PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 68–74 (1982)

Replication of Dengue Virus in Cultured Mosquito Cells at Suboptimal Temperature (41399)

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Abstract. Cultured *Aedes albopictus* mosquito cells were infected at optimal (28°) and suboptimal (20°) temperatures with dengue virus type-3 at multiplicities of infection of 2.0 and 0.01. Extracellular and intracellular virus titers were determined over an interval of 6 weeks by fluorescent focus assay using hyperimmune mouse ascities fluid as a source of antibody. Upon infection at a multiplicity of 2.0, cultures at 28° reached peak virus titers in 3 days, while cultures at 20° required 14 days. At a lower infection multiplicity of 0.01, peak titers were reached in 5 days at 28°, but required 3 weeks at 20°. Viral titers maintained after the peak was reached were the same at both infection multiplicities and temperatures tested. At both 28° and 20° *A. albopictus* cultures became persistently infected with 20–30% of the cells containing immunofluorescent cell-associated viral antigen. These results indicate that the time required to reach peak infectious virus titers for dengue-3-infected *A. albopictus* cell cultures is temperature dependent. In contrast, the viral titers maintained after the peak was reached, the release of virus from infected cells, and the establishment of persistent infection were the same at both 28° and 20°.

Dengue virus is a group B togavirus which replicates in, and is transmitted by mosquitoes of the *Aedes* genus. Dengue virus also replicates in cultured cells of various *Aedes* species (1, 2). Because of the worldwide distribution of this virus and it's association with epidemics of dengue fever in man in the western hemisphere (3), an understanding of the regulation of dengue virus replication in mosquitoes or cultured mosquito cells is important. One factor known to affect the replication of dengue virus in live mosquitoes is low ambient air temperature. Air temperatures from 18 to 25°, which are well within the temperature range for survival of *Aedes* species, can inhibit dengue virus replication and transmission in laboratory-reared mosquitoes (4, 5), a phenomenon originally reported for yellow fever virus (6, 7) and additionally demonstrated for other arboviruses (5, 8, 9). Detailed studies of the exact mechanism of this temperature-dependent regulation of viral replication, however, have not been done in live mosquitoes. In reported studies on dengue virus replication in cultured mosquito cells the incubation temperatures of choice have been 28°–30° (1, 2, 10–12) and 37° (10). No information is available on the effect of incubation temperatures below 28° upon the replication of dengue virus or any other group B togavirus, although in nature, mosquitoes are frequently subjected to temperatures below 28°. In this investigation we have compared the course of a dengue virus (type-3) infection in cultured *A. albopictus* (A. alb) cells at their optimal incubation temperature (28°), and at a suboptimal temperature (20°). Specifically, we have determined the effect of the suboptimal incubation temperature upon infectious virus output, release of virus from infected cells, development of immunofluorescent cell-associated viral antigen, and growth rates of infected cultures.

Materials and Methods. *Virus.* Dengue virus type-3 (Caribbean stain PR-6, mouse brain passage 6) was obtained from Philip Russell (Walter Reed Army Institute of Research, Washington, D.C.). Virus stocks were prepared and passed in suckling mouse brain (SMB) by standard procedures (13), and stored as a 20% SMB suspension in 0.05 *M* borate-buffered saline, pH 9.0, at −70°.

Cell cultures. Baby hamster kidney cells (BHK-21) were grown in Eagle's miminal

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essential medium (MEM) (Flow Laboratories, McLean, Va.) containing 10% tryptose phosphate broth, 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cultures were maintained at 37° in a humidified, 5% CO_2 atmosphere. A. alb cells were obtained from the American type culture Collection (No. CCL-126). These cells were conditioned to grow in A. alb medium: MEM containing 10% Mitshuhashi and Maramarosch medium (14) 16 m*M* *N*-2-hydroxyethylpiperazine-*N*″,2-ethanesulfonic acid (Hepes) buffer, 10% heat-inactivated (56° for 30 min) fetal bovine serum, 50 units/ml penicillin, and 50 mg/ml streptomycin. Cultures were maintained in humidified incubator at 28° unless otherwise indicated.

Cell growth curves. A. alb cells were seeded at 2×10^5 cells/35-mm dish in 2 ml of A. alb medium and immediately incubated as indicated. Cell counts from duplicate dishes were determined using a hemocytometer with cells dispersed in 2 ml of PBS (0.05 *M* sodium phosphate buffer, pH 7.4, 0.15 *M* NaCl) after removal of the original medium. The media on all cultures during the course of growth experiments was replaced every 2 days.

