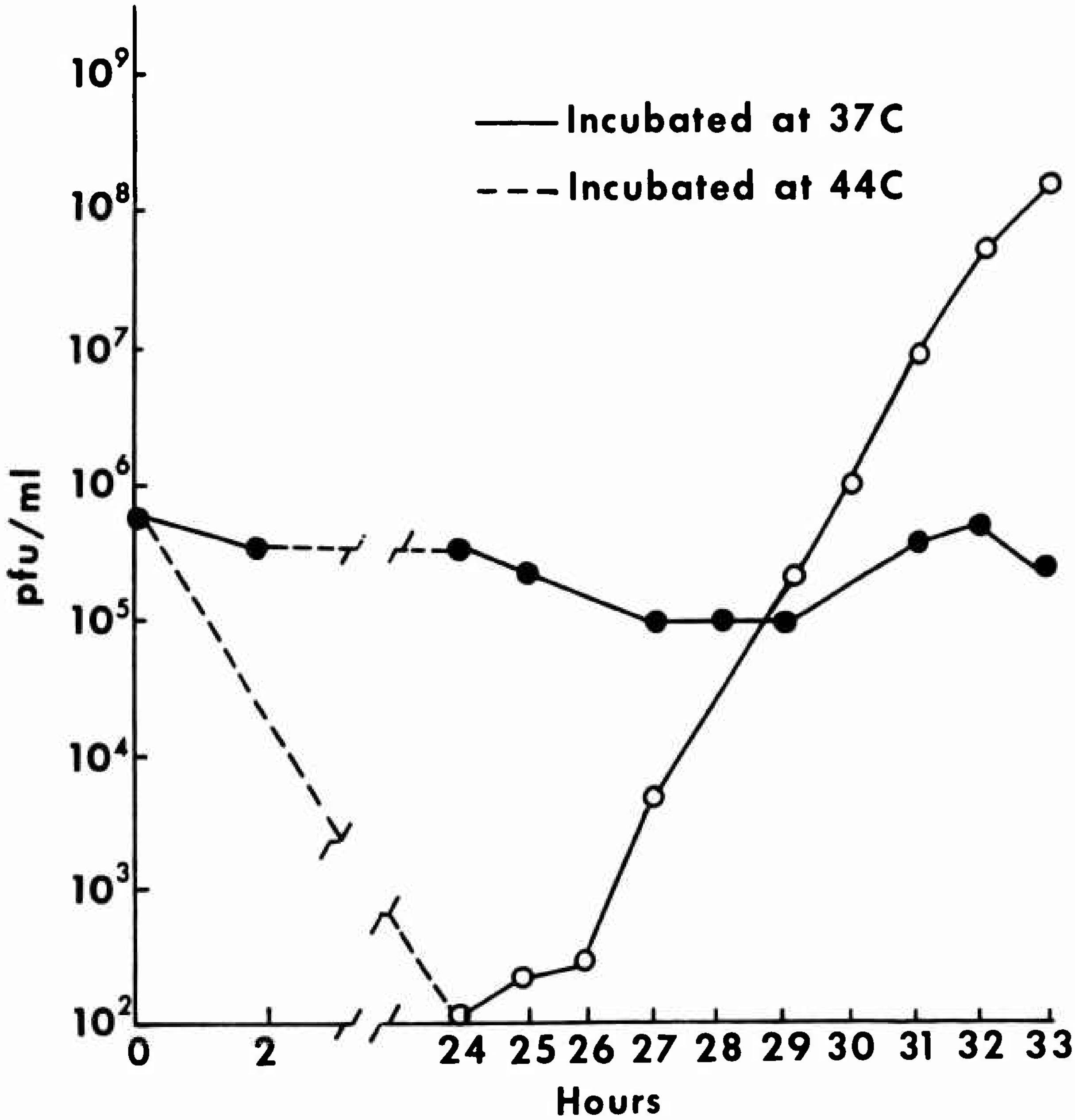

Figure 5. Effect of Pre-Incubation at 37 and 44 C Upon VEE Virus Growth Response at 37 C.

The information obtained in this experiment, and that obtained in the two previous experiments, suggests that there are at least two temperature-sensitive steps involved in the synthesis of VEE virus. The first step occurs early in the replication sequence, as indicated by the completely reversible inhibition of virus growth of cultures placed initially at 44 C. The second step occurs later in the virus infection, as demonstrated by the irreversible inhibition of virus growth at 44 C following pre-incubation at 37 C. If the early step of virus synthesis were the only temperature-sensitive step involved, then pre-incubation at 37 C would by-pass the critical event and virus could replicate at 44 C. Evidence will be presented later to demonstrate that pre-incubation at 37 C did by-pass the first temperature-sensitive step, but virus growth was still strongly inhibited at the high temperature.

