

UNCLASSIFIED

AD NUMBER
AD476498
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; Dec 1965. Other requests shall be referred to Army Biological Labs., Fort Detrick, Frederick, MD 21701.
AUTHORITY
Biological Defense Research Lab ltr dtd 28 Sep 1971

THIS PAGE IS UNCLASSIFIED

AD

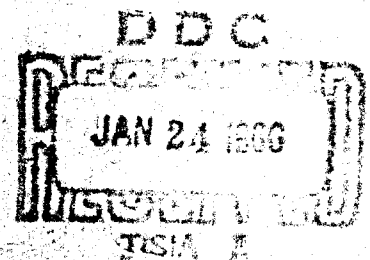
TECHNICAL MANUSCRIPT 266

AD 476498

VENEZUELAN EQUINE ENCEPHALITIS VIRUS :
A DOUBLY BLOCKED
CONDITIONAL LETHAL VIRUS

Eugene Zebovitz

DECEMBER 1965



UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

Best Available Copy

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 266

VENEZUELAN EQUINE ENCEPHALITIS VIRUS: A DOUBLY BLOCKED
CONDITIONAL LETHAL VIRUS

Eugene Zebowitz

Virus and Rickettsia Division
DIRECTORATE OF BIOLOGICAL RESEARCH

Project 1C014501B71A01

December 1965

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

ABSTRACT

The replication of Venezuelan equine encephalitis (VEE) virus is completely inhibited at 44 C. The inhibited steps were analyzed by shifting the incubating temperatures up or down, and determining the rate and extent of infectious RNA synthesis, intact virus synthesis, and complement-fixing antigen formation during the shifts. The inhibition appears to be due to the presence of two temperature-sensitive steps involved in the synthesis of VEE virus in chick fibroblast cells. The first step of inhibition at 44 C occurs early in virus replication and can be completely reversed simply by returning cultures to 37 C. The time of inhibition appears to be some point between the time when the virus enters the cell and is uncoated and the beginning of viral RNA synthesis. The second temperature-sensitive step in VEE virus synthesis is irreversible and occurs at a point after the synthesis of viral RNA and before the formation of virus structural protein as measured by complement-fixing antigen.

VENEZUELAN EQUINE ENCEPHALITIS VIRUS: A DOUBLE BLOCKED CONDITIONAL LETHAL VIRUS

A conditional lethal virus has been defined by others as one that infects a cell and fails to make complete virus under one set of conditions but replicates normally under another set of conditions. Viruses that are sensitive to elevated temperatures after infection fall into this category. Such viruses or their mutants are being used for many purposes including the elucidation of the biochemical and morphological pathways of virus synthesis. Most of the viral studies thus far have been made with phage, but last year I* reported preliminary studies on the temperature sensitivity of our parent Venezuelan equine encephalitis (VEE) virus, Trinidad strain; Burge and Pfefferkorn** recently reported studies on temperature-sensitive mutants of Sindbis virus, another Group A arbovirus.

Incubation temperatures of 44 C or greater completely inhibit the growth of the parent VEE virus in chick fibroblast (CF) cell cultures. The temperature effect seems to be specifically upon the growth of the virus rather than upon the host cells. Chick fibroblast cells have unusual heat tolerance and can survive temperatures up to about 46 C for 48 hours. In addition, another Group A arbovirus, eastern equine encephalitis (EEE) virus, can produce close to maximal titers at 44 C and form plaques up to 46 C, indicating that the host cells are still competent to produce virus at elevated temperatures.

The data presented in this report show that there are two temperature-sensitive steps involved in the growth of VEE virus in CF cells.

The growth response of VEE virus, Trinidad strain, at 37 and 44 C is shown in Figure 1. Chick fibroblast cell monolayers were infected at a multiplicity of 100 plaque-forming units (pfu) per cell. The virus was allowed to adsorb for 15 minutes and the monolayers were washed twice with phosphate-buffered saline to reduce the residual virus titer in the supernatant medium. The cultures were overlaid with a lactalbumin hydrolysate medium and placed in either a 37 or 44 C incubator. At two-hour intervals one plate was removed from each incubator and the supernatant growth medium assayed for virus titer.

