AD NUMBER

AD808909

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Biological Defense Research Lab ltr dtd 28 Sep 1971

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TECHNICAL MANUSCRIPT 355

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Sidney Halle
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JANUARY 1967

DEPARTMENT OF THE ARMY
Fort Detrick
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STUDIES ON THE LIMITED GROWTH OF TEMPERATURE-SENSITIVE VEE VIRUS AT 44 C

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Project 1C014501B71A

January 1967

STUDIES ON THE LIMITED GROWTH OF TEMPERATURE-SENSITIVE VEE VIRUS AT 44 C

ABSTRACT

By using a special sampling method, a significant, but limited, increase in Venezuelan equine encephalitis (VEE) virus titer can be detected during early 44 C incubation of VEE-infected chick embryo (CE) cells. The maximum titer is 0.1 to 1% that of cultures incubated at 37 C. This magnitude of the increase in titer is not a function of size of virus inoculum. The limitation of the increase was not due to lack of adsorption or to medium alteration at 44 C. The best conditions for demonstrating the phenomenon were determined. Effective removal of excess inoculum and medium replacement at 2-hour intervals were optimal for demonstrating early 44 C growth of VEE in CE monolayers. Experiments with metabolic inhibitors and other selective conditions suggest that the increase is due to true de novo viral synthesis and not to elution or recoating of the input viral genome. The limited virus growth at 44 C is especially sensitive to low levels of puromycin compared with growth at 37 C. Possible mechanisms involved are discussed.

Recent reports by Zebovitz* and Zebovitz and Brown** have shown that Venezuelan equine encephalitis (VEE) virus, unlike eastern equine encephalitis (EEE) virus, was an apparent doubly blocked conditional lethal virus that failed to multiply at 44 C on primary chick embryo (CE) cell cultures. The first block occurred early, i.e., after virus uncoating but before viral RNA synthesis; the second block occurred late, i.e., after synthesis of viral RNA and apparently before viral protein was made. The present work describes in detail a limited but distinct early growth of VEE virus at 44 C that is revealed under certain conditions.

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* Zebovitz, E. 1966. Venezuelan equine encephalitis virus: A doubly blocked conditional lethal virus. Bacteriol. Proc. 110.

** Zebovitz, E.; Brown, A. November 1966. Venezuelan equine encephalitis virus: An apparent doubly blocked conditional lethal virus, (Technical Manuscript 321). Virus and Rickettsia Division, Fort Detrick, Frederick, Maryland.

The standard sampling method for assay of viral growth in CE monolayer culture was followed with the pooled supernatants of duplicate plates removed at each sampling time from a large identical group of plates established at the start of the experiment. The possibility that this sampling method might be masking some viral growth was explored by a change in procedure. The modification consisted of assaying the virus, which accumulated in the culture supernatant during the interval between samplings, by removing the supernatant fluid for assay and replacing it with fresh culture medium at each sampling time from a single set of cultures. By this method, a limited but distinct early increase in titer of VEE at a "restrictive" temperature (44 C) was revealed. For simplicity and brevity this method of sampling will be called, henceforth, the supernatant replacement method.

With this method, during the initial and early intervals (0 to 2 hours, 2 to 3 hours) at 44 C a decrease in titer of the residual inoculum virus was observed (Fig. 1). A base level was subsequently reached from which the first increase in titer was usually observed during the 3- to 5-hour interval. This titer increased further during the 5- to 7-hour interval (often 1,000- to 10,000-fold above base level) and then declined during overnight incubation until 24 hours. Control cultures at 37 C showed similar growth kinetics, but the yields were more than 1,000-fold higher and no significant declines were noted at 24 hours. Note also that the rate of virus multiplication at 44 C was approximately equal to that at 37 C; only the peak yield was lower at 44 C.

Table 1 shows that the absolute value of the peak titer was approximately proportional to the inoculum size; however, the magnitude of the increase in titer was relatively constant and independent of inoculum size. A 100-fold difference in multiplicity of infection resulted in less than a threefold difference in the "index of increase," i.e., the difference between the maximum titer and the minimum titer. Appropriate temperature controls made it improbable that the increase in titer resulted from inadvertent lowering of the temperature to conditions permissive for growth. Media and wash fluids were kept at temperatures corresponding to the incubation temperature, manipulations were minimized, and control cultures for the standard sampling methods were handled in an identical manner with respect to temperature conditions.

