

Effects of Temperature on the In vitro Replication of Dengue Viruses

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OBJECTIVE :

1. To determine the kinetics of replication and the patterns of thermo-inactivation, at different temperatures, of selected dengue viruses that originated from dengue virus infected humans and mosquitoes.

2. To determine the plating efficiency, at different temperatures, of dengue viruses isolated directly from humans with a clinical diagnosis of dengue hemorrhagic fever.

BACKGROUND : This is a continuation of an investigation that was initiated during 1978 to determine if the virulence properties of dengue viruses could be assessed on the basis of the degree of replication at different temperatures. Preliminary findings indicated that the pattern of replication of selected serotypes and strains of dengue viruses in LLC-Mk2 cells at different temperatures did not differ significantly. Studies to define the plating efficiency of wild dengue viruses at different temperatures were hindered by the failure to reisolate viruses from the original specimens. However, limited data suggested that the plating efficiency of dengue virus type-3 strains was greater at 32°C than at 35 and 39°C. This report includes the results of observations made on additional serotypes and strains of dengue viruses. In addition, selected serotypes and strains of dengue viruses were characterized in regard to their rates of thermo-inactivation at different temperatures.

METHODS : Dengue viruses employed in virus replication and thermoinactivation studies originated from humans or from *Aedes aegypti* mosquitoes. Stock viruses were prepared in LLC-Mk2 cells, suckling mice, and in *Toxorhynchites splendens*. Viruses of human origin included serotypes and strains that were associated with human disease that varied in severity from grades I to IV. A list of dengue viruses selected for investigation is presented in Table 1.

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The plating efficiency of dengue viruses was determined on the basis of the number of plaque forming units (PFU) obtained directly from plasma and cellular components of the blood of dengue fever and dengue hemorrhagic fever (DHF) patients diagnosed at Children's Hospital during 1978. Suspensions of plasma and cellular components were assayed by the direct plaque technique in LLC-Mk2 cells at 32, 35, and 39°C. Separation of cellular components into leukocyte sub-population and platelets was performed as reference described elsewhere in this Annual Report¹. Viruses were identified by plaque reduction neutralization tests (PRNTS) employing dengue virus specific antisera prepared in Rhesus monkeys.

Virus replication curves were performed for dengue viruses at 32, 35, and 37°C in LLC-Mk2 cells. Screw cap tubes were seeded with 1.0 ml of a cell suspension that contained $10^{5.0}$ cells per ml. After 4 days at 37°C, medium-free monolayers were inoculated with a dilution of virus to yield a multiplicity of infection (MOI) of 0.1. Cultures with inoculum were incubated for one hour at 32, 35, and 37°C. After the absorption period, 1.0 ml of RPMI 1640 medium was added to each culture. Medium contained 10% fetal calf serum (FCS), 200 units of penicillin per ml and 200 ug per ml of streptomycin.

At 12 hours post inoculation and at 12 hour intervals thereafter, 3 cultures per temperature were harvested for virus assay. After removing cells from the tube wall by vigorous aggitation with glass head, 0.5 ml of FBS was added to each suspension. Aliquots of 0.5 ml of each suspension were stored at -70°C. Suspensions were assayed for virus by a micro-plaque assay employing 24 hole disposable plastic plates. \log_{10} dilutions were made in RPMI 1640 medium and inoculated onto medium-free cell monolayers, 0.05 ml per well. After a 60 minute absorption at 35°C, each culture received 0.5 ml of an agar overlay. Cells were incubated at 35°C for 5 days and 0.5 ml of a second agar overlay that contained a 1:1000 dilution of neutral red was added to each cell culture. PFUs were counted and recorded on day 6 post inoculation.

Thermo-inactivation rate of the infectivity of dengue viruses was determined at 37 and 41°C. One ml of each stock virus suspension was added to 9.0 ml of preheated RPMI 1640 medium and/or human serum that was shown to be free of dengue virus antibody by plaque reduction neutralization test (PRNT). A 0.5 ml aliquot of each virus suspension was obtained immediately and at 2, 4, and 8 hours of incubation. \log_{10} dilutions were assayed for virus by PRNT in LLC-Mk2 cells.

RESULTS : The infectivity titers attained by strains of dengue virus types 1, 2, 3 and 4 in LLC-Mk2 cells that were maintained at 32, 35, and 37°C are presented in Figures 1, 2, 3, and 4. Strains of dengue virus type-2 and 4 exhibited comparable titers at each temperature. The magnitude of replication of the grade I strain of dengue virus type 1 was slightly higher than that of grade II strain at 32, 35, and 37°C. Of the two strains of dengue virus type-3 the grade IV strain exhibited significantly higher titers than that of the grade I strain. In addition, the latent period of the grade I strains was 48 to 60 hours longer than that of grade IV strains.

As a result of problems encountered in the preparation of stock viruses, both cell culture and mouse brain propagated viruses were employed in virus replication curves. The possibility that the source of the virus stocks influenced the results was considered by conducting virus replication studies with dengue virus type-1 and type-2 stocks that were prepared in both cell culture and suckling mice. As shown in Figures 5 and 6, the latent period and infectivity titers exhibited by dengue virus types-1 and 2 were comparable regardless of the source of the stock virus.

Since the thermo-stability of the infective properties of viruses can be affected by the type and content of media, an experiment was conducted to compare human serum with RPMI 1640 medium, 10% FCS, for use in thermo-stability studies of dengue viruses. The results presented in Figures 7 showed the rate and pattern of inactivation of dengue virus type 2 to be approximately the same for the two media. On the basis of these results, RPMI 1640 medium was selected for thermo-stability studies. As shown in Figures 8 and 9, the infectivity of each strain of dengue virus types 1 and 3 had reached undetectable levels after 4 hours of incubation at 37 and 41°C. No apparent difference was observed in the rate of loss of infectivity for the grade I and grade IV strains at different temperatures. In contrast, the dengue virus types 2 and 4 strains varied considerably in the rate of thermal inactivation, (Figures 10 and 11). These viruses were more thermo-stable than dengue viruses types 1 and 3, and the grade III and IV strains of dengue-2 and dengue-4 respectively, were more stable than the grades I strains of the latter dengue virus serotypes.

The results of thermal inactivation studies of dengue virus types 2 that originated from field collected *Ae. aegypti* are presented in Figures 12 and 13. The infectivity of both dengue virus strains was rapidly inactivated at 37°C and 41°C. The rate of decrease in infectivity was approximately the same for virus stocks that were prepared in *T. splendens* and for the same strains that were subsequently propagated in LLC-Mk2 cells

The yield of dengue virus type-2 that was obtained from plasma and cellular component of blood of patients with different grades of illness is presented in Table 2. No apparent relation was observed between the yield of virus and the grade of illness of the patient. The yield of virus varied according to temperature and the type of specimen. Although the viruses replicated at different temperature, only strain D78-135 appeared to be temperature sensitive. The distribution of viruses according to source and temperature permissive for replication is presented in Table 3.

REFERENCES :

1. Nisalak, A., Watts, D.M., Burke, D.S., and Nimmannitya, S., 1978-1979 Isolation of Dengue Virus from Plasma and Cellular Components of the Blood of Dengue Patients. Annual Progress Report, AFRIMS.

Table 1. Dengue virus serotypes and strains employed in virus replication and thermolability studies.