Viral replication curves. Monolayers of A. alb cells in 24-well tissue culture plates were rinsed once with PBS and then inoculated with 0.1 ml of diluted dengue-3-infected SMB. After absorption for 2 hr at 20°, the cultures were rinsed twice with PBS, followed by the addition of 1.0 ml of A. alb medium. Cultures were then incubated at either 20° or 28°, as indicated.

At each interval during the experiments, medium was removed from duplicate wells, centrifuged at 2000*g* for 10 min, and the supernatant stored at −70° until assayed for extracellular virus titer (EV). For determination of cell associated virus titer (CAV), the remaining cells in the wells were rinsed three times with PBS, removed by vigorous rinsing with 1.0 ml of A. alb medium, and stored at −70°. Prior to assay, the samples were frozen and thawed rapidly three times in a dry ice–acetone bath, centrifuged at 2000*g* for 10 min, and the supernatant collected.

Indirect immunofluorescence (IF) staining. Antibody to dengue-3 viral antigen for use in IF staining was prepared in mouse hyperimmune ascites fluid (HMAF) as described by Cardiff *et al.* (15), using infected SMB as the immunogen. To reduce nonspecific staining the HMAF was exhaustively absorbed with acetone-fixed, uninfected A. alb cells. For IF staining, cells cultured on either glass coverslips or eight-well tissue culture slides (Lab-Tek, Miles Laboratories, Napierville, Ill) were rinsed three times with PBS, and fixed in cold (−20°) acetone for 15 min. The cells were then rinsed once with PBS, flooded with diluted HMAF, and incubated for 45 min at room temperature. After rinsing three times with PBS, the cells were flooded with fluorescein-conjugated, rabbit anti-mouse IgG (Miles Laboratories, Napierville, Ill.) for 45 min at room temperature. After rinsing three times with PBS the slides were mounted and then examined using a Leitz Ortholux fluorescent microscope with an HBO 200-W mercury lamp, BG-38 heat absorbing, BG-12 exciter, and K-510 suppression filters.

Infectious virus titration. Infectious virus was quantitated in BHK-21 cells by the fluorescent focus assay described by Igarashi (16). Briefly, 0.1 ml of virus sample was absorbed to a monolayer of BHK-21 cells in an eight-well tissue culture slide for 2 hr at 37°. The wells were then washed three times with PBS and 0.3 ml of BHK medium containing 5% fetal bovine serum was added. After 72 hr of incubation, the slides were stained using the IF technique with a 1/40 dilution of HMAF described above and the number of focus-forming units (FFU) counted.

Results. *Effect of temperature upon viral replication curves.* Cultures of A. alb cells were inoculated with dengue-3-infected SMB at a multiplicity of infection (m.o.i.) of approximately 0.01, and incubated at either 20 or 28°. Media on all cultures was changed on Day 7. A peak in EV titer of approximately 7×10^3 FFU/ml was obtained after 5 days at 28° (Fig. 1). After Day 5, virus titers steadily declined until they reached 2×10^3 FFU/ml on Day 8. Further change in titer from Day 14 was negligible.