C. SYNTHESIS OF INFECTIOUS RNA AND COMPLETE VIRUS

The next series of experiments were designed to obtain further information on the early and late temperature-sensitive steps in the replication of VEE virus by following the synthesis of both infectious RNA and complete virus. It was shown by Wecker[19] and confirmed by Colon and Idoine[20] that by extracting with cold phenol one can obtain infectious RNA from infected cells that is not associated with mature virus. This permits a simple differentiation between infectious RNA in some form(s) of virus precursor(s) and the mature virus.

The results of a representative experiment are shown in Figure 6. In cultures infected with virus and maintained at 37 C, infectious RNA (IRNA) synthesis appeared to increase without an appreciable lag period and reach maximal titer at 8 hours (Curve D). Virus synthesis, detected after a 2-hour lag period, increased for approximately 8 hours (Curve A). Cultures incubated at 44 C exhibited neither IRNA synthesis (Curve F) nor virus growth (Curve C). Furthermore, it was shown in previous experiments that if the cultures were infected with infectious RNA instead of intact virus and incubated at 44 C, there was no increase in either virus titer or IRNA synthesis, both of which increased in controls incubated at 37 C. This suggests that the first block at 44 C occurs at a point between uncoating of virus and infectious RNA synthesis.

The early temperature block was by-passed by incubating infected cultures for 2 hours at 37 C before shifting them to 44 C for an additional 8 hours. The results in Figure 6 (Curve E) show that the IRNA titer began to increase during the first 2 hours at 37 C and continued to increase even after the cultures were placed at 44 C, at about the same rate as cultures held only at 37 C; later, the rate of IRNA synthesis decreased and the final yield was lower than that of those held only at 37 C (Curve D). In several experiments the final yield at 10 hours at 44 C varied between 30 and 50% of that at 37 C. It appears that pre-incubation at 37 C by-passed the early temperature-sensitive step and permitted significant synthesis of viral RNA (compare Curves D and E) without a corresponding synthesis of mature virus (compare Curves A and C). The lower terminal rates and yield of IRNA synthesis after shifting to 44 C may be due to the inhibition of further polymerase synthesis that began during the 37 C pre-incubation and/or perhaps to the labile nature of the IRNA made at 44 C, which is not incorporated into infectious virus particles.

