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Effect of suspending media on freeze-drying and preservation of vaccinia virus

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SUMMARY

Unpurified and purified smallpox vaccines were prepared from calf dermal pulp, or chorioallantoic membrane (CAM) of hen eggs infected with vaccinia virus, and freeze-dried. The protective effect of various suspending media was investigated both in the course of the freeze-drying and in the period of subsequent storage of the dried product at different temperatures, including 100° C.

Single media consisting of either sodium glutamate or peptone were effective in the preservation of both unpurified and purified vaccines prepared from calf dermal pulp or CAM. It was shown that there was an optimal concentration of sodium glutamate for the preservation of the vaccine preparations, especially of the purified vaccine.

Combined media, consisting of soluble starch, polyvinylpyrrolidone or sodium carboxymethyl cellulose with sodium glutamate, were effective with the purified vaccine when the concentration of sodium glutamate exceeded the optimum necessary for preservation.

INTRODUCTION

It is now generally accepted that in order to prevent the reduction of biological activity during both the freeze-drying process and the preservation of biological products, the addition of some suspending medium to the product is very important (Harris, 1954).

With regard to viral products, Collier (1955) reported that peptone acted protectively in the freeze-drying and the preservation of smallpox vaccine. However, with peptone there is a possibility of an unfavourable side reaction. On the other hand, sodium glutamate is a pure crystalline compound and exerts a powerful protective influence on the viability of dried BCG vaccine (Cho & Obayashi, 1956; Obayashi & Cho, 1957). No sufficient work, however, has been undertaken on the effect of sodium glutamate on viral activity during and after the freeze-drying.

The present paper deals with the effect of the suspending media on vaccinia virus preparations both in the freeze-drying and in subsequent preservation.

MATERIALS AND METHODS

Virus vaccine source

Calf dermal pulp and chorioallantoic membrane (CAM) of developing hen eggs infected with vaccinia virus (strain IKEDA) were used.

Unpurified calf dermal pulp smallpox vaccine

Calf dermal pulp was suspended in the proportion of 1 g. to 3 ml. of 0·004 M McIlvaine buffer (pH 7·3), and homogenized in a Waring blender. The virus suspension was filtered through 80 mesh wire screen. After centrifugation at 2,000 rev./min. for 20 min., three volumes of the supernatant obtained were added to one volume of the suspending medium.

Unpurified CAM smallpox vaccine

The infected CAM was suspended in the proportion of 1 g. to 0·5 ml. of 0·004 M McIlvaine buffer (pH 7·3), and homogenized in a Waring blender. The following procedure was the same as in the preparation of the unpurified calf dermal pulp vaccine.

Purified calf dermal pulp smallpox vaccine

The calf dermal pulp was purified with 33% fluorocarbon (trichlorotrifluoroethane) buffer mixture by the method of Epstein (1958), and the suspending medium was added to an equal volume of the vaccine.

Purified CAM smallpox vaccine

The vaccine, that is, the elementary body suspension, was prepared from the infected CAM by the 10% extract method of Collier (1955) using fluorocarbon. The suspending medium was added to an equal volume of the vaccine.

Suspending media

In the first group of media a number of simple substances, such as sodium glutamate or peptone, were used alone dissolved in 0·004 M McIlvaine buffer (pH 7·3). In the second group, sodium glutamate was used in combination with soluble starch, polyvinylpyrrolidone (PVP) or sodium carboxymethyl cellulose (SCMC). According to Obayashi, Ota & Arai (1961) the first group of substances exert a sublimation-retarding effect, whereas the second group of substances, such as PVP and SCMC, rather have a sublimation-promoting property.

Freeze-drying

The liquid vaccine suspension was pipetted in 0·5 ml. volumes into ampoules which were frozen in a chamber of the drying apparatus at below −30° C. for about 2 hr., and dried for 8 to 11 hr. An example (batch no. 115) of the freeze-drying process in the present experiments is shown in Fig. 1. The maximum temperature of the vaccine in the final drying stage was controlled at about 20° C. After the drying, each ampoule was sealed under vacuum.

Residual moisture content

Residual moisture content was measured by Abderhalden's method. The dried products were heated at 60° C. for 3 hr. under a pressure of 0·1 mm Hg in an apparatus specially made for this purpose, in order to evaporate the residual moisture still contained. Residual moisture content of dried products was calculated by the following equation:

$$\text{Residual moisture content } (\%) = \frac{A-B}{A} \times 100,$$

where A is the initial weight of the dried product and B is the weight after 3 hr. desiccation. Statistical significance level ($P < 0{\cdot}05$) between the residual moisture contents obtained in a preliminary test was 1·19 %.

