PURIFICATION AND CONCENTRATION OF THE VIRUS OF FOOT-AND-MOUTH DISEASE BY COMBINED CENTRIFUGATION AND ULTRAFILTRATION METHODS[1]

By I. A. GALLOWAY and M. SCHLESINGER

From the National Institute for Medical Research, London, N.W. 3

(With 2 Figures in the Text)

In a previous paper, Galloway & Elford (1936), it was reported that preparations of purified foot-and-mouth disease virus, giving a negative reaction in the test for proteins (sulphosalicylic acid) and having a potency equivalent to the stock filtrates, could be obtained by repeated washing on graded collodion membranes of suitable porosity. It has been found now that purified concentrates, i.e. of *increased* virus content, can be made by combining ultrafiltration through graded collodion membranes with two other methods. The first of these is a modification of the Bechhold-König ultrafiltration technique which, although it is not selective and therefore cannot be used for purification, makes it possible to reduce large volumes of virulent liquid to a very small amount rapidly and without serious interference by blocking effects. Porcelain filter candles coated with acetic acid collodion are employed with special glass containers. One of us (M. S.) has found these collodion-coated filter candles to be useful also for the concentration of bacteriophage. The second is a centrifuging method. The Sharples-Super Centrifuge, with the closed cylinder coated with a thin agar layer, is used in the way already outlined by Schlesinger (1936). It has been found that by spinning the virus into the agar and subsequently extracting the gel in fresh medium it is possible to reduce the protein content of undiluted vesicle lymph sufficiently to permit subsequent washing on graded collodion membranes (Elford) without dilution of the virus suspension. The application of this spinning method to the study of the virus of foot-and-mouth disease has been described already by Schlesinger & Galloway (1937). There remain to be reported the results of two series of experiments, one in which concentration of the virus was effected by the use of porcelain filter candles coated with acetic acid collodion and the other in which a combination of the different methods of centrifugation and ultrafiltration were used to obtain purified virus concentrates.

[1] This work was done on behalf of the Foot-and-Mouth Disease Research Committee who have kindly given their permission for the publication of this paper. One of us (M. S.) is indebted to the Academic Assistance Council for a grant.

Our results were reported in general terms to the 2nd International Congress for Microbiology held in London, July 1936 (see *Proceedings*).

Concentration of virus on porcelain filter candles coated with acetic acid collodion

Methods

Preparation of filters. A porcelain filter candle (Doulton) is dipped into glacial acetic acid. It is then immersed in 6–9 per cent collodion[1] in glacial acetic acid, slight suction being applied for about 15–30 sec. The filter candle is removed and held in a vertical position so that the surplus collodion may drain off and then immediately inverted and rotated to allow of the formation of a homogeneous thin layer of collodion. It is essential that the candle be completely covered and especially the junction of the unglazed and glazed portion. The collodion is coagulated by immersion in water. The following day the acetic acid is removed by filtration of large amounts of tap water. In order to remove the last traces of acetic acid and any calcium salts which might cause trouble when filtering virus suspended in buffered phosphate solution the final washing of the filters is made with a very weak solution of sodium phosphate in distilled water. Before use the surface of the collodion membrane is rinsed carefully with distilled water to remove any deposit of calcium phosphate or other foreign matter which may have formed during the process of washing. For work with small quantities of liquid the smallest sizes of Chamberland L 5 filter candles are coated with acetic acid collodion in a similar way.

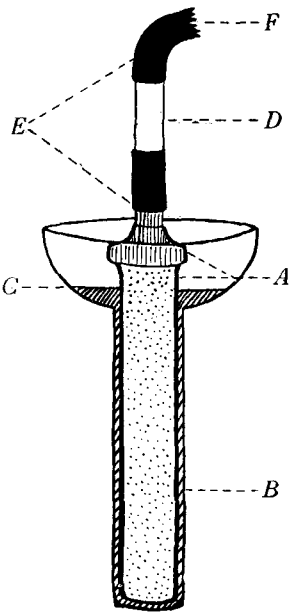

Fig. 1. A = coating of collodion; B = special glass container; C = highest level of filtering liquid; D = glass connecting tube; E = rubber tubing; F = connexion to negative pressure.

