AN EVALUATION OF METHODS FOR THE CONCENTRATION AND PURIFICATION OF INFLUENZA VIRUS*

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The concentration and purification of influenza virus are of special importance in connection with studies on the fundamental chemical and physical properties of the virus itself. The lack of knowledge of such properties is emphasized at the present time by the disagreement concerning so simple and yet so fundamental a property as the true size of the infectious unit of influenza virus. For some years it was generally accepted that the virus was represented by a sphere about 80 to 120 mμ in diameter (1, 2). Recently, however, Chambers and coworkers (3) and Bourdillon (4) secured data, some of which reasonably could be interpreted as indicating that at least a portion of the virus possesses a size of the order of 10 mμ. Friedewald and Pickels (5) and Taylor and associates (6), on the other hand, consider, on the basis of recent experiments, that the size of the virus cannot be 10 mμ and is more nearly that accepted earlier. This general situation, which is illustrative of the lack of definite knowledge of the fundamental physicochemical properties of influenza virus, emphasizes the necessity for studies on the concentration and purification of the virus, for with the preparation of purified virus it should become possible to conduct experiments in which a given physical entity is correlated with virus activity. The direct positive correlation of virus activity with a given physical entity on fractionation of that entity by different procedures would provide good evidence that the preparation is essentially pure and hence suitable for studies on its fundamental physicochemical properties.

The concentration and purification of influenza virus also appear to be of importance in connection with the prevention or control of influenza. Numerous investigators (7–17) have shown that the administration of active or inactive virus results in an increase in circulating antibodies, and Francis (18, 19) has demonstrated that this increase is accompanied by an enhancement of the virus-inactivating capacity of the nasal secretions. Irrespective of the mechanism by means of which a solid immunity to influenza is achieved, the importance of securing concentrated virus preparations is indicated by the findings of Hirst and coworkers (20) that the average antibody response of human beings is

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directly related, though not strictly proportional, to the amount of virus administered. The widespread use of the chick embryo for the cultivation of viruses (21) and the current increase in the use of infectious materials from this source for human vaccination make it prudent to attempt to purify influenza virus obtained from chick embryos in order to avoid or reduce difficulties due to sensitization of human beings to chick embryo proteins. If not used directly, such concentrates could be used for the production of antisera which could be employed after the manner described by Smorodintseff and associates (22).

In 1936 Elford and Andrewes (2) demonstrated that influenza virus could be concentrated by high-speed centrifugation, and this process has subsequently been used for this purpose by other workers. Recently Hirst (23) and McClelland and Hare (24) showed that influenza virus in the chorioallantoic fluid of the chick embryo can be adsorbed by the red cells of the embryo or by adult chicken red cells. It was found that under the proper conditions much of the virus could be eluted from the red cells. These workers have also demonstrated that the precipitate which forms when infectious allantoic fluid is frozen and then thawed contains the bulk of the active virus (25, 26). Francis and Salk (27) have used adsorption on and elution from embryonic red cells as a means of effecting approximately a tenfold concentration of influenza virus. It appeared desirable to study each of these general methods in some detail in order to determine the relative efficiency of each and the nature of the purified materials yielded by the methods. The results of studies of the four general methods, together with a study of the concentration and purification of virus on a fairly large scale by means of centrifugation in a Sharples centrifuge are described in the present report.

Materials and Methods

Source of Virus.—The PR8 strain of influenza virus, generously supplied by Dr. T. Francis, Jr., of the University of Michigan, was used in the present work. When received in the form of frozen and dried allantoic fluid, the strain had been isolated from ferret 198, passed 70 times in mice, 717 times in tissue culture, and 23 times in chick embryos. It was passed twice in chick embryos in this laboratory, and a pool of allantoic fluid from the second passage was distributed in a large number of sealed ampules which were stored in a CO_2 ice box. At weekly or bimonthly intervals, single ampules of this stock inoculum were thawed, allowed to stand at room temperature for about 30 minutes with occasional stirring, and a 10^{-5} dilution was prepared with sterile 0.1 M phosphate buffer at pH 7. The residual stock inoculum was stored at 4°C. for subsequent use and 0.1 or 0.2 cc. portions of the diluted inoculum were injected through a small opening in the shell above the air sac into the allantoic sac of White Leghorn chick embryos brought to 10 days of age at 39°C. The infected embryos were incubated at 36°C. for 36 to 48 hours (28), then chilled for 2 or more hours at 4°C., and the allantoic fluid was harvested and used as starting material.