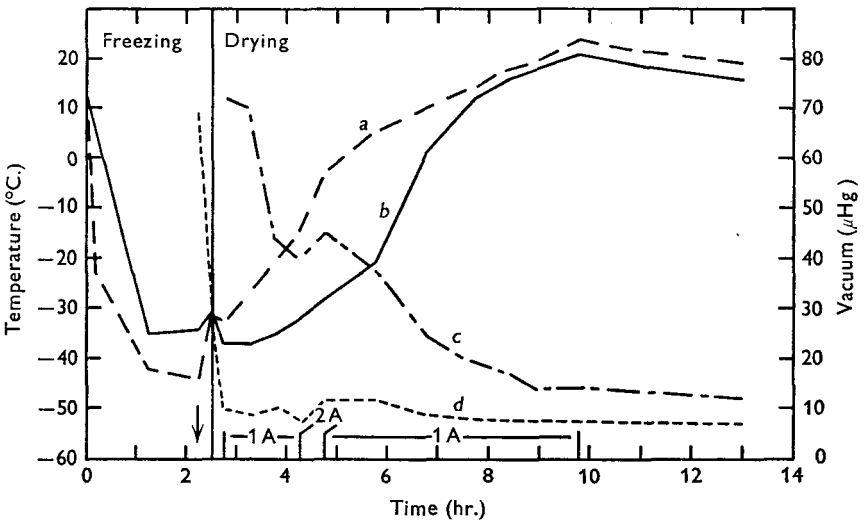

Fig. 1. An example of the freeze-drying process in the present experiments (batch no. 115). (*a*) Temperature of shelf; (*b*) temperature of vaccine; (*c*) Pirani gauge reading; (*d*) temperature of condenser. 1A and 2A, Heater current (amp.). ↓, Changing point of cooling solvent flow from chamber to condenser. Freeze-drying apparatus: Kyowa RL-500S type, made by Kyowa Vacuum Engineering Co. Ltd., Tokyo, Japan.

Virus titration

After the reconstitution of dried vaccine with 0·5 ml. phosphate buffered saline (PBS, pH 7·4) serial tenfold dilutions were made, and the virus titre was measured by the method of Westwood, Phipps & Boulter (1957) using CAM of developing hen eggs. The titre was expressed as log pock-forming units per millilitre (log PFU/ml.) of the reconstituted virus suspension. Statistical significance level ($P < 0{\cdot}05$) between the titres obtained in a preliminary test was 0·66 log PFU/ml.

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Boiling test

In order to observe the effect of the suspending media on the stability of virus titre, the dried vaccine was heated in a boiling water bath for a certain period of time.

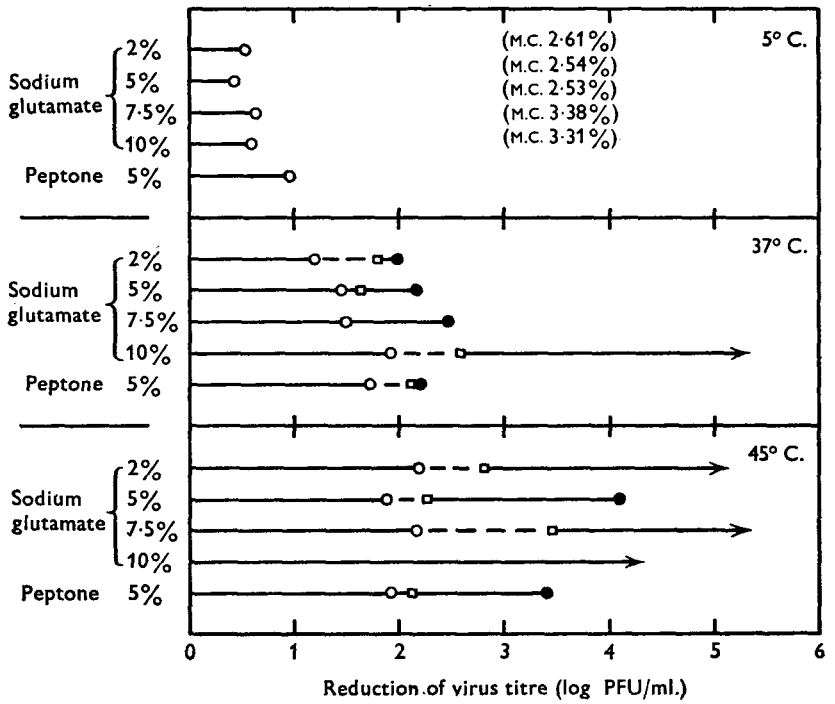

Fig. 2. Effect of single suspending media on preservation of dried unpurified calf dermal pulp vaccine (batch no. 109). ○, Preserved at 5° C. for 43 months; preserved at 37° C. for 12 months; preserved at 45° C. for 6 months. □, Preserved at 37° C. for 36 months; preserved at 45° C. for 21 months. ●, Preserved at 37° C. for 48 months; preserved at 45° C. for 36 months. →, Larger reduction of virus titre than the given point. M.C. Residual moisture content of dried vaccine obtained immediately after freeze-drying.