Dengue Virus	Host-Passage	Grade of Illness	LLC-Mk ₂ Cells (PFU/1.0 ml)	SMLD ₅₀ **
Den-1 75-001	Mk ₂ -5	I	5.7*	5.7
-001	SM-3	I	6.1	6.3
74-112	Mk ₂ -5	IV	5.5	5.2
-112	SM-3	IV	7.2	7.2
Den-2 77-132	Mk ₂ -4	I	4.3	NA
3379	SM-5	II	6.5	8.3
77-248	Mk ₂ -4	IV	6.8	5.2
-248	SM-3	IV	7.9	8.6
Den-3 77-2877	SM-5	I	6.3	5.2
77-2797	SM-5	IV	6.2	7.4
Den-4 77-092	Mk ₂ -4	I	3.7	3.5
-092	SM-3	I	7.3	7.1
77-380	Mk ₂ -4	III	4.2	NA
Den-2 189-17A	<i>T. splendens</i> -1	-	3.7	-
Den-2 189-17A	Mk ₂ -1	-	4.4	-
Den-2 189-18A	<i>T. splendens</i> -1	-	4.0	-
Den-2 189-18A	Mk ₂ -1	-	4.0	-

* =Log₁₀ Plaque forming unit/1.0 ml.

** =Suckling mouse lethal dose₅₀/1.0 ml.

NA =Not adapted to suckling mice.

T. splendens = *Toxorhynchites splendens*.

SM =Suckling mice.

Mk₂=Monkey kidney cells.

Table 2. The yield of dengue virus type 2 obtained from plasma and leukocyte fractions that were assayed at different temperatures.

Specimen Number of	Plasma			Mononuclear Cells			Polymorpho- nuclear Cells			Grade of Virus	
	32°	35°	37°	32°	35°	37°	32°	35°	37°	Illness	Serotype
D-78-078	00	04*	00	TNTC	TNTC	TNTC	04	00	00	III	Den-2
D-78-081	TNTC	00	00	---	-	--	00	05	00	I	Chikungunya
D-89-091	-	-	-	08	82	66	-	-	-	PUO	Den-2
D-78-099	TNTC	TNTC	TNTC	-	-	-	-	-	-	III	Den-2
D-78-112	00	52	53	00	01	04	00	00	02	II-III	Den-2
D-78-114	TNTC	TNTC	TNTC	24	TNTC	TNTC	03	05	00	PUO	Den-2
D-78-117	C	TNTC	TNTC	08	TNTC	10	?	TNTC	07	PUO	Den-2
D-78-132	03	05	04	04	01	00	-	-	-	PUO	Den-2
D-78-133	09	37	40	00	01	00	03	00	00	PUO	Den-2
D-78-135	28	10	20	80	10	17	22	10	05	II	Den-2
D-78-136	-	-	-	00	TNTC	162	-	-	-	III	Den-2
D-78-156	-	-	-	00	03	00	-	-	-	III	Den-2
D-78-157	00	74	59	00	02	04	-	-	-	PUO	Den-2
D-78-159	00	28	27	00	03	00	-	-	-	III	Den-2
D-78-168	-	-	-	00	04	00	-	-	-	II	Den-2
Total	7	10	9	7	13	8	4	4	3		

* Plaque forming units/0.3 ml.

** Not done.

Table 3. The distribution of dengue virus type-2 strain isolations by source and by temperature of primary isolation culture.

Source of virus	Temperature of primary isolation culture						Total
	32-35-37°C	32-35°C	35-37°C	32°C	35°C	37°C	
Plasma*	5	0	3	0	1	0	09
Mononuclear cells	5	1	3	0	4	0	13
Polymorphonuclear cells*	1	1	0	2	1	1	06

* D-78-117 isolate not included as results were inconclusive for 32°C.

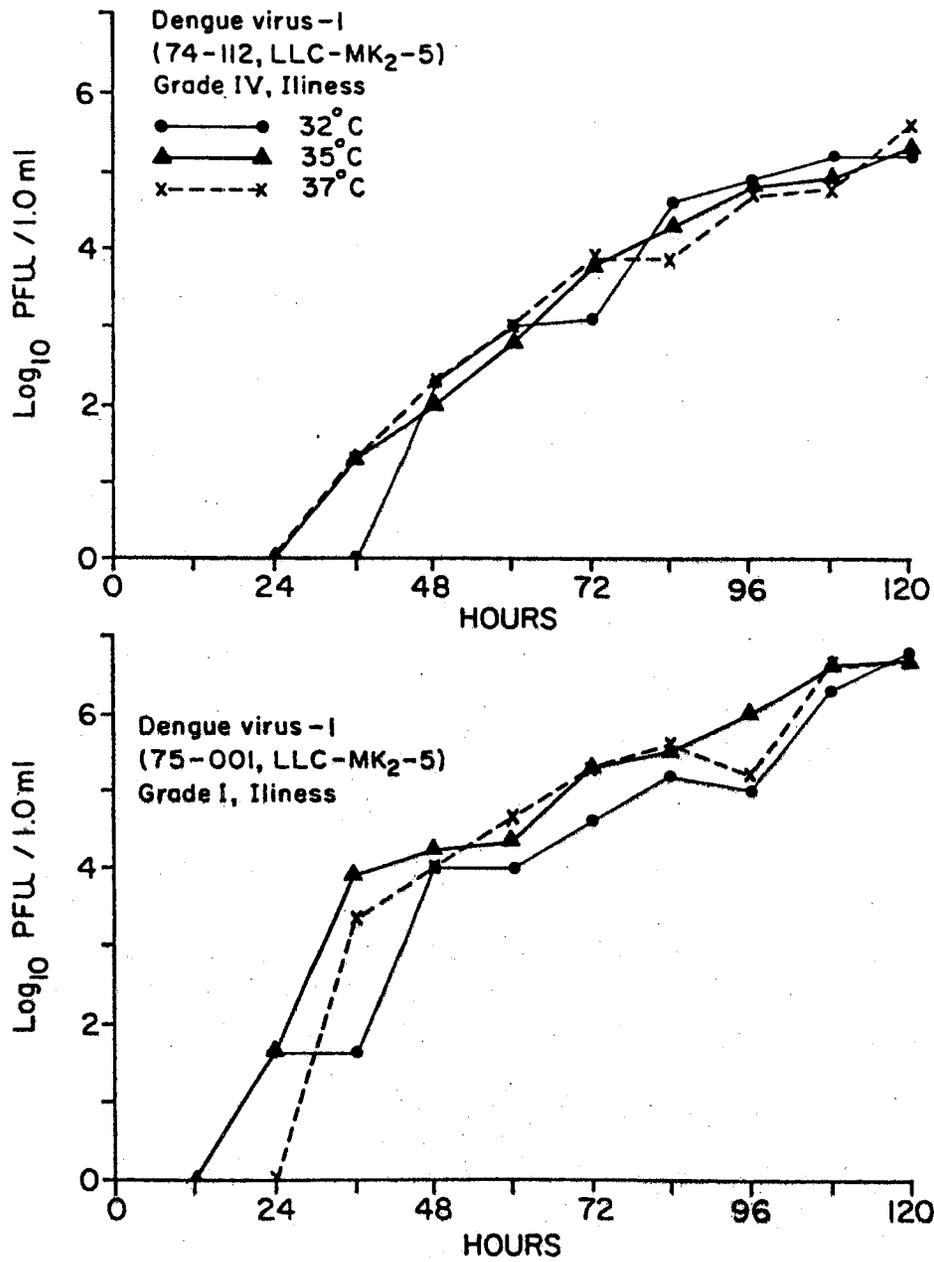


Figure 1. Replication of dengue virus type I strains in LLC-MK₂ cells at different temperatures.

