STUDIES ON HERPES SIMPLEX VIRUS

I. THE STABILITY AND PRESERVATION OF EGG-ADAPTED HERPES SIMPLEX VIRUS

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In animal tissues the virus of herpes simplex is very stable and can be preserved at refrigerator temperature or in the frozen state for many months. Egg-adapted herpes virus, on the other hand, is said to be relatively unstable so that it cannot be satisfactorily preserved and stored (Scott, 1948a,b). Two recent standard texts on viruses state that "egg virus appears harder to keep, very little surviving for 6 months even in a dry-ice cabinet" (Scott, 1948a) and "as egg material, however, only 1 out of 7 specimens were recoverable after 7 months in the dry-ice box. This deterioration is apparently not influenced by quick or slow freezing or by any of the three recommended diluents." When frozen from the moist state and kept at -20 C the activity of the egg virus should not be relied upon for more than 7-10 days" (Scott 1948b). These statements might imply that the physical, chemical, or even biological properties of herpes virus in egg material differ greatly from those of the virus in animal tissues. As an alternative the possibility must be considered that the chemical and physical properties of the menstruum greatly influence the stability of the virus. In this respect the complexity of animal tissues might well have advantages over simpler suspending media.

In this report it is shown that certain suspending media permit the adequate storage of egg-adapted herpes simplex virus for many months. Quantitative data are given indicating the relative efficiency of several menstrua in the preservation of such virus at low temperatures. In addition, storage in the dry-ice cabinet (-70 C) is compared with storage in the electrically operated mechanical refrigerator (-20 C).

MATERIALS AND METHODS

Viruses. Several passages of two different egg-adapted strains of herpes simplex virus were employed. Strain HR was originally obtained from Dr. Harry Rose in the form of infected mouse brain, and strain KER was isolated in this laboratory on the chorioallantoic membrane (CA) from the fresh vesicle fluid of a patient's herpetic lesion. Both strains underwent six CA passages before being adapted to the yolk sac (YS) route by the method of Rose and

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1 Supported in part by a grant from the Committee on Research of the University of California School of Medicine.

2 Diluents: 0.5 per cent gelatin in buffered saline, nutrient broth, physiologic saline plus 10 per cent normal rabbit serum.
Molloy (1947). The 14th YS passage of strain KER and the 19th to 22nd YS passages of strain HR were used in the experiments.

**Virus suspensions.** Stock virus, diluted $10^{-4}$ in peptone broth, was inoculated in 0.5-ml amounts into the YS of embryonated eggs that had been preincubated at 38°C for 7 days. Incubation was continued at 35.6 to 36°C for 4 to 5 days. The eggs were then chilled for 1 hour at $-20$°C or for 18 hours at 4°C and the clear amniotic and allantoic fluids harvested and pooled. After centrifugation at 2,000 rpm for 10 minutes antibiotics were added to ensure bacterial sterility. The final concentration of penicillin was 100 units per ml and of streptomycin 50 μg per ml. This pooled embryonic fluid constituted the virus suspension for each test.

**Methods of assay.** (a) CA titration: The number of infectious units was determined on the CA of 12- to 13-day-old embryonated eggs by the method of Burnet and Faris (1942). Herpetic pocks were enumerated after 48 to 72 hours' incubation at 36°C. Usually four membranes were used for each dilution of material. (b) YS titration: Virus titers were estimated by measuring the LD_{50} of suspensions inoculated by the YS route into groups of 7-day-old embryonated eggs, 6 to 8 eggs per group. Five-tenths ml of tenfold dilutions of material in unbuffered saline were injected within 5 minutes of thawing the frozen ampoules. The inoculated eggs were incubated at 36°C and candled at least once daily; deaths were recorded. Deaths occurring in the first 48 hours after injection were discounted and attributed to trauma. Some eggs of each lot were cultured to test for bacterial sterility. The LD_{50} of the thawed suspension was calculated according to the method of Reed and Muench (1938). Either method of titration was accurate to within 0.5 log or better.

**Suspending media and storage.** The influence of the following menstrua on the survival of egg-adapted herpes virus in cold storage was tested:

(a) Skim milk, sterilized by autoclaving at 10 pounds pressure for 10 minutes.

(b) Buffered saline, containing 67 ml of 0.2 m Na_{2}HPO_{4}, 30 ml of 0.2 m KH_{2}PO_{4}, and 1,900 ml of 0.85 per cent solution of NaCl, sterilized by autoclaving (Scott, 1948b).

(c) Gelatin, 0.5 per cent in buffered saline (b), sterilized by autoclaving at 15 pounds pressure for 15 minutes (Scott, 1948b).