Chicken Red Cells.—Red cells obtained from adult chickens by severing the cervical vessels were prepared as described by Hirst and Pickels (29). The cells were stored

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at 4°C. in a 15 to 20 per cent suspension in saline and were used within 1 week of preparation.

Red Cell Agglutination Titrations.—The agglutination titrations were carried out by Hirst's method (29) as modified for use in this laboratory (30). Unfortunately, during the major portion of the present work a virus standard was not available for purposes of comparison, hence the values reported in this paper are not standardized.

Chick Embryo Titrations.—The titrations in chick embryos were carried out as described by Hirst (31), except that sterile 0.1 M phosphate buffer at pH 7 was used as a diluent in the place of saline (32).

Nitrogen Determinations.—Since essentially all of the nitrogen in solutions of materials sedimented two or more times at high speed was found to consist of protein nitrogen, total nitrogen determinations by the Nessler method (28) were made on such solutions and the values obtained were regarded as protein nitrogen values. In all other cases the samples were treated with an equal volume of hot 10 per cent trichloroacetic acid, then quickly cooled, and the precipitate collected by centrifugation. The precipitate was dissolved in a small volume of 0.2 N sodium hydroxide and precipitated with 5 per cent trichloroacetic acid. Analyses by the Nessler method were made on the washed precipitates. Because of the possibility that different preparations might contain different percentages of nitrogen, the concentrations of protein are usually expressed in terms of protein nitrogen. However, preliminary results indicate that approximate protein concentrations may be obtained from the protein nitrogen values by multiplying by the factor 10.

High-Speed Centrifugation.—Two vacuum centrifuges of the air turbine type described by Bauer and Pickels were used (33). The centrifuge heads, which were 8 inches in diameter, each accommodated 14 Lusteroid tubes 3 inches in length and 0.75 inch in diameter at an angle of 33° from vertical. About 10 to 15 minutes were required for acceleration or deceleration, and in the present experiments centrifugation was continued for 2 hours at 24,000 R.P.M. However, it should be noted that in subsequent experiments reported elsewhere (34) centrifugation for 20 to 30 minutes was found sufficient to sediment influenza virus. During the latter portion of the work, a commercially available Sharples laboratory centrifuge was used. The centrifuge was equipped with a celluloid liner and a cooling coil, and the experiments were conducted in a manner similar to that used in work with tobacco mosaic virus (35).

Sedimentation Constants.—Determinations of sedimentation constants of the purified virus preparations were kindly made by Dr. M. A. Lauffer and Mr. H. K. Schachman by means of a Bauer-Pickels type ultracentrifuge (36, 37) equipped with a Svensson-Philpot optical system (38, 39). The solutions used usually contained about 2 to 3 mg. of protein per cc. in 0.1 M phosphate buffer at pH 7. The constants corrected to water at 20°C. are given in Svedberg units. A Svedberg unit, symbolized by S, is a sedimentation rate of 10^{-13} cm. per second per unit centrifugal field.

EXPERIMENTAL

General Methods

Following numerous preliminary experiments on each of the general methods, a series of experiments was carried out in which portions of the same starting

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material were concentrated and purified by four general methods involving (*a*) differential centrifugation, (*b*) adsorption on and elution from adult chicken red cells, (*c*) elution of the precipitate formed on freezing and thawing of allantoic fluid, and (*d*) adsorption on and elution from embryonic chick red cells. In a typical test, 356 10-day chick embryos were each inoculated with 0.1 cc. of a 10^{-6} dilution of stock PR8 allantoic fluid. After 36 hours' incubation at 36°C., ¾ of the embryos were chilled for 4 hours and the allantoic fluid harvested, centrifuged at low speed for 5 minutes, and equal portions used for Methods A, B, and C. The remaining embryos were used for Method D.

