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# IN VITRO GROWTH KINETICS AND THERMOSTABILITY OF THE GOLDEN SHINER VIRUS

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*Abstract:* In vitro replication of the golden shiner virus (GSV) occurred at 20 C to 30 C in fathead minnow cells; GSV did not replicate at 15 C or 35 C. Optimal replication temperature was approximately 30 C, where the latent period was 8 hr and viral yield was 113 infectious units per cell. Maximum cytopathic effect coincided with maximum released viral titer. Suitable storage temperatures for GSV were -70 C and 4 C. Viral infectivity was rapidly lost at temperatures above 20 C and at normal freezer temperature (-15 C).

## INTRODUCTION

The golden shiner virus (GSV) was first isolated from *Notemigonus crysoleucas* in the summer of 1977 (Plumb et al., 1979). The virion is reo-like with double stranded RNA, icosahedral symmetry, no envelope and a 70 nm diameter. There are morphological, biochemical and physical characteristics shared with infectious pancreatic necrosis virus (IPNV) (Cohen et al., 1973; Malsberger and Cerini, 1963); however, GSV is serologically distinct (Schwedler and Plumb, 1980).

Fish viruses, unlike viruses of homeotherms replicate at a wide range of temperatures. Variations in pathogenicity have been correlated with temperature for at least two fish viruses. Channel catfish virus (CCV) is less pathogenic at temperatures below 20 C (Plumb, 1973) while infectious hematopoietic necrosis virus is less pathogenic at temperatures above 18 C (Amend, 1970).

The success of in vitro isolation of poikilothermic animal viruses is often dependent on incubation temperature. Therefore, it is imperative to incubate inoculated cell cultures near the optimal temperature for virus replication to insure detection of the suspected virus.

The optimal storage temperatures for a virus must also be known to insure viability during storage. The thermostability of fish viruses can vary even between strains of a specific virus, i.e. IPNV (McMichael et al., 1975; Wolf and Quimby, 1971).

The objectives of these studies were to determine the optimum in vitro replication temperature of GSV in fathead minnow cells (FHM) (Gravell and Malsberger, 1965) and to evaluate its thermostability. Experiments were designed to construct single-step growth curves and correlate the onset of cytopathic effect (CPE) at selected incubation temperatures and determine suitable storage temperatures for GSV.

## MATERIALS AND METHODS

Isolate #AL-77-131 of GSV (8th Passage) from the Southeastern Cooperative Fish Disease Laboratory (SCFDL) was used in these experiments. The virus was propagated in monolayer cultures of FHM cells at 30 C in Eagle's minimum essential medium sup-

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plemented with 10% fetal bovine serum (MEM-10), 100 mg streptomycin; 100 IU penicillin and 50 μg gentamicin/ml. The virus suspension was harvested at 48 hr post-infection and clarified by filtration through a 0.45 μm membrane. Triplicate samples of the viral suspension were titered in microcultures of FHM cells (Gratzek et al., 1973). Three 0.1 ml aliquots of serial 10-fold dilutions of GSV in Hanks' balanced salt solution (HBSS) were placed in separate wells of a microculture plate (Linbro). To each well enough FHM cells were added to provide a 90% confluent monolayer. The cultures were incubated at 30 C for 72 hr fixed with 10% formalin and stained with 1% crystal violet. The cultures were examined for CPE and the titers in tissue culture infectious doses -50% ($TCID_{50}$/ml) were calculated (Reed and Muench, 1938).

Single-step growth curves were constructed for virus release at 35 C, 30 C, 25 C, 20 C and 15 C. FHM cells were removed from 75 $cm^2$ flasks using a 0.25% trypsin solution, and resuspended in MEM-10. Titered virus was added to the cell suspension at an estimated rate of 50-100 $TCID_{50}$ per cell. The virus cell suspension was incubated for 15 min at 25 C in an Erlenmeyer flask with a teflon magnetic bar on an electronic stirrer plate. After absorption, cell numbers were estimated by counting 10 samples of the suspension in a hemacytometer. The virus-infected cell suspension was dispensed into multiwell culture plates at 1.0 ml per 2.0 $cm^2$ well and allowed to settle and attach at appropriate test temperatures. After one hr the cells were firmly attached, forming a 90-100% confluent monolayer. The MEM-10 containing unabsorbed virus was removed and the cell sheet was washed twice with 1 ml volumes of MEM-2 (2% fetal bovine serum). A third 1 ml volume of MEM-2 added to the cultures was allowed to remain on the cells and the cultures were returned to the appropriate incubators. The removed MEM-10 plus the two washings were combined and titrated to determine unadsorbed GSV. The amount of virus absorbed was calculated by subtracting the titer in the unadsorbed fraction from the initial titer.

