Preservation of $T_2$ Bacteriophage With Liquid Nitrogen

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ABSTRACT

MEYLE, JANET S. (University of Illinois at the Medical Center, Chicago), and J. EMERSON KEMPF. Preservation of $T_2$ bacteriophage with liquid nitrogen. Appl. Microbiol. 12:400–402. 1964.—There have been few reports on the preservation of viruses at the temperature of liquid nitrogen (-196°C). In this study, factors affecting the survival of phage were observed. Phage lysates in broth did not lose titer after a storage period of 4 weeks in liquid nitrogen. The rate of freezing was not critical, but freezing in nitrogen vapor was not satisfactory. When the virus was partially purified and suspended in buffered saline solution, it rapidly lost titer. Of seven protective agents recommended in the literature, only gum acacia seemed to demonstrate significant protection of purified virus against loss of viability. The temperature of thawing of phage suspended in broth did not affect its titer after freezing and storing in liquid nitrogen.

As pointed out by Clark, Horneland, and Klein (1962), it is desirable to find a means of preserving infectious, genetic, and other properties of viruses for periods longer than those attainable by present methods. These investigators initiated a study of the preservation of viruses with the use of liquid nitrogen. Believing confirmation and extension of this work to be desirable, we studied factors affecting the viability of $T_2$ phage during freezing and storage in liquid nitrogen.

MATERIALS AND METHODS

Virus. The virus used was the $T_2$ strain of phage, propagated and titered by the soft agar layer method described by Adams (1959).

Freezing. Quantities (1 ml) of phage suspension were dispensed into 1.2-ml prescored glass ampules (T. C. Wheaton Co., Chicago, Ill.), which were then sealed with an oxygen torch. Samples of phage were slow-frozen in a Linde BF-3 freezer (Linde Co., Tonawanda, N.Y.), in which the cooling rate was 1 to 2°C per min; rapidly frozen by clipping the ampules into metal racks, plunging them into liquid nitrogen, and removing when boiling ceased; or placed in a metal container which was held for 2 to 3 hr in the nitrogen vapor just above the level of liquid nitrogen in a thermos flask.

RESULTS

Effects of thawing rate on the preservation of $T_2$ phage. Before the effects of freezing and storage could be studied, it was necessary to determine whether variation in the thawing rate affected the titer of phage. To avoid the possible protective effect of large numbers of virus particles, low concentrations of phage were used. (It was subsequently shown, however, that $10^9$ particles per ml had no protective effect.)

Freshly harvested, unfiltered phage lysates were diluted in nutrient broth to contain $2.5 \times 10^3$ infectious particles per ml, slowly frozen, stored in liquid nitrogen for 8 hr, and then thawed at 4°C, 22°C (room temperature), or in water baths at 37 or 50°C. Thawing time varied from 3 to 8 min. Two or four samples were used at each temperature. In all cases, the assays of phage after thawing did not differ more than 0.1 log from that of the original samples.

Effect of rate of freezing on the viability of phage lysates in nutrient broth stored in liquid nitrogen. A freezing rate of 1 degree per min is considered a satisfactory rate for freezing tissue prior to storage in liquid nitrogen. It seemed of interest to determine whether this rate would be satisfactory for $T_2$ phage.

In a typical experiment, fresh lysates of phage in nutrient broth were diluted to a concentration of $2 \times 10^3$ to $2.5 \times 10^3$ plaques per ml, and 1-ml samples were sealed in ampules. These samples were then frozen at a rate of 1°C per min, or rapidly frozen by plunging into liquid nitrogen. They were then stored for 4 weeks and titered after thawing at 37°C. The results (Table 1) indicate that there was no appreciable difference in titer on preparation for storage by rapid or slow freezing.

Variable results were obtained after freezing the samples in liquid nitrogen vapor; however, since this method was time-consuming and cumbersome, it was not studied further.

Comparison of the effects of storing phage at 4°C, −54°C, or in liquid nitrogen. Duplicate samples of phage lysates in nutrient broth were frozen in liquid nitrogen and stored either in liquid nitrogen or at −54°C in an electric refrigerator. A third set of samples was not frozen and was stored at 4°C. Plaque assays were made at 2 and 4 weeks. It was found that no decrease occurred in 4 weeks when virus was stored in liquid nitrogen, but in 2 weeks the titer had already decreased 2.4 logs at −54°C (Table 2). This finding was unexpected, since no loss occurred at 4°C.

Survival in liquid nitrogen of partially purified phage suspended in phosphate-buffered saline (PBS) solution. It is recognized that small amounts of protein and other materials are protective to various biologicals, so it was
postulated that, although titers of phage lysates stored in liquid nitrogen showed no loss, there might be a decrease when the phage was partially purified and stored in PBS. Accordingly, we concentrated virus from a freshly grown broth suspension by two cycles of centrifugation at 2,000 rev/min for 10 min and 20,000 rev/min for 90 min (36,000 × g) in a no. 20 rotor of a Spinco model L preparative centrifuge; the virus was then suspended in 0.01 M PBS, placed in ampules which were sealed, frozen in liquid nitrogen, stored in liquid nitrogen for 8 hr, and then titered. In all cases, the virus lost 0.7 to 1.6 logs from the original titer of 2.7 × 10⁶, indicating that to store partially purified T₂ phage in liquid nitrogen without decrease in titer a protective agent is needed.