(d) Rabbit serum, 10 per cent, in buffered saline (b). The serum was passed through a Seitz filter and added aseptically to the sterile saline (Scott, 1948b).

(e) Yolk, harvested aseptically from 12-day-old embryonated eggs and used within 1 week, after bacteriological sterility had been established.

(f) Allantoic fluid, harvested aseptically from 12-day-old embryonated eggs and used within 1 week, after bacteriological sterility had been established.

Suspensions were prepared for storage by adding the pooled embryonic fluids (10 or 50 per cent by volume) to the sterile menstruum and, after thorough mixing by pipette, distributing them into ampoules or tubes in amounts of 0.3 to 1.0 ml. The materials were quick-frozen in mixtures of dry ice and alcohol. The frozen suspensions were stored in sealed glass ampoules at $-70$°C in the
dry-ice chest or in rubber-stoppered small test tubes at -20 C in the electric “deep freeze.” At intervals containers were removed from storage and rapidly thawed in warm water, and the suspensions were titrated as outlined.

RESULTS

Effects of various menstrua on the viability of egg-adapted herpes virus stored at -70 C. HR virus (19th YS passage) with an estimated original titer of $10^{4.8}$ infectious units per ml was mixed in a concentration of 10 per cent with various menstrua and stored at -70 C. The CA titers obtained after periods up to 10 months are given in table 1. Skim milk and egg yolk were most efficient in preserving viability under these conditions. In a suspension of 9 parts of skim milk and 1 part of egg fluids containing virus the concentration of infectious units decreased from $10^{4.8}$ to $10^{3.3}$ per ml during 319 days of storage. In the same period the loss of viable virus was much greater in allantoic fluid and in saline with 10 per cent rabbit serum, and no viable virus at all could be recovered from either buffered saline alone or from buffered saline containing 0.5 per cent gelatin.

Effects of various menstrua on the viability of egg-adapted herpes virus stored at -20 C. HR virus (22nd egg passage) with an original LD$_{50}$ titer of $10^{-4.6}$ by YS inoculation was mixed in a concentration of 10 per cent with the same menstrua as above. The LD$_{50}$ titers obtained after storage in the electric “deep freeze” at 20 C for various periods of time are indicated in table 2. Titration performed 1 hour after freezing suggested a marked loss of viability only in the buffered saline and the 10 per cent serum in saline. However, in the subsequent 4 weeks of storage there was a sharp drop in titer of the virus in all menstrua except in milk and in yolk. After 110 days of storage the titer in milk and yolk showed little decrease, whereas no viable virus was recovered from the other suspending media. The estimated rates of loss of viability on storage in the various menstrua are shown graphically in figure 1.

TABLE 1

<table>
<thead>
<tr>
<th>MENSTRUUM</th>
<th>DAYS OF STORAGE AT -70 C</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>76</td>
</tr>
<tr>
<td>Skim milk</td>
<td>4.8*</td>
</tr>
<tr>
<td>Buffered saline</td>
<td>2.7</td>
</tr>
<tr>
<td>0.5% gelatin in buffered saline</td>
<td>3.6</td>
</tr>
<tr>
<td>10% rabbit serum in buffered saline</td>
<td>2.8</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>4.5</td>
</tr>
<tr>
<td>Allantoic fluid</td>
<td>—</td>
</tr>
</tbody>
</table>

* Logarithm of estimated number of infectious units per ml.
† No lesions on lowest dilution examined.
The stability of egg-adapted herpes virus in 50 per cent skim milk, at -20 C and -70 C. In view of the inherently low titers of the available egg fluid pools the

TABLE 2
Relative stability of egg-adapted herpes simplex virus (HRYS2) frozen in 10 per cent concentration in various menstrua and stored at -20 C
(Assayed by lethal effect following inoculation into the yolk sac. Before freezing, LD50: 10^{-4.4})

<table>
<thead>
<tr>
<th>MENSTRUUM</th>
<th>DAYS OF STORAGE AT -20 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0*</td>
</tr>
<tr>
<td>Skim milk</td>
<td>4.3†</td>
</tr>
<tr>
<td>Buffered saline</td>
<td>3.8</td>
</tr>
<tr>
<td>0.5% gelatin in buffered saline</td>
<td>4.3</td>
</tr>
<tr>
<td>10% rabbit serum in buffered saline</td>
<td>4.0</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>4.5</td>
</tr>
<tr>
<td>Allantoic fluid</td>
<td>4.1</td>
</tr>
</tbody>
</table>

* Titrated 1 hour after freezing.
† Negative logarithm of dilution of material giving 50 per cent deaths in chick embryos.