At 35 C, 30 C and 25 C three infected cultures were assayed at 2 hr intervals up to 12 hr, at 4 hr intervals up to 24 hr and at 12 hr intervals up to 72 hr post infection. At 20 C and 15 C triplicate cultures were assayed at 4 hr intervals up to 24 hr, 8 hr intervals up to 48 hr and 12 hr intervals up to 96 hr post infection. The MEM-2 was removed with a Pasteur pipet from each well and placed in individual centrifuge tubes and centrifuged at 13,500 g for 5 min. The supernatant from each sample was titrated in microcultures of FHM cells and individual titers were calculated and averaged.

At 30 C, in addition to released virus (RV) titer, the cell associated virus (CAV) titer was determined. Pellets from the 30 C samples and cells removed from the well were suspended in HBSS. The HBSS suspension of cells was removed and quick frozen at -70 C. Upon thawing, the cell suspension was sonicated at 105 kHz at 105 acoustical W for 30 s using the microtip of a Model 300 dismembrator (Fisher). The samples were titrated separately in microcultures of FHM cells and individual titers were calculated and averaged.

Prior to collection of the RV samples the individual cultures were examined for CPE. An estimate of the percentage of the cell sheet demonstrating CPE was made.

The latent period for the 30 C single-step growth experiment was determined graphically. Virus yield at 30 C was calculated as the number of progeny virus produced per cell.

The thermostability test was initiated by dividing a virus suspension into 200 aliquots of 1 ml each. Sealed ampules were stored (40 ampules each) at 30 C, 20 C, 4 C, -15 C and -70 C. Initial virus

titer was determined by triplicate titration in FHM cell microcultures. At predetermined time intervals for up to 6 mo, three samples from each temperature were removed and the virus titrated. Frozen samples were thawed quickly in a water bath to 25 C.

## RESULTS

At 35 C, the multiplicity of infection (MOI) was 56 $TCID_{50}$/cell. The cells adsorbed all but 20 $TCID_{50}$/cell, giving an adsorbed multiplicity of infection (MOIa) of 36 $TCID_{50}$/cell. There was no characteristic GSV CPE through 96 hr post-infection, however, a considerable number of rounded cells sloughed from the cell sheet at approximately 18 hr post-infection. The monolayer remained confluent. There was no detectable release of virus through 96 hr (Fig. 1). Control cultures did not demonstrate cell sloughing.

At 30 C and 25 C, the MOI was 56 $TCID_{50}$/cell. All but 11 $TCID_{50}$/cell were adsorbed, giving an MOIa of 45 $TCID_{50}$/cell. At 30 C, CPE first appeared at 10 hr post-infection and CPE progressed to include 100% of the cell sheet by 16 hr post-infection. The RV titer at 30 C began to increase after an 8 hr latent period. Viral release continued logarithmically through 16 hr post-infection and leveled off at $10^{7.0}$ $TCID_{50}$/ml (Fig. 1). The CAV production appeared to be in the logarithmic phase of replication of 2 hr post-infection, therefore, no estimation of the eclipse stage could be made. The titer of the CAV increased logarithmically through 8 hr post-infection where it leveled off at approximately $10^{7.5}$ $TCID_{50}$/ml (Fig. 1). The total virus production per ml was $10^{7.6}$ $TCID_{50}$. The number of cells per well was $10^{5.5}$ thus the resulting viral yield was about 113 progeny virus per cell.

At 25 C, CPE was first observed at 10 hr post-infection. The CPE progressed to include 100% of the cell sheet by 20 hr post-infection. The RV titer began logarithmic increase after 10 hr post-infection and continued through 20 hr post-infection when the titer leveled off at $10^{7.0}$ $TCID_{50}$/ml (Fig. 1).

At 20 C and 15 C, the MOI was 125 $TCID_{50}$/cell, with a MOIa of 92 $TCID_{50}$/cell. At 20 C, CPE was first observed at 40 hr post-infection. The initial increase in RV titer was at approximately 24 hr post-infection. At 20 C, viral release continued at a more gradual rate than at 25 C or 30 C. The titer increased through 48 hr where it leveled off at approximately $10^{7.0}$ $TCID_{50}$/ml. At 15 C, no CPE or increase in RV titer was observed through 96 hr post-infection (Fig. 1).

