INTRODUCTION

In a previous paper, we have reported with respect to a titration of herpes simplex viruses by the plaque technique. Herpes simplex virus is possibly more or less inactivated by heat even during the course of the procedure for titration because of its thermolabile nature. Until now a number of studies related to the thermal inactivation of herpes simplex virus were reported. According to Wallis and Melnick, herpes simplex virus was stabilized by 1 M Na$_2$HPO$_4$ and Na$_2$SO$_4$ from heating at 50°C. On the other hand, the virus was made thermosensitive in the presence of isotonic salt concentrations at pH 7.2 or above. In distilled water, the virus was thermostable but sensitive in Earle's salt solution. Comparative studies using MS strain of herpes simplex virus with respect to the thermoinactivation of intracellular and extracellular virus were performed by Plummer and Lewis at different temperatures. Extracellular virus harvested from the supernatant culture fluid was more stable than intracellular virus obtained by sonic disintegration of the infected cells.

We have been studied that the biological properties between HF strain, a standard herpes simplex virus, and M strains (in preparation). The latter strain was isolated from a patient with herpes labialis by Maruta. The purpose of this paper was to compare the thermosensitivity between HF and M strains under various conditions.

MATERIALS AND METHODS

Cells Established African grivet monkey kidney (GMK) cells were grown in mixed growth medium of Melnick-Hank's solution and Eagle's minimal essential medium (7:3) supplemented with 10% bovine serum.

Viruses Two strains of HF and M of herpes simplex virus were used. Each strain propagated in GMK cells was maintained with 0.5% skim milk,
0.125% lactoalbumin hydrolysate and 0.225% sodium bicarbonate in Earle's saline (MLE). Infected cells were harvested the next day. The whole culture was frozen and thawed, and sonicated at 20 kc/sec. for three minutes in ice water. The suspended virus was stored at –80°C until used. HF and M strains used in this experiment were the stocks which were serially passed thirteen and eleven times in GMK cells, respectively. The stocked virus titer was an order of 10^7 PFU/ml.

**Heating methods** The tubes containing samples were incubated at 37°C, 45°C or 50°C and were removed at the start and then at different intervals of time and were stocked in an ice water bath. Skim milk, bovine serum, glycerin, dimethyl sulfoxide, MgCl₂ and Na₂SO₄ were dissolved in sterilized PBS (0.01 M phosphate buffer saline, pH 7.0). Distilled water was neutralized by a 0.1 M NaOH solution. The reagents were autoclaved except for bovine serum and dimethyl sulfoxide. Equal volumes of the virus dilution in PBS and the each reagent were mixed and heated in shaking tubes in a water bath.

**Virus assay** The titer of survival viruses was assayed by counting the number of plaques in GMK monolayers. Three to five 2 oz bottles were used for one titration. The cell monolayers were washed twice by sterilized PBS and inoculated with 0.2 ml. of heated virus suspension. The detailed methods used in this study have been fully described in the previous paper. The results reported were those obtained by carrying out repeatedly at least two times. Essentially the same results were obtained.

**RESULTS**

**Characterization of HF and M strains**

The biological properties were different in the two strains. HF strain virus formed hemorrhage pocks on the chorioallantoic membrane, but the M strain caused a formation of thicked transparent pocks with a white spot in the center. The pock size of HF and M strains was almost same. The feature of cytopathogenicity of HF strain was the formation of polynucleated giant cells, yet M strain was the formation of rounded and clumped cells as previously described. The plaque size of HF and M strains was about 1.0 to 4.5 mm. and 0.5 to 1.5 mm. on the fourth day, respectively. The burst size of HF strain was larger than M strain in various established cell lines. More detailed reports will be appeared in the following papers.

**Thermostability of virus dilution by MLE at different temperatures**

The stock viruses of HF and M strains were diluted 10-fold in MLE, and heated at 50°C for 2.5, 5 and 10 minutes. The results are shown in Fig. 1 by semilogarithmic form. The thermoinactivation curves are practically straight lines in a semilogarithmic plot. The titers of HF and M strains decrease to about 1/100 for 3 and 6 minutes, respectively. Fig. 1 shows that HF strain was thermoinactivated more rapidly than M strain.
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Fig. 1. Thermal inactivation of HF and M strains in MLE at 50°C.

Fig. 2. Thermal inactivation of HF and M strains at 45°C. The viruses were suspended in MLE.

Fig. 3. Thermal inactivation of HF and M strains suspended in MLE at 37°C.
A lag phase or plateau was not observed in every present experiment. The inactivation at 50°C was extremely quick, therefore the following experiments were performed at 45°C.

Fig. 2 shows the thermosensitivity of two herpes virus strains in MLE at 45°C. The surviving virus titers of HF and M strains decrease to 1/10 for about 20 and 40 minutes, respectively. The resistance of M strain to heat was greater than HF strain.