As shown in Figure 1, the growth of VEE virus was completely inhibited at 44 C. Over a period of 24 hours, the virus titer in the growth medium dropped three logs.

* Zebovitz, Eugene. January 1965. Growth of VEE and EEE viruses in chick fibroblast cultures at high temperatures, (Technical Manuscript 176). Virus and Rickettsia Division, U.S. Army Biological Laboratories, Frederick, Maryland.

** Burge, B.W.; Pfefferkorn, E.R. 1965. Conditional-lethal mutants of an animal virus: Identification of two cistrons. Science 148:959-960.

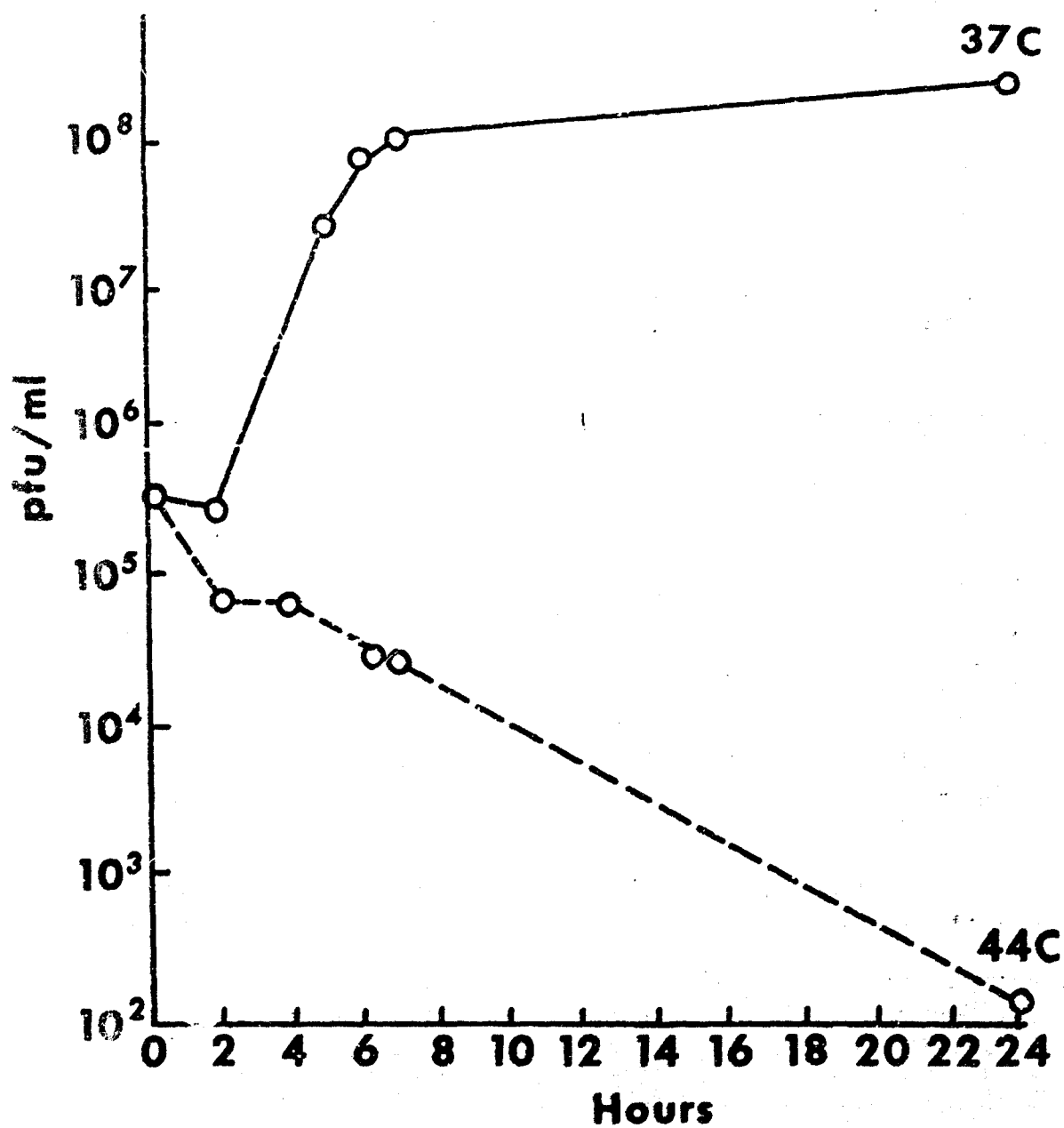


Figure 1. Growth Response of VEE Virus at 37 and 44 C.

Figure 2 shows the effect of transferring cultures held at 44 C for 24 hours to a 37 C incubator. After a two-hour lag period, virus began to be produced at a rate that closely approximated the growth response of control cultures held only at 37 C. Similar experiments were also performed in which cultures were transferred to 37 C at different times during the first 24 hours at 44 C. The results indicated that it was possible for the virus to resume growth when the cultures were placed at 37 C.

The data presented to this point suggest that a virus-induced enzyme system that is temperature-sensitive at 44 C or greater is involved in the synthesis of VEE virus. However, this system does not seem to be irreversibly damaged at high temperatures, because transferring the cultures to 37 C results once again in a typical growth curve.

Figure 3 illustrates the effect of two incubation conditions upon the growth response of VEE virus. One set of infected CF 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 two 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 data presented on Figure 3 show that the virus growth of cultures held only at 44 C was not inhibited and rose to maximal titer when placed at 37 C. However, when similar cultures were first 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.

The information obtained in this experiment, plus that obtained in the previous experiment, suggests that there are at least two temperature-sensitive steps involved in VEE virus synthesis. 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 the 37 C pre-incubation did by-pass the first temperature-sensitive step, but virus growth was still strongly inhibited.

The next series of experiments was designed to obtain further information on the early and late temperature-sensitive steps by following the sequence of infectious RNA synthesis as well as virus formation.