Preservation test

In this test the dried vaccines were preserved at 5°, 37° and 45° C., to evaluate the stability of the dried vaccine.

RESULTS

Experiment 1. *Unpurified smallpox vaccine*

Effect of single suspending media on unpurified calf dermal pulp vaccine (batch no. 109)

Using 2, 5, 7·5 and 10% sodium glutamate, and 5% peptone as single media their effect was investigated in freeze-drying and in preservation at 5°, 37° and 45° C.

Virus titre of the dried vaccine after freeze-drying was between 8·09 and 8·3

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log PFU/ml., and the reduction of virus titre by the freeze-drying was between 0·22 and 0·56 log PFU/ml.

During 43 months preservation at 5° C., only a slight difference was observed in the reduction of virus titre among the different suspending media mentioned (Fig. 2). After storage at 45° C. for 36 months, the best results were obtained with 5 % peptone and 5 % sodium glutamate.

These results indicated that peptone and sodium glutamate were effective for the preservation of the unpurified calf dermal pulp vaccine. However, the use of sodium glutamate in concentrations as high as 7·5 % and 10 % clearly resulted in a considerable reduction in virus titre, particularly after long preservation at high temperature.

This experiment was chiefly made from a practical viewpoint in order to observe the stability of dried smallpox vaccine at high temperatures for several years, and in the following study comparison of the effect of different kinds and concentrations of suspending media on stability of dried products was made after preservation for several months.

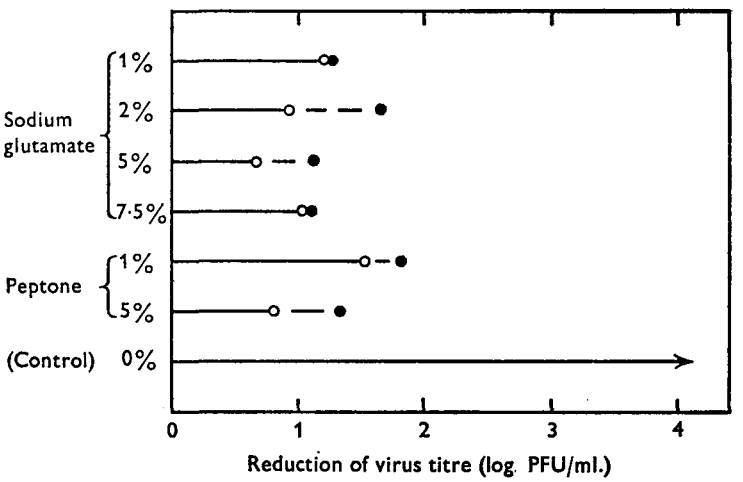

Fig. 3. Effect of single suspending media on preservation of dried unpurified CAM vaccine (batch no. C-1). ○, Preserved at 45° C. for 2 months. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

Effect of single suspending media on unpurified CAM vaccine (batch no. C-1)

Unpurified CAM vaccine was freeze-dried with 1, 2, 5 and 7·5 % sodium glutamate and 1 and 5 % peptone as single media.

Virus titre immediately after freeze-drying was between 6·99 and 7·44 log PFU/ml., and the reduction of virus titre by the freeze-drying was below the significance level (0·66 log PFU/ml.).

The reduction of virus titre after the preservation of the resulting vaccines is shown in Fig. 3. After preservation for 2 and 5 months at 45° C., all of these vaccines were far more stable than the control without any suspending medium, and no significant difference in survival was observed among different concentrations of sodium glutamate and peptone.

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Effect of combined suspending media on unpurified calf dermal pulp vaccine (batch no. 114)

These combined media consisted of 5 and 7·5 % sodium glutamate as the first group, with various concentrations of soluble starch, PVP or SCMC as the second group.