Method of employing the filters. Special glass containers (see Fig. 1) have been made so that when the filter candles are placed in them there remains a space of only 1 or 2 mm. between the collodion filter membrane and the wall of the glass vessel. By this arrangement a large filtering surface is in use continuously until nearly the whole of the liquid being filtered under negative pressure is removed. The upper part of the glass container is cup-shaped and serves as a reservoir for the virus suspension to be concentrated. It is advisable in filling not to raise the level of the liquid in the reservoir too high in case there may be imperfect covering of the candle with the collodion at the junction of the glazed and unglazed portions. The time necessary for concentration is longer for unpurified virus suspensions containing considerable amounts of protein than for purified preparations, but even in the former case about 100 c.c. can be filtered in $\frac{1}{2}$–1 hour. In order to recover and resuspend the virus gathered on the surface of the collodion membrane, sufficient medium (in the case of foot-and-mouth disease virus, buffered phosphate solution at pH 7·6) is introduced with a capillary pipette into the interior of the filter candle to fill it completely and a few drops of liquid are allowed to filter in the reverse direction under gravity. While the inverse filtration is proceeding, the surface of the collodion may be wiped gently on the wall of the glass container or with the side of a pipette. The smallest final volume of resuspended virus which can be obtained conveniently when using the Doulton collodion coated filter candle is about 1 c.c. By transferring this amount to smaller glass containers made for use with the small Chamberland filter candles it can then be reduced by a similar procedure even to 0·1 c.c.

[1] Owing to variations in different batches of collodion it may be necessary to increase the concentration to 9 per cent. In the present experiments, however, 6 per cent acetic acid collodion was quite satisfactory.

https://doi.org/10.1017/S0022172400035178 Published online by Cambridge University Press

Table I. *Concentration of virus on porcelain filter candles coated with acetic acid collodion*

Date	Material	Titre of stock						Titre of concentrate						Volume of stock in c.c.	Volume of concentrate in c.c.	Degree of concentration effected
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}			
22. v. 36	Fresh filtrate of unpurified virus	+ +	+ +	+ +	00	00	—	+ +	+ +	+ +	+0	00	—	100	1·0	×100
4. vi. 36	ditto	+ +	+ +	+0	00	00	—	+ +	+ +	+ +	+ +	00	—	100	1·0	×100
11. vi. 36	ditto	+ +	+ +	+0	00	00	—	+ +	+ +	+ +	+ +	00	—	30	0·3	×100
19. vi. 36	ditto	+ +	+ +	+ +	00	00	—	+ +	+ +	+ +	+0	00	—	10	0·1	×100
14. v. 36	Stored filtrate of unpurified virus	+ +	+ +	+0	+0	00	—	+ +	+0	+0	00	—	—	20	1·0	None
29. v. 36	ditto	+ +	+ +	+ +	+ +	00	—	+ +	+ +	+ +	00	00	—	100	1·0	None
11. vi. 36	ditto	+ +	+ +	+0	00	00	—	+ +	+ +	+ +	+ +	00	—	50	0·5	×100
26. vi. 36	ditto	+ +	+ +	00	00	00	—	+ +	+0	+0	00	00	—	100	1·0	×100?
4. vi. 36	Stored filtrate of purified virus	+ +	00	00	00	—	—	+ +	+ +	00	00	—	—	50	0·5	×100
11. vi. 36	ditto	+0	00	00	00	—	—	+ +	+0	00	00	—	—	50	0·5	×100
19. vi. 36	Fresh filtrate of purified virus	+ +	+ +	+ +	00	00	00	+ +	+ +	+ +	+0	00	00	20	0·2	×100
15. vii. 36	ditto	+ +	+ +	+ +	+ +	+ +	00	+ +	+ +	+ +	+ +	+ +	+ +	10	0·1	×100

+ + = both guinea-pigs inoculated developed lesions.
+0 = one guinea-pig out of two inoculated developed lesions.
00 = neither of two guinea-pigs inoculated developed lesions.
— = no test made.