Figure 1. Estimated rates of deterioration of egg-adapted herpes virus stored in 10 per cent concentration in various menstrua at -20 C.

proportion of virus to menstruum was increased. It was found convenient to mix equal parts of egg fluids and milk for storage. The results obtained with several egg passages of two virus strains are shown in table 3. Materials pre-
erved either in the "deep freeze" or the dry-ice chest in this fashion were sufficiently stable to be used as stock pools of virus in neutralization tests for 4 to 6 months.

Skim milk has likewise been employed for the stabilization of herpes virus in specimens obtained from patients. Vesicle fluid or corneal scrapings in skim milk have been kept at -20 C for 2 to 6 weeks, with subsequent recovery of the virus. Several strains of egg-adapted herpes virus have been successfully lyophilized in skim milk suspension and have retained viability for at least 18 months.

### TABLE 3

Stability of different strains of egg-adapted herpes simplex virus frozen in 50 per cent concentration in skim milk

(Assayed by lethal effect following inoculation into the yolk sac)

<table>
<thead>
<tr>
<th>HERPES VIRUS POOL</th>
<th>TITERS BEFORE FREEZING</th>
<th>STORAGE TEMPERATURE</th>
<th>DAYS OF STORAGE IN THE FROZEN STATE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20-22</td>
</tr>
<tr>
<td>HR YS21</td>
<td>4.8</td>
<td>-70 C</td>
<td>4.4*</td>
</tr>
<tr>
<td>KER YS14</td>
<td>4.7</td>
<td>-70 C</td>
<td>4.3</td>
</tr>
<tr>
<td>HR YS22†</td>
<td>5.5</td>
<td>-70 C</td>
<td>5.1</td>
</tr>
<tr>
<td>HR YS22†</td>
<td>5.5</td>
<td>-20 C</td>
<td>-</td>
</tr>
</tbody>
</table>

* Negative logarithm of dilution of material giving 50 per cent deaths in chick embryos.
† Same pool of virus stored at -20 C and -70 C.

### DISCUSSION

The data presented in this paper indicate that egg-adapted herpes virus can be preserved satisfactorily for long periods, provided proper suspending media are employed. Skim milk and egg yolk have both been found useful for this purpose, but the ease of handling, storage, and sterilization of skim milk makes it more desirable than yolk. Yolk furthermore tends to become viscous with storage and is therefore less convenient.

Skim milk has previously been shown to be a good stabilizing medium for rickettsiae (Topping, 1940; Anderson, 1944). Hornibrook (1950) has recently reviewed the literature and has described a lactose-salts mixture resembling dialyzed milk as a useful menstruum for lyophilizing viruses and bacteria. Others (Bovarnick, Miller, and Snyder, 1950) have investigated the influence of salts, sugars, amino acids, and proteins on the stability of rickettsiae. The use of sera and proteins as stabilizers and diluents for viruses has been reviewed by Dick and Taylor (1949), who advocated plasma albumin for this purpose.

The menstrua, milk and yolk, found effective here in the preservation of egg-adapted herpes virus are both complex substances, containing carbohydrates, proteins, and salts described as efficient stabilizers in the studies just mentioned. No attempt has been made here to assign a specific role to one or more chemical constituents that may be responsible for the stabilizing effect of milk and yolk. Emphasis is placed, however, on the essential similarity between egg-grown and
tissue-grown herpes virus with respect to stability in storage, if proper stabilizing menstrua are employed. Whereas skim milk satisfactorily preserved the viability of egg-adapted herpes virus in cold storage, such diluents as gelatin or serum in buffered saline (Scott 1948a,b) were found unsuitable for this purpose.

The data comparing storage of herpes virus at -20 C and -70 C do not permit a final conclusion on the relative merits of these temperatures for long periods. However, for periods of 4 to 6 months, storage in the electric "deep freeze" at -20 C can be just as adequate as storage in the dry-ice chest. This finding agrees with the results of Olitsky et al. (1949) on a number of other viruses.

SUMMARY

Egg-adapted herpes simplex virus, although said to be unstable and difficult to preserve, can be stored at low temperatures for at least 10 months with little loss in infective titer, provided proper stabilizing menstrua are employed.

Skim milk and egg yolk are good stabilizing agents for this purpose. Milk is convenient to use and serves also as a suitable menstruum for lyophilizing egg-adapted herpes virus.

When frozen in milk, egg-adapted herpes virus remains viable for many months in storage at either -20 C or -70 C.

REFERENCES