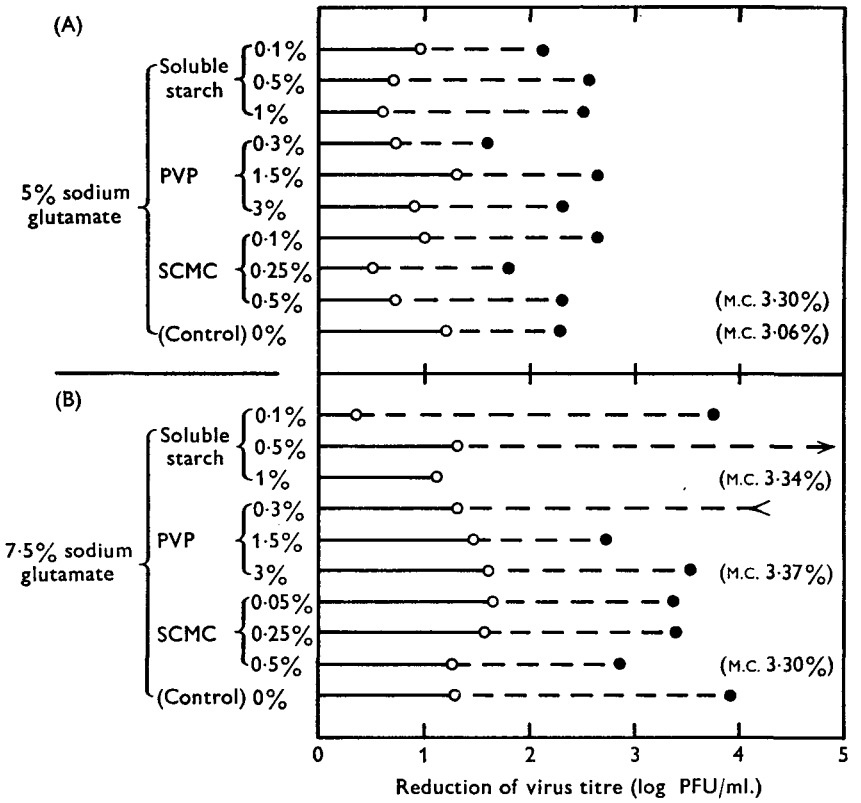

Fig. 4. Effect of combined suspending media on preservation of dried unpurified calf dermal pulp vaccine (batch no. 114). ○, Preserved at 45° C. for 1 month. ●, Preserved at 45° C. for 9 months. ≺, Smaller reduction of virus titre than the given point. →, Larger reduction of virus titre than the given point. M.C. Residual moisture content of dried vaccine obtained immediately after freeze-drying.

Virus titre of the dried vaccines after freeze-drying was between 7·45 and 8·09 log PFU/ml. with batch no. 114 (A), and between 7·66 and 8·32 log PFU/ml. with batch no. 114 (B).

The virus titre of the dried vaccine was tested after preservation at 45° C. for 1 and 9 months as shown in Fig. 4. The addition of the media of the second group showed no stability-enhancing effect, the degree of reduction of virus titre being almost similar to that of the control vaccines dried with sodium glutamate alone.

Experiment 2. Purified smallpox vaccine

Effect of single suspending media on purified calf dermal pulp vaccine (batch no. 115)

The protective effects on the purified vaccine of sodium glutamate and peptone were compared. In order to clarify the effect of the concentration of sodium glutamate, a wide concentration range from 0·1 to 7·5 % was tested.

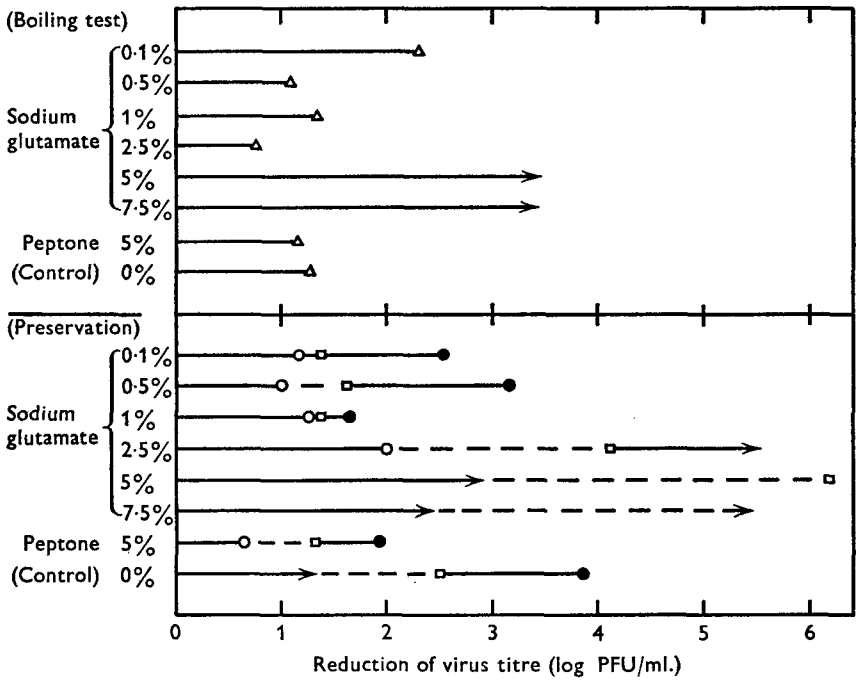

Fig. 5. Effect of single suspending media on preservation of dried purified calf dermal pulp vaccine (batch no. 115). △, Heated in a boiling bath for 10 min. ○, Preserved at 45° C. for 1 month. □, Preserved at 45° C. for 3 months. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

Virus titre of the dried vaccine after the freeze-drying was between 6·30 and 7·56 log PFU/ml., and the degree of reduction of virus titre by the freeze-drying was rather slight except with 0·1 % sodium glutamate, and in the control.