Twelve experiments were made with collodion-coated filter candles. The results are given in Table I. It will be seen that in the case of fresh unpurified virus the increase in titre after concentration always corresponded to the reduction of the volume of liquid, i.e. the degree of concentration of the virus effected was to the limit of expectancy. In the case of unpurified virus, which had been stored for 10 days or more at 0° C., less regular results were obtained. That no concentration effect could be demonstrated in some cases with stored unpurified virus may have been due rather to incomplete resuspension of the material gathered on the filtering surface than to an actual loss of virus. The results with both stored and fresh preparations of purified virus were consistently good and similar to those obtained with fresh unpurified virus. As already explained, this method of concentration is not selective, for not only the virus but the lymph and tissue proteins and haemoglobin contained in unpurified preparations of virus are likewise concentrated. However, the possibility of concentrating the proteins provides a more sensitive control of the degree of purity of washed preparations of virus than the immediate application of the sulphosalicylic acid test. Thus it could be shown that preparations of virus washed on graded collodion membranes until the sulphosalicylic acid test had just given a negative result again gave a positive reaction when concentrated. On the other hand, concentrates gave no reaction with the sulphosalicylic acid test when made from preparations of virus which had been subjected to further washing after the protein test had first become negative.

Purification and concentration of the virus by combined centrifugation and ultrafiltration methods

In these experiments two methods of washing the virus were used, viz. (*a*) by centrifugation in the Sharples-Super Centrifuge; (*b*) by ultrafiltration on collodion membranes of suitable porosity and in addition the method of concentrating on porcelain filter candles coated with acetic acid collodion just described. The lymph was collected from the pad vesicles of guinea-pigs which had been infected by intradermal inoculation. In Exp. I an anticoagulant (Heparin) was used and the lymph was diluted 1 : 6 in $M/45$ buffered phosphate solution at *p*H 7·6. As the titre of this vesicle lymph was lower than usual, in Exp. II the vesicle fluid was defibrinated instead before being diluted 1 : 2 in the buffered phosphate solution. This material was centrifuged first in the Sharples-Super Centrifuge; the agar lining was extracted in fresh buffered phosphate solution. This procedure reduced the protein content sufficiently to allow of repeated washing on a graded collodion membrane of A.P.D. 25 mμ which has been shown previously is the filtration end-point for the virus of foot-and-mouth disease. Before the washing process on the graded collodion membrane, the agar extract was filtered through a membrane of A.P.D. 0·70μ to remove all traces of the gel. After two washings on the membrane

A.P.D. 25 mμ, a sample of the virus was removed from the filter and was found to give no reaction for protein with the sulphosalicylic acid test. A third washing of the virus was then made. Finally the washed virus was concentrated on filter candles coated with acetic acid collodion. In Exp. I titrations of the virus by inoculation of guinea-pigs were made at the different stages of purification to determine whether after any particular manipulation a drop had occurred, but this was not proved to be the case. This precaution was omitted in Exp. II and only the original vesicle lymph and the purified concentrate were titrated. The results are recorded in Table II. They show that it is possible

Table II. *Purification and concentration of the virus of foot-and-mouth disease by combined centrifugation and ultrafiltration methods*

	Original material	Titre	Titre of extract of agar-lining tube	Titre of filtrate of agar extract Membrane A.P.D. 0·70 μ	Titre of washed virus (3 washings) Membrane A.P.D. 25 mμ	Sulphosalicylic acid test on washed virus	Amount and titre of concentrated washed virus (collodion-coated filter candle)
Exp. I	24 c.c. of diluted vesicle lymph (1 : 6) in *M*/45 buffered phosphate at *p*H 7·6	10^{-4}	10^{-4}	10^{-4}	10^{-4}	Negative after 2nd washing	20 c.c. reduced to 0·2 c.c. 10^{-6}
Exp. II	10 c.c. of lymph diluted 1 : 2 in *M*/45 buffered phosphate at *p*H 7·6	10^{-6}	—	—	—	Negative after 2nd washing	10 c.c. reduced to 0·1 c.c. 10^{-8}