Protective effect of various agents on the survival of partially purified virus in liquid nitrogen. Because the titer of partially purified virus decreased markedly in a simple buffer, we attempted to find an agent suitable for protecting the virus.

A variety of agents have been recommended for protection of viruses during storage at low temperatures. Those selected for study were gum acacia (Campbell-Renton, 1941; Rivers and Ward, 1935), glycerine (Clark et al., 1962; Porterfield and Ashwood-Smith, 1962), dimethyl sulfoxide (Porterfield and Ashwood-Smith, 1962), cysteine hydrochloride (Labzoffsky, 1946), glycine (Weil et al., 1948), lactose (Kroeger and Kempf, 1959), and bovine serum albumin (Dick and Taylor, 1949; Olitsky, Yager, and Murphy, 1950).

To rule out toxicity to phage, samples were prepared in which the virus and the various agents were combined in the same proportions as used in the subsequent storage experiments; the samples were allowed to stand for 8 hr at 4°C, and then were titered. In two experiments, none of the agents caused a drop in titer of more than 0.3 log₁₀.

Samples of phage and the various protective agents were then prepared, frozen in liquid nitrogen, and assayed after 8 hr of storage in liquid nitrogen. The virus in low concentration, when purified and suspended in a variety of agents, appeared to be protected only by 2.5% gum acacia and possibly by 10% glycerine (Table 3). Slow or rapid freezing had no effect on the results.

Effect of virus concentration on survival of virus in liquid nitrogen. It was thought that if virus were present in high concentration, the virus particles might be mutually protective. To determine whether this was true, partially purified virus was placed in a series of ampules in a concentration of 10⁹ particles per ml in one group and 2.68 × 10⁸ in a second group. One-half of the samples was slowly frozen and the other half rapidly frozen; all were stored in liquid nitrogen for 8 hr and then titered. It was found that both concentrated and diluted samples lost about 1 log in titer, and that there was no difference in titer whether the samples were rapidly or slowly frozen (Table 4). In subsequent experiments, it was found that gum acacia had a greater protective effect for high concentrations of virus than for low.

### Table 3. Effect of protective agents on partially purified phage stored in liquid nitrogen for 8 hr

<table>
<thead>
<tr>
<th>Protective agent*</th>
<th>Final conc of agent</th>
<th>Titer of phage after storage (X 10²)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (phosphate-buffered saline)</td>
<td>—</td>
<td>3.5</td>
</tr>
<tr>
<td>Gum acacia</td>
<td>2.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Glycerine</td>
<td>10.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>2.5</td>
<td>3.4</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>0.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.08</td>
<td>1.1</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>20.0</td>
<td>0.45</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.0</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Four experiments were performed for each agent.
† The titer of phage before freezing was 2.7 × 10⁹.

### Table 4. Comparison of the viability of purified phage in concentrated or dilute suspension after 8-hr storage in liquid nitrogen

<table>
<thead>
<tr>
<th>Rate of freezing</th>
<th>Titer of phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before storage</td>
<td>After storage*</td>
</tr>
<tr>
<td>Slow</td>
<td>2.7 × 10¹</td>
</tr>
<tr>
<td>Rapid</td>
<td>2.7 × 10¹</td>
</tr>
</tbody>
</table>

* Each titer is an average of four experiments.


**DISCUSSION**

It appears that a short-term experiment of 30 days could be used in a selection of materials and procedures to be tested further for their effect on the preservation of viruses for prolonged periods.

Some of our results, such as the lack of protection by glycine, cysteine, and serum albumin, did not confirm the findings of others. This was not unexpected, since differences may result from such factors as the strain of virus used, methods of growing it, preparation for storage, conditions of assay, and other factors. The protective effect of gum acacia may have been physical or, in part, a result of its neutralization of the alkalinity resulting from loss of CO₂ on freezing.

It might be expected that virus particles would exert a mutually protective effect at a concentration of 10¹⁰ per ml. However, when we observed preparations of this concentration of phage on an electron microscope, we found the particles not touching. It is possible that a protective effect exists in greater concentrations.

A somewhat unexpected finding was that phage suspended in broth survived poorly at −54 C as compared with −196 or 4 C. This would suggest that −54 C is less satisfactory for maintaining virus than is 4 C; however, it is well known that the reverse is true. The explanation may be that the virus in our experiments was prepared for storage by freezing at −196 C and then "warmed" to −54 C on storage, causing recrystallization to occur.

**LITERATURE CITED**