The thermonactivation curves of HF and M strains at 37°C in MLE are shown in Fig. 3. It took more than 6 hours for the decrease of survivors to 1/10.

The following experiments were performed to compare the thermal inactivation of herpes simplex virus in different dilutions. Two strains of viruses were diluted with Puck's salt solution, MLE and 1% skim milk in Earle's medium and heated at 45°C for suitable periods. The results on HF strain were shown in Fig. 4a and M strain in Fig. 4b. In both strains, the virus preservation was most excellent in MLE and 1% skim milk in Earle's medium was better than Puck's salt solution. Puck's salt solution was much inferior among the three medium in both strains. The decrement period of surviving virus titer to 1/10 in MLE medium was about three times as long as that in Puck's salt solution. In these diluents, HF strain was more thermosensitive than M strain.

![Fig. 4a](image-url). Thermal inactivation of herpes simplex virus at 45°C. Fig. 4a: HF strain. Fig. 4b: M strain. The viruses were suspended in MLE (1), 1% skim milk in Earle's saline (2) and Puck's saline (3).
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It was considered that virus preservation was affected by pH of virus diluent. Therefore, the effects of pH were studied using 0.01 M phosphate buffered saline. PBS was adjusted to desired pH by changing the ratio of Na₂HPO₄ and KH₂PO₄. Fig. 5a and 5b show the results of the thermostabilization at 45°C. HF strain shows excellent preservation at pH 7.0 - 7.2 and M strain shows superior thermostability at pH 6.6 - 7.2. M strain had more comprehensive range of the thermal stabilization than HF strain with regard to optimal pH. M strain was considerably resistant to heat as compared with HF strain at each pH point.

Fig. 5 a. 
Fig. 5 b.
The thermal inactivation of HF (Fig. 5 a) and M (Fig. 5 b) strains at different pH. The viruses were suspended in PBS and heated at 45°C for the time indicated.

The thermostabilizing effects of the several reagents on HF and M strains

Hitherto, many investigations in respect to herpes virus preservation have been reported. Proteins, for example, serum, skim milk and egg yolk or amino acids or glycerin have a stabilizing effect on the various virus preservation. However, Wallis and Melnick \(^{11}\) showed that the proteins counteract in part thermosensitizing effects of the salts contained in the virus suspension. On the other hand, herpes virus was stabilized by 1 M Na₂SO₄, but was inactivated by isotonic salt concentrations or Earle’s salt solution. In contrast, purified herpes virus was thermostable when suspended in distilled water. Wallis and Melnick \(^{10}\), Yamamoto and Shingu \(^{12}\) reported that enteroviruses were protected by 1 M MgCl₂. It is recognized that glycerin and dimethyl sulfoxide (DMSO) of 10 % protect the cell cultures from freezing and thawing. In addition, DMSO protects the virus possessing envelope from those treatment.
Each sample of the control was reserved in an ice water bath. Experimental samples were heated at 45°C for 10 minutes in the water bath. At the end of the heating period, the samples were transferred into an ice water bath. Preservations of two herpes virus strains to these reagents are shown in Table 1. The numbers represent the percentage of survival viruses of experimental groups in each control group. Experiments were carried out twice with similar results. For preservation of herpes virus, 1% skim milk and 10% bovine serum were especially excellent. If the heating periods were prolonged, these strains survived for long periods in 1% skim milk and 10% bovine serum solutions. HF and M strains were preserved pretty well in 10% glycerin. When the viruses were suspended in distilled water and PBS, HF strain had little resistance to heating. 10% DMSO and 1 M Na₂SO₄ was much inferior to the above mentioned reagents in the thermostabilizing effects. Survival virus of HF strain was not observed by the plaque method, but 19 and 18% of M strain virus survived in 10% DMSO and 1 M Na₂SO₄ solution. The protections of HF and M strains were most inferior in 1 M MgCl₂ solution and survivors were not already found. The effects of the virus protection to those medium were usually superior in M strain. It is suggested that HF strain in various reagents and conditions is more thermolabile than M strain.

### TABLE 1

<table>
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<th>reagents</th>
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<tr>
<td>PBS</td>
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<td>skim milk (1%)</td>
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<td>bovine serum (10%)</td>
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<td>glycerin (10%)</td>
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<td>0%</td>
</tr>
<tr>
<td>Na₂SO₄ (1 M)</td>
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<td>18%</td>
</tr>
<tr>
<td>dist. water</td>
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### DISCUSSION

The thermosensitivity of HF and M strains of herpes simplex virus was compared under various conditions. The results obtained were in agreement or disagreement with other author’s results.

Hoggan and Roizman showed that the thermal inactivation curves of herpes simplex virus had a lag phase or plateau varying in duration depending on the temperature. In the present experiments the thermoinactivation curves
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were straight lines in both strains at 37°C, 45°C and 50°C. It was considered that the existence of a lag phase in the thermoinactivation curve was due to the difference in virus strains or suspending medium.