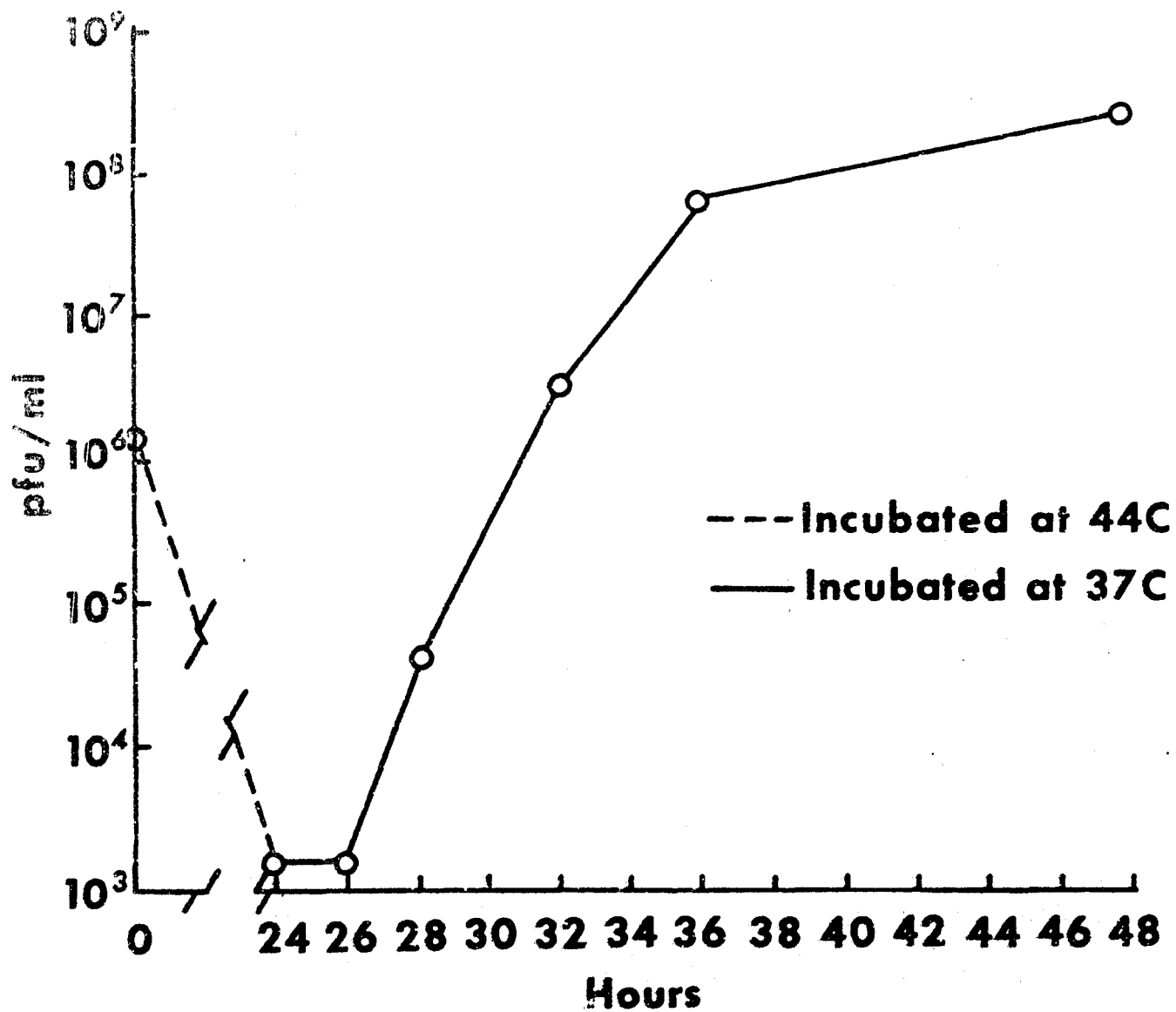


Figure 2. Effect of Transferring VEE-Infected Chick Fibroblast Cell Cultures from 44 to 37 C.