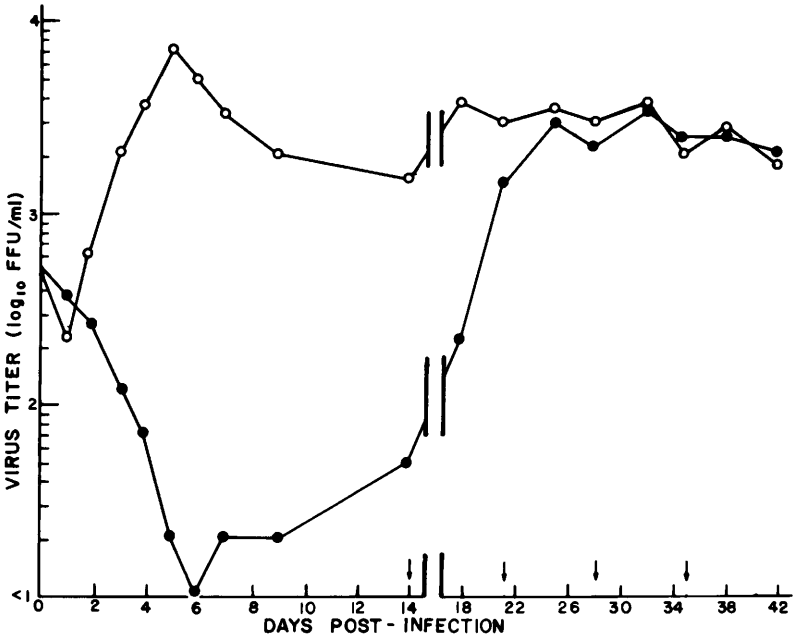

FIG. 1. Comparison of EV titers after infection of A. alb cell cultures with dengue-3 virus at an m.o.i. of approximately 0.01, and incubation at either 20° (●) or 28° (○). Virus titers are mean values from a single experiment. Arrows indicate subculturing of the cells at a passage ratio of 1:4.

In cultures incubated at 20° after inoculation the EV titers steadily declined until virus was no longer detected on Day 6. By Day 14 the EV titer increased to 50 FFU/ml.

Cells at both temperatures were subcultured at a passage ratio of 1:4 on Day 14 and at weekly intervals thereafter, with a medium change 4 days after each passage. Although samples were taken only from confluent cultures, cell counts were performed on parallel cultures to determine the total number of cells per well at each temperature. These cell counts did not differ by more than 10%, which is well within the statistical limits of the hemocytometer counting technique used. In addition, no cytopathic effect was detected during daily observation of the cultures.

From Day 14, the EV titer at 20° steadily increased and reached a peak at approximately 21 to 25 days (Fig. 1). The EV titer maintained at both temperatures for the remainder of the experiment (Days 25–42) were approximately the same. The continued release of infectious EV from the infected cultures, in addition to the absence of detectable cell destruction or cytopathic effect, indicates that persistent infections were established at both 28° and 20°.

When cultures were infected at a m.o.i. of approximately 2.0, peak EV titer (5×10^3 FFU/ml) was observed after 3 days at 28° (Fig. 2). The titers steadily declined thereafter until they reached 1.5×10^3 FFU/ml on Day 10. At Day 10, the EV titers were the same at both temperatures. At this time, on Day 14, and weekly intervals thereafter, the cells were subcultured as previously described. On Day 14, the EV titer at 20° reached a peak of 2.5×10^3 FFU/ml. Again, the EV titers maintained at both temperatures were approximately the same for the rest of the experiment (Days 14–42). In addition, no cell destruction or cytopathic effect was detected at this multiplicity, indicating that persistent infections were established at both 20° and 28°.

CAV titers were also assayed during this experiment to determine if the delay in reaching peak EV titer at 20° was caused by nonrelease of the virus, resulting in an ac-

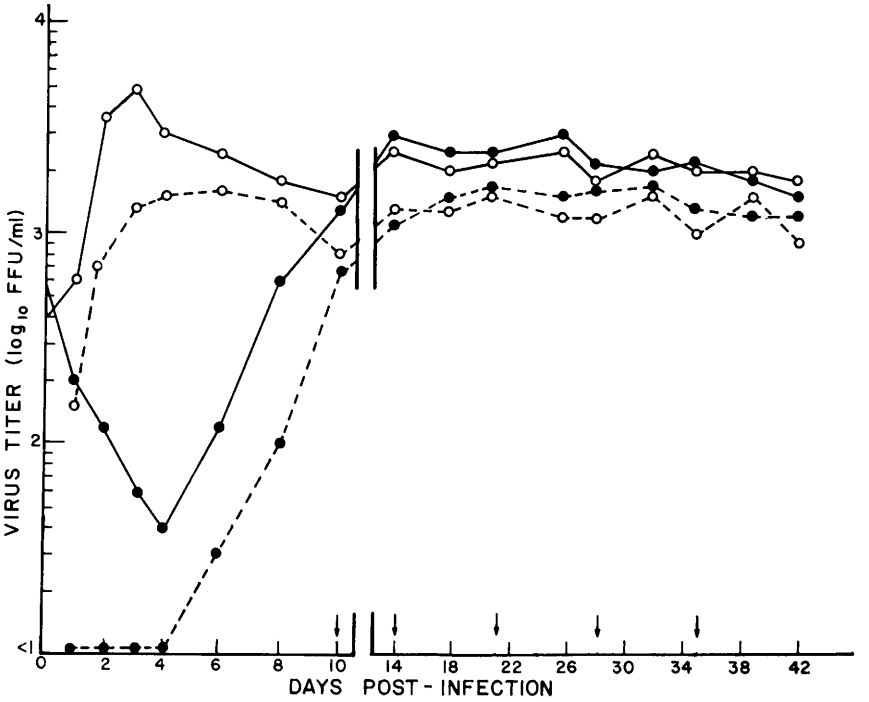

FIG. 2. Comparison of EV (solid lines) and CAV (dotted lines) titers after infection of A. alb cell cultures with dengue-3 virus at an m.o.i. of approximately 2, and incubation at either 20° (●) or 28° (○). Virus titers are mean values from a single experiment. Arrows indicate subculturing of the cells at a passage ratio of 1:4.