Method A.—320 cc. of infectious allantoic fluid containing 0.128 mg. of protein nitrogen and 150 chick cell agglutination (CCA) units per cc. were centrifuged at 24,000 R.P.M. for 2 hours. The upper ⅔ of the supernatant liquid was removed and found to contain 0.118 mg. of protein nitrogen and four CCA units per cc. These results indicate that the protein represented by the 0.118 mg. of protein nitrogen per cc. which remained in the supernatant liquid contained less than 3 per cent of the CCA activity, whereas the protein represented by the 0.010 mg. of protein nitrogen per cc. which was sedimented contained over 97 per cent of the CCA activity. The pellets of sedimented protein were suspended in 35 cc. of 0.1 M phosphate buffer at pH 7 and centrifuged at a low speed of 3000 R.P.M. in order to remove about 10 mg. of insoluble protein which in 5 cc. possessed 200 CCA units per cc. The 35 cc. of supernatant liquid were again centrifuged for 2 hours at 24,000 R.P.M. The upper ⅔ of the supernatant liquid was removed and found to contain 0.024 mg. of protein nitrogen per cc. and no CCA activity. The sedimented protein was suspended in 5.4 cc. of buffer and on centrifuging at slow speed approximately 0.6 mg. of insoluble protein possessing no CCA activity was removed. The 5.4 cc. of supernatant liquid contained 0.272 mg. of protein nitrogen and 6100 CCA units per cc.; hence the purified material possessed a specific CCA activity of 22,500 units per mg.of protein nitrogen.[1] In the ultracentrifuge the material was found to be fairly homogeneous and to have a sedimentation constant of about 600 S. Based on the CCA activity of the starting material, about a 70 per cent recovery of this activity was achieved. The yield of this material was about 0.04 mg. per cc. of allantoic fluid.

Method B.—To 320 cc. of allantoic fluid similar to that used in Method A and cooled to 4°C. were added 14 cc. of a 23 per cent suspension of adult chicken red cells. After standing for 5 hours at 4°C., the preparation was centrifuged for 10 minutes at low speed to separate the red cells. The supernatant liquid was removed and found to have 46 CCA units per cc.; hence about 70 per cent of the original activity was adsorbed on the red cells. The red cells were eluted with 32 cc. of 0.1 M phosphate buffer at pH 7 for 2½ hours at 37°C. and then centrifuged at low speed. The eluate contained 0.208 mg. of protein nitrogen and 312 CCA units per cc. The cells were again treated

[1] This value and other values of 25,000, 22,000, and 20,600 CCA units per mg. of protein nitrogen recorded later in this paper are probably abnormally low, for in subsequent work values approximately 50 per cent higher have been obtained regularly for PR8 virus preparations obtained by differential centrifugation.

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in a similar manner to yield a second eluate which contained 0.008 mg. of protein nitrogen and 26 CCA units per cc. The supernatant allantoic fluid from the first treatment with cells was cooled to 4°C. and treated in a similar manner with fresh red cells. The supernatant liquid from these cells possessed 30 CCA units per cc. The eluate from these cells contained 0.099 mg. of protein nitrogen and 160 CCA units per cc.

Method C.—The remaining 320 cc. portion of allantoic fluid was transferred to celluloid centrifuge tubes of 90 cc. capacity, frozen overnight in a CO_2 ice box, and then thawed at room temperature during the course of about 2½ hours. Care was taken that the temperature of the liquid did not rise above about 3°C. The fluid was centrifuged at 3000 R.P.M. in a cold room and the precipitate was collected and extracted with 32 cc. of 0.1 M phosphate buffer at pH 7 for 30 minutes at about 23°C. Following centrifugation at low speed, the residual precipitate was again extracted as described above to yield a second extract. The first extract contained 0.138 mg. of protein nitrogen and 1034 CCA units per cc. The second extract contained 0.024 mg. of protein nitrogen and 66 CCA units per cc. The residual insoluble material following the two extractions contained 2.4 mg. of nitrogen and practically no CCA activity. The supernatant allantoic fluid from the original precipitate contained 9 CCA units per cc. In other comparable experiments this fraction was found to contain 10 to 20 per cent of the original activity.

Method D.—The remaining 89 infected embryos were taken directly from the incubator following 36 hours' incubation, opened, and the blood vessels torn as described by Francis and Salk (27). The bloody allantoic fluid was harvested within a few minutes and collected in iced centrifuge tubes. The 570 cc. of fluid which were obtained were allowed to stand at 4°C. for 5 hours and then centrifuged at low speed in the cold room. The embryonic red cells were eluted twice for 2½ hours at 37°C. with two 57 cc. portions of buffer. The final volume of embryonic red cells was 3.5 cc.; hence the effective concentration of red cells in the original allantoic fluid was at least 0.62 per cent. The first and second eluates of the embryonic red cells contained 0.173 and 0.032 mg. of protein nitrogen and 1038 and 170 CCA units per cc., respectively.