Under "restrictive" temperature conditions the increase in titer usually reached a plateau at about 7 to 9 hours after infecting the cultures. The maximum titers in the supernatant of CE cultures seem to be limited to about 10^5 to 10^6 pfu per ml. These limitations were not due to lack of normal adsorption at 44 C. As shown in Figure 2, growth kinetics at 37 C or at 44 C of cultures initiated by viral adsorption at 44 C were similar to those of control cultures in which adsorption took place at 37 C.

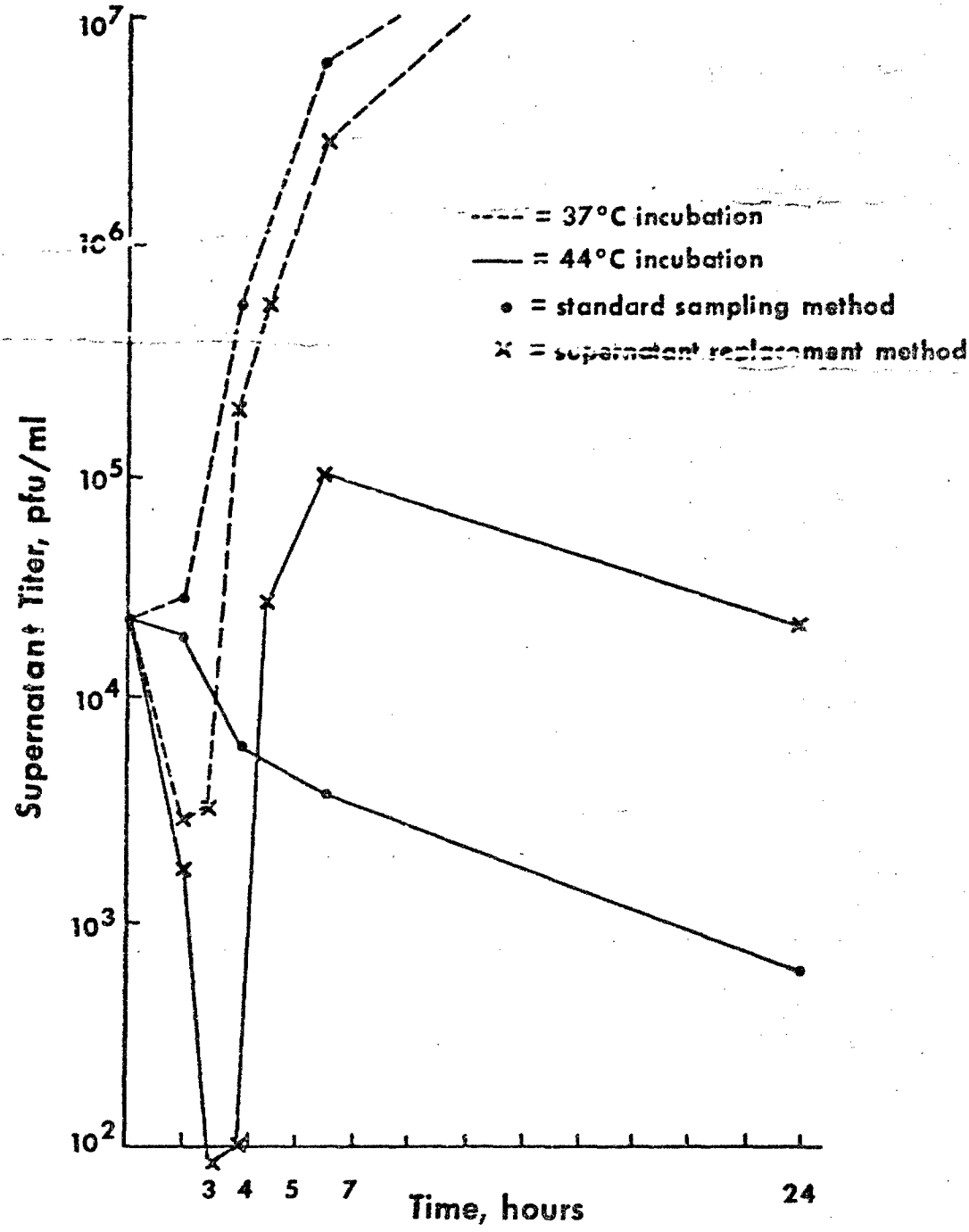

Figure 1. Limited Virus Growth at 44 C Revealed by Supernatant Replacement Sampling Method.