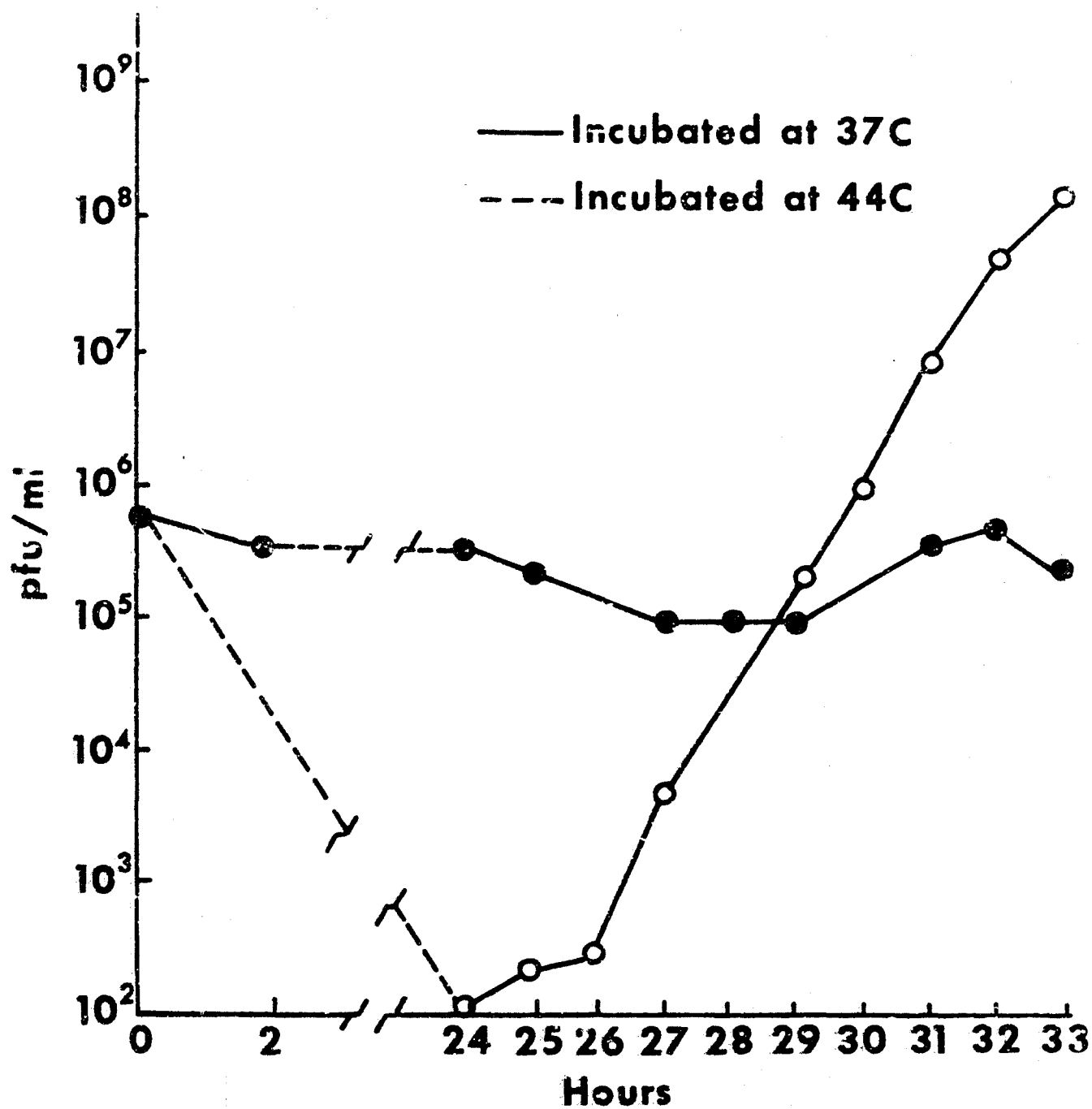


Figure 3. Effect of Pre-Incubation at 37 and 44 C upon VEE Virus Growth Response at 37 C.

Figure 4 shows the effect of incubation at 37 C upon virus growth and infectious RNA synthesis. The viral RNA was extracted with cold phenol from infected CF cells derived from the monolayers grown in ten 60-millimeter petri plates. The RNA was precipitated with ethanol and assayed on CF monolayers made hypertonic with 1 molar sodium chloride.

In cultures infected with virus and maintained at 37 C, infectious RNA synthesis appeared to increase without an appreciable lag period and reach maximal titer at 8 hours. Mature virus synthesis was detected after a two-hour lag period, increased for approximately 10 hours and remained fairly constant thereafter. Cultures incubated at 44 C exhibited neither infectious RNA synthesis nor virus growth. Furthermore, if the cultures were infected with infectious RNA instead of intact virus and incubated at 44 C, there was no increase in either virus growth or infectious RNA synthesis. This suggests that the first block occurs at a point between uncoating of virus and infectious RNA synthesis.

An attempt was made to by-pass the first temperature block by incubating VEE-infected cell cultures for 2 hours at 37 C before shifting them to 44 C for an additional 22 hours. As shown in Figure 5, virus titers did not change during the total 24-hour period but the infectious RNA 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 in much the same manner as cultures held only at 37 C.

It appears that pre-incubation at 37 C by-passed the early temperature-sensitive step and permitted the synthesis of viral RNA without a corresponding synthesis of mature virus.