The supernatant liquid from the original allantoic fluid, after adsorption with embryonic cells, was found to possess 76 CCA units per cc. In order to recover this activity, the preparation was treated with 25 cc. of a 23 per cent suspension of adult chicken red cells for 5 hours at 4°C. The red cells were then removed by centrifugation at low speed and eluted with 57 cc. of buffer for 2½ hours at 37°C. The eluate contained 0.128 mg. of protein nitrogen and 420 CCA units per cc. The supernatant liquid from these cells possessed 26 CCA units per cc.

Further Purification by Differential Centrifugation of Products Yielded by Methods B, C, and D.—The most active fractions yielded by Methods B, C, and D possessed 1600, 7500, and 6000 CCA units, respectively, per mg. of protein nitrogen, whereas Method A gave a product possessing 22,500 CCA units per mg. of protein nitrogen. It is obvious therefore that Method A yielded a product which per milligram of protein nitrogen possessed three or more times the CCA activity of the products obtained by the other methods. Since the

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low specific activity of the materials yielded by Methods B, C, and D could be due to the presence of large amounts of inactive low molecular weight protein, it appeared desirable to subject these preparations to further purification by differential centrifugation.

Accordingly, the first eluates from the first and second treatments with red cells in Method B were combined to give 64 cc. of a solution containing 0.154 mg. of protein nitrogen per cc., and this was centrifuged for 2 hours at 24,000 R.P.M. The supernatant liquid contained 0.128 mg. of protein nitrogen per cc. and possessed no CCA activity. The pellets of sedimented protein were dissolved, sedimented at low speed, and then at high speed, to yield a final preparation of 1.4 cc. containing 0.224 mg. of protein nitrogen and 5600 CCA units per cc. The specific CCA activity of this material was therefore 25,000 units per mg. of protein nitrogen. The product was found to have a sedimentation constant of about 600 S. The over-all yield of purified material in this experiment is probably abnormally low, since in comparable experiments yields two or three times larger were obtained. However, in general, adsorption on and elution from adult chicken red cells has been found to give low yields of purified material. The fact that the combined eluates from the red cells contained about 1 mg. of protein per cc. which was not sedimented and possessed no CCA activity indicates that such eluates of red cells can contain over 80 per cent of non-virus protein. This large amount of non-specific protein which arises from the allantoic fluid or the red cells could be used as an argument against the direct use of such eluates as vaccines.

The 32 cc. of first extract of the precipitate obtained in Method C, which contained 0.138 mg. of protein nitrogen and 1034 CCA units per cc., were centrifuged for 2 hours at 24,000 R.P.M. The upper ⅔ of the supernatant liquid was found to contain 0.106 mg. of protein nitrogen per cc. and no CCA activity. The pellets of sedimented protein were dissolved in buffer, centrifuged at low speed, and then at high speed for 2 hours to yield a final preparation of 2.5 cc. containing 0.40 mg. of protein nitrogen and 8800 CCA units per cc. The specific CCA activity was therefore 22,000 units per mg. of protein nitrogen. The yield of this material, which was found to have a sedimentation constant of about 600 S, was approximately 0.025 mg. per cc. of allantoic fluid used as starting material. The recovery of CCA activity based on that of the allantoic fluid was about 50 per cent.

The first eluate from the embryonic red cells and the eluate from the adult chicken cells which were obtained in Method D were combined to yield a solution containing 0.150 mg. of protein nitrogen per cc. and subjected to two differential centrifugation cycles. The upper ⅔ of the supernatant liquid from the first high-speed centrifugation contained 0.133 mg. of protein nitrogen and two CCA units per cc. The final preparation of 5 cc. contained 0.513 mg. of protein nitrogen and 10,600 CCA units per cc.; hence, the specific CCA activity was 20,600 units per mg. of protein nitrogen. The material was found to have a sedimentation constant of about 600 S. The recovery of CCA activity was about 60 per cent and the yield of purified material was 0.036 mg. per cc. of allantoic fluid used as starting material.