cumulation of CAV. The CAV titer at 28° peaked on approximately Day 3. At 20°, CAV first appeared on Day 6 and steadily increased through Day 10. After subculturing, the CAV titer reached a peak on Day 14. CAV titers were approximately the same at both temperatures for the rest of the experiment (Days 14–42). In general, CAV titers were parallel to the EV titers, but the number of infectious CAV units was always lower.

Effect of temperature upon cell-associated viral antigen. Cultures incubated at either 20° or 28° after infection were subcultured weekly, and examined over a 4-week period for immunofluorescent cell-associated viral antigen to determine the fraction of cells containing antigen (Table I).

After infection at a m.o.i. of approximately 2, viral antigen was detected in 20 to 30% of the cells after 2 days at 28°, and 7 days at 20°. An an m.o.i. of 0.01, at 28°, viral antigen was detected in less than 1% of the cells. The fraction of cells containing antigen increased to 20–30% after 3 weeks.

Effect of temperature upon growth rate of cells. Cell counts were performed each time samples were taken during the viral

TABLE I. EFFECT OF m.o.i. AND INCUBATION TEMPERATURE UPON THE PERCENTAGE OF CELLS DEVELOPING IMMUNOFLUORESCENT CELL-ASSOCIATED VIRAL ANTIGEN IN DENGUE-3 INFECTED *A. albopictus*, CELL CULTURES

Days after infection	m.o.i. 2		m.o.i. 0.01	
	20°	28°	20°	28°
1	0	0	0	0
2	0	20–30	0	<1
3	0	20–30	0	5–10
4	0	20–30	0	20–30
7	20–30	20–30	<1	20–30
14	20–30	20–30	<1	20–30
21	20–30	20–30	20–30	20–30
28	20–30	20–30	20–30	20–30

growth curve experiments to ensure that samples were obtained only from cultures of the same cell density at each temperature (i.e., confluent cultures). This was done because subconfluent cultures maintained at each of the incubation temperatures did not demonstrate the same cell growth rates (Fig. 3). The doubling time of uninfected cultures was approximately 48 hr at 20°, and 24 hr at 28° during 1 to 4 days after seeding. Cultures that had been infected for a period of 8 weeks (eight passages) were also tested to compare their cell growth rates with the uninfected cultures at each of the temperatures. It was conceivable that viral infection might disrupt necessary cell metabolic functions, resulting in a decreased rate of growth when compared to uninfected cells. However, the growth rate of infected cultures did not differ significantly from that of the uninfected cells at either temperature. The cell morphology over the course of the experiments and the cell density after 7 days were the same at both temperatures for both infected and uninfected cells. Cell counts of cultures maintained for more than 7 days at 20° did not significantly increase.

Discussion. In this study we have demonstrated that incubation of A. alb cell cultures at a suboptimal temperature (20°) after infection with dengue-3 virus caused a delay in reaching peak infectious EV titer when compared to cultures incubated at 28°. This delay was independent of the infection multiplicity (m.o.i. of 2.0 or 0.01), and was not associated with an accumulation of CAV at 20°. In contrast, after the peak virus titer was reached, the suboptimal incubation temperature did not affect the amount of infectious virus released into the culture medium or the establishment of persistent infection.