TABLE 1. INFLUENCE OF INOCULUM SIZE ON INCREASE IN VEE VIRUS TITER AT RESTRICTIVE TEMPERATURE (44 C)

	Sampling Interval	pfu per ml	
		Low Inoculum	High Inoculum
Inoculum titer	0 hr	2.1×10^2	2.2×10^4
Base titer	2-3 hr	$<1 \times 10^1$	8.0×10^1
Peak titer	5-7 hr	4.2×10^3	1.0×10^5
Index of increase[a/]		4.2×10^2	1.3×10^3

a. The difference between the maximum (peak) titer and the minimum titer, a measurement for comparing increments.

Toxicity of culture media or nutrient depletion that may have been caused by the higher temperatures were not involved as limiting factors. The medium used was lactalbumin hydrolyzate containing 10% calf serum. Media conditioned by 24 hours' preincubation over CE monolayers at 44 C or 37 C could be used to demonstrate typical virus growth at 37 C or the increase in titer at 44 C as effectively as fresh, unconditioned media. Furthermore, the ability of virus-infected cultures held at 44 C to reinitiate normal growth when placed at 37 C, as described by Zebovitz, and Zebovitz and Brown makes the interpretation of the limitation based on some nonspecific effect of medium rather unlikely.

In the course of these studies we found that if excess inoculum were not removed the titer increase at 44 C would not be noticed. For example, the wash immediately after adsorption of inoculum did not remove sufficient residual virus to show new viral growth. The removal of residual virus to a satisfactory base level was best achieved by an additional two or three washes with phosphate-buffered saline at 2 hours postinfection. The maximum yield of virus was also affected by the frequency of sampling; a sampling interval of 1½ to 2½ hours was optimal. Long assay intervals (e.g., overnight) for 44 C cultures showed titer losses that could not be explained by the inactivation ordinarily observed for the virus at this temperature in the absence of cells.