to make concentrates of purified virus giving a negative sulphosalicylic acid reaction in tests for protein and having a 100-fold higher titre than the original vesicle lymph. In the second experiment a purified concentrate was obtained with a titre of 10^{-8} from virulent vesicle lymph diluted 1 : 2 having a titre of 10^{-6}. The time necessary for such an experiment was about 12 hours. However, a purified concentrate having a titre of 10^{-8} by no means represents the limit of the efficiency of the combined methods of purification and concentration now available. In the experiments just referred to, only 5 c.c. of virulent vesicle lymph were available, whereas 20 c.c. could be dealt with in one spinning of the Sharples-Super Centrifuge, and by repeating the centrifugation while using the same agar lining the virus contained in 100 c.c. or more of undiluted vesicle lymph could be collected in about 5 c.c. of the gel. The feasibility of concentrating the virus contained in a large amount of liquid, of as high protein content as vesicle lymph, in a small quantity of agar-gel was demonstrated in the following experiment: 2 c.c. of vesicle lymph were diluted in 110 c.c. of cow serum. This virus suspension was centrifugalized at a low speed and then filtered through a sand and pulp filter to remove the coarser particles. A small sample of the virus was removed for subsequent tests for

virus potency. Five amounts of 20 c.c. were then centrifugalized successively for 30 min. in the Sharples cylinder lined with 5 c.c. of agar, the same gel being employed throughout. The supernatant fluid from each spinning was collected separately. Finally the agar-gel was extracted in fresh buffered phosphate solution. A comparison was then made, by titration on guinea-pigs, of the virus potency of the stock virus suspension, the third and fifth supernatant liquids and the agar extract. A hundredfold drop in titre of both the third and fifth supernatants was detected, i.e. in each case about 99 per cent of the virus had been thrown down. Further a comparison of the virus titre of the agar extract with that of the original virus suspension showed that a tenfold concentration of the virus had been effected, corresponding to the reduction of the volume of liquid from 100 to 10 c.c. (5 c.c. buffered phosphate solution and 5 c.c. of gel).

Thus, provided a sufficient amount of vesicle lymph or similar starting material be available, purified virus concentrates infective in a limiting dilution of 10^{-9}, or even higher, could be prepared.

Discussion

It has been shown that it is possible by filtration through filter candles coated with acetic acid collodion to reduce large quantities of virulent liquid to a very small amount rapidly and without serious interference by blocking effects. The degree of concentration of virus effected by this process was to the limit of expectancy, i.e. the increase in titre after concentration corresponded to the reduction of the volume of liquid. Further, by the use of the closed agar-lined cylinder of the Sharples-Super Centrifuge, the protein content of virulent vesicle lymph (diluted 1 : 2) could be reduced without loss of virus to a degree which allowed of continued purification by repeated washing on a graded collodion membrane without dilution. By a combination of the three methods, viz. washing once in the Sharples centrifuge, repeatedly on a graded collodion membrane of suitable porosity, A.P.D. 25 mμ, and final concentration on a filter candle coated with acetic acid collodion, a purified preparation was obtained having a titre of 10^{-8}, i.e. a limiting infective dilution a hundredfold higher than the original vesicle lymph. There is no previous record in the literature of foot-and-mouth disease of preparations of this virus content, much less in a purified state. It has been shown, however, in an experiment in which virus was suspended in blood serum that it is possible to concentrate the virus contained in a large amount of liquid of as high protein content as vesicle lymph in a small quantity of agar-gel. Therefore if a sufficient amount of starting material be available, purified virus concentrates infective in a limiting dilution of 10^{-9} or even higher could be prepared by the combined methods of purification and concentration now available. In Fig. 2 a suggested scheme for the purification and concentration of the virus of foot-and-mouth disease is set out.