A consistent property of most togavirus-infected, cultured mosquito cells at incubation temperatures of 28–30° is an absence of cytopathic effect and the establishment of persistently infected cultures (10, 17–19). Persistently infected cultures have also been reported for Sindbis virus (a group A togavirus) at a suboptimal temperature (15°) (20). For dengue virus, an ab-

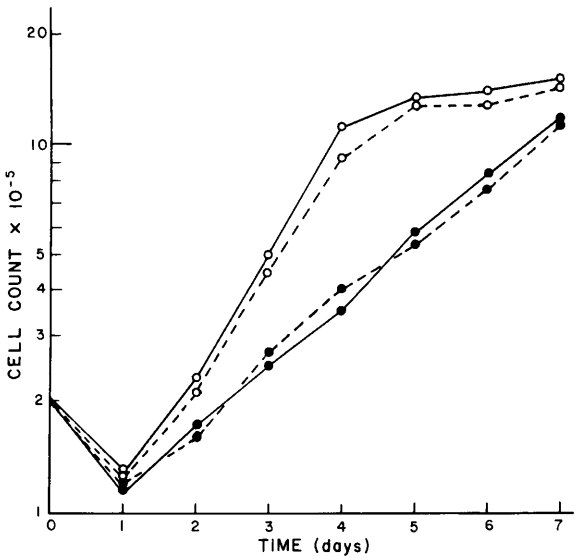

FIG. 3. Comparison of growth rates of A. alb cell cultures. Cultures of 2×10^5 cells/35-mm petri dish of unifected cells (solid lines), and cells persistently infected for 8 weeks with eight passages (dotted lines), were incubated at 20° (●) or 28° (○). Cell counts are an average of the total number of cells per dish of duplicate dishes.

sence of cytopathic effect and persistent infection in cultured mosquito cells are not consistently observed at 28°, since cytopathic effect with cell destruction has been reported (11, 12, 19, 21). In view of these reports, it was conceivable that incubation of dengue-infected cultures at a suboptimal temperature, where viral replication is partially inhibited, could affect the establishment of persistent infection or development of cytopathic effect when compared to cultures at 28°. Our results, however, show that persistent infection occurred with dengue virus at both 20 and 28°, since cultures produced virus for the duration of the experiments and demonstrated persistence of cell-associated viral antigen. In addition, no cytopathic effect was observed, and viral infection did not change the growth rate of cell cultures, indicating no cell destruction or major disruption of essential cellular metabolism.

Another property of togavirus persistently infected cultures is that only a fraction of the cells contain viral antigen (18, 22–25). This fraction can also be variable (22, 24). The effect of suboptimal incubation temperature upon the fraction of cells containing viral antigen, however, has not been reported for any togavirus persistently infected culture. Our study shows that the fraction of cells containing dengue viral antigen in persistently infected cultures is consistent (20–30%), and independent of the incubation temperature (20 or 28°).

The effect of suboptimal incubation temperature upon dengue virus infection in cultured A. alb cells is consistent with the findings of others using togavirus-infected laboratory-reared mosquitoes. Coleman and McLean (4), and McLean *et al.* (5) reported that dengue-infected mosquitoes required an extended incubation period at temperatures below 80°F (27°C), before virus could be detected or reached peak titer. This is consistent with our finding of a delay in reaching peak virus titer at 20° after primary infection of cultured mosquito cells with dengue virus.

In the mosquito, dengue virus infection is lifelong, productive, and without detectable effect upon the insect's health. Incubation of dengue-infected mosquitoes at suboptimal temperature does not affect this outcome (4). We found an analogous infection outcome in mosquito cell cultures where dengue virus infection resulted in the establishment of persistently infected cell lines at both 28° and 20°. In addition, the titers of virus maintained at each incubation temperature after the cultures became persistently infected were the same. This phenomenon demonstrates that after persistent infection is established (peak virus titer is reached) the virus output of infected cultures is independent of the incubation temperature (28° or 20°). Extrapolation of this result in tissue culture to dengue infection of live mosquitoes would indicate that in a persistently infected mosquito, a consistent, transmissible quantity of virus can be maintained at any temperature allowing survival of the mosquito.

In conclusion, our results obtained using dengue-infected A. alb cell cultures suggests that these cultures will be a suitable model for further study of the effect of temperature upon dengue virus replication in the mosquito.

This work was supported by Public Health Service Grant 5R22-AI-13380-03 from the National Institute of Allergy and Infectious Diseases, and by Grant 88M from the Council for Tobacco Research, USA, Inc.