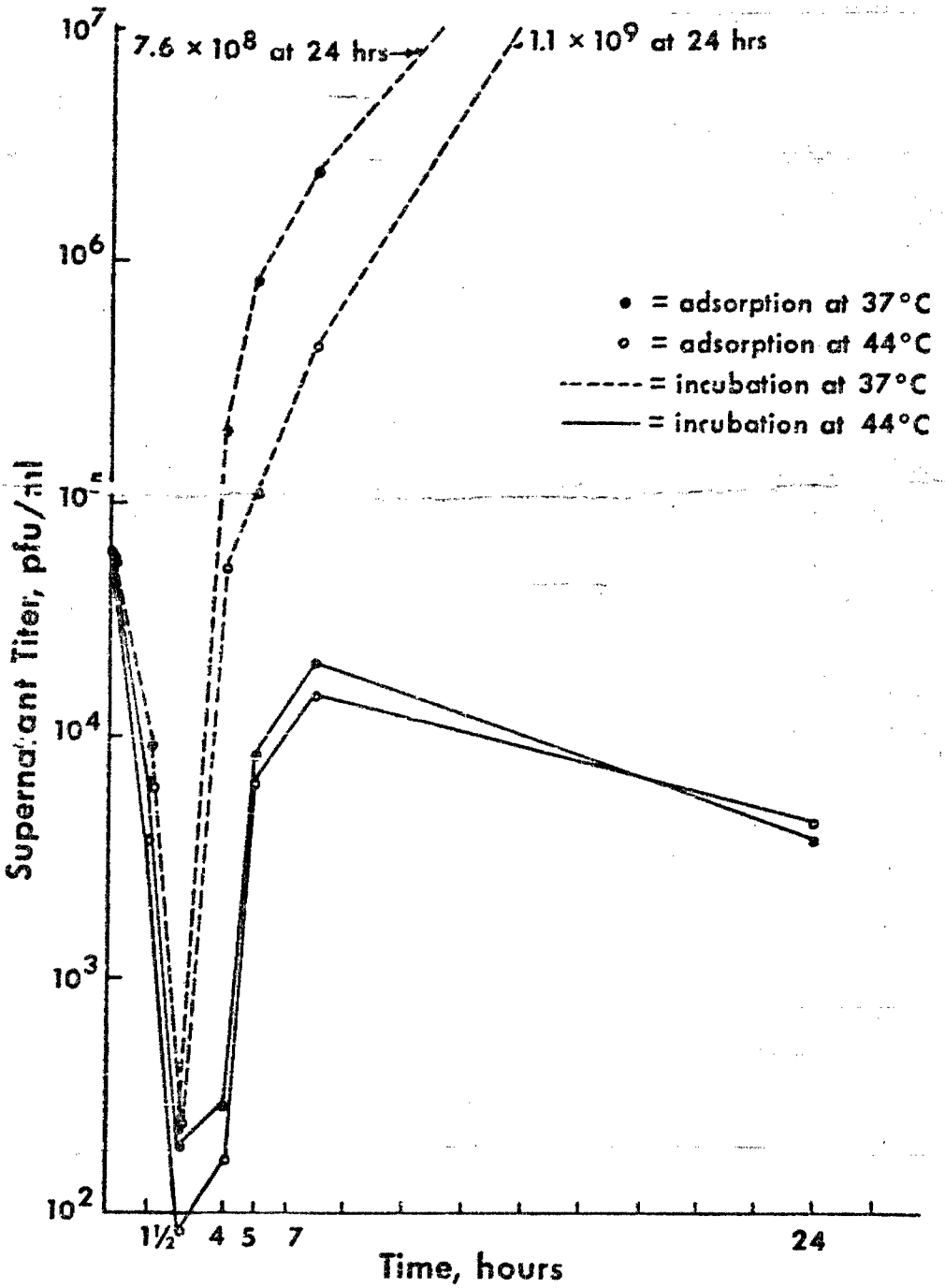

Figure 2. Effect of Adsorption Temperature on VEE Cultures at 37 and 44 C.

When conditions were finally standardized, a concerted effort was made to arrive at a reasonable explanation for the limited virus growth at 44 C. An obvious explanation would be elution of adsorbed virus. The similarity in kinetics of the limited growth at 44 C to that observed at 37 C made this explanation unlikely. However, to check this possibility, cultures were set at 6 C, which presumably should not affect the demonstration of simple nonenzymatic elution but might suppress a dynamic metabolic process. These cultures were analyzed by the supernatant replacement method, which might increase the probability of nonenzymatic elution. These experiments showed, however, that simple nonenzymatic elution could not account for the increase in titer seen at 44 C because virus titer did not increase at 6 C.

Evidence thus accumulated to strengthen the view that the titer increase at 44 C might be true de novo viral synthesis. Enzymatic or temperature-dependent elutions, however, were not yet excluded as explanations for the increase in titer. When 60 to 100 µg of fluorophenylalanine (FPA) were incorporated per ml of minimal medium described by Zebovitz* growth of VEE in CE monolayer culture at 37 C was significantly inhibited and the limited growth at 44 C was completely abolished. Possible in vitro inactivation by FPA (i.e., in the absence of cells) was not found to be responsible. To check the possibility of a specific effect of FPA on release, rather than growth, experiments were performed that showed the effect of the drug on both the supernatant and the cell-associated virus produced at 44 C. As seen in Table 2, the presence of 60 µg of FPA per ml in minimal medium culture completely inhibited the normal yield observed at 6 hours postinfection of supernatant and cell-associated viral components.

TABLE 2. INHIBITION OF VEE VIRUS GROWTH BY FPA AT 44 C

Time, hours	Supernatant Virus, pfu/ml		Cell-Associated Virus, pfu per monolayer	
	FPA Concentration		FPA Concentration	
	0	60 µg/ml	0	60 µg/ml
0	4.0×10^{8}	-	1.7×10^{6}	-
1	9.0×10^{8}	6.0×10^{2}	2.8×10^{3}	3.2×10^{3}
6	2.2×10^{8}	2.5×10^{8}	1.2×10^{8}	1.2×10^{8}

* Zebovitz, Eugene. June 1964. Defined medium for maintaining chick fibroblast monolayers, (Technical Manuscript 142). Virus and Rickettsia Division, U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland.

As shown in Figure 3, 1, 5 or 25 µg of puromycin per ml, when present in CE cultures only during the 1st hour after infection with VEE virus (i.e., cultures were washed three times after the 1st hour to remove puromycin), inhibited only slightly the rate of virus growth at 37 C. Moreover, these inhibitions were not detectable by 24 hours. At 44 C, however, these drug concentrations completely inhibited growth at 6 hours. At 24 hours this inhibition was completely reversed only in that culture treated with 1 µg of puromycin per ml. Growth at 44 C of the culture treated with 5 µg of puromycin per ml was only partially reversed: growth of the culture treated with 25 µg per ml remained completely inhibited 23 hours after the drug had been removed. Note that this level of puromycin had, at best, only a temporary effect on virus growth at 37 C and showed no direct virucidal effect. Thus, it can be seen that there is an early step (0 to 1 hour) in growth of VEE virus at 44 C during which it is unusually sensitive to puromycin.

