# A SEDIMENTABLE COMPONENT OF ALLANTOIC FLUID AND ITS RELATIONSHIP TO INFLUENZA VIRUSES*

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PLATE 6

The isolation and purification of animal viruses has been considerably complicated in many cases by the apparent inability to separate cleanly the disease agent from cellular debris or to distinguish it chemically and physically from "heavy" components of normal tissues (1–3). These difficulties are serious in that they impede the characterization of such disease agents, and render chemical and physical analyses ambiguous. Recently it has been shown that each of several strains of influenza virus can be concentrated and purified by differential centrifugation of the allantoic fluids of infected chick embryos (4–11). Such preparations are remarkable for their high infectivities and apparent low content of extraneous protein or cellular debris. While it has been reported that normal chick embryo tissue contains extractable macromolecular components (13, 14) there has been no description of macromolecular material in normal allantoic fluid. Recently, during experiments with normal allantoic fluid, it was observed that a measurable amount of proteinaceous material was sedimented under conditions comparable to those employed earlier in the isolation of influenza viruses. Therefore, the present investigation was undertaken to determine the yield under various conditions and some of the chemical, physical, and serological properties of the material obtained by high speed centrifugation of normal allantoic fluid. It appeared that such a study might help in the evaluation of the nature and purity of preparations of influenza virus obtained under similar conditions from infectious allantoic fluid and in the eventual development of methods for the production of pure influenza virus.

## EXPERIMENTAL

*Preparation of Material.*—Normal embryonated white Leghorn eggs, incubated for 10 to 14 days at 39°C. were chilled overnight at 4° to minimize bleeding during the removal of the allantoic fluids. The allantoic fluids were harvested in the customary manner and centrifuged for 5 to 10 minutes in an angle centrifuge at about 4000 R.P.M. The clarified fluid was then spun in a Bauer-Pickels type air-driven high-speed centrifuge at 24,000 R.P.M. for about

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90 minutes. The small gelatinous pellets thus obtained were suspended in 0.1 M phosphate or borate buffer at pH 7, or in distilled water, depending upon the subsequent use of the material. Insoluble matter was removed by low-speed centrifugation and the cycle of high- and low-speed centrifugation was repeated one or more times. This procedure was modified slightly in some cases.

The PR8 and the Lee strains of influenza virus were cultured in embryonated eggs which had been incubated for 10 days at 39°C. Both strains were originally supplied through the generosity of Dr. T. Francis, Jr., of the University of Michigan, and their identities have been checked subsequently by means of red cell agglutination inhibition tests (15, 16). Following inoculation, the eggs were incubated for 48 to 72 hours at 36°C., chilled overnight at 4°, and the allantoic fluids were harvested in the customary manner. Purified preparations of virus were obtained from the allantoic fluids by the centrifugation procedure described by Stanley (10). A 20 minute period of high-speed centrifugation was used in most cases.

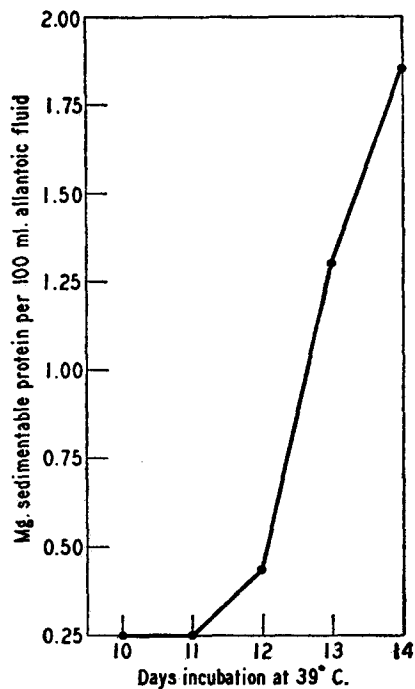### Relation of Yield of Material to Age of Embryo

Ordinarily 9 to 12 day old embryos are employed for culturing or testing strains of influenza virus and the inoculated eggs are incubated for an additional 24 to 72 hours before harvesting the allantoic fluids. Thus the embryos generally used come within the age range of 10 to 14 days. Hence, allantoic fluids were harvested from groups of 10 to 14 day old embryos and the sedimentable material was removed by 2 to 3 centrifugation cycles as described under Preparation of material.[1] The amounts of protein obtained were estimated from nitrogen values obtained by the Nessler method (17) by use of the factor 10. In some cases, 200 eggs were employed and 40 eggs were chilled and harvested on each of 5 days beginning with the 10th day of incubation. In other instances, larger groups were harvested on one of the days between the 10th and 14th. Protein values representing from 4 to 10 groups of eggs were averaged to give a value for each day.