1. Singh KRP, Paul SD. Multiplication of arboviruses in cell lines from *Aedes albopictus* and *Aedes agypti*. Curr Sci 37:65–67, 1968.
2. Varma MGR, Pudney M, Leake CJ. Cell lines from larvae of Aedes (stegomyia) Malayensis and Aedes (s) Pseudoscutellaris (Theobald) and their infection with some arboviruses. Trans R Soc Trop Med Hyg 68:374–382, 1974.
3. Russell PK. Dengue in the Caribbean, 1977. Washington D.C., Pan American Health Organization, Scientific publication No. 375, pp1–9, 1979.
4. Coleman JC, McLean DM. Dengue virus transmission by Aedes agypti mosquitoes following intrathoracic inoculation. Amer J Trop Med Hyg 22:124–129, 1973.
5. McLean DM, Clarke AM, Coleman JC, Montalbetti CA. Skidmore AG, Walters TE, Wise R. Vector capability of *Aedes aegypti* mosquitoes for California encephalitis dengue viruses at various temperatures. Canad J Microbiol 20:255–262, 1974.
6. Nelson CD. The effect of various temperatures in

modifying the extrinsic incubation period of the yellow fever virus in *Aedes agypti*. Amer J Hyg **16**:163–167, 1932.
7. Bates M, Roca-Garcia M. Development of virus of yellow fever in Haemagogus mosquitos. Amer J Trop Med **26**:585–605, 1946.
8. Chamberlain RW, Sudia WD. The effects of temperatures upon the extrinsic incubation of eastern equine encephalitis in mosquitoes. Amer J Hyg **62**:295–305, 1955.
9. McLean DM, Grass PN, Miller MA, Wong KSK. Arbovirus growth in *Aedes aegypti* mosquitoes throughout their viable temperature range. Arch Virol 49:49–57, 1975.
10. Sinarachatanant P, Olson LC. Replication of dengue virus type 2 in *Aedes albopictus* cell culture. J Virol **12**:275–283, 1973.
11. Suitor EC Jr, Paul FL. Syncytia formation of mosquito cell cultures mediated by type 2 dengue virus. Virology **38**:482–483, 1969.
12. Sweet BH, Unthank HD. A comparative study of viral susceptibility of monolayers and suspended mosquito cell lines. Curr Top Microbiol Immunol **55**:150–154, 1971.
13. Smith TJ, Brandt WW, Swanson JL, McCown JM, Buescher EL. Physical and biological properties of dengue-2 virus and associate antigens. J Virol **5**:524–532, 1970.
14. Mitsuhasi J, Maramorosch K. Leafhopper tissue culture; embryonic, hymphol and imaginal tissues from aseptic insects. Contrib Boyce Thompson Inst **22**:435–460, 1964.
15. Cardiff RD, Brandt WE, McCloud TG, Shapiro D, Russell PK. Immunological and biophysical separation of dengue-2 antigens. J Virol **7**:15–23, 1971.
16. Igarashi A, Mantani M. Rapid titration of dengue virus type 4 infectivity by counting fluorescent foci. Biken J. **17**:87–93, 1974.
17. Igarashi A, Koo R, Stollar V. Evolution and properties of *Aedes albopictus* cell cultures persistently infected with Sinbis virus. Virology **82**:69–83, 1977.
18. Singh KRP. Growth of arboviruses in *Aedes albopictus* and *Aedes agypti* cell lines. Curr Top Microbiol Immunol **55**:127–133, 1971.
19. Yunker CE. Arthropod tissue culture in the study of arboviruses and rickettsiae: A review. Curr Top Microbiol Immunol **55**:113–126, 1971.
20. Peleg J, Pecht M. Adaptation of an Aedes agypti mosquito cell line to growth at 15°C and its response to infection by sindbis virus. J Gen Virol **38**:231–239, 1978.
21. Paul SD, Singh KRP, Bhat UKM. A study of the cytopathic effect of arboviruses on cultures from *Aedes albopictus* cell line. Indian J Med Res **57**:339–348, 1969.
22. Davey MW, Dennet DP, Delgarno L. Semliki Forest virus replication in cultured *Aedes albopictus* cells: Studies on the establishment of persistence. J Gen Virol **24**:453–463, 1974.
23. Igarashi A. Isolation of a Sindbis *Aedes albopictus* clone sensitive to dengue and Chikungunya viruses. J Gen Virol **40**:531–544, 1978.
24. Riedel B, Brown DT. Novel antiviral activity found in the media of Sindbis virus persistently infected mosquito (*Aedes albopictus*) cell cultures. J Virol **29**:51–60, 1979.
25. Davey MW, Dennet DP, Delgarno L. The growth of 2 togaviruses in cultured mosquito and vertebrate cells. J Gen Virol **20**:225–232, 1973.

Received September 23, 1981. P.S.E.B.M. 1982, Vol. 170.