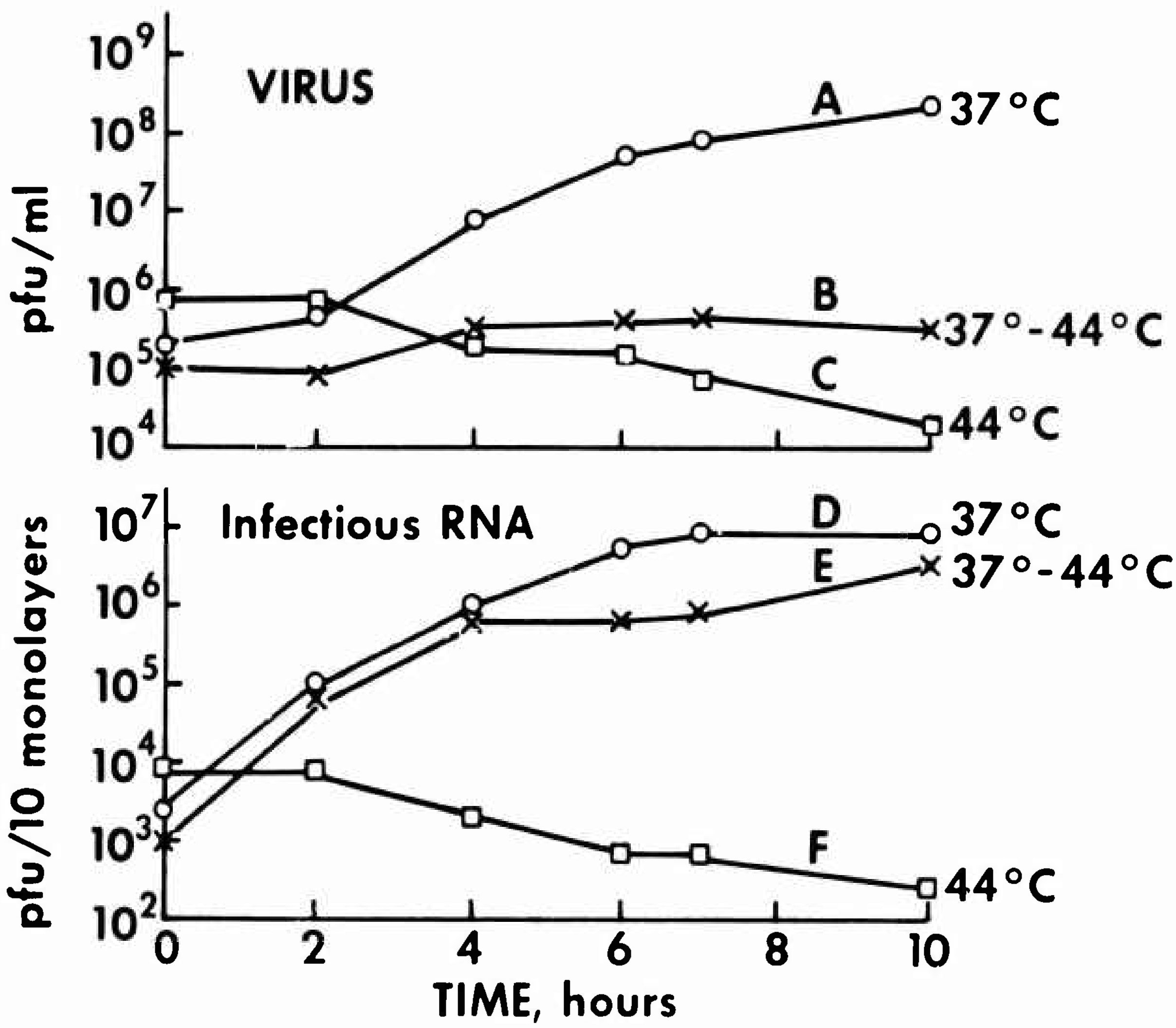

Figure 6. Effect of Different Incubation Conditions Upon Replication of Virus and Infectious RNA.

Because synthesis of mature infectious virus was strongly inhibited, it was of interest to test whether virus-specific protein measured as complement-fixing antigen could be detected by antibody made against infectious virus. Monolayers of CE cells in petri plates were infected with VEE virus and subjected to different temperatures of incubation. At various times during the incubation periods, in groups of five plates each, the monolayers were washed twice, suspended in PBS, and disrupted in a sonic oscillator. The cell debris was removed by centrifugation and the cell extract was analyzed for complement-fixing (CF) antigen.

The results in Table 2 show that virus CF antigen was not detectable at 44 C before or after pre-incubation for 2 hours at 37 C, although it was detectable in controls. These results suggest that virus-specific protein, either in a structurally intact virus particle[21] or in an incomplete form (e.g., the core[22]) was not made in significant amounts at 44 C.

TABLE 2. EFFECT OF DIFFERENT TEMPERATURES UPON FORMATION OF VEE VIRUS ANTIGEN IN CHICK EMBRYO CELLS

	1/CF Titer[a]		
Time, hr	37 C	44 C	37 C for 2 Hours, 44 C for 22 Hours
0	0	0	0
2	0	0	0
4	2	0	0
6	4	0	0
8	16	0	0
10	128	0	0
24	128	0	0

a. Reciprocal of titer showing complete fixation of complement.

IV. DISCUSSION

The results of this study indicate that VEE virus, Trinidad strain, is apparently a doubly blocked temperature-sensitive (conditional lethal) virus. Table 3 lists the characteristics of the two distinct blocks.

TABLE 3. CHARACTERIZATION OF THE TEMPERATURE-SENSITIVE STEPS INVOLVED IN THE SYNTHESIS OF VEE VIRUS

Temperature-Sensitive Step I	Temperature-Sensitive Step II
1. Virus multiplication reversibly inhibited at 44 C.	1. Virus multiplication irreversibly inhibited at 44 C.
2. No infectious RNA synthesis.	2. Infectious RNA is synthesized.
3. No virus CF antigen detected.	3. No virus CF antigen detected.

The results of experiments in which RNA synthesis was inhibited at high temperature after cells were infected by either intact virus or its IRNA indicate that the early block occurs after uncoating and before IRNA synthesis. The fact that the early temperature-sensitive step was reversible with approximately 100% efficiency suggests that the IRNA assumes a stabilized form in the cell. The possibility exists, therefore, that this system allows the accumulation of an intermediate product in the early stages of virus replication that should lend itself to isolation and further analysis.

