PRESERVATION OF VIRUSES OF THE PSITTACOSIS-LYMPHOGRANULOMA VENERUM GROUP AND HERPES SIMPLEX UNDER VARIOUS CONDITIONS OF STORAGE

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It is well known that most viral agents lose some of their viability—often rapidly—if stored in ordinary diluents such as buffered saline or nutrient broth. It is also known that this loss of viability is usually dependent on the temperature at which the preparation is stored.

The effect of temperature on the storage of certain neurotropic viruses was studied by Olitsky et al. (1950) who found that in the presence of normal rabbit serum at −25 to −30 C most of these agents could be recovered on animal passage after 9 to 12 months storage, though the titers were markedly reduced. Horsfall (1940) has shown that at −70 C most viruses retain their infectivity for long periods of time.

Many investigators have shown that some substances, particularly proteins, have a stabilizing or protecting effect on viruses, rickettsiae, and bacteria when used as the suspending menstruum for these agents. Anderson (1944) and Topping (1940) have found sterile skim milk an ideal medium in which to store or lyophilize certain rickettsiae. Dick and Taylor (1949) found that higher infectivity titers were obtained with some viruses, including the virus of yellow fever in the presence of small concentrations of bovine albumin. Cook and Hudson (1937) and Bauer and Mahoffy (1930) have shown that the presence of some types of serum in the suspending medium has a protective effect on the viruses of yellow fever and St. Louis encephalitis. Bovarnick, Miller, and Snyder (1950), while looking for a defined medium in which to study purified preparations of rickettsiae, have shown that a medium made up of glutamate high in potassium ion and containing a small amount of serum albumin favored the survival of those strains of rickettsiae studied.

When working with a viral agent in the laboratory it is desirable to find an easily prepared medium and the optimal temperature which favor the maintenance of the viability or infectious property of that agent. In our experience, the problem of preservation of members of the psittacosis-lymphogranuloma venerum group and herpes simplex virus was especially critical. It is the purpose of this report to describe the effect of several conditions of storage on the preservation of infectivity of meningopneumonitis and herpes simplex viruses. No

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attempt was made to break down the substances used, which are for the most part chemically complex, to find specifically the reason for the protective or stabilizing effect which was exhibited.

While this work was in progress, a report by Speck, Jawetz, and Coleman (1951) indicated that normal egg yolk and milk had excellent stabilizing effect on egg adapted herpes simplex virus. As will be shown later, our results confirm their findings.

**I. MENINGOPNEUMONITIS**

**MATERIALS AND METHODS**

*Virus.* The California 10 strain of meningopneumonitis virus was employed in this study; it was received from Dr. Wong of Lederle Laboratories. Stock virus was produced by inoculating a suitable dilution of virus into the allantoic cavity of 8½ day embryonated hen's eggs. After 6 days' incubation at 36 C the allantoic fluids were harvested from all living embryos. Fluids from 10 eggs were pooled. Smears and sterility checks were made on each pool, and the smears were stained by the Macchiavello method. Arbitrarily, the number of elementary bodies seen were rated 1+ to 4+, and all 3+ or 4+ pools which were bacteriologically sterile were combined to make the stock virus used in these experiments.

*Normal yolk.* Yolk was removed from 8 day embryonated hen's eggs with a sterile pipette. Several pools were made, and those which were bacteriologically sterile were used.

*Milk.* Fresh skim milk obtained from a local dairy was used. It was sterilized by autoclaving.

*NORMAL rabbit serum.* Serum was obtained aseptically from a normal rabbit, inactivated at 62 F for 20 minutes, and made up to 10 per cent concentration by volume using 0.02 M phosphate buffered water (pH 7.2).

*Glutamate medium.* This was prepared according to the method of Bovarnick, Miller, and Snyder (1950). Because of the subsequent dilution of the menstruum by the virus, the concentrations of the various salts in part 1 of the medium and the serum albumin in part 2 were three times that used by Bovarnick et al. (1950).

Virus and the menstruum to be tested were mixed in the proportion of 2 parts infected allantoic fluid to 1 part of the material tested. Each mixture was dis-

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1 The concentrations of materials used were:

Part 1: 22.38 g sucrose, 0.15 g KH₂HPO₄, 0.39 g K₂HPO₄, 0.22 g l-glutamic acid in 100 ml distilled water. The reaction of the medium was adjusted to pH 7.0 with 0.2 M KOH. Solution was sterilized by autoclaving.