Another question to be answered was - If mature infectious virus was not made at high temperature, was virus structural protein made? We attempted to answer this by looking for complement-fixing antigen in a system in which the antibody used was made against intact virus. Monolayers of CF cells in petri plates were infected with VEE virus and subjected to different incubation conditions. At various times during the incubation period, 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 antigen. The antiserum was prepared in guinea pigs using killed virus grown on hamster cells. With this antiserum there was no cross-reaction with normal chick embryo cell components.

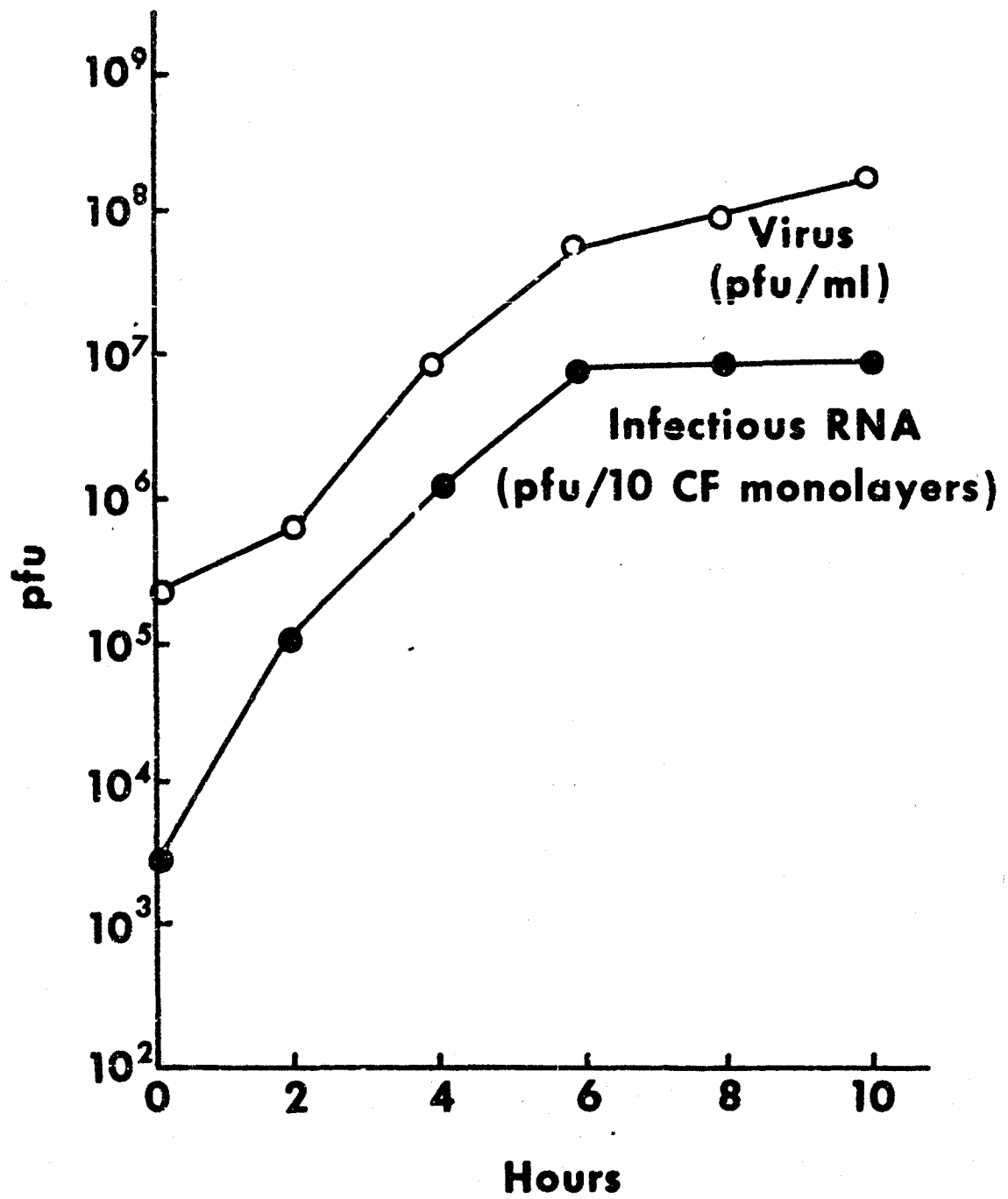


Figure 4. VEE Virus and Infectious RNA Formation at 37 C.

