Stability of Pseudorabies Virus During Freeze-Drying and Storage: Effect of Suspending Media

EILEEN M. SCOTT* AND W. WOODSIDE

Department of Pharmacy, The Queen's University of Belfast, Medical Biology Centre, Belfast BT9 7BL, Northern Ireland

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The effect of suspending media on the stability of pseudorabies virus upon freeze-drying and subsequent storage was studied. A variety of media was tested, including: sodium glutamate; sucrose; lactose; lactalbumin hydrolysate; peptone; a combination of sucrose, dextran, and glutamate; and various combinations of sucrose, glutamate, and potassium phosphates. Suspending media containing glutamate, either alone or in combination with sucrose and either dextran or phosphates, afforded the greatest degree of protection during the freeze-drying process and upon storage. Some possible functions of these additives in preventing injury to the virus during freezing and drying have been suggested.

Pseudorabies (Aujeszky's disease) is an acute and often fatal nervous condition of domestic and wild animals caused by a virus of the herpes group (4). The pig is the natural host, and in severe attacks of the disease mortality can often exceed 80%. Several attenuated strains of virus have been produced, and their use for vaccination has been reported (5, 20, 22). A naturally occurring strain of low virulence was isolated by Bartha (3) and has been extensively used in Hungary. A freeze-dried vaccine from the strain isolated by Bartha has been produced in Budapest by Kucsera (13), who showed that it could produce good immunity in pigs, although high-titer virus was required and freeze-drying adversely affected potency. Other workers showed that the virus could be freeze-dried with a small drop in titer if protected by suitable additives (24). The nature of the suspending medium has clearly been shown to affect the retention of infectivity of many viruses upon freeze-drying and subsequent storage (2, 17, 19, 23). In this study a variety of additives was investigated to determine their suitability as suspending media for pseudorabies virus during freeze-drying.

MATERIALS AND METHODS

Virus strains. The NIA-1 strain of pseudorabies virus, grown in monolayers of Vero cells, was used (9, 14). Cells were grown in Eagle medium with 10% fetal bovine serum, which was replaced by Eagle medium containing 1% fetal bovine serum shortly before the cells were infected. The frozen and thawed cultures were clarified by centrifugation at $1,500 \times g$ for 5 min and stored at -80°C until re-

quired. The K strain (3) was also used and was grown and harvested in the same way.

Suspending media. The following solutions were used: sucrose, 5%; lactose, 5%; peptone, 5%; sodium glutamate, 2%; lactalbumin hydrolysate, 2%; SDG, a combined medium consisting of 5% sucrose, 4% dextran, and 1% sodium glutamate; SPG, a stabilizer used by Bovarnick et al. (6) consisting of sucrose (0.218 M), K_2HPO_4 (0.0071 M), KH_2PO_4 (0.00376 M), and potassium glutamate (0.0049 M); SPGA, SPG with 1% bovine albumin; and other modifications of the SPG formula.

The sucrose, lactose, and KH_2PO_4 used in this investigation were analar grade; K_2HPO_4 and peptone were general purpose grade. The sodium and potassium salts of L-glutamic acid, bovine albumin powder (Cohn fraction V), and dextran (clinical grade; molecular weight, 60,000 to 90,000) were supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks, England. Lactalbumin hydrolysate was supplied by Difco Laboratories, Detroit, Mich.

Freeze-drying. A modified Edwards EF3 freeze-dryer (Edwards High Vacuum, Crawley, Sussex) was used. The virus suspension was added to the suspending medium (media 1 in 10, see Results) and 5-ml amounts were pipetted into vials, which were then placed on the shelf of the freeze-dryer. The vials were cooled to -40°C on the shelf and vacuum was applied. After 6 h of drying, heat was applied to the shelf at a heating rate of 30°C and drying continued for 20 h, by which time the pressure inside the chamber had attained that of the isolated pump (10^{-2} torr) and the temperature had risen to 20°C . Vials were sealed under vacuum and capped with a metal ring. Two vials were assayed immediately for infectivity, and the remaining vials were stored at -80 , 4 , 20 , and 37°C for assay at intervals of 1, 4, 13, 26, and 52 weeks. Samples for assay were reconstituted to the original volume by addition of 5 ml of

sterile water. In all samples reconstitution was rapid and complete.

Virus assay. Tenfold virus dilutions were prepared using Eagle medium with 1% fetal bovine serum as diluent, and 0.1 ml of each dilution was inoculated onto Vero cells in microtiter plates (four wells per dilution). The number of wells infected after 3, 5, and 7 days was recorded, and the 50% tissue culture infective dose per milliliter was calculated by the method of Reed and Muench (18).

RESULTS

Results of initial drop in titer after freeze-drying are presented in Fig. 1. Total drop in titer after storage at 4, 20, and 37°C is shown in Fig. 2, 3, and 4, respectively.

The K strain of pseudorabies virus was freeze-dried using SPGA as protecting medium. Results are shown in Fig. 5.