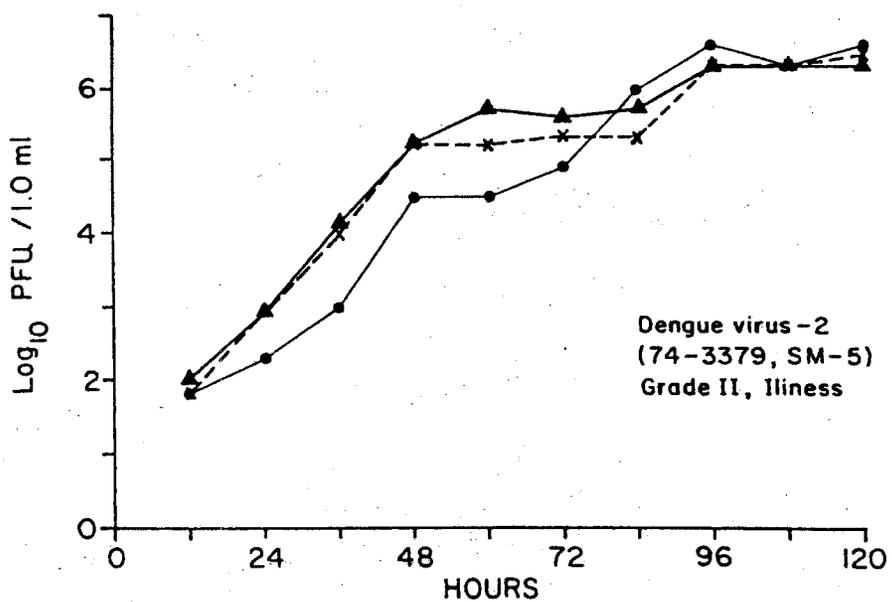
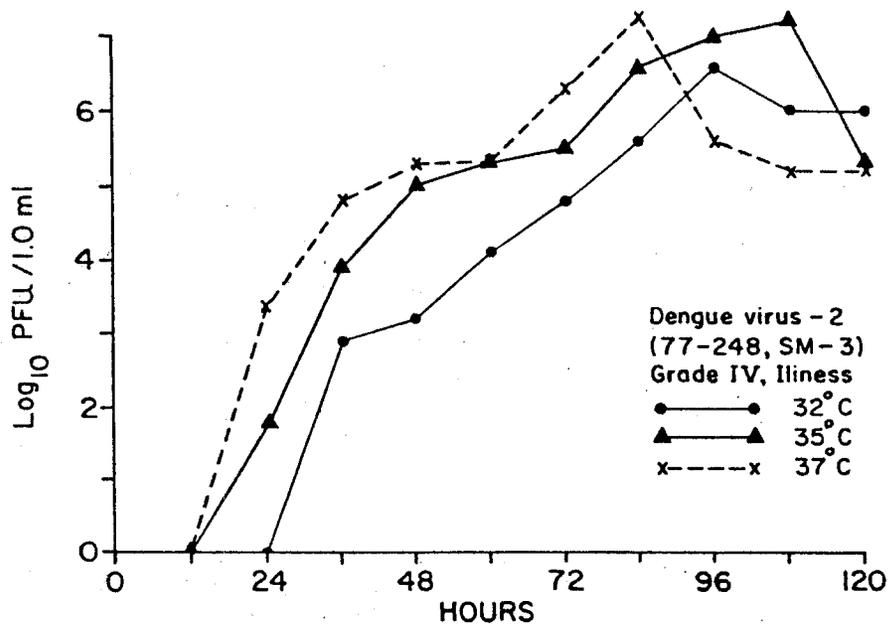


Figure 2. Replication of dengue virus type 2 strains in LLC-MK₂ cells at different temperatures.

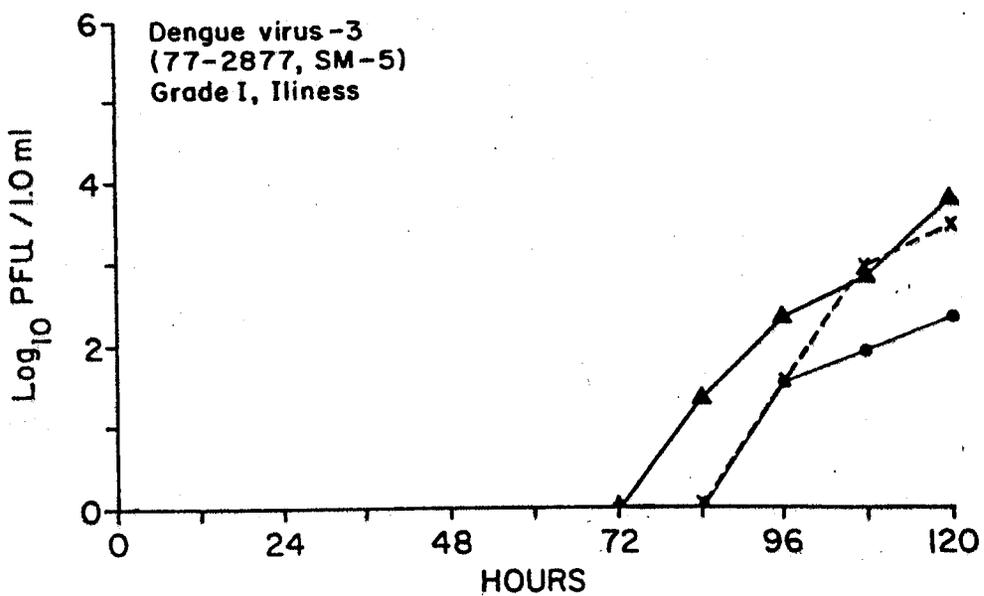
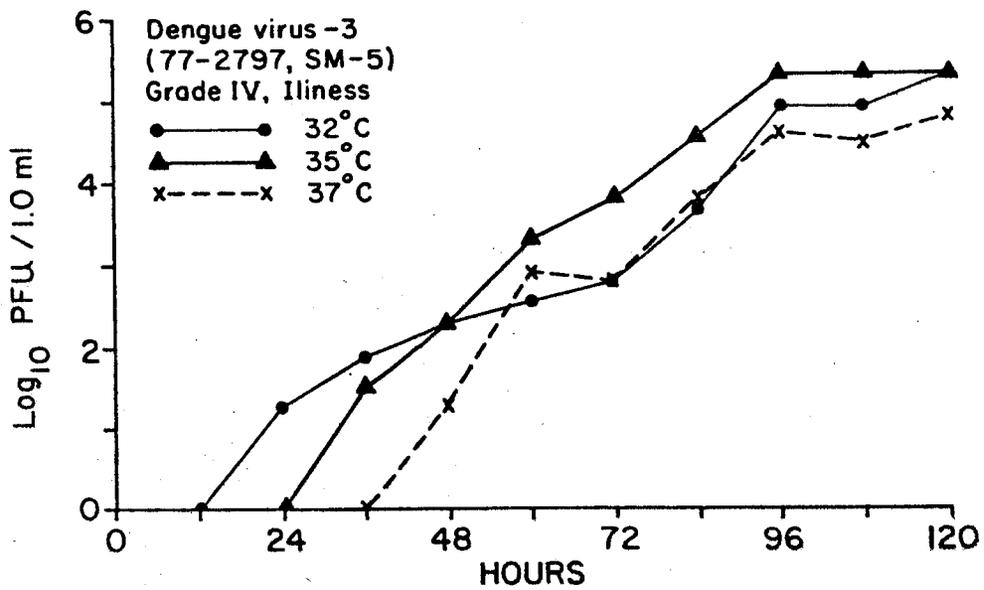


Figure 3. Replication of dengue virus type 3 strains in LLC-MK₂ cells at different temperatures.

