Thermostabilization and Thermosensitization of Herpesvirus

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ABSTRACT

WALLIS, CRAIG (Baylor University College of Medicine, Houston, Tex.), AND JOSEPH L. MELNICK. Thermostabilization and thermosensitization of herpesvirus. *J. Bacteriol.* 90:1632-1637. 1965.—Herpesvirus, long considered as one of the most thermolabile of viruses, was stabilized by 1 M Na_2SO_4 or Na_2HPO_4 so that it withstood heating at 50 C, but the virus was not protected by 1 M MgCl_2 , MgSO_4 , or KH_2PO_4 , or 2 M KCl or NaCl; 1 M Na_2SO_4 also stabilized herpesvirus at 25 and 37 C. In contrast, herpesvirus was made extremely thermosensitive in the presence of isotonic salt concentrations or of isotonic tris(hydroxymethyl)aminomethane buffer, especially at pH 7.2 or above. Partially purified virus was relatively thermostable when suspended in distilled water at pH 7.2, but in Earle's salt solution the virus immediately became thermosensitive. As found in tissue culture harvests, herpesvirus was thermolabile, but the virus was rendered stable at 50 C by simple dilution in distilled water. Protection by proteins or amino acids, generally accepted as virus-stabilizing agents, did not seem to be the result of a direct effect upon herpesvirus. The present data suggest that the added proteins counteract in part thermosensitizing effects of the salts contained in the virus harvest.

Previous studies have shown that high concentrations of salts affect viruses in different ways. For example, enteroviruses are stabilized to thermal inactivation at 50 C by MgCl_2 but not by MgSO_4 (Wallis and Melnick, 1962; Wallis, Melnick, and Rapp, 1965), and myxoviruses are stabilized by MgSO_4 but not by MgCl_2 (Rapp, Butel, and Wallis, 1965; Wallis et al., 1965). Since herpesvirus has long been considered to be one of the most labile viruses, we have studied the problems associated with its thermostabilization.

This report is concerned with studies of the conditions under which salts either stabilize or labilize herpesvirus. In the course of the investigation, factors were discovered which render the virus even more thermosensitive than usual.

MATERIALS AND METHODS

Rabbit kidney (RK) cells. Kidneys from immature New Zealand rabbits were trypsinized and grown in 1-oz (30 ml) prescription bottles with 10% calf serum and Eagle's medium.

Herpesvirus. The JES strain propagated in RK cells was maintained with 5% calf serum in Eagle's medium. The stock used had a titer of 10^8 plaque-forming units (PFU)/ml.

Crude virus harvest. A single-cycle yield of herpesvirus, grown in RK cells infected with a multiplicity of 10 PFU/cell, was harvested 12 hr

later. Cultures were frozen and thawed, and the harvest was clarified by centrifugation at 2,000 rev/min for 30 min. The supernatant fluid was dispensed in ampoules that were stored at -90 C.

Partially purified virus. The crude harvest was centrifuged at $18,000 \times g$ for 1 hr. The pellet was suspended to its original volume in distilled water, and the virus suspension was centrifuged twice more at $18,000 \times g$; each time, the pellet was resuspended to its original volume in distilled water. Such preparations will be referred to as "partially purified" virus. They were always used on the day of preparation.

Virus assay. Herpesvirus was assayed by determining the number of PFU in RK monolayers. The overlay medium consisted of Eagle's medium with 5% calf serum, 0.4% NaHCO_3 , 1:40,000 neutral red, and 1.5% agar (Difco).

Inorganic salts. All salts were dissolved in distilled water and were sterilized by boiling. Stocks were neutralized by HCl or NaOH, so that the final pH of all samples after virus dilution was at 7.2 unless otherwise indicated.

Heating methods. Herpesvirus was diluted 10-fold in distilled water or salt solutions as indicated. Samples were brought to pH 7.2 with HCl, NaOH, or NaHCO_3 , and then representative specimens were heated at 50 C. Specimens to be heated were delivered to the bottom of tubes, and the top of each tube was flamed thoroughly to sterilize at least 2.5 cm of the uppermost surface of the tube. All test tubes used were uniform in