In the boiling test no particular stability-enhancing effect was observed either in peptone or in sodium glutamate (Fig. 5). The reduction of virus titre was rather more marked with 5 % and 7·5 % of sodium glutamate. However, in the preservation at 45° C. the reduction of the virus titre was lowest in 1 % sodium glutamate and in 5 % peptone, higher concentrations of sodium glutamate resulting in a larger reduction of titre than the control.

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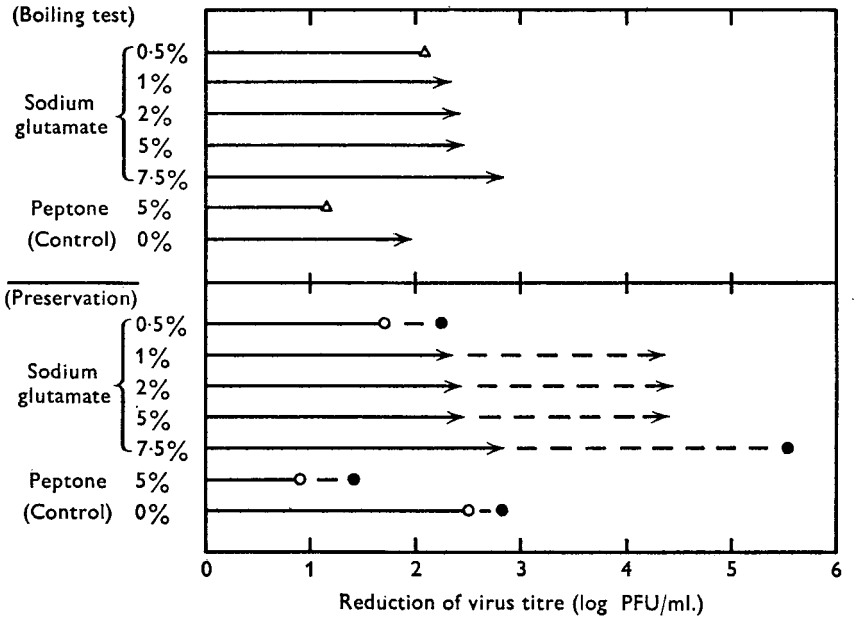

Fig. 6. Effect of single suspending media on preservation of dried purified CAM vaccine (batch no. C-2). △, Heated in a boiling bath for 30 min. ○, Preserved at 45° C. for 1 month. ●, Preserved at 45° C. for 2 months. →, Larger reduction of virus titre than the given point.

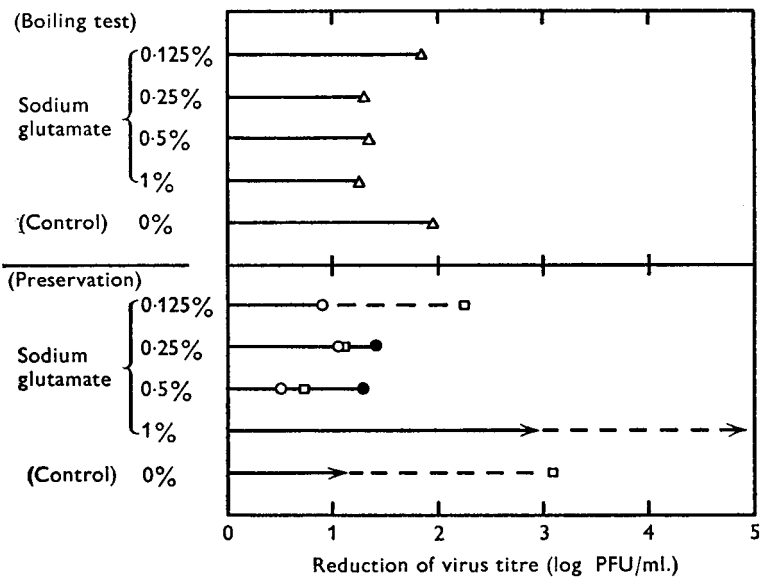

Fig. 7. Effect of single suspending media on preservation of dried purified CAM vaccine (batch no. C-5). △, Heated in a boiling bath for 30 min. ○, Preserved at 45° C. for 1 month. □, Preserved at 45° C. for 3 months. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

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Effect of single suspending media on purified CAM vaccine (batch no. C-2 and C-5)

In Figs. 6 and 7 the effect of different concentrations of sodium glutamate ranging from 0·5 to 7·5 % (batch no. C-2) and from 0·125 to 1 % (batch no. C-5) upon the stability of dried purified CAM vaccine was examined.