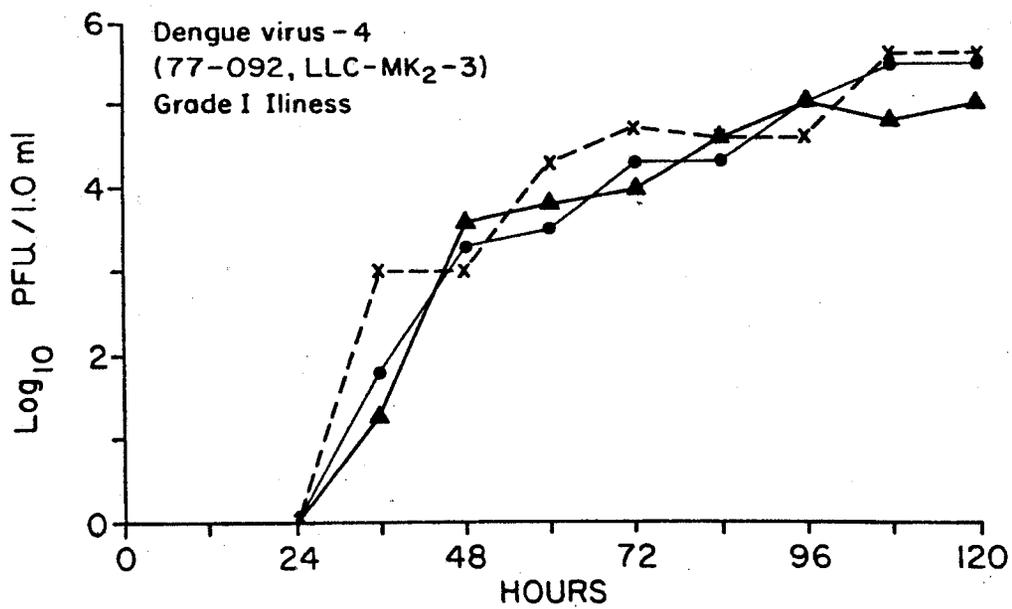
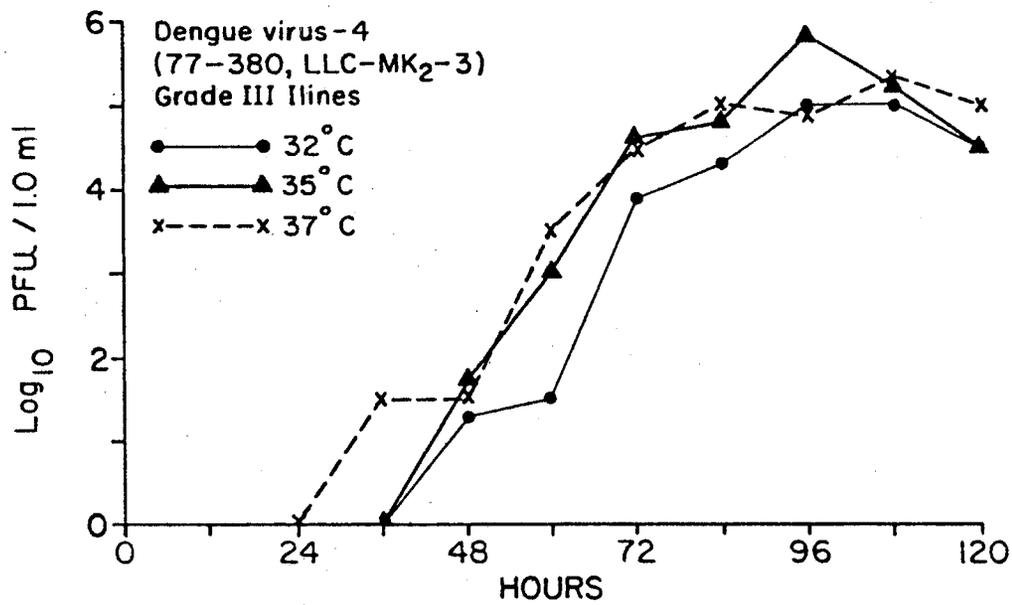


Figure 4. Replication of dengue virus type 4 strains in LLC-MK₂ cells at different temperatures.

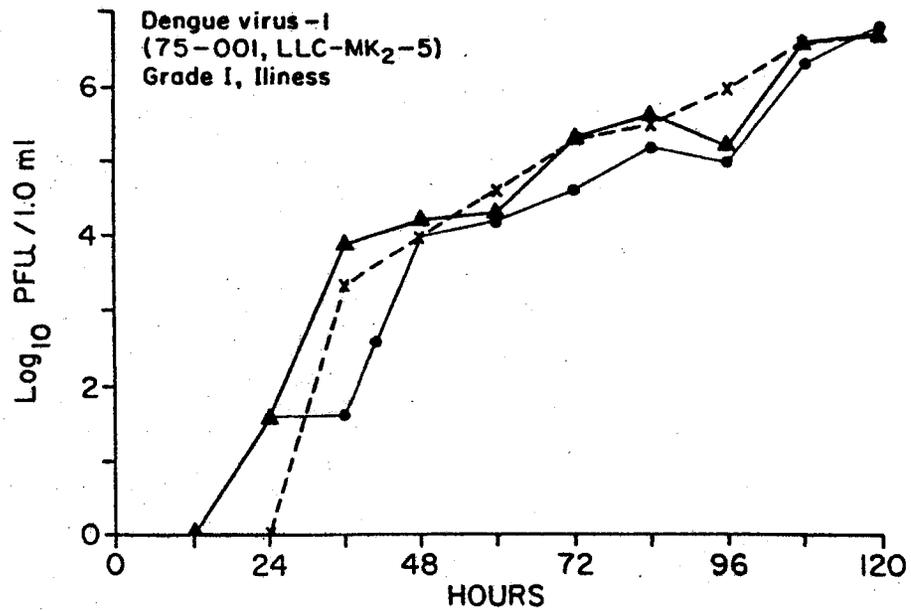
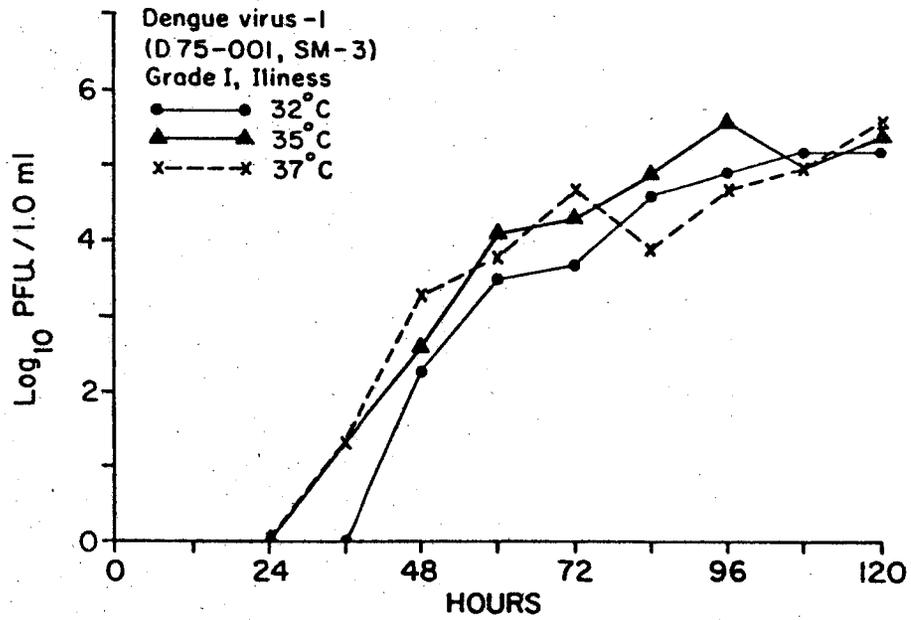


Figure 5. Replication of dengue virus type I suckling mouse brain passaged seed and a cell culture propagated seed virus, in LLC-MK₂ cells at different temperatures.