TABLE 1. *Effects of inorganic salts on stability of herpesvirus*

Herpesvirus diluent*	Log ₁₀ decrease in titer (PFU/ml) after heating at 50 C for 15 min
Distilled water.....	2.0
MgCl ₂ , 1 M.....	>6.0
MgSO ₄ , 1 M.....	3.0
NaCl, 2 M.....	>6.0
Na ₂ HPO ₄ , 1 M.....	0.2
Na ₂ SO ₄ , 1 M.....	0.1
KH ₂ PO ₄ , 1 M.....	>6.0
KCl, 2 M.....	>6.0

* The virus was diluted 10-fold in diluents indicated, and samples were heated at 50 C. All samples were adjusted to pH 7.2 before heating.

size and thickness, measuring 13 by 100 mm, with an 0.8-mm wall. The tube was then rubber-stoppered and plunged into a water bath, at a depth sufficient to cover the tube to within 6 mm of the stopper. At the end of the heating period, the tubes were quickly transferred to an ice-water bath.

RESULTS

Effects of salts on the stability of herpesvirus at 50 C. The crude virus stock was diluted 10-fold in distilled water and in 1 or 2 M concentrations of the salts indicated in Table 1. Representative samples were then heated at 50 C for 15 min. All samples heated in Cl⁻ or K⁺ (MgCl₂, NaCl, KH₂PO₄, KCl) were inactivated to a greater degree than were samples heated in distilled water (Table 1). MgSO₄ did not stabilize the virus, nor enhance its inactivation; Na₂SO₄ spared the virus at 50 C. Similarly, Na₂HPO₄ stabilized herpesvirus at 50 C. These two salts were then studied further.

The following experiment was performed to determine the effect of heating for longer periods. The crude virus stock was diluted 10-fold in distilled water, in 1 M Na₂HPO₄, and in 1 M Na₂SO₄. Representative samples were heated at 50 C for 15 to 120 min. Herpesvirus heated at 50 C in distilled water was inactivated markedly after 15 min, and was completely destroyed after 60 min (Fig. 1). In Na₂HPO₄, the virus was significantly stabilized, with only an 0.5 log₁₀ decrease in titer after 15 min, 0.7 to 1.2 decrease at 30 min, 1.7 to 2.2 at 60 min, and 3.6 to 4.0 at 120 min. Herpesvirus was stabilized to a greater degree in Na₂SO₄. Threefold greater stabilization occurred in Na₂SO₄ at 15 min as compared to Na₂HPO₄; 10-fold greater at 30 and 60 min; and almost 100-fold greater at 120 min.

Effects of salt concentration. The crude virus

FIG. 1. *Stabilization of herpesvirus by 1 M Na₂SO₄ and Na₂HPO₄. Herpesvirus was diluted 10-fold in distilled water, Na₂SO₄, and Na₂HPO₄, and samples were heated at 50 C for the times indicated.*

stock was diluted 10-fold in distilled water and in different concentrations of salts, and representative samples were heated at 50 C for 15 min. Biphasic curves were obtained with both of these salts (Fig. 2). In 1 M Na₂HPO₄, virtually no loss in infectivity occurred, but at 0.1 M enhanced inactivation occurred, with a decrease in titer of 6 log₁₀. This may be compared to a loss of only 3 log₁₀ when the virus was heated in distilled water. As the molarity of Na₂HPO₄ was further decreased, the degree of inactivation approached that of the samples heated in water. A similar biphasic curve was obtained in Na₂SO₄, but the break at 0.1 M was not as sharp.

Stabilizing effects of Na₂HPO₄ and Na₂SO₄ on herpesvirus at other temperatures. The crude virus stock was diluted 10-fold in distilled water, in 1 M Na₂HPO₄, and in 1 M Na₂SO₄, and representative samples were held at 25 and at 37 C for as long as 1 month. Control samples were held at -90 C.

Na₂SO₄ proved to be a stabilizing agent at both 25 and 37 C. After 30 days at 25 C, only 1 log₁₀ decrease in titer occurred in Na₂SO₄, 2.0 log₁₀ in distilled water, and >6.0 log₁₀ in Na₂HPO₄ (Table 2). At 37 C, a similar pattern was evident, with Na₂SO₄ stabilizing the virus to a greater degree

FIG. 2. Effects of different concentrations of Na_2SO_4 and Na_2HPO_4 on herpesvirus at 50 C. The virus was diluted in distilled water, or in various salt concentrations, and was then heated.