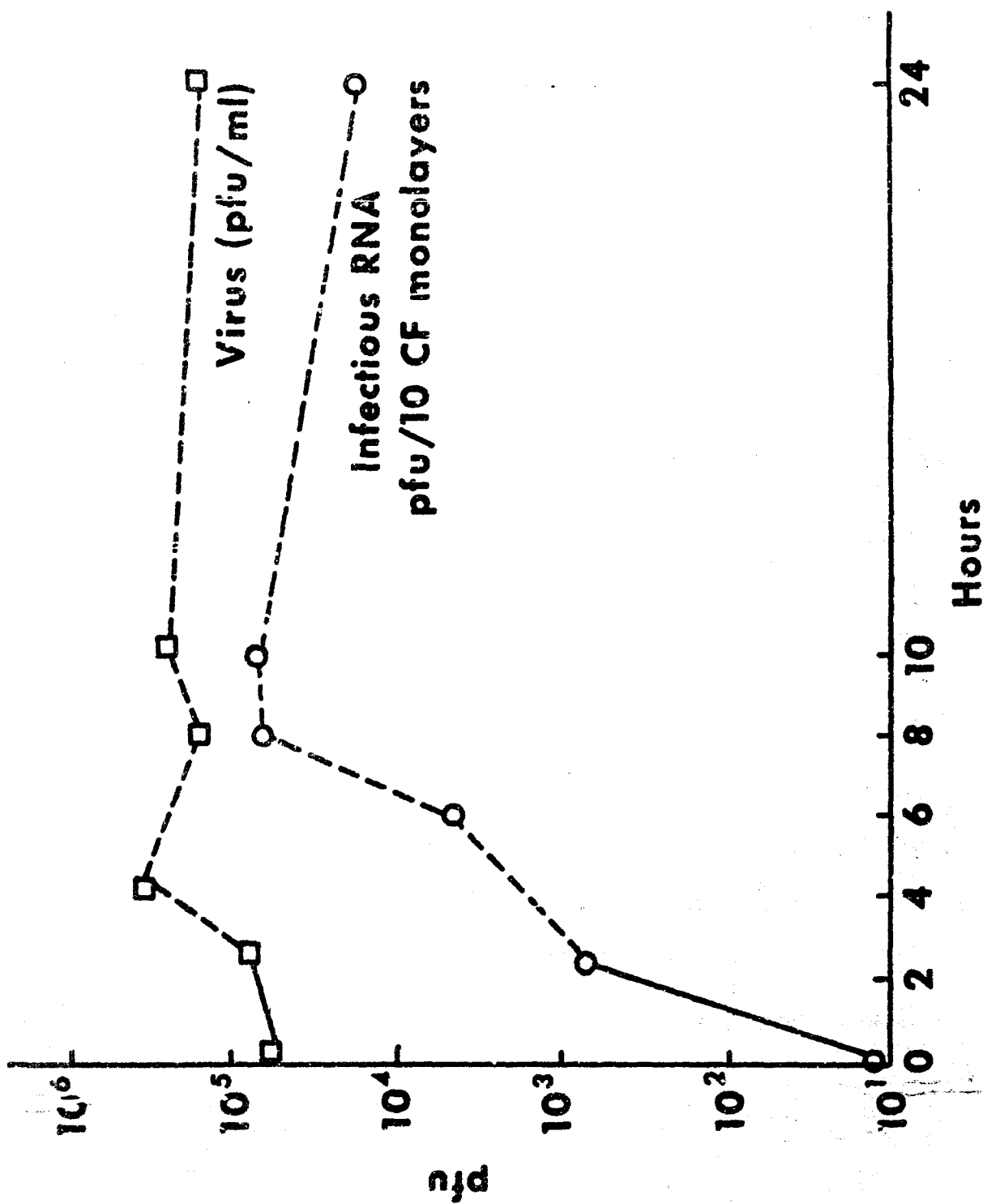


Figure 5. VEE Growth Response and Infectious RNA Synthesis at 44 C after Pre-Incubation at 37 C for 2 Hours.