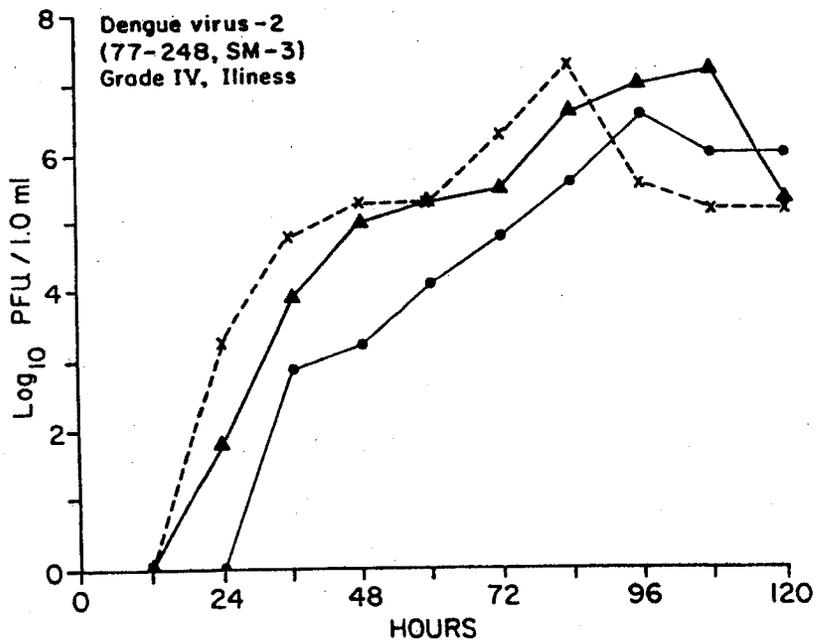
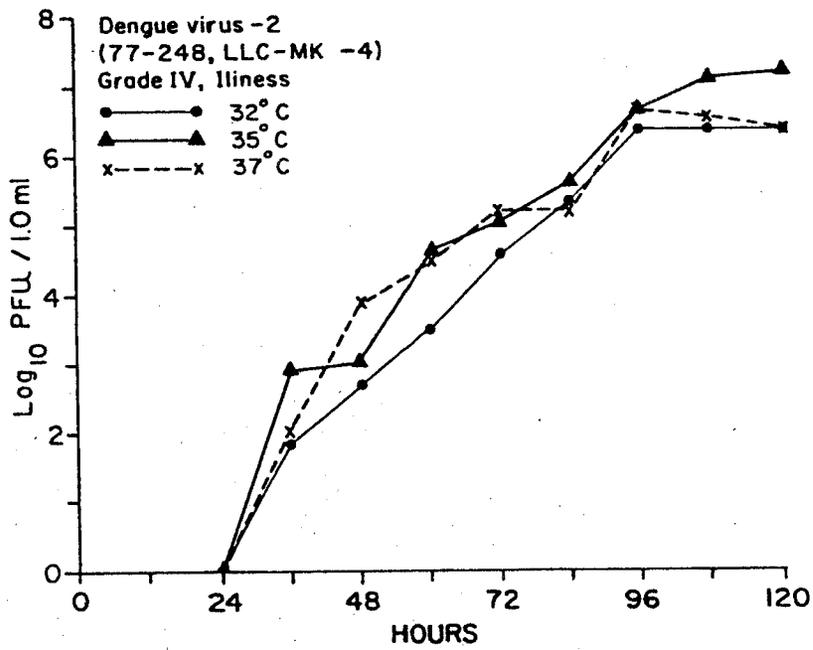


Figure 6 Replication of dengue virus 2 suckling mouse brain passaged seed and a cell culture propagated seed virus, in LLC -MK₂ cells at different temperatures.

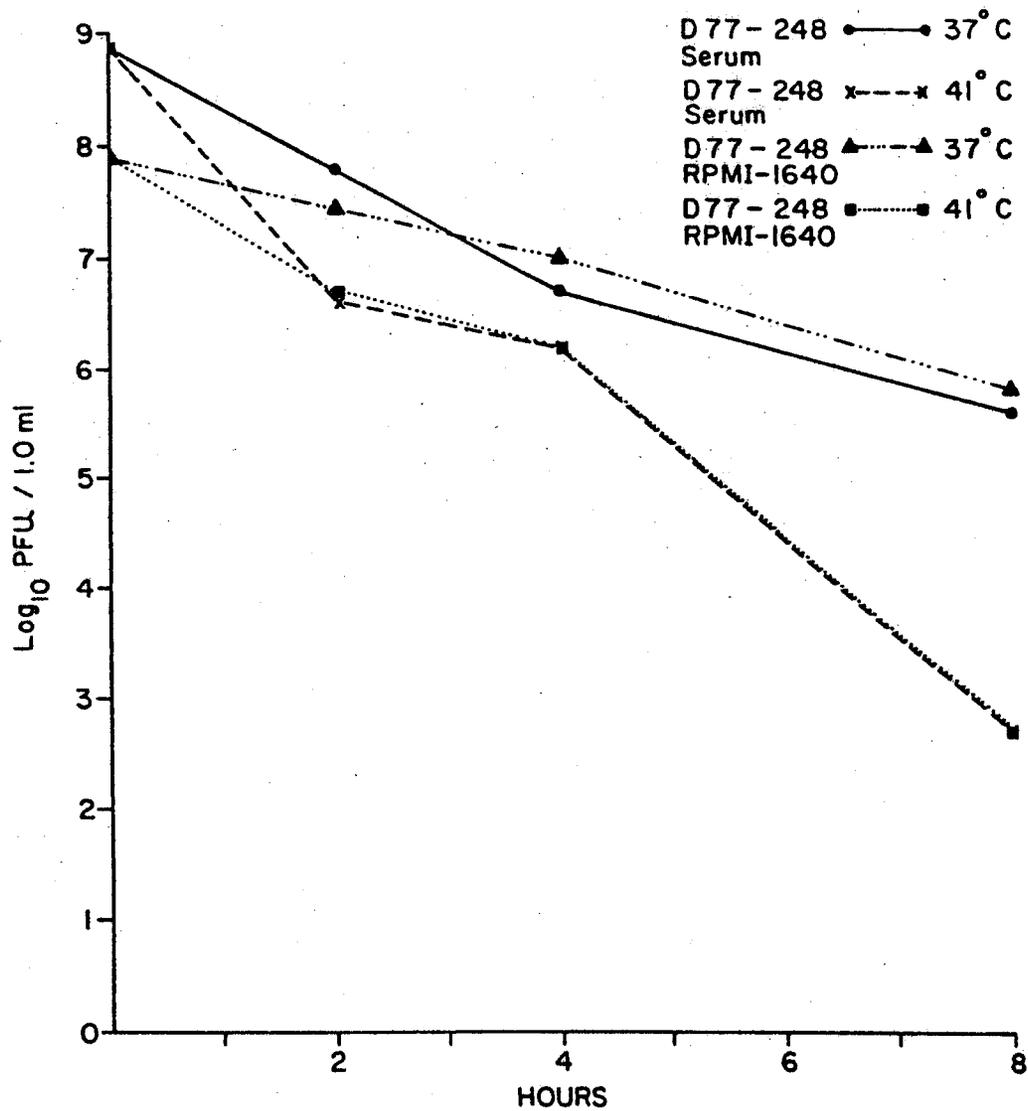


Figure 7. Thermolability of dengue virus type 2 in human serum and in RPMI 1640, 10 % fetal calf serum medium.