The effect of puromycin is further demonstrated by the data shown in Table 3. In this experiment, puromycin at 5 µg per ml was present during the first 2 hours in the culture incubated at 44 C. The cells from 10 CE monolayer cultures at each sampling time were assayed for cell-associated virus and for infectious RNA (IRNA) extracted with hot phenol. Note again that there was inhibition of the synthesis of virus at 44 C and also of the associated IRNA in the presence of puromycin. Also note the increase in both virus and IRNA at 44 C in the control cultures. The increase in IRNA suggests true virus replication. Experiments to compare extraction of IRNA with hot or cold phenol have not demonstrated the presence of any unusual population of incomplete infectious viral forms that were incapable of natural reinfection. The possibility was considered that recoating of viral nucleic acid from the inoculum with new viral protein could lead to an "apparent" increase in titer, as described in the case of vaccinia virus by Easterbrook.* This seems improbable because IRNA was shown to replicate. More conclusive evidence is expected when results are available from further experiments with specific RNA inhibitors.

Some of the probable mechanisms involved to explain limited growth of VEE virus at 44 C are: (i) Viral growth at restrictive temperatures may reflect a single cycle of viral product that fortuitously is demonstrable under these special conditions of assay; in addition, the progeny may be incapable of natural reinfection at 44 C. (ii) The limited virus growth may be the normal yield from a minute fraction (approximately 1/1000) of CE cells that possess a suppressor gene that reversed the conditional lethal property of VEE virus and thus permitted normal growth in such cells at 44 C. (It is important to remember that most of the CE cells permit normal growth of EEE virus at 44 C.) (iii) The special conditions of the

* Easterbrook, K.B. 1963. Conservation of vaccinial DNA during an abortive cycle of multiplication. Virology 21:508-510.