A word should be added with regard to the use of the centrifugation technique, described in this paper, as a means of purifying virus. The method should be used only to reduce the protein content of virus suspensions to a point when they can be washed repeatedly on graded collodion membranes without dilution. In this way the use of the centrifuge speeds up the purification process. Although more complete purification of virus suspensions could be effected by repeated washing in the centrifuge, there is a possible danger of adsorption of the virus by the agar if the washing is carried too far. It has been shown previously by us, Schlesinger & Galloway (1937) that highly purified preparations of virus are adsorbed by the agar-gel.

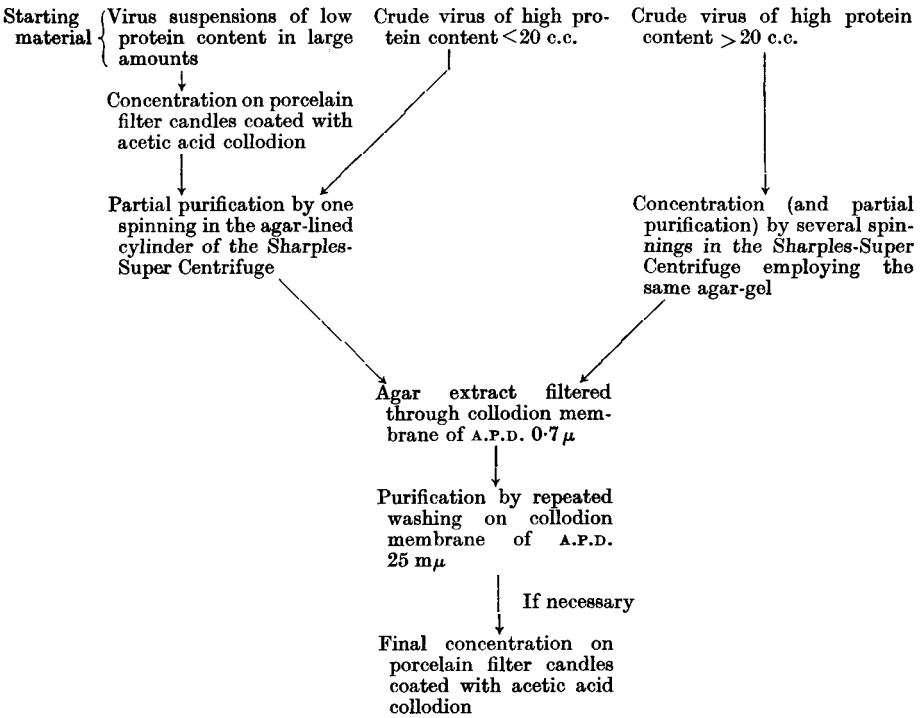

Fig. 2. Suggested scheme for the purification and concentration of the virus of foot-and-mouth disease.

SUMMARY

1. It is possible to concentrate the virus of foot-and-mouth disease on filter candles coated with acetic acid collodion. The degree of concentration of the virus effected was to the limit of expectancy, i.e. the increase in titre after concentration corresponded to the reduction of the volume of the liquid.

2. By a combination of the two methods of purification of the virus, viz. washing once in the closed agar-lined cylinder of the Sharples-Super Centrifuge

and repeatedly on a graded collodion membrane of A.P.D. 25 mμ, very potent purified concentrates of virus can be rapidly obtained, especially if the final concentration is made on filter candles, coated with acetic acid collodion. In this way a concentrate of virus with a titre of 10^{-8} and a negative sulphosalicylic acid test for protein has been prepared in one day from virulent vesicle lymph (diluted 1 : 2) having a titre of 10^{-6}.

3. The possibilities of the different methods of purification and concentration of the virus are discussed and a scheme suggested for their application.

REFERENCES

GALLOWAY, I. A. & ELFORD, W. J. (1936). *Brit. J. exp. Path.* **17**, 187.
SCHLESINGER, M. (1936). *Nature*, **138**, 549.
SCHLESINGER, M. & GALLOWAY, I. A. (1937). *J. Hygiene*, **37**, 445.

(*MS. received for publication* 9. II. 1937.—Ed.)