Table 1 shows that at 37 C virus antigen was first detected at 4 hours and increased to a maximum titer at 10 hours. No detectable virus antigen was formed in cultures held only at 44 C after adsorption. Moreover, virus antigen was not detectable in cultures held at 44 C, which by-passed the first temperature-sensitive step by pre-incubation for 2½ hours at 37 C. These data indicate that the formation of virus structural protein that would be detectable by complement fixation was inhibited in the second temperature-sensitive step.

TABLE 1. EFFECT OF DIFFERENT TEMPERATURES
UPON FORMATION OF VEE VIRUS ANTIGEN
IN CHICK FIBROBLAST CELLS

Time, hr	Temperature		
	37 C	44 C	37 C for 2 hours 44 C for 22 hours
0	0	0	0
2	0	0	0
4	2 ^a /	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.

VEE virus, Trinidad strain, appears to be a doubly blocked conditional lethal virus. There are at least two temperature-sensitive steps involved in the synthesis of this virus in CF cells. The first step of inhibition at 44 C occurs early in virus replication and is reversible, suggesting that some factor introduced by the virus is held intact or is otherwise stabilized despite long incubation periods (up to 24 hours) at high temperature. The time of inhibition appears to be located at some point between the time when the virus enters the cell and is uncoated and the beginning of viral RNA synthesis. The second temperature-sensitive step in VEE virus synthesis is irreversible and occurs at a point after the synthesis of viral RNA and before the formation of virus structural protein as measured by the complement fixation test.

~~Unclassified~~
Security Classification

DOCUMENT CONTROL DATA - R&D		
(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)		
1. ORIGINATING ACTIVITY (Corporate Author)		2a. REPORT SECURITY CLASSIFICATION
U.S. Army Biological Laboratories Fort Detrick, Frederick, Maryland 21701		Unclassified
		2b. GROUP
3. REPORT TITLE		
VENEZUELAN EQUINE ENCEPHALITIS VIRUS: A DOUBLY BLOCKED CONDITIONAL LETHAL VIRUS		
4. DESCRIPTIVE NOTES (Type of report and inclusive dates)		
5. AUTHOR(S) (Last name, first name, initial)		
Zebovitz, Eugene NMI		
6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
December 1965	16	2
8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)	
b. PROJECT NO.	Technical Manuscript 266	
c. LC014501B71A01	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
d.		
10. AVAILABILITY/LIMITATION NOTICES		
Qualified requestors may obtain copies of this publication from DDC. Foreign announcement and dissemination of this publication by DDC is not authorized. Release or announcement to the public is not authorized.		
11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY	
	U.S. Army Biological Laboratories Fort Detrick, Frederick, Maryland 21701	
13. ABSTRACT		
The replication of Venezuelan equine encephalitis (VEE) virus is completely inhibited at 44 C. The inhibited steps were analyzed by shifting the incubating temperatures up or down, and determining the rate and extent of infectious RNA synthesis, intact virus synthesis, and complement-fixing antigen formation during the shifts. The inhibition appears to be due to the presence of two temperature-sensitive steps involved in the synthesis of VEE virus in chick fibroblast cells. The first step of inhibition at 44 C occurs early in virus replication and can be completely reversed simply by returning cultures to 37 C. The time of inhibition appears to be some point between the time when the virus enters the cell and is uncoated and the beginning of viral RNA synthesis. The second temperature-sensitive step in VEE virus synthesis is irreversible and occurs at a point after the synthesis of viral RNA and before the formation of virus structural protein as measured by complement-fixing antigen.		

DD FORM 1473
1 JAN 64

Unclassified
Security Classification