The relation of yield to the age of the embryo is shown graphically in Text-fig. 1. It is apparent that the amount of sedimentable protein in normal allantoic fluid through 12 days of incubation is small compared to that of embryos infected with the PR8 strain of influenza virus, since 8 to 16 times as much protein can be isolated from the latter (9). This fact is in accord with the results of Chambers, Henle, Lauffer, and Anderson (18) who found that centrifugation of small volumes of allantoic fluid of normal 11 day embryos failed to sediment a measurable amount of protein. It should be noted that the values shown in Text-fig. 1 probably represent minimum ones, for the losses involved in the centrifugation of such small amounts of material can be considerable. It can be seen from Text-fig. 1 that on the 13th day the yield of sedimentable protein rises sharply and on the 14th day reaches a value of about 0.02 mg. per ml. of allantoic fluid. After 14 days, the embryos are so large and the urates and similar products so plentiful that it becomes impractical to harvest the allantoic fluid cleanly.

[1] Although the starting fluid is referred to throughout this paper as allantoic fluid, it should be noted that little attempt was made to exclude an occasional harvest of amniotic fluid. Since the sedimentable material had the general character of and gave common color tests for protein, it was assumed to be composed at least in part, of protein and is, therefore, referred to as the normal protein.

*Agglutination and Infectivity Tests*

The purified normal protein showed no red cell agglutinating activity under the test conditions ordinarily employed in this laboratory (19) even when used at a concentration of 1 mg. per ml. Nitrogen analyses on the supernatant fluids of normal protein-chicken red cell mixtures indicated that no adsorption of normal protein by the red cells occurred either at room temperature or at



TEXT-FIG. 1. The relation of yield of sedimentable protein from normal allantoic fluid to age of embryo.

4°. This was also true of the small components isolated from virus preparations which will be described later. The material obtained from chicken red cells by incubating at 37° in 0.1 M phosphate buffer at pH 7 for about 5 hours did not react visibly with antiserum to the normal protein. Also the normal protein failed to exhibit any signs of infectiousness when instilled into mouse lungs or inoculated into chick embryos at $10^{-3}$ to $10^{-6}$ gm. per ml.

*Relation of Time of Centrifugation to Yield of Normal Protein*

It has been shown that high speed centrifugation of infectious allantoic fluid for 20 to 30 minutes will sediment the PR8 virus activity almost quantitatively

(6, 10, 11). Therefore, it was considered of interest to determine how much normal protein would sediment in 30 minutes as compared with the yield obtained in the usual 90 minute period.

Allantoic fluid from 14 day embryos was divided into 2 lots. One lot was subjected to four successive 30 minute and the other to four successive 90 minute periods of high-speed centrifugation. After each centrifugation the supernatant liquids were immediately decanted and the pellets were taken up in equal volumes of 0.1 M phosphate buffer at pH 7. In the first of 2 experiments, 1.5 times as much material was obtained at 90 minutes as at 30 minutes and in the second test, 2.5 times as much. In each case, only one-fourth to one-fifth the amount of material was obtained after 4 centrifugations as was obtained after 2 centrifugations. This loss of material accompanying repeated centrifugation has also been observed with preparations of PR8 virus.

It is clear that appreciable amounts of normal protein sediment to form pellets during 30 minutes at 24,000 R.P.M.