The results provide convincing evidence that the products yielded by Methods B, C, and D contain large amounts of inactive protein of low molecular

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weight, for in each instance a single sedimentation at 24,000 R.P.M. gave a supernatant liquid possessing little or no CCA activity but containing about 80 per cent of the protein. In experiments conducted in connection with other studies, the removal of CCA activity from the supernatant fluid on high-speed centrifugation was found to be accompanied by a corresponding removal of infectivity as measured in mice or in chick embryos (34). The removal of the inactive protein was accompanied by an increase in the specific CCA activity of the material obtained in the sedimented pellets, for following two differential centrifugation cycles the purified products possessed 25,000, 22,000, and 20,600 CCA units, respectively, per mg. of protein nitrogen. Since all gave a fairly homogeneous boundary in the ultracentrifuge and possessed a sedimentation constant of about 600 S, they appear to be quite comparable to the product yielded by Method A. However, in view of the different procedures used to obtain the final purified preparations and in order to secure additional evidence correlating CCA activity with virus activity, the preparations were titrated in chick embryos by the method described by Hirst (31). Dilutions containing from 10^{-11} to 10^{-15} gm. of the purified preparations per 0.1 cc. in 0.1 M phosphate buffer were prepared and 0.1 cc. portions were used for each of five embryos at each dilution. The 50 per cent infectivity end points of the final preparations obtained by Methods A, B, C, and D, including the two differential centrifugation cycles, were $10^{-14.2}$, $10^{-14.3}$, $10^{-13.8}$, and $10^{-13.5}$ gm., respectively.

Concentration and Purification by Means of the Sharples Centrifuge

The ease with which an appreciable degree of concentration and purification of influenza virus was achieved by means of differential centrifugation demonstrates the advantage of employing this process. However, because of the limitations of the vacuum type high-speed centrifuge for large scale work, it appeared desirable to determine if the Sharples laboratory centrifuge in which a continuous flow is used could be employed for the centrifugation of virus in allantoic fluid.

Accordingly, 1500 cc. of freshly harvested PR8 allantoic fluid clarified by centrifugation at low speed for 5 minutes and possessing 287 CCA units per cc. were passed at a rate of about 15 cc. per minute through a cooled Sharples centrifuge equipped with a bowl operating at 50,000 R.P.M. and containing a celluloid liner. The liner was removed and the thin white film of material was dissolved in the bowl contents and centrifuged at low speed to remove insoluble matter. The 300 cc. of clarified solution were found to contain 1000 CCA units per cc., whereas the effluent of the Sharples centrifuge contained but 62 CCA units per cc. The recovery in the concentrate of 70 per cent of the CCA activity originally present in the allantoic fluid demonstrates that the Sharples centrifuge may be employed profitably for the concentration and purification of influenza virus in allantoic fluids.

In a large scale experiment, the Sharples centrifuge was employed with a concentrate

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of PR8 influenza virus obtained from allantoic fluid by the freezing and thawing method. Lots of 500 cc. or more of freshly harvested PR8 allantoic fluid were clarified by centrifugation at low speed for 5 minutes, then frozen in a CO_2 ice box and stored at $-12°$ or $-70°$C. for 2 to 90 days. The frozen fluids were removed at the same time and allowed to thaw. Care was taken that the temperature of the fluids did not rise above about 3°C. The cold fluids, which totaled 13.3 liters in volume, were then filtered with suction during the course of 4 hours in a cold room on two Buchner funnels containing single layers of No. 1450½ C. S. and S. filter paper. The precipitate was suspended in 1650 cc. of 0.1 M phosphate buffer at pH 7. After standing for 30 minutes, the preparation was clarified by centrifugation at low speed for 5 minutes. The supernatant liquid was found to contain 376 CCA units per cc. and 0.79 mg. of nitrogen per cc., of which 0.17 mg. per cc. was protein nitrogen. The precipitate was again extracted with 350 cc. of buffer and this extract was found to contain 252 CCA units per cc. and 0.74 mg. of nitrogen per cc., of which 0.10 mg. per cc. was protein nitrogen. Since the CCA activity of all lots of allantoic fluid making up the pool was not determined, it is not known whether the low CCA activity of the extracts was due to poor allantoic fluid or to a subsequent loss of virus. It was found that small volumes of allantoic fluid passed the No. 1450½ C. S. and S. filter paper without significant loss of CCA activity. The combined extracts, which contained about 700,000 CCA units and about 2 gm. of protein, were passed through a cooled Sharples centrifuge at a rate of 15 cc. per minute, as described in the preceding experiment. Then, without stopping the centrifuge, 500 cc. of 0.1 M phosphate buffer were passed into the centrifuge at the same rate. The effluent from the centrifuge was found to contain 62 CCA units per cc. and 0.62 mg. of nitrogen per cc., of which 0.08 mg. was protein nitrogen. The thin film, about 1 mm. thick, of material on the celluloid liner was suspended in the 270 cc. of 0.1 M phosphate buffer contained in the bowl and much insoluble matter was removed on centrifuging at low speed for 5 minutes. The precipitate was extracted with 50 cc. of buffer and the combined extracts were found to contain 676 CCA units, 0.42 mg. of total nitrogen, and 0.064 mg. of protein nitrogen per cc. Although the centrifugation permitted a reduction in protein from about 2000 mg. to 164 mg., the recovery of CCA activity was only about 30 per cent, or about one-half that achieved in the preceding experiment in which allantoic fluid was centrifuged directly. The results indicate that the efficiency is decreased considerably when concentrates obtained by the freezing and thawing method are used in place of untreated allantoic fluid in the Sharples centrifuge. Such concentrates contain large amounts of non-protein nitrogen as well as extraneous protein which on centrifugation form large precipitates, and much CCA activity appears to be lost on this insoluble matter.