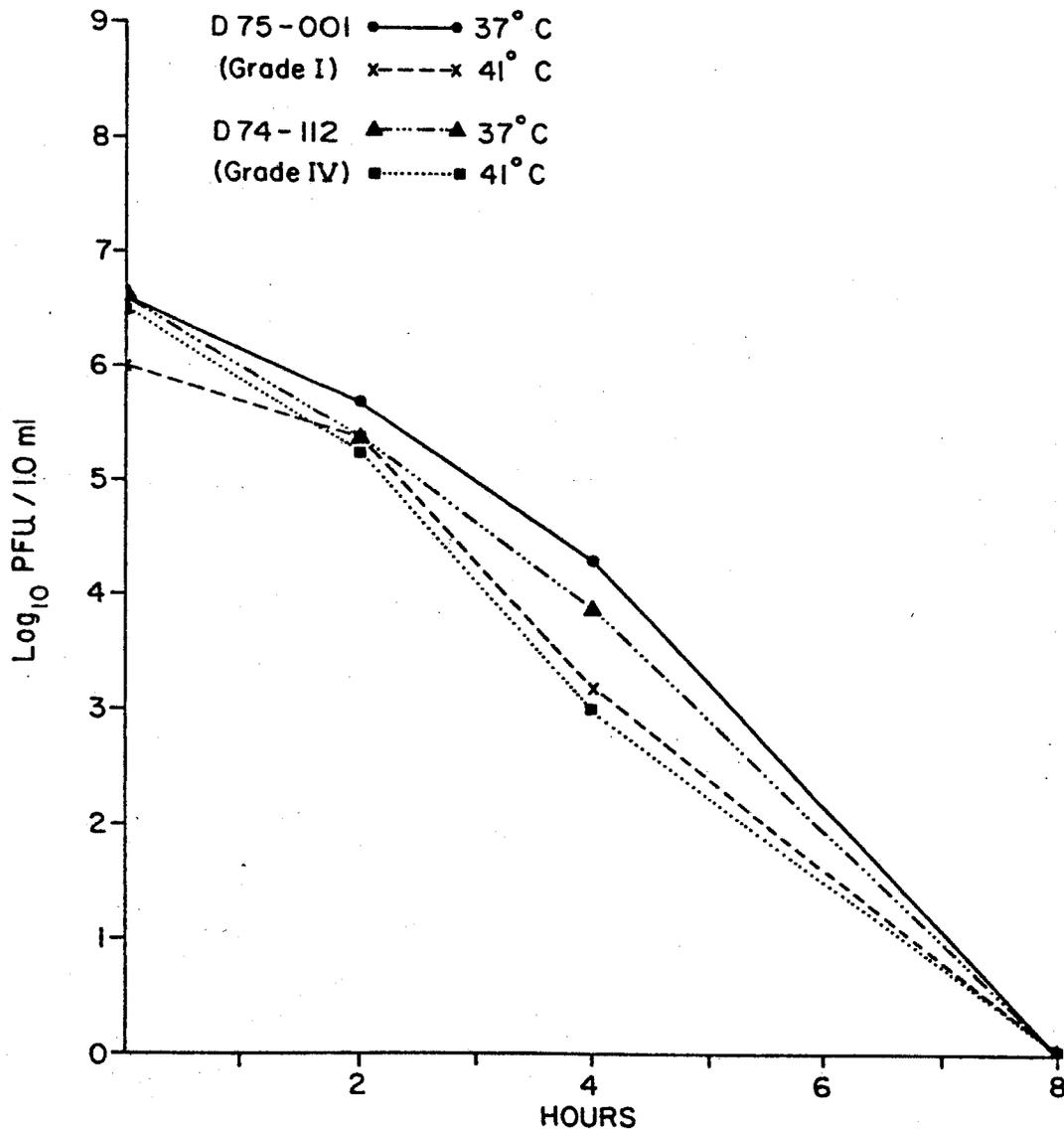


Figure 8. Thermolability of dengue virus type I strains.

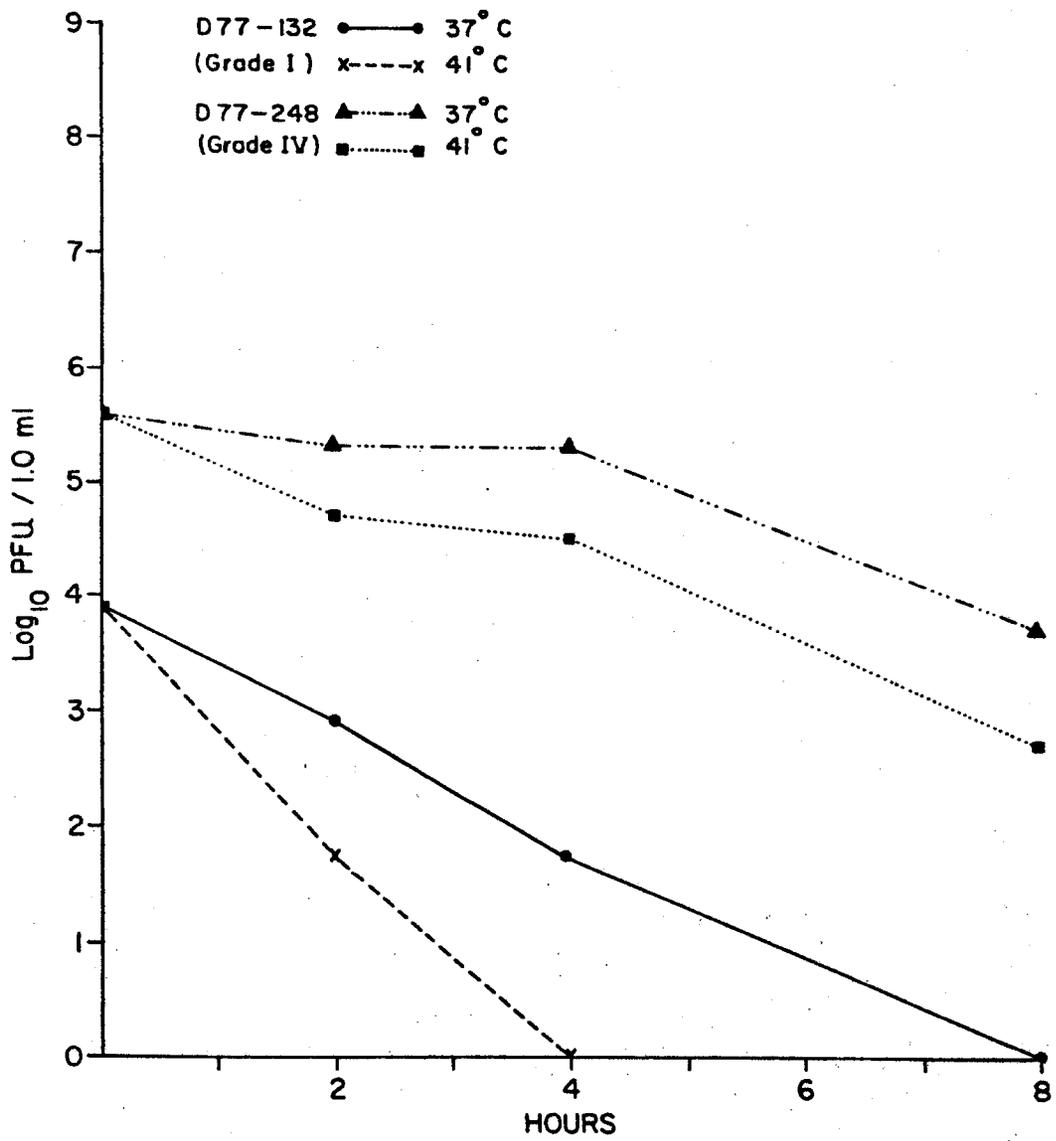


Figure 9. Thermolability of dengue virus type 2 strains.