Part 2: 5 ml human serum albumin 25 per cent (Harvard preparations) was dialyzed against 10 parts K-N solution (K-N solution = KCl 0.122 M and NaCl 0.023 M). Time of dialysis was 6 hours. The dialyzing fluid was changed every 1½ hours. The volume of the serum increased from 5 ml to 6 ml during dialysis. This was further diluted with K-N solution to final volume of 42 ml (protein concentration = 0.020 g/ml). This solution was filtered through a Seitz filter.

The two were mixed in the proportion of 7 ml part 1 to 3 ml of part 2 to make the glutamate medium tested in these experiments.
distributed into several small rubber stoppered tubes to be stored at 4°C and −20°C and into small glass vials which were sealed with a flame for storage at −65°C to −70°C following quick freezing in dry ice-alcohol bath. The object in storing each preparation in several small quantities was to have a separate tube and vial for each titration interval, thus, insuring greater uniformity by avoiding freezing and thawing a single sample. This practice may cause virus inactivation.

**Titrations.** Titrations were made in the allantoic cavity of the embryonated hen’s eggs immediately after mixing these preparations and after 2 weeks, 11 weeks, and 16 weeks of storage to determine the amount of virus infectivity present at these intervals.

The method of titration was to make suitable dilutions from a single vial or tube of each preparation and inoculate each dilution in 0.2 ml quantity into the allantoic cavity of embryonated hen’s eggs. After 6 days’ incubation, smears from the allantoic fluid of each living egg were made, stained with Macchiavello stain, and examined microscopically for the presence or absence of elementary bodies. The ID<sub>50</sub> was calculated for each preparation by the method of Reed and Muench (1938).

**RESULTS**

Figure 1 shows that at −70°C there was very little if any decrease in the virus infectivity of meningopneumonitis virus over the period of eleven weeks, even when no protective substance was added to the infected allantoic fluid. The virus in allantoic fluid, as harvested from the egg, decreased only approximately 0.3 to 0.5 logs in titer.

As shown in figure 2, which represents the −20°C to −25°C temperature range, there occurred a considerable decrease in titer of the untreated virus. Also at this temperature very little protective effect was exerted by normal yolk or the 10 per cent normal rabbit serum, though the yolk may have been somewhat more protective than the serum. Both were definitely inferior to the skim milk and glutamate medium preparations. In the case of the glutamate medium there was almost no demonstrable loss in the infectivity titer.
In figure 3, it can be seen that when these preparations were stored in the refrigerator (4°C) the decrease in titer during the first 2 weeks was not great, in the vicinity of 1.0 to 1.5 logs, which was only slightly greater than that of the same materials, in that same period of time, which were stored at −25°C. However, after 11 weeks at 4°C the only preparation which contained any demonstrable active virus was the one stored in skim milk.

II. HERPES SIMPLEX VIRUS

MATERIALS AND METHODS

Virus. The seed virus used in this experiment was the 277th egg passage of strain HF. After 48 hours' growth on the chorioallantoic membranes of 12-day old embryonated hen's eggs, the membranes were removed and stored 6 days in 50 per cent glycerine (pH 6.9 to 7.2) at −20°C, then washed in buffered gelatin saline to remove the glycerine, and ground in a mortar with sterile alundum. A 20 per cent suspension by weight of these ground chorioallantoic membranes was prepared using buffered gelatin saline as described by Scott (1948). Follow-

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3 Originally supplied by the Rockefeller Institute, New York City.
4 This diluent was used in diluting all preparations in the experiments for titration and contained 500 units of penicillin and 100 μg of streptomycin per ml. The buffer used was a phosphate buffer with pH of 7.2.
ing centrifugation at 1,800 rpm for 10 minutes to remove coarse particles, the supernatant was diluted to $10^{-3}$ using buffered gelatin saline. This virus dilution in 0.05 ml was put onto the chorioallantoic membranes of 11-day old eggs by the false air sac technique described by Coriell et al. (1949) and incubated at 36 to 37 C for 48 hours.

Following incubation, the chorioallantoic membranes from those eggs which were previously inoculated by the false air sac route were removed, and each membrane was cut into four pieces. Four pools (for uniformity, each pool consisted of one piece of every membrane) of infected membranes were prepared. Three of the pools were separately weighed and ground in a chilled Waring blender for 10 minutes. Each was then suspended in one of the following menstrua as a 20 per cent suspension by weight:

1. Homologous allantoic fluid. This was obtained from the eggs inoculated by the false air sac route before removing the allantoic membranes.