In a third experiment 500 chick embryos, incubated for 10 days at 39°C., were each inoculated with 0.2 cc. of a 10^{-5} dilution in 0.1 M phosphate buffer of stock PR8 allantoic fluid and then further incubated at 36° for 40 hours. The embryos were chilled at 4° for 6 hours and the allantoic fluid was then harvested. The 3000 cc. of turbid and slightly red-colored fluid, which were found to contain 240 CCA units and 0.383 mg. of protein nitrogen per cc., were clarified by passage through a cooled Sharples centrifuge at a rapid rate of about 180 cc. per minute. Then 275 cc. of 0.1 M phosphate buffer at pH 7 were passed into the centrifuge at the same rate. The 2900 cc. of effluent liquid which were obtained were found to contain 110 CCA units and 0.346

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mg. of protein nitrogen per cc.; hence, over 50 per cent of the CCA activity was removed despite the high rate of flow of the allantoic fluid. It seemed possible that adsorption of virus on the few embryonic red cells present might be responsible for a large part of the decrease in CCA activity. Accordingly, the material on the celluloid liner was suspended in the 260 cc. of fluid contained in the centrifuge bowl and allowed to stand at 37° for 90 minutes. The preparation was then spun at 3000 R.P.M. in a clinical centrifuge for 5 minutes to yield an opalescent supernatant liquid which was found to contain 1526 CCA units and 0.171 mg. of protein nitrogen per cc. Practically all of the CCA activity removed from the allantoic fluid on passage through the Sharples centrifuge at 180 cc. per minute was thus recovered.

The 2900 cc. of effluent liquid obtained from the run at 180 cc. per minute were passed into the Sharples centrifuge at a rate of about 15 cc. per minute and followed by 275 cc. of 0.1 M phosphate buffer. The effluent liquid was found to contain 8.8 CCA units and 0.336 mg. of protein nitrogen per cc.; hence, the sedimentation of 0.01 mg. of protein nitrogen per cc. was accompanied by the removal of over 90 per cent of the CCA activity. The material on the celluloid liner was dissolved in the contents of the centrifuge bowl and made up to 600 cc. After clarification in a clinical centrifuge for 5 minutes the solution was found to contain 320 CCA units and 0.084 mg. of protein nitrogen per cc. This solution was combined with the 260 cc. of extract of material obtained from the run at 180 cc. per minute and then passed through the Sharples centrifuge at a rate of about 15 cc. per minute. The preparation was followed by 275 cc. of 0.1 M phosphate buffer. The 800 cc. of effluent liquid were found to contain 41 CCA units and 0.040 mg. of protein nitrogen per cc. The loss of CCA activity in the effluent liquid was therefore less than 10 per cent of that contained in the 860 cc. which were passed into the centrifuge. The material on the celluloid liner was dissolved in the liquid contained in the centrifuge bowl and the preparation was clarified by centrifugation for 5 minutes in a clinical centrifuge. The 275 cc. of opalescent fluid which were obtained were found to contain 1800 CCA units and 0.088 mg. of protein nitrogen per cc. The results indicate that, in each of the two centrifuge runs at a flow rate of about 15 cc. per minute with allantoic fluid and with a concentrate, the loss of activity in the effluent liquid was less than 10 per cent and that about 70 per cent of the CCA activity present in the original allantoic fluid was recovered in the final preparation. The material in the final preparation was found to contain a component with a sedimentation constant of about 600 S, but, since much of the material sedimented more rapidly than the 600 S component, the preparation apparently contained considerable aggregated matter. However, the specific CCA activity of about 21,000 units per mg. of protein nitrogen, which was possessed by the final preparation, indicates that a marked degree of purification was effected by means of the Sharples centrifuge.