Virus titre of the dried vaccine after the freeze-drying was between 7·32 and 7·80 log PFU/ml. except in the control in batch no. C-2, and 6·38 and 6·91 log PFU/ml. except in the control in batch no. C-5. The control vaccine without medium decreased to a virus titre of 5·88 log PFU/ml. after the drying in the former batch, and 5·08 log PFU/ml. in the latter.

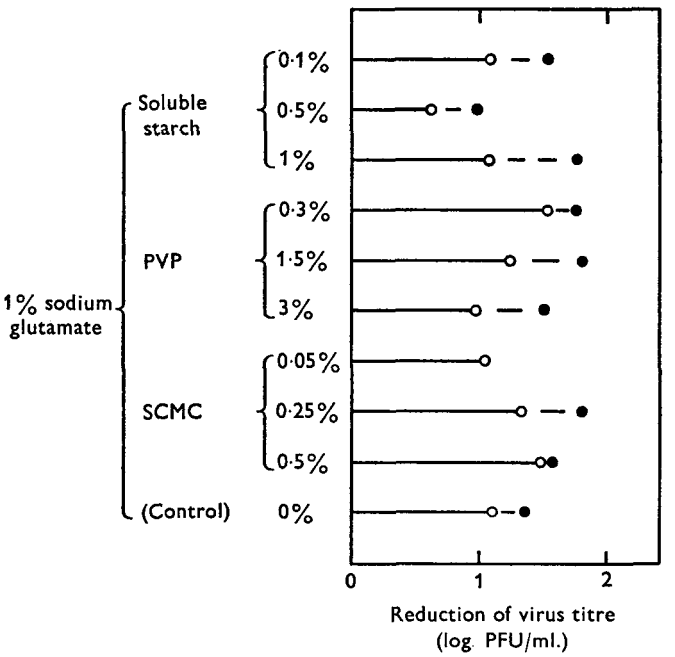

Fig. 8. Effect of combined suspending media on preservation of dried purified calf dermal pulp vaccine (1) (batch no. 118). ○, Preserved at 45° C. for 2 months. ●, Preserved at 45° C. for 5 months.

Reduction of virus titre by the freeze-drying ranged from 0·18 to 0·50 log PFU/ml. in batch no. C-2, and from 0·14 to 0·36 log PFU/ml. in batch no. C-5 except in the two controls.

In the preservation test, the best result was obtained with 0·5 % sodium glutamate followed by 0·25 %, and 5 % peptone also revealed a good protective property.

Effect of combined suspending media on purified calf dermal pulp vaccine (batch no. 118 and 119)

Since the addition of the medium of the second group to sodium glutamate exerted no beneficial effect in the case of unpurified calf dermal pulp vaccine, this point was further examined by using purified vaccine. As shown in Fig. 8, soluble

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starch, PVP or SCMC was added to 1 % sodium glutamate which was confirmed as the optimum concentration when used alone (Fig. 5).

Dried vaccines from 6·51 to 7·20 log PFU/ml. in batch no. 118, and from 7·15 to 7·42 log PFU/ml. in batch no. 119 were preserved.

The reduction of virus titre by the freeze-drying, and after 5 months preservation at 45° C., was similar to the reduction in the controls containing sodium glutamate alone. Thus when sodium glutamate was used in its optimal concentration, the addition of the medium of the second group exerted no added protection.