No appreciable loss in viral infectivity occurred within 6 mo in the ampules stored at 4 C and -70 C. A gradual decline in viral infectivity to zero at 148 days took place in the ampules stored at 20 C. There was a rapid decline in viral infectivity to zero at 7 days in the ampules stored at 30 C, and a complete loss of viral infectivity by day 3 for the ampules stored at -15 C (Fig. 2).

## DISCUSSION

Optimal replication temperature for GSV in FHM cells appears to be around 30 C, with no replication at 35 C and 15 C. At all temperatures where GSV replicated, approximately the same maximum titer ($10^{7.0}$/ml) was obtained. However, the time to attain maximum titer varied with temperature.

Since the number of cells per culture was relatively constant while MOIa and temperature varied considerably, we postulate that a theoretical maximum of progeny virus was produced. The viral yield of 113 $TCID_{50}$/cell appeared to represent maximum GSV production in FHM cells. We infer that while temperature regulates the rate of viral replication, the cell can produce only a finite number of progeny virus. The viral yield of GSV in FHM cells of 113 $TCID_{50}$/cell is over twice the reported 40-

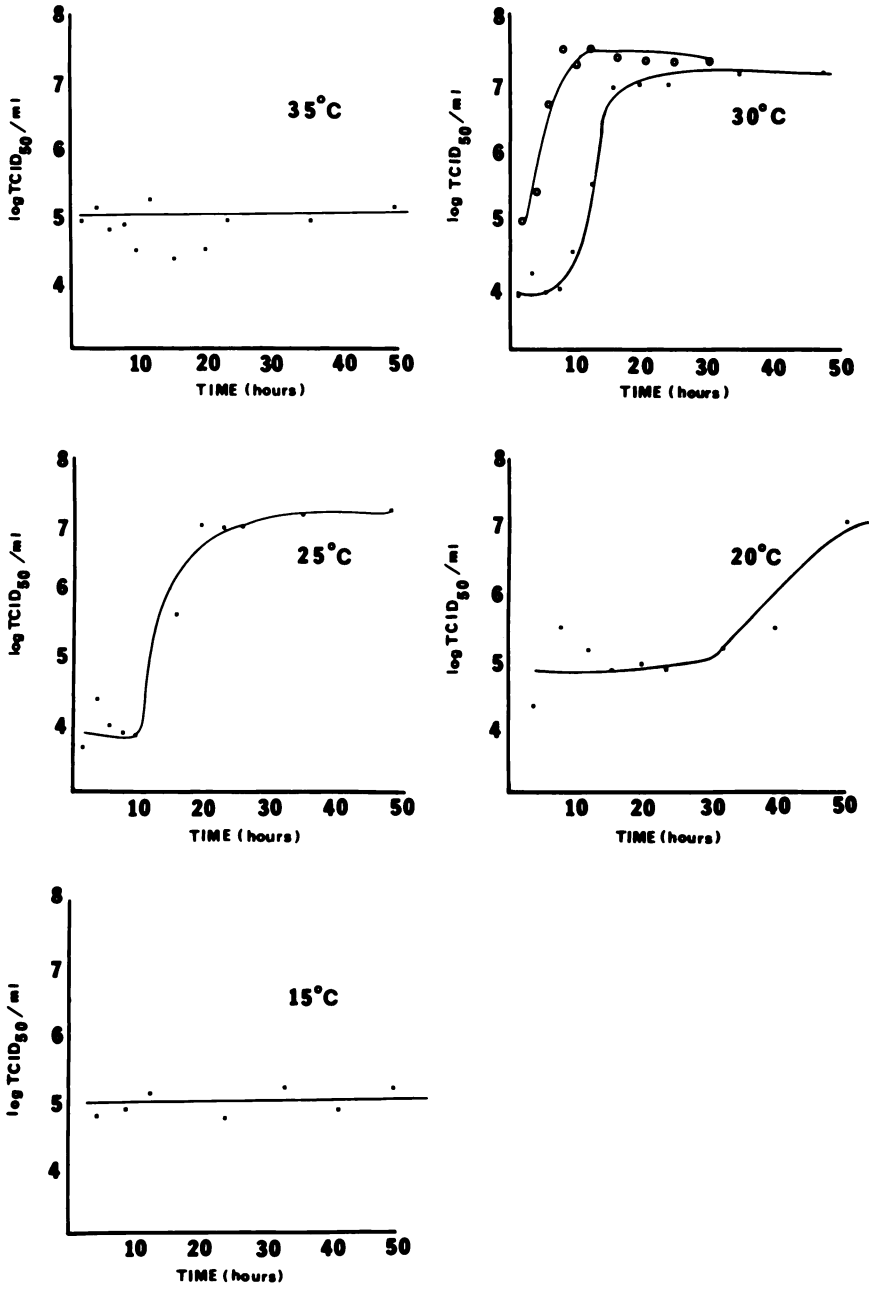


FIGURE 1. Single-step growth curves for golden shiner virus in fathead minnow cells at various temperatures: cell associated (o——o), released virus (•——•).

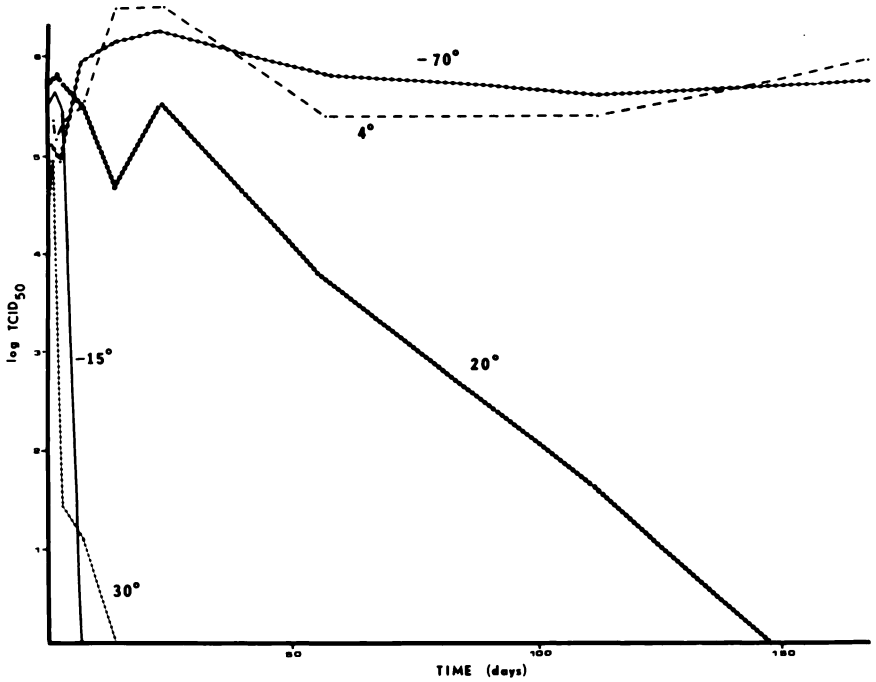


FIGURE 2. The infectivity ($TCID_{50}$/ml) of golden shiner virus after storage in Eagle's minimum essential medium with 10% fetal calf serum at various temperatures.