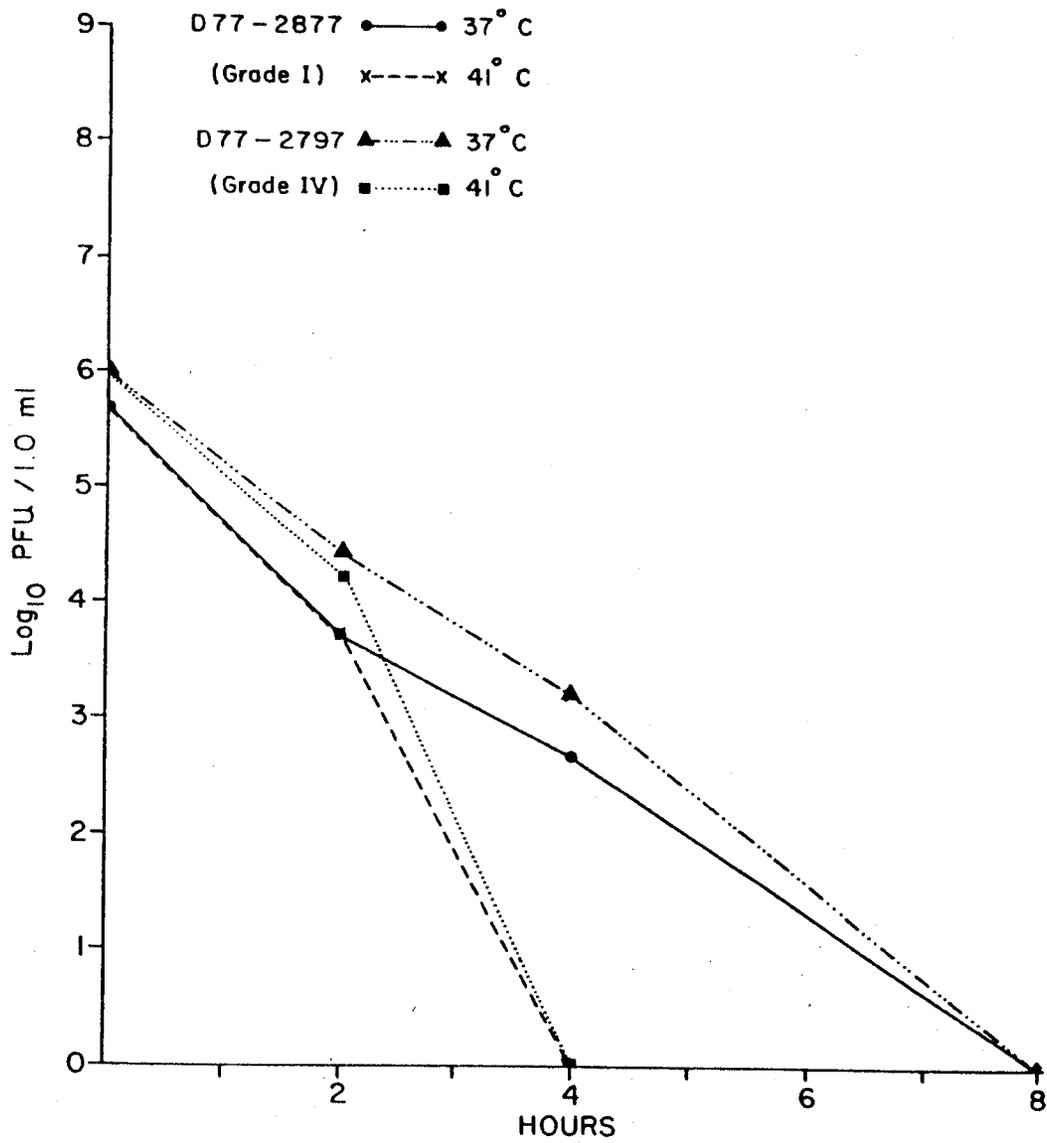


Figure 10. Thermolability of dengue virus type 3 strains.

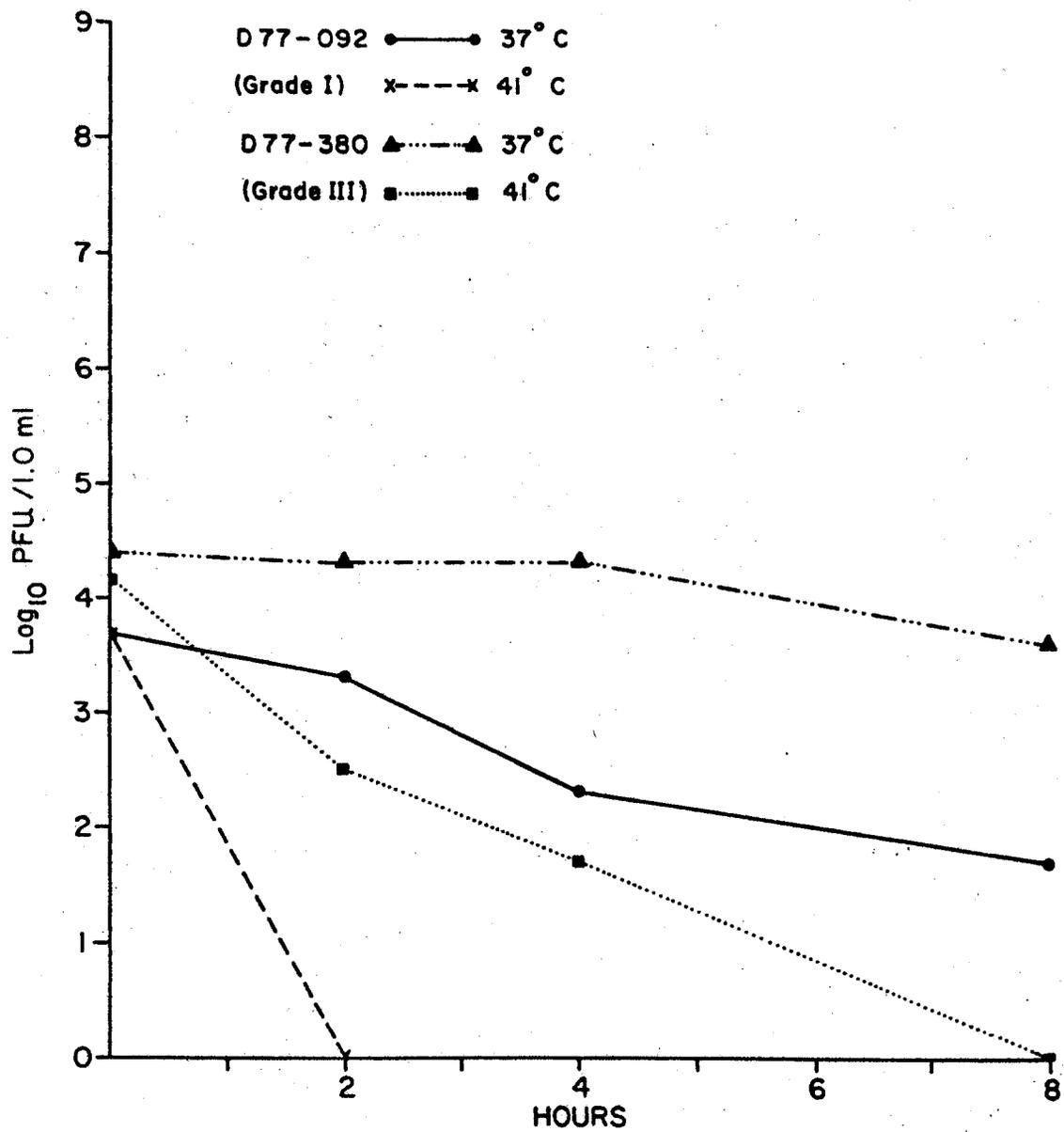


Figure II. Thermolability of dengue virus type 4 strains.