TABLE 2. Effects of Na_2HPO_4 and Na_2SO_4 on herpesvirus at 25 and 37 C*

Temp	Time	Log ₁₀ decrease in titer (PFU/ml)		
		Distilled water	1 M Na_2HPO_4	1 M Na_2SO_4
C	days			
	25			
	5	0.5	0.0	0.0
	15	1.3	2.5	0.6
	30	2.0	>6.0	1.0
37	1	0.5	0.3	0.0
	4	3.0	>6.0	2.3
	6	>6.0	>6.0	3.1

* Herpesvirus was diluted 10-fold in diluents indicated, and samples were held at 25 or 37 C for up to 30 days.

than distilled water, and with Na_2HPO_4 causing a greater loss of virus than occurred in the controls in distilled water.

Effects of tissue culture constituents on the thermostability of herpesvirus. The fact that Na_2SO_4 and Na_2HPO_4 at low concentrations could thermosensitize herpesvirus suggested that the salts present in the tissue culture harvest might be responsible for the thermosensitivity of the virus. To test this possibility, the salt concentration of the crude virus stock was reduced by simple dilution, and the virus was tested for thermoresistance. Partially purified salt-free virus was also included as a control.

The undiluted, crude virus sample heated at

FIG. 3. Effect of dilution on the thermostability of herpesvirus. Herpesvirus in the form of crude harvest or in the form of purified virus was diluted in distilled water, and samples were heated at 50 C.

50 C for 15 min decreased in titer 2.4 log₁₀ (Fig. 3). The sample at 10⁻¹ dilution lost only 1.6 log₁₀ and at 10⁻² to 10⁻⁴, only 0.9 log₁₀. The partially purified stock was decreased in titer to the same degree (0.8 to 1.0 log₁₀), regardless of the dilution. These results indicated that a 100-fold dilution of the materials present in tissue culture harvests was sufficient to reduce the thermosensitizing factor to an ineffective concentration.

Effects of Earle's salt solution (ESS) and its components on viral thermosensitivity. In an attempt to identify the thermosensitizing factor in the virus harvest, the effects of ESS were studied. Partially purified herpesvirus was diluted 10-fold, to contain about 10⁷ PFU/ml in distilled water, in ESS, and in the components of ESS. The pH of each sample was adjusted to 7.2 with very small amounts of NaHCO_3 , and samples were heated at 50 C for 15 min.

The sample heated in distilled water was decreased in titer 0.9 log₁₀, but that heated in ESS was decreased >5.0 log₁₀ (Table 3). Samples heated in certain components of ESS (KCl, MgCl_2 , MgSO_4 , and glucose) were inactivated to about the same degree as the corresponding sample in distilled water. However, samples in NaCl, CaCl_2 , and NaH_2PO_4 were thermosensitized, and were inactivated to a much greater extent than were samples heated in distilled water.

Effect of pH. On a number of occasions, it was

TABLE 3. *Effects of Earle's salt solution and components thereof on the thermosensitivity of herpesvirus*

Herpesvirus diluent*	Log ₁₀ decrease in titer (PFU/ml) after 15 min at 50 C
Distilled water.....	0.9
Earle's salt solution (ESS).....	>5.0
<i>Components of ESS</i>	
NaCl, 0.116 M.....	>5.0
KCl, 0.0054 M.....	1.0
CaCl ₂ , 0.0018 M.....	3.5
MgCl ₂ , 0.001 M.....	1.2
MgSO ₄ , 0.001 M.....	1.4
NaH ₂ PO ₄ , 0.0011 M.....	3.2
Glucose, 0.0055 M.....	0.8

* Purified herpesvirus was diluted 10-fold in the diluents indicated and heated at 50 C. The pH of all samples was 7.2 at time of heating.

observed that herpesvirus derived from late harvests (multiple-cycle yields) was more thermostable than early harvests (single-cycle yields). The pH of early harvests was 7.2 to 7.4, whereas late harvests were at pH 6.8 to 7.0 (because of the accumulation of metabolic acids). When early and late harvests were adjusted to the same pH (7.2) and heated, both harvests were similar in viral thermosensitivity. This led us to suspect that the thermosensitizing effects of ESS, described above, might be pH-dependent. This possibility was examined as follows. Crude virus stock was diluted 10-fold in NaHCO₃-free ESS. NaHCO₃ was added to bring the pH to 6.8, and the sample was then divided into two parts. One part was immediately stoppered to retain pH 6.8; the second part was allowed to aerate until pH 7.2 was attained, and then was stoppered. Samples were then heated at 50 C. Herpesvirus heated in ESS at pH 6.8 lost only 1.2 log₁₀ titer, as compared to >5.0 log₁₀ decrease in titer of the corresponding sample heated in ESS at pH 7.2.