2. Skim milk. This was obtained from a local dairy and sterilized by autoclaving for 10 minutes at 15 pounds pressure.

3. Normal rabbit serum. This was made as a 10 per cent concentration with buffered water and inactivated at 56 C for 30 minutes.

Glycerine. The fourth pool of chorioallantoic membranes (unground) was placed in sterile 50 per cent glycerine (pH 6.9 to 7.2).

Homologous yolk. To make the yolk sac preparation, the same $10^{-3}$ dilution of seed virus which was used to prepare the infected chorioallantoic membranes was inoculated in 1.0 ml quantities into the yolk sac of 9-day old eggs which were then incubated at 36 to 37 C for 48 hours. Following incubation, the yolk sacs were removed without rinsing, but a considerable quantity was allowed to remain adherent to the sacs. The sacs were weighed and then ground in a chilled Waring blender for 10 minutes. The volume of buffered water added to make the suspension equalled the weight of tissue ground. These preparations are referred to as homologous yolk preparations.

All preparations with the exception of the membranes in glycerine were stored at 4 C, −20 to −25 C, and −65 to −70 C. The glycerine preparation was kept at −20 C only.

Titration. Titrations on all of the virus preparations were done before storage and also one and four months later. Those with homologous allantoic fluid, skim milk, normal rabbit serum, and glycerine were titrated on the chorioallantoic membrane of 12-day old eggs using the plaque counting method described by Burnet and Faris (1942). Four eggs were used for each dilution. The chorioallantoic membranes in 50 per cent glycerine were washed in buffered gelatin saline, ground, and titrated as before. (Diluent was added to make a 20 per cent suspension by weight.) The eggs were incubated for not more than 40 hours to avoid secondary plaques. The chorioallantoic membranes were removed and placed in petri dishes containing 0.5 per cent formalin fixative, and the plaques were counted. Arbitrarily, chorioallantoic membranes containing three or more plaques were recorded as positive, and less than 3 as negative. The ID$_{50}$ was determined for each of the preparations by the method of Reed and Muench (1938).
STORAGE OF HERPES SIMPLEX VIRUS IN THE CO₂ ICEBOX -70°C

- Homologous yolk
- Homologous allantoic fluid
- Skim milk
- Normal rabbit serum (10%)

Figure 4

STORAGE OF HERPES SIMPLEX VIRUS IN THE DEEP FREEZER -20°C

- Homologous allantoic fluid
- Skim milk
- Normal rabbit serum (10%)
- Glycerine (50%)

Figure 5

STORAGE OF HERPES SIMPLEX VIRUS IN THE REFRIGERATOR +4°C

- Homologous yolk
- Homologous allantoic fluid
- Skim milk
- Normal rabbit serum (10%)

Figure 6
The virus titer of the yolk sac preparations is determined by inoculating 1.0 ml of the diluted material into the yolk of 9-day old eggs. Six or seven eggs were inoculated with each dilution, re-incubated, and candled twice daily. The LD50 was based on numbers of embryos dying between 48 to 96 hours following the inoculation of the virus suspension. Deaths occurring earlier than 48 hours were attributed to trauma and were not included.

RESULTS

Figures 4, 5, and 6 reveal the changes in the infectivity of the herpes simplex virus following storage under different conditions. In the experiment reported there was no change in infectivity at -70 C. The only small decrease at -20 C occurred in the serum preparation. In an experiment not shown in this paper, there was a more marked reduction in the infectivity of the preparations kept at -20 C. At 4 C there was a rapid decrease in active virus over the course of 4 months when the membranes were suspended in allantoic fluid or normal rabbit serum. The decrease was much less rapid, however, in preparations stored in yolk or skim milk over the same period of time.

SUMMARY

A temperature of -70 C proved best for the storage of both meningopneumonitis and herpes simplex viruses in all preparations tested.

At -25 C meningopneumonitis virus was best preserved in either skim milk or glutamate medium.

A temperature of 4 C was most unsatisfactory for storage of meningopneumonitis virus for a prolonged period of time. Sterile skim milk was, of the substances tested, the only one which exhibited any preserving effect at this temperature.

In infected chorioallantoic membranes, stored either whole in glycerine or ground with skim milk, the herpes virus did not seem to decrease in titer over a 4 month period at -20 C.

At 4 C the infectivity titer of herpes virus decreased over this 4 month period, but the decrease was minimal in skim milk and yolk preparations.

At all temperatures tested, yolk and skim milk showed the best protective action on egg adapted herpes simplex virus.

REFERENCES