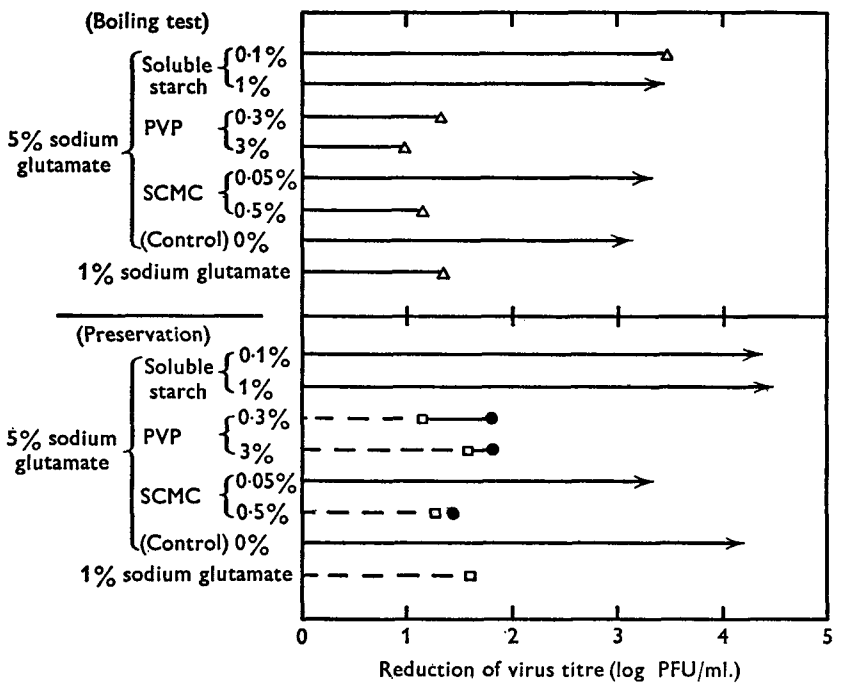

Fig. 9. Effect of combined suspending media on preservation of dried purified calf dermal pulp vaccine (2) (batch no. 119). △, Heated in a boiling bath for 10 min. □, Preserved at 45° C. for 3 months. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

However, when the concentration of sodium glutamate was increased to 5 %, the addition of PVP and a high concentration of SCMC effected an increase of protection up to the level of 1 % sodium glutamate alone (Fig. 9). But soluble starch and a lower concentration of SCMC did not show such protective effect. It thus became clear that the addition of PVP and SCMC produced added protection only when sodium glutamate was used in higher concentration than the optimum one.

Effect of combined suspending media on purified CAM vaccine (batch no. C-4)

The effect of the addition of media of the second group in the purified CAM vaccine using 2 % sodium glutamate was investigated.

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Dried vaccines from 6·34 to 7·14 log PFU/ml. were tested, and the reduction of virus titre by the freeze-drying was not great.

As shown in Fig. 10, the addition of PVP and a high concentration of soluble starch and SCMC to sodium glutamate again showed a certain protective effect.

DISCUSSION

The role of suspending media is concerned firstly with the protection of biological products from the damaging effect of freeze-drying. In this respect, however, the degree of the reduction of virus titre by the freeze-drying is relatively

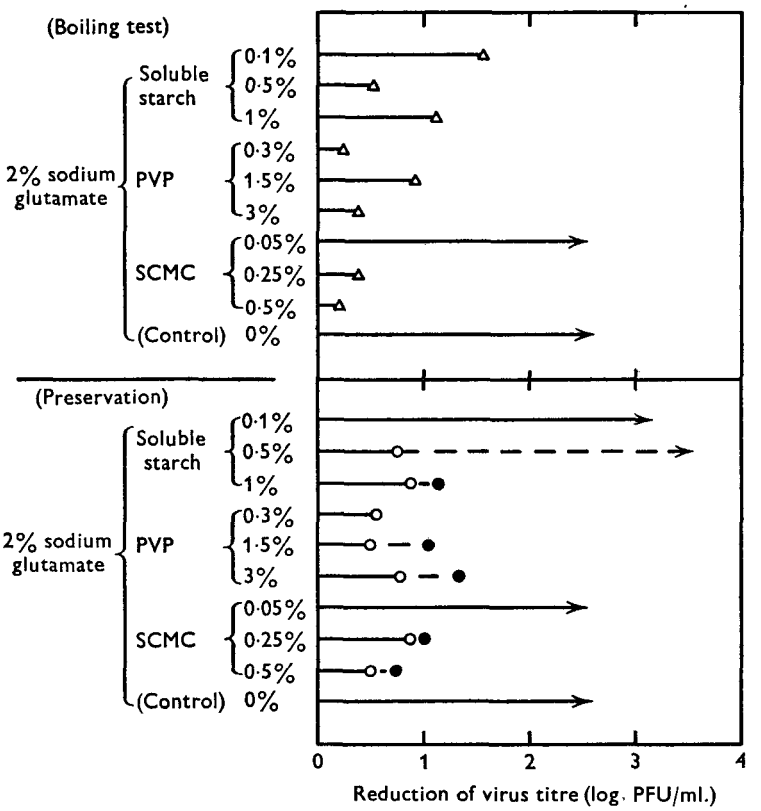

Fig. 10. Effect of combined suspending media on preservation of dried purified CAM vaccine (batch no. C-4). △, Heated in a boiling bath for 30 min. ○, Preserved at 45° C. for 1 month. ●, Preserved at 45° C. for 5 months. →, Larger reduction of virus titre than the given point.

smaller in the case of vaccinia virus as compared with that of poliovirus (Kraft & Pollard, 1954) or Coxsakie virus (Tyrrel & Ridgewell, 1965). Therefore, in the present study the effect of suspending media was chiefly examined with regard to the preservation process.