Repeated tests showed that by adjusting herpesvirus in ESS to pH 6.5 to 6.9, the virus was rendered thermoresistant. Below pH 6.5 or at pH 7.0 to 8.0, herpesvirus was rendered thermosensitive. At pH 6.5 to 6.9, the average decrease in titer was 1.4 log₁₀ at 50 C. At pH 7.0 to 7.1, variations of 1 to 3 log₁₀ occurred. However, at pH 7.2, the virus was consistently rendered thermosensitive, with >5.0 log₁₀ decrease in titer. At higher levels (pH 7.3 to 8.0), the virus also decreased in titer >5.0 log₁₀ upon heating.

Effects of organic medium on the stability of herpesvirus. Experiments were performed to determine the effects of Eagle's maintenance medium and the organic components of the medium

TABLE 4. *Effects of organic ingredients on the stability of purified herpesvirus*

Virus diluent*	Log ₁₀ decrease in titer (PFU/ml) after heating at 50 C for 15 min
Distilled water.....	1.0
Earle's salt solution (ESS).....	>5.0
Eagle's tissue culture medium (including 5% calf serum, amino acids, vitamins, ESS, and RK cell extract).....	2.7
<i>Components of Eagle's medium</i>	
Calf serum (5%) in distilled water..	1.1
Calf serum (5%) in ESS.....	3.0
Amino acids in distilled water....	1.3
Amino acids in ESS.....	2.8
Vitamins in distilled water.....	1.0
Vitamins in ESS.....	>5.0
RK cell extract in distilled water...	1.4
RK cell extract in ESS.....	>5.0

* Purified herpesvirus containing 10^{7.7} PFU/ml was diluted 10-fold as indicated and heated to 50 C.

on the stabilization of the virus. Partially purified herpesvirus was freshly prepared in distilled water, to give a preparation having a titer of 10^{7.7} PFU/ml. This stock was diluted 10-fold in distilled water, in Eagle's complete tissue culture medium, and in components thereof. Samples were heated at 50 C for 15 min.

Herpesvirus suspended in amino acid or protein solution was not more stable than virus in water alone (Table 4). Neither the vitamin solution nor an extract obtained from uninoculated RK cells was effective in stabilizing the virus. Not only were the organic materials ineffective by themselves, but they were also unable to reverse the thermosensitizing effect of ESS. However, 5% calf serum and the amino acids had some effect, although slight, in preventing the thermosensitization of herpesvirus by ESS. These data emphasize that proteins and amino acids do not directly effect the stabilization of herpesvirus, but counteract in part the deleterious action of salts.

Since the amino acids could be tested individually, the following experiment was performed to determine which of the amino acids in Eagle's medium were responsible for the effects described above. The partially purified virus was diluted 10-fold in 0.15 M NaCl to thermosensitize the virus, and then was diluted 10-fold further in the different amino acids of Eagle's medium, each at the concentration recommended (Eagle, 1959). Samples were then heated at 50 C for 15 min. All the amino acids, with the exception of

TABLE 5. *Effects of Tris buffer on thermosensitivity of herpesvirus**

Virus sample	Log ₁₀ decrease in titer after heating for 15 min at 50 C
Extracellular virus in Eagle's medium with 5% calf serum.....	2.4
<i>Intracellular virus in</i>	
Eagle's medium with 5% calf serum.....	2.5
Distilled water.....	0.8
Tris buffer.....	>5.0

* Intracellular herpesvirus in packed RK cells was released into the diluents indicated, by freezing and thawing.

arginine and lysine, were capable of counteracting the thermosensitizing effects of NaCl. The mitigation of the thermosensitivity can be seen in the following figures. Virus heated in distilled water lost 0.9 log PFU/ml; in 0.15 M NaCl, >5.0 log PFU/ml; but, in the individual amino acids, only 2.4 to 3.3 log PFU/ml.