DISCUSSION

High-speed centrifugation of infectious allantoic fluid appears to result in the almost quantitative removal of CCA activity from the supernatant fluid, whereas treatment with adult or embryonic chicken red cells or the freezing and thawing method effects only a 50 to 90 per cent removal of CCA activity. A second elution of red cells or of the precipitate obtained on freezing and thawing

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or a second treatment of the supernatant allantoic fluid with fresh red cells or by the freezing and thawing method yields only a small amount of activity; hence, such additional processing of allantoic fluid appears to be of no practical importance. Since the eluates of red cells and the extract of the precipitate obtained on freezing and thawing of allantoic fluid contained over 80 per cent of non-specific protein, it is obvious that such eluates or extracts should not be used directly for vaccination purposes or for studies on the physicochemical properties of influenza virus. Differential centrifugation provides a direct means of obtaining a concentrated and purified preparation, presumably free of large amounts of inactive protein, and therefore appears to represent the method of choice for the large scale preparation of purified virus for purposes of vaccination or for the study of physicochemical properties. This method can, of course, be used in conjunction with the other methods and when so used appears to yield a uniform product with respect to particle size and CCA activity. The over-all yield based on the CCA activity of the allantoic fluid used as starting material was about 70 per cent when differential centrifugation was employed alone and about 50 per cent when used in conjunction with concentrates obtained by the freezing and thawing method or by adsorption on embryonic red cells. Somewhat smaller over-all yields were obtained when adsorption on red cells after the manner described in Method B was included as a step in the purification process.

It may be noted that in experiments reported in another paper (34) centrifugation at 24,000 R.P.M. for 20 minutes rather than for the 2 hours used in the present work was found to be sufficient to effect almost quantitative sedimentation of the virus and CCA activities. It is possible to prepare fairly large amounts of purified virus by means of one or two centrifuges of the vacuum type, especially in view of the decreased length of time of centrifugation that is required. However, for the preparation of purified virus on a very large scale, it appears that the Sharples centrifuge may be preferable despite the fact that in the present work, as in earlier studies on tobacco mosaic virus (35), the Sharples centrifuge was found to be somewhat less efficient than the vacuum type centrifuge. The removal of about 80 per cent of the CCA activity from the effluent liquid from the Sharples centrifuge and the recovery in the extract of the material on the centrifuge liner of about 70 per cent of the original activity in the allantoic fluid at a flow rate of about 1 liter an hour, which were achieved in the present work, provide an idea of the efficiency and capacity of the equipment.

SUMMARY

The concentration and purification of influenza virus by means of differential centrifugation in a vacuum type centrifuge, by adsorption on and elution from adult chicken red cells, by elution of the precipitate formed on freezing and

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thawing of allantoic fluid, by adsorption on and elution from embryonic chick red cells, and by combinations of the first method with each of the three succeeding methods, have been studied. Over-all yields of virus of about 50 to 70 per cent were obtained by these methods and combinations of methods except for somewhat lower yields when adsorption on and elution from adult chicken red cells was employed. However, the purified products obtained by methods involving only the use of red cells or the freezing and thawing technique were found to contain about 80 per cent of non-virus protein. The purified products obtained when differential centrifugation was used either alone or in combination with any one of the other methods were found to be indistinguishable and to consist of a fairly homogeneous component having a sedimentation constant of about 600 S. Such preparations possessed about 22,000 chicken red cell agglutinating units per mg. of protein nitrogen and solutions containing only about 10^{-14} gm. of the materials gave 50 per cent infectivity end points in chick embryos. The Sharples centrifuge was found to be almost as efficient as the vacuum type centrifuge for the concentration and purification of influenza virus and, because of its larger capacity, is recommended for the preparation of purified virus on a large scale.

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