Scott, McLeod and Tokumaru \(^3\) studied about GP and P strains of herpes simplex virus. GP strain causes the formation of syncytial giant cells and P strain produces rounding and piling up of the cells with a few small giant cells. The infective titers of GP and P strains in the tissue culture medium reduced by half for 1.5 hours at 37°C. The GP and P strains were similiarly inactivated by heat. However, the half life times of HF and M strains at 37°C in our experiments were about 120 and 140 minutes, respectively. Kaplan \(^3\) reported that the survivors of herpes simplex virus were less than 1% at 37°C for 24 hours. Plummer and Lewis \(^3\) showed that 1/10 decrement of the titer of extracellular and intracellular viruses was about 16 and 8 hours at 36°C, respectively. Katori \(^4\) reported that the thermosensitivity of herpes simplex virus was changed by the different host passages. According to him, HF strain was passaged in rabbit kidney cell, rabbit brain, mouse brain and chorioallantoic membrane of a chick embryo. The titer of the virus except the passage of the virus in mouse brain reduced to about 1/10 at 37°C for 1.5 hours. The survivors of the passaged virus in the mouse brain was less than 1/1,000 at 37°C for 30 minutes. Each of the different passaged viruses was inactivated by heat at different rates. However, it was considered that the virus suspending medium was different in the content of virus stabilizing agents such as proteins.

The decrement of the virus titer was about 1/10 in MLE medium at 37°C for 7 hours in the present experiment. In the thermoinactivation at 50°C, the titers of HF and M strains decrease to about 1/100 for 3 and 6 minutes, respectively. Plummer and Lewis \(^5\) showed that MS strain of herpes simplex virus was inactivated to 1/100 for about 10 minutes at 50°C. Schneweis \(^7\) showed a loss of infectivity of 5 to 6 log. for type 2 but only 0.5 to 1.5 log. for type 1 strain of herpes simplex virus, when the viruses were held at 4°C for 100 days.

A distinct difference was shown in the thermal stability of the HF and M strains of herpes simplex virus under the present experimental conditions. Therefore, it was cleared that the thermosensitivity of herpes simplex virus was different among the strains. The thermostability at 37°C, 45°C and 50°C of HF strain was greater than that of M strain. The range of the thermostability of HF and M strains at different pH of PBS was pH 7.0 - 7.2 and 6.6 - 7.2, respectively. The decrement of the titer of M strain in PBS and MLE medium was about 34% and 27%, respectively at 45°C for 20 minutes. The titer of HF strain suspended in PBS and MLE medium decreased to about 22% and 9% for 20 minutes at 45°C, respectively. The range of optimal pH for the thermostability of M strain was more comprehensive and the rate of the thermal inactivation was slower than HF strain. It was in agreement with the results of Farnham and Newton \(^1\). They showed that the thermal inactivation of HFEM strain of herpes simplex virus was promoted when the pH was off from
the range of pH 6.8 to 7.4. They reported that herpes virus is more stable at pH 6.8 than at 7.4 at 37°C, and most stable at pH 7.0. The skim milk, the bovine serum and the yolk of an egg etc. were known as the stabilizing agents of the viruses in general. The bovine serum and the skim milk showed a good preservation for herpes simplex virus at 45°C for 10 minutes. HF and M strains were pretty well preserved by 10% glycerin. In contrast to the studies of Wallis and Melnick¹¹), the virus preservation in 1 M Na₂SO₄ and distilled water was inferior as compared to PBS, MLE and Puck's solution. These differences of the thermal inactivation in HF and M strains may be due to the difference in herpes virus strains. It is recognized that the viruses with the envelope like herpes viruses are protected by DMSO. However the preservation in 10% DMSO of HF and M strains at 45°C was fairly poor. The effect of the protection by 1 M MgCl₂ at 45°C for 10 minutes was most inferior and no virus remained infectious. The thermostability of M strain against experimented agents was much better than HF strain. It was concluded that the thermal resistance for HF and M strains of herpes simplex virus was dependent upon the composition of the virus suspending medium at certain temperatures.

SUMMARY

A comparison was made for HF and M strains of herpes simplex virus for their thermosensitivity in various conditions. The infectivity of herpes viruses were preserved well by 10% bovine serum and 1% skim milk in PBS at 45°C for 10 minutes. The titers of HF and M strains showed pretty well preservations by PBS, MLE and 10% glycerin in PBS. The thermostabilizing effects of 10% DMSO, 1 M MgCl₂, 1 M Na₂SO₄ in PBS and distilled water were fairly inferior. The thermostability of M strain under the various conditions was greater than HF strain. The range of optimal pH for high thermostability was more comprehensive in M strain as compared to HF strain.

REFERENCES

6) PLUMMER, G. and LEWIS, B.: Thermoinactivation of herpes simplex virus and cyto-
Thermoinactivation of Herpes Virus