Effects of tris(hydroxymethyl)aminomethane (Tris) buffer on thermosensitivity of herpesvirus. Plummer and Lewis (1965) reported that extracellular herpesvirus was more stable than intracellular virus. However, the intracellular virus was released into isotonic Tris-buffered saline, and from our present work it appears that they may well have thermosensitized their virus with the salts present in Tris buffer. To elucidate this problem, the following experiment was performed. Cultures of RK cells were inoculated with herpesvirus, with an input of 5 PFU/cell; after adsorption for 2 hr, the cells were washed five times with Eagle's medium, and then Eagle's medium containing 5% calf serum was added to the cultures. After an incubation period of 18 hr at 35 C, the fluid phase was obtained and centrifuged, and the supernatant fluid was frozen. The infected monolayer cultures were washed gently five times with physiological saline, and the cells were removed from the glass with a rubber policeman. After low-speed centrifugation, the packed cells were suspended in Eagle's medium, in distilled water, and in Tris-buffered saline. The intracellular virus suspensions were frozen and thawed, and clarified by centrifugation. Each virus sample was diluted 10-fold in the same solution in which the cells had been suspended, and all were tested for thermostability.

Intracellular and extracellular virus, when contained in Eagle's medium, had the same thermosensitivity (Table 5). However, intracellular

virus showed greater inactivation in Tris buffer than in Eagle's medium. In distilled water, the intracellular virus proved to be even more stable than the extracellular virus harvest in Eagle's medium. The intracellular virus in distilled water was similar in thermoresistance to partially purified virus (Table 4).

DISCUSSION

Herpesvirus has generally been regarded as an exceedingly thermolabile virus, but by manipulating its environment we have learned how it can be stabilized, even at 50 C. This was readily accomplished by the use of 1 M Na₂HPO₄ and Na₂SO₄. However, as the concentration of salts was decreased to isotonicity, the inactivation rate became greater than in samples heated in distilled water. As the salt concentration was further decreased, the inactivation rates decreased and approached those of samples heated in distilled water. Nakamura and Ueno (1964) also showed biphasic inactivation curves with Japanese encephalitis virus in MgSO₄ solutions. This virus is also stable in distilled water. The enhanced inactivation of herpesviruses in the presence of isotonic salt concentration was found to be due to a direct effect of the salts upon the virus.

Once removed from the isotonicity of the tissue culture medium in which it was grown, herpesvirus turned out to be a relatively stable virus. However, the addition of isotonic concentrations of salts such as those contained in ESS, Eagle's medium, or Tris buffer (pH 7.2) rendered the virus thermosensitive. At pH 6.8, little thermosensitization was evident. Farnham and Newton (1959) have shown that herpesvirus is more stable at 37 C at pH 6.8 than at 7.4. The stabilizing effects of proteins (Tokumaru and Scott, 1964) have been shown in this current study to be an indirect action. Proteins and amino acids failed to stabilize partially purified virus in water, but were able in part to counteract the thermosensitizing effects of salts on herpesvirus.

Recently, Plummer and Lewis (1965) reported that intracellular herpesvirus was more labile than extracellular virus. To obtain intracellular virus, packed cells containing herpesvirus were suspended in Tris buffer and then disrupted to release the virus into the buffer. Our results show that both intracellular and extracellular herpesvirus have the same thermal characteristics when the intracellular virus is released into Eagle's medium, the nutrient fluid in which the extracellular virus had also been liberated. However, intracellular virus released into Tris-buffered

saline is rendered highly thermosensitive, whereas herpesvirus released into distilled water is relatively thermostable. Thus, in Plummer and Lewis' experiments the virus was being thermosensitized by the Tris-buffered saline. Vonka and Benyesh-Melnick (*J. Bacteriol.*, *in press*) found another member of the herpesvirus group, cytomegalovirus, to be more stable in distilled water than in saline diluents; they also found cytomegalovirus to be more rapidly inactivated at pH levels above 7.0.

In this study we have shown that herpesvirus is decreased in titer only 0.5 log₁₀ after being held at 37 C for 24 hr. Other workers (Kaplan, 1957; Hoggan and Roizman, 1959; Scott, McLeod, and Tokumaru, 1961) obtained 100 to 3,000 times more inactivation under these conditions. However, in our experiments, the virus was diluted in distilled water and then incubated at 37 C, whereas the workers referred to above either incubated the virus in the form of the tissue culture harvest or they diluted the virus in tissue culture medium. It is now evident that the differences between our results and those reported by others can be explained by the thermosensitization of herpesvirus by the salts contained in their suspending media. Some years ago, Munk and Ackermann (1953) reported herpesvirus to be more stable in distilled water than in allantoic fluids at 4 C, but this finding does not appear to have been utilized.

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