Since IRNA synthesis at 44 C continues once it begins after pre-incubation at 37 C, it appears that enzyme (polymerase?) activity may be stable at high temperature and that some step preceding the formation of the active enzyme may be inhibited in the early temperature-sensitive step.

When the first step was by-passed by a short pre-incubation period at 37 C before shifting to 44 C, considerably more than 99% of the viral synthesis was inhibited. IRNA synthesis, however, continued at a rate comparable to that of 37 C controls, then the rate decreased until a 50 to 70% lower final yield was reached. The lower rates may be due to the inhibition of new polymerase synthesis and/or the greater heat lability of newly synthesized viral RNA that was not incorporated into intact virus particles. As opposed to controls at 37 C, significant amounts of CF antigen were not detected at 44 C with or without a pre-incubation period at 37 C. Thus CF antigen in mature[21] or incomplete virus particles[22,23] was not made in significant amounts at 44 C. To determine which, if any, virus-specific proteins are made at high temperature will require other analytical techniques.

As shown by Burge and Pfefferkorn,[10] who made use of differentially blocked temperature-sensitive mutants of Sindbis virus, one may suggest that physiologically, two cistrons have been demonstrated in the doubly blocked VEE virus. It remains to be seen whether the cistrons can be demonstrated and mapped genetically.

On alternative explanation to account for the apparent double block in VEE virus replication at elevated temperatures is that a translational control type of mutation exists in this virus that prevents all virus-genome-directed protein synthesis at high temperature. Such a mutation, for example, could be one involving a modulation area on the virus genome (analogous to modulator areas on m-RNA[24]) that tells the ribosome where to attach and begin translation. According to this idea, these sites on VEE IRNA cannot bind to ribosomes at 44 C, although EEE IRNA can. The IRNA of both viruses should bind to ribosomes at 37 C. Such an idea could explain the apparent double block. Thus, viral replication and IRNA synthesis are both inhibited early, and the IRNA synthesis that continues when the early blocked step is by-passed can be accounted for by the relative stability at high temperatures of the enzyme(s) involved in IRNA synthesis. No virus (presumably structural) protein is made that is measurable as CF antigen because of the temperature-sensitive modulator area on the virus genome; since the IRNA could not bind to ribosomes at 44 C, all new virus-directed protein synthesis is inhibited whenever the shift is made to 44 C. This hypothesis, which can be tested, is analogous to others that have been proposed for RNA viruses that involve translational control of protein synthesis by a polycistronic viral genome.[25,26]

V. SUMMARY

In contrast to eastern equine encephalitis virus, the replication of Venezuelan equine encephalitis virus was strongly inhibited at 44 C in CE cells. The inhibited steps were analyzed by shifting the incubating temperatures up or down, and determining during the shifts the rate and extent of IRNA synthesis, intact virus synthesis, and formation of complement-fixing antigen. The inhibition appeared to be due to two temperature-sensitive steps involved in the synthesis of VEE virus in chick embryo cells. The first step of inhibition at 44 C occurred early

in virus replication and could be completely reversed simply by transferring cultures to 37 C. The inhibition appeared to take place at some point between the time when the virus entered the cell and was uncoated and the beginning of viral RNA synthesis. The second temperature-sensitive step in VEE virus synthesis was irreversible and occurred at a point after the synthesis of viral RNA and before the formation of virus protein measured as complement-fixing antigen.

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13. ABSTRACT The replication of Venezuelan equine encephalitis (VEE) virus is completely inhibited at 44 C. The inhibited steps were analyzed by changing the incubating temperatures and determining the rate and extent of infectious RNA synthesis, intact virus synthesis, and complement-fixing antigen formation during the changes. The inhibition appears to be due to the presence of two temperature-sensitive steps involved in the synthesis of VEE virus in chick embryo cells. At 44 C the first step occurs early in virus replication and can be completely reversed by returning cultures to 37 C. The site of inhibition appears to be located at some point beyond the time when the virus enters the cell and is uncoated and the beginning of viral RNA synthesis. The efficiency of reversing the inhibition after 24 hours' incubation at 44 C is approximately 100%, and suggests that the viral genome exists in a stabilized form in the cell. The second temperature-sensitive step is irreversible and occurs late in the virus synthesis process, probably between synthesis of viral RNA and formation of virus protein as measured by complement-fixing antigen, or as antigen that would be detectable by the fluorescent antibody technique. The question of whether VEE virus is fundamentally a singly or doubly blocked conditional lethal virus is discussed.

14. Key Words

*VEE	Extracting	*Synthesis	Plaque
Chick embryo	Assay	Temperature	RNA
Inhibiting	Antigen	Replication	Complement fixing

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