### Effect of Freezing and Thawing on Yield of Normal Protein

In the preparation of plant viruses, it was found that freezing infected plants or infective juice denatured normal proteins and hence permitted a ready separation of these materials from the relatively unaffected virus proteins (1). The effect of freezing and thawing on the normal protein in allantoic fluid was tested in the following manner:—

About 400 ml. of normal allantoic fluid were centrifuged in an angle centrifuge at 5000 R.P.M. for 5 minutes. The clear supernatant fluid was divided into 2 equal volumes. One lot was stored at 4°, and the other was frozen and stored overnight at −12° and thawed on the following day. A fluffy white precipitate appeared in the thawed fluid which failed to dissolve readily even when the fluid reached room temperature. Both lots of fluid were centrifuged in an angle centrifuge at 5000 R.P.M. for 5 minutes and the clear supernatant fluids were subjected to 2 cycles of differential centrifugation. The precipitate obtained upon angling the frozen and thawed fluid was extracted once with 0.1 M phosphate buffer and the extract was also subjected to 2 cycles of differential centrifugation. The pellets obtained in the high speed centrifugations were dissolved each time in 0.1 M phosphate buffer. After 2 cycles of centrifugation each fraction was analyzed for protein. In 2 experiments, the yields of sedimentable protein obtained from frozen and thawed fluids were 50 and 60 per cent, respectively, of those obtained from unfrozen fluids. However, these yields were raised to 83 and 90 per cent when augmented by the protein recovered by extraction of the precipitate in the thawed fluids. The latter procedure appeared to be justified since the extracted protein proved to be practically indistinguishable serologically from that obtained directly by centrifugation of unfrozen fluid.

It appears that the sedimentable protein of normal allantoic fluid is not appreciably affected by freezing and thawing under the conditions just described.

### Sedimentation Constant

Determinations of sedimentation constants of the purified normal protein preparations were kindly made by Dr. M. A. Lauffer and Mr. H. K. Schachman by means of a Bauer-

Pickels type ultracentrifuge equipped with a Svenssen-Philpot optical system. Eight preparations, containing 2 to 12.8 mg. of protein per ml., were examined. Seven of these were in 0.1 M phosphate buffer, and the eighth was in 0.1 M borate buffer, all at pH 7.

The sedimentation constants obtained are listed in Table I and can be compared with the values of 600 to 800 reported for PR8 virus particles (6, 9, 12). A single boundary, which was usually quite sharp, was observed with preparations containing 2 to 3 mg. of protein per ml. However, with more con-

TABLE I

*Sedimentation Constants of the Normal Protein in 0.1 M Phosphate Buffer at pH 7*

| Preparation | Mg. per ml. | $s_{20}^0$ |
|---|---|---|
| 1 | *ca.* 2 to 3 | 28 |
| 2 | " | 34 |
| 3 | " | 54 |
| 4* | " | 58 |
| 5 | 5<br>2.5 | 33 and 58<br>*cir.* 90 |
| 6 | 5<br>2.5 | 20 and 51<br>25 and 125 |
| 7 | 6.6<br>2.2 | 19 and 38<br>30 and 121 |
| 8 | 12.8<br>6.4<br>3.2 | 13 and 16<br>17 and 36<br>36 and 65 |

* In 0.1 M borate buffer at pH 7.

centrated preparations, double boundaries were almost invariably observed and these usually persisted upon dilution of the concentrates. As can be noted in Table I, there is a pronounced dependence of sedimentation constant on concentration of material. This and the fact that solutions of the material have been found to possess a very high intrinsic viscosity (30) can be regarded as evidence that the particles of the normal protein are anisometric like those of tobacco mosaic virus (20). However, preliminary examinations of such preparations with the electron microscope have revealed a preponderance of essentially spherical particles (Fig. 1) with only occasional suggestive rod-like fragments of material. It is probable that anisometric particles are present

but are too small in one dimension to be distinguished readily by means of the electron microscope.   In any event, it should be noted that the unusually large intrinsic viscosity of the normal material (190 to 270) can account, at least in part, for the multiple sedimentation values listed in Table I since these were not corrected for the viscosity of the preparations.   When such corrections are made, apparent differences in sedimentation constants tend to vanish.   For example, a fresh preparation of normal material in 0.1 M phosphate buffer was sedimented in the analytical ultracentrifuge at concentrations of 5.3 and 2.65 mg. per ml., respectively, and viscosity determinations were made.   Double boundaries were observed in the centrifuge with components corresponding to 21 S and 41 S, and 38 S and 83 S for the larger and smaller concentrations of material, respectively.   However, when these values were corrected for viscosity (30) they became 85 and 170, and 77 and 169, respectively.   The major component, constituting an estimated 80 per cent of the material in this case, was that with a corrected constant of 170 S.