For the preservation of dried vaccinia virus, sodium glutamate and peptone were effective in both the unpurified and purified vaccines, while the existence of an optimal concentration of sodium glutamate was observed clearly only in the purified vaccines owing to the relative absence of the extraneous non-viral sub-

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stances which were present in the unpurified vaccine. The existence of an optimal concentration of sodium glutamate was essentially similar to that seen in BCG and *Lactobacillus bifidus* (Cho & Obayashi, 1956; Obayashi & Cho, 1957; Ota, 1959; Obayashi *et al.* 1961). Obayashi *et al.* (1961) suggested that the concentration of sodium glutamate is related to the residual moisture content of the final product, and that for the preservation of the product there existed an optimum residual moisture content. They observed that the increase in the concentration of sodium glutamate tended to depress the sublimation during the drying in contrast to soluble starch and PVP, in which no such diminution of sublimation occurred with increase of the concentration.

Also in the present study, soluble starch, PVP and SCMC in the combined media raised the protective activity up to that attained by using the optimal concentration of sodium glutamate alone. In this case therefore the role of the suspending media of the second group seems to lie in their sublimation-promoting effect when too high a concentration of sodium glutamate retards the sublimation.

Greiff & Rightsel (1968) observed a certain degree of correlation between the residual moisture content of dried influenza virus and its stability at high temperatures. So far in our experiment with unpurified calf dermal pulp vaccines dried in either sodium glutamate or peptone, no correlation was found between the residual moisture content below about 4% and the reduction of virus titre after preservation or by the boiling test (Suzuki, 1969). However, this point should be further examined with the purified smallpox vaccines, since the presence of an extraneous non-viral substance might obscure the relation between these two components.

The major role of suspending medium for dried bacterial products, such as protection of living bacilli during both the freeze-drying process and subsequent period of storage, was observed also in the case of vaccinia virus when sodium glutamate and peptone were used as suspending medium. As to the mechanism of this protection, the sublimation-retarding activity of sodium glutamate and sublimation-promoting activity of the media of the second group, such as soluble starch, PVP and SCMC, was considered to play some part. As to this point further studies are required, especially with purified virus. However, for practical purposes the present study may provide some useful information for those engaged in the freeze-drying of virus vaccines.

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REFERENCES

Cho, C. & Obayashi, Y. (1956). Effect of adjuvant on preservability of dried B.C.G. vaccine at 37° C. *Bulletin of the World Health Organization* **14**, 657.

Collier, L. H. (1955). The development of a stable smallpox vaccine. *Journal of Hygiene* **53**, 76.

Epstein, M. A. (1958). An investigation into the purifying effect of a fluorocarbon on vaccinia virus. *British Journal of Experimental Pathology* **39**, 436.

Greiff, D. & Rightsel, W. A. (1968). Stability of suspensions of influenza virus dried to different contents of residual moisture by sublimation in vacuo. *Applied Microbiology* **16**, 835.

Harris, R. J. C. (1954). The preservation of viruses. In *Biological Applications of Freezing and Drying*. P. 436. Ed. R. J. C. Harris. New York: Academic Press.

Kraft, L. M. & Pollard, E. C. (1954). Lyophilization of poliomyelitis virus. Heat inactivation of dry MEF 1 virus. *Proceedings of the Society for Experimental Biology and Medicine* **86**, 306.

Obayashi, Y. & Cho, C. (1957). Further studies on the adjuvant for dried B.C.G. vaccine. *Bulletin of the World Health Organization* **17**, 255.

Obayashi, Y., Ota, S. & Arai, S. (1961). Some factors affecting preservability of freeze-dried bacteria. *Journal of Hygiene* **59**, 77.

Ota, S. (1959). Studies on freezing and freeze-drying of *Lactobacillus bifidus* (4). *Ochanomizu Igaku Zassi* **7**, 2968.

Tyrrel, D. A. J. & Ridgewell, B. (1965). Freeze-drying of certain viruses. *Nature* **206**, 115.

Suzuki, M. (1969). Relation between reduction of vaccinia virus titer and residual moisture content. In *Freezing and Drying of Microorganisms*. P. 111. Ed. T. Nei. Tokyo: University of Tokyo Press, and Baltimore & Manchester: University Park Press.

Westwood, J. C. N., Phipps, P. H. & Boulter, E. A. (1957). The titration of vaccinia virus on the chorioallantoic membrane of the developing chick embryo. *Journal of Hygiene* **55**, 123.