The media code used is as follows: (i) suspending media—1, SPG; 2, SDG; 3, sodium glutamate (2%); 4, lactalbumin hydrolysate (2%); 5, sucrose (5%); 6, lactose (5%); 7, peptone (5%); and (ii) SPG-based suspending media—8, SPGA; 9, SPG (sodium salts); 10, SPG (minus glutamate); 11, SPG (sucrose, 20%); 12, SPG (sucrose, 5%); 13, SPG (glutamate, 5%); 14, SPG (glutamate, 2%); 15, SPG (glucose, 0.218 M, replacing sucrose).

Summary of results. Media containing glutamate, either alone or in combination with

other substances, as in SPG (6) or SDG, afforded best protection during the freeze-drying process, although 2% lactalbumin hydrolysate and 5% sucrose were only slightly less effective (Fig. 1). Greatest retention of infectivity on storage at 4°C was observed in samples suspended in media containing glutamate. At higher storage temperatures good retention of infectivity was obtained when SPG or SDG was used as the suspending medium. Addition of sucrose to the suspending medium, as in SPG and SDG, was important for survival. Although sucrose alone was much less effective, it afforded good protection during storage at higher temperatures. Lactalbumin hydrolysate and lactose, although less effective than SPG and SDG during the freeze-drying process, provided reasonable protection upon storage at 4°C. Poor results were obtained when 5% peptone was used as the suspending medium.

Since the Bovarnick SPG afforded better protection than most of the other additives during the freeze-drying process, various modifications of the formula were investigated in an attempt to further improve survival of virus. SPGA was the most successful of these media; improved survival of virus was obtained upon freeze-drying and subsequent storage at all temperatures. Increased concentration of glutamate in the SPG formulation did not improve initial survival of virus upon freeze-drying, although a

FIG. 1. Effect of suspending medium on initial drop in titer (log 50% tissue culture infective dose [TCID₅₀] per milliliter) of pseudorabies virus (NIA-1 strain) upon freeze-drying. See Results for media code.

FIG. 2. Effect of suspending medium on total drop in titer ($\log TCID_{50}$ per milliliter) of pseudorabies virus (NIA-1 strain) after freeze-drying and subsequent storage at $4^{\circ}C$. See Results for media code.

FIG. 3. Effect of suspending medium on total drop in titer ($\log TCID_{50}$ per milliliter) of pseudorabies virus (NIA-1 strain) after freeze-drying and subsequent storage at $20^{\circ}C$. See Results for media code.

slight improvement upon storage at 4 and $37^{\circ}C$ was noted. An increase in the concentration of sucrose to 20% did not improve survival, and it was also noted that the appearance of the product had altered due to "puffing." Replacement of sucrose by glucose had an adverse effect; loss of infectivity of virus increased significantly at all temperatures. A slight decrease in titer was

obtained when sodium salts were used instead of potassium salts in the SPG medium.

The effect of freeze-drying and subsequent storage of the Bartha strain of pseudorabies virus, suspended in SPGA, was similar to results for the NIA-1 strain, indicating that freeze-drying affected both strains in a similar manner.

FIG. 4. Effect of suspending medium on total drop in titer (log TCID₅₀ per milliliter) of pseudorabies virus (NIA-1 strain) after freeze-drying and subsequent storage at 37°C. See Results for media code.

FIG. 5. Effect of freeze-drying and subsequent storage on the titer (log TCID₅₀ per milliliter) of pseudorabies virus (K strain) suspended in SPGA.

DISCUSSION

The presence of glutamate in the suspending medium had a significant effect on survival of pseudorabies virus during freeze-drying and storage, a concentration of 1%, as in SPG, being the most effective. This is in agreement with other workers who found that it enhanced the survival of a variety of organisms (8, 15, 21). It was suggested by Greaves (11) that sodium glutamate reacted preferentially with carbonyl groups in the medium, thus protecting viral protein, which in the freeze-dried state is sensitive to denaturation by carbonyl groups. Thus, the addition of carbonyl-containing compounds such as glucose would reduce survival upon freeze-drying, especially if prolonged storage at high temperatures was required. This was found to be the case when glucose was used in

the SPG formulation to replace sucrose, survival being significantly affected in all cases. It is likely that the glucose was inhibiting neutralization of carbonyl groups by sodium glutamate under storage conditions.

A possible function of sucrose and lactose is prevention of damage during freezing. Herber and Santarius (12) suggested that sugars are cryoprotectants by virtue of their ability to retain water by hydrogen bonding to that water or by substituting for water in structures sensitive to dehydration. Dextrose and albumin also act as cryoprotectants by providing a protective coat for membranes (10).

The combination of sucrose and glutamate in phosphate buffer, SPG, which was first used by Bovarnick et al. (6) to increase stability of rickettsiae, proved to be effective for freeze-drying pseudorabies virus and for retention of infectivity upon storage particularly, at 20 and 37°C. This medium was also used successfully by Calnek et al. (7) for freeze-drying Marek's disease virus. The addition of bovine serum albumin to SPG improved the protective effect of this medium. Serum albumin has frequently been used to increase stability of viruses, both for low-temperature storage (16) and for freeze-drying (19). It was suggested by Adams (1) that virus particles become inactivated when they reach the gas-liquid interface and that the loss of infectivity could be prevented by saturating the gas-liquid interface with another protein, thereby preventing virus access to the surface. Bovine albumin may exert its effect in this way.

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