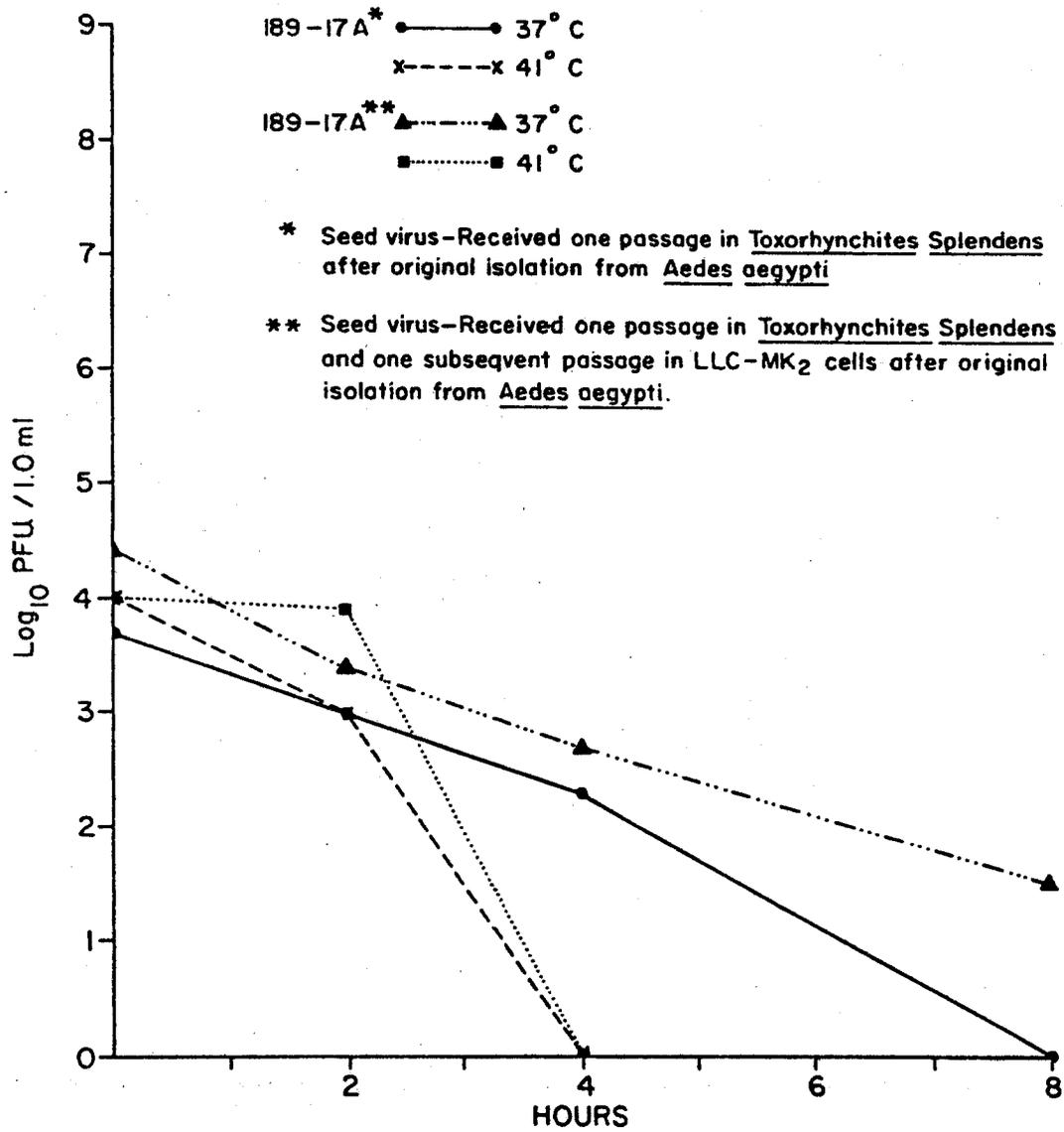


Figure 12. Thermolability of dengue virus type 2 (189-17A) that was isolated from Aedes aegypti

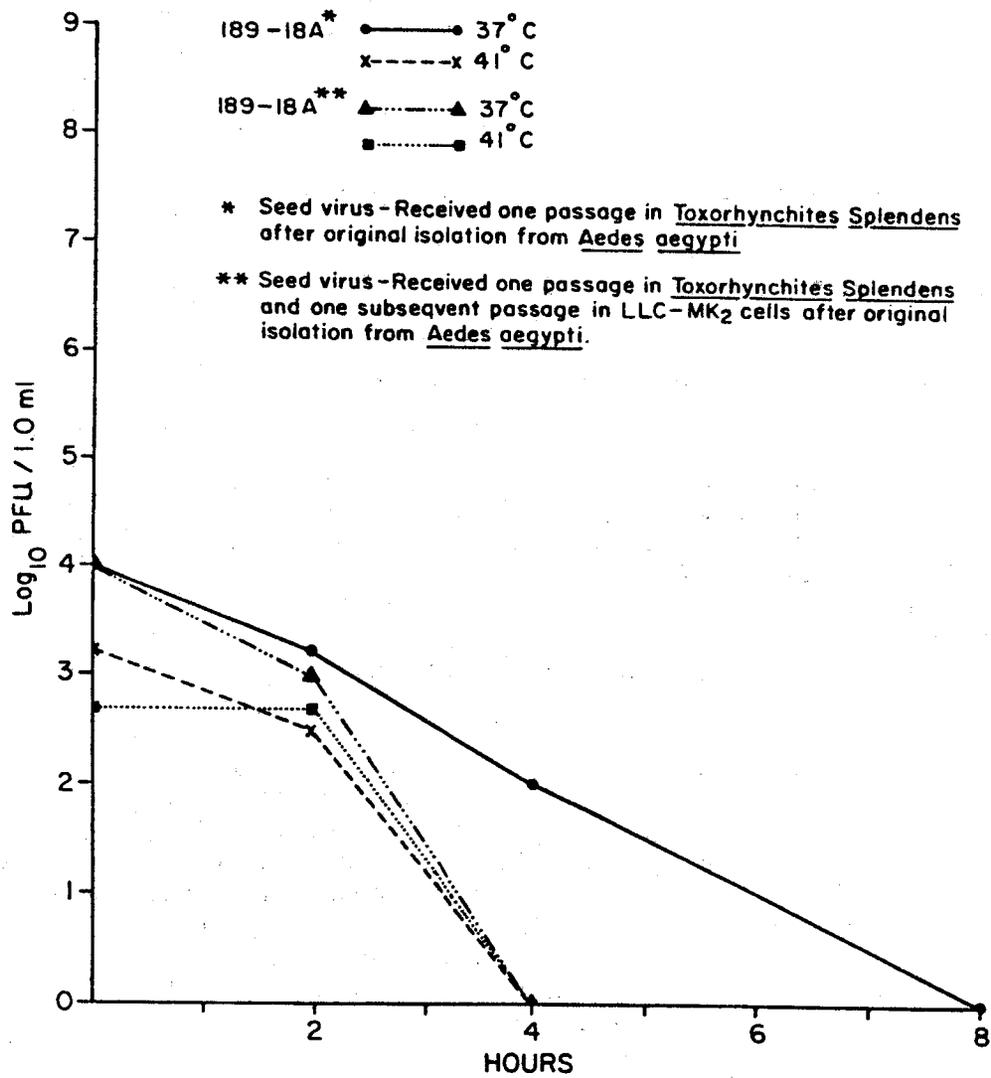


Figure 13. Thermal inactivation of dengue virus type 2 (189-18A) that originated from Aedes aegypti.