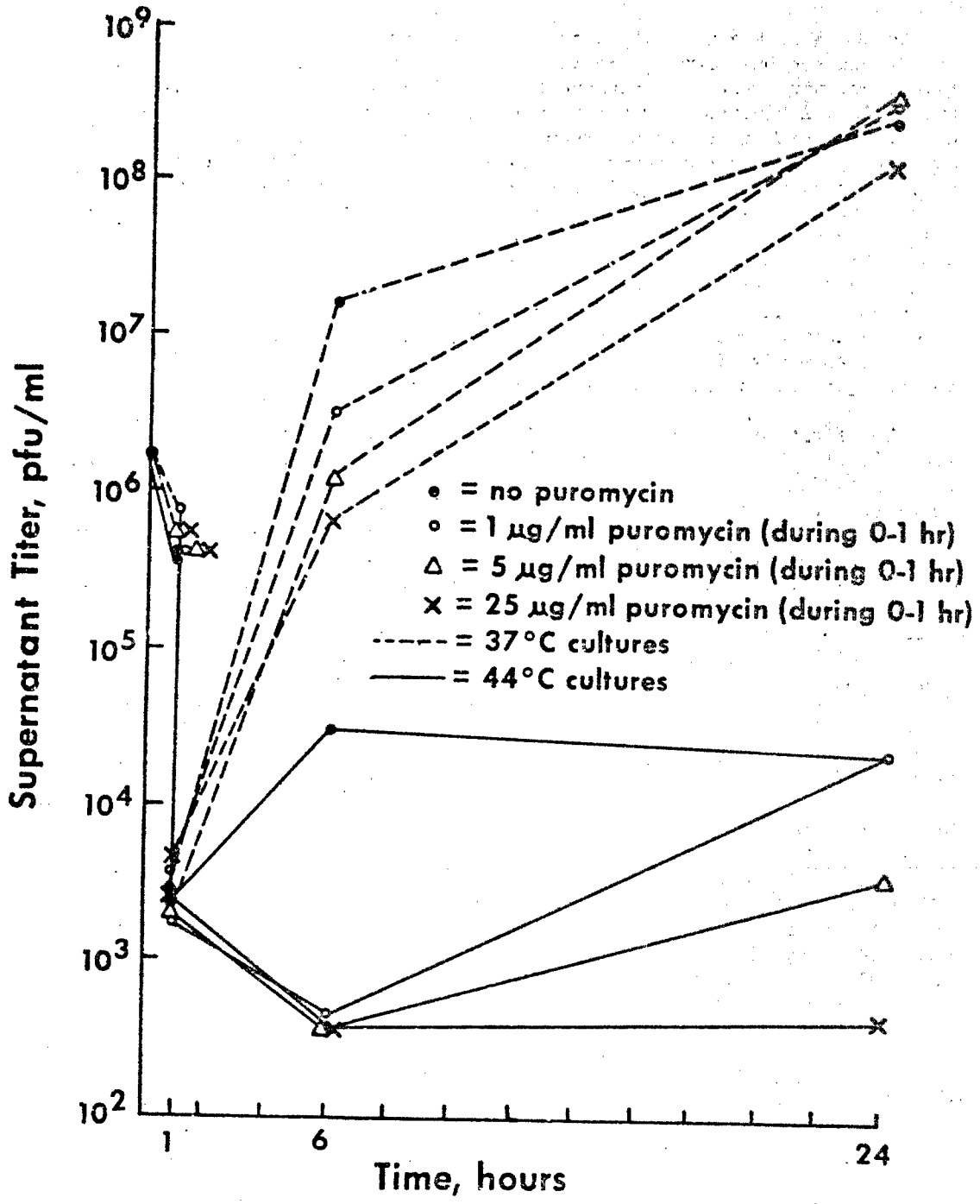

Figure 3. Puromycin Inhibition of VEE Virus Growth at 37 and 44 C.

supernatant replacement method of sampling may cause a metabolic imbalance or a shift of equilibrium that may stimulate a low level of virus growth. The extent of this growth may be controlled by the level of a limiting enzyme (thus phenotypically leaky). The limited growth may also be facilitated by removal (via medium replacement) of a possible inhibitor produced during 44 C incubation of VEE-infected cultures. For example, the (incomplete) removal of a physical entity, i.e., auto-interfering live virus (thus the necessity for washing) or virus-specific product would also account for the limited virus growth observed.

TABLE 3. EFFECT OF PUROMYCIN ON THE TITERS OF INTACT VIRUS AND INFECTIOUS RNA FROM VEE VIRUS[a]

Time, hours	Cell-Associated Virus, pfu/ml		IRNA[b], pfu/10 monolayers	
	Puromycin Concentration[c]		Puromycin Concentration[c]	
	0	5 µg/ml	0	5 µg/ml
0	3.2×10^{7}	-	4.6×10^{3}	-
2	6.3×10^{8}	3.4×10^{3}	6.3×10^{3}	1.2×10^{2}
7	1.4×10^{8}	7.5×10^{3}	3.7×10^{4}	1.8×10^{2}

a. Propagated in chick embryo monolayer cultures at 44 C.
b. Extracted with hot phenol.
c. Puromycin present only during period 0 to 2 hours postinfection.

To summarize, by using a special sampling method a significant but limited increase in VEE virus titer could be detected early (5 to 7 hours) during 44 C incubation of chick embryo cells infected with VEE virus. The maximum titer was 0.1 to 1% that of cultures incubated at 37 C. The magnitude of the increase in titer was not a function of size of virus inoculum. The limitation of the increase was not due to lack of adsorption or to changes in medium that might have resulted from incubation at 44 C. Effective removal of excess inoculum and medium replacement at 2-hour intervals were optimal for demonstrating the limited growth. Experiments with metabolic inhibitors and other selective conditions suggest that the increase is due to true de novo viral synthesis and not to elution or recoating of the input viral genome. The limited virus growth at 44 C is especially sensitive to low concentrations of puromycin; growth at 37 C, by comparison, is relatively insensitive to the low concentrations of puromycin.

It is hoped that experiments in progress will provide sufficient information to define completely the nature of the limited VEE virus growth observed at 44 C under certain experimental conditions.

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) Department of the Army Fort Detrick, Frederick, Maryland 21701	2a. REPORT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3. REPORT TITLE

STUDIES ON THE LIMITED GROWTH OF TEMPERATURE-SENSITIVE VEE VIRUS AT 44 C

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (Last name, first name, initial)

Halle, Sidney (NMI)
Zebovitz, Eugene (NMI)

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
January 1967	14	3

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1C014501B71A	Technical Manuscript 355
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
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13. ABSTRACT

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14. Key Words

*Venezuelan equine encephalitis	Chick embryos	Culture media
*Growth	Puromycin	Replication (viruses)
*High temperature	Adsorption	
Temperature	Sampling (physical)	
Titer		

DD FORM 1 JAN 64 1473

Unclassified
Security Classification