50 $TCID_{50}$/cell for IPNV in red swordtail (SWT) cells (Kelly and Loh, 1975).

The onset of CPE at 25 C and 30 C corresponded with initial viral release. The complete CPE of the cell sheet at these temperatures corresponded with maximum RV titer. At 20 C CPE was noted at 40 hr post infection, 10 hr after an increase in RV titer was noted. Complete CPE however corresponded with maximum RV titer.

Although the optimal replication temperature for GSV in FHM cells is around 30 C, high titer production of GSV is suggested at 25 C. Maximum titer is only slightly delayed at 25 C and released virus particles are more stable at cooler temperatures.

The in vitro optimum temperature of GSV correlates with the seasonal occurrence of GSV. At the SCFDL all isolates of GSV occurred in the late summer to early fall when water temperatures are usually in excess of 25 C.

Storage at two of the five storage temperatures tested (-70 C and 4 C) were superior in preventing loss of infectivity. GSV appear to be sensitive to warmer temperatures. A rapid loss of infectivity at -15 C was probably due to the slow freezing and thawing cycle similar to a complete loss in viral infectivity in European IPNV isolates in a single thaw cycle (Wolf and Quimby, 1971). Long range storage of GSV in growth medium with minimal loss of infectivity can be achieved at either normal refrigeration or ultrafreezer temperatures.

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