## Electron Micrographs

Electron micrographs of typical preparations of purified normal protein and of purified PR8 virus were made through the courtesy of the RCA Laboratories at Princeton with the kind assistance of Dr. James Hillier.   Material was generally applied to the mounts at a concentration of about $10^{-4}$ gm. per ml.   In one instance, the normal material was examined at $10^{-3}$ gm. per ml.

The micrograph of the material examined at $10^{-3}$ gm. per ml. shows too dense a concentration of material (Fig. 1) to distinguish individual particles, but there are in a few places definite indications of material drawn out in fibrous strands. At a lower concentration of material such strands are no longer apparent and spherical images like those shown in the central picture of Fig. 1 are predominant.   Measurements of the images of 245 such particles indicated that 39 had diameters of about 40 m$\mu$, 52 had diameters of about 20 m$\mu$, and 154 had diameters of about 10 to 15 m$\mu$.   This indicates that approximately 80 per cent by weight of the visible particles are represented by those having a diameter of about 40 m$\mu$.   If these particles correspond with the major component of the sedimentation studies, it is necessary to assume that the anisometric particles presumed to be responsible for the high viscosity of preparations of normal material constitute a small additional fraction whose particles are not readily distinguished in the electron microscope.   The micrograph of the preparation of PR8 virus shows particles of somewhat greater uniformity in size and measurement of the diameters of 31 of the most distinct particles yields an average figure of about 100 m$\mu$.

## Chemical Tests and Analyses

Elementary analyses were made by Dr. A. Elek of The Rockefeller Institute on a typical dried preparation of normal protein and on two similar preparations of PR8 influenza virus.

Both materials were obtained by subjecting the appropriate allantoic fluids to 2 centrifugation cycles. The pellets were suspended in distilled water after each centrifugation and the final solutions were frozen and dried *in vacuo* to give white fluffy material. This was further dried *in vacuo* over $P_2O_5$ at 60–100°C. before analyses were made. Some additional phosphorus analyses were made on solutions and dried preparations of normal protein and of virus by the colorimetric method of King (21). Qualitative tests for pentose and desoxypentose were made with Bial and Dische reagents respectively (22, 23) and quantitative estimates of carbohydrate were obtained by the method of Tillmans and Philippi (24). An estimate of total lipid was made by extraction of 15 to 50 mg. samples with 3:1 alcohol-ether, and of phospholipid, by extraction of similar samples or of the residues from the alcohol-ether extractions with 30–60° petroleum ether.

Too few analyses have been made for a conclusive comparison of the chemical compositions of normal protein and PR8 virus. However, it is apparent from data obtained in the present analyses that there is a marked similarity in the compositions of current preparations of the two materials. Both contain about 52 per cent of carbon, 7.7 per cent of hydrogen, 10 per cent of nitrogen, 1 per cent of phosphorus, 1 per cent of sulfur, 25 per cent of alcohol-ether extractable and 15 per cent of petroleum ether extractable material, and 6 per cent of carbohydrate. Preparations of both materials, either in solution or in the dry state, gave positive Bial reactions. In this respect, the preparations of PR8 virus differ from those described by Taylor and coworkers (6). Positive Dische tests for desoxypentose were obtained with both normal protein and virus when 10 to 20 mg. were used, and the results of the test were particularly unequivocal when made on fat-free material. These results suggest that preparations of the normal protein and of the virus are composed in part of nucleoprotein, although nucleic acid has not yet been isolated from either material. Further investigations of the chemical compositions of the normal protein and of preparations of various strains of influenza virus are now in progress.

### Isoelectric Point

The isoelectric point of a typical preparation of the normal protein was kindly determined by Dr. G. L. Miller using a microelectrophoretic technique (25). The value obtained was at pH 2.3, which is considerably more acid than the values around pH 5 found for mouse lung or chick embryo preparations of PR8 virus (25, 26). The small components separable from preparations of the F12 and Lee strains of influenza virus by centrifugal methods (10, 29) were found to behave electrophoretically as though they were identical with the normal protein.

### Serological Tests

Antisera to preparations of the normal protein and to similar preparations of PR8 virus were obtained from the blood of rabbits 8 to 10 days after the last of five spaced intravenous

injections of a total of 0.1 to 25 mg. of material.[2] Semiquantitative precipitin tests were made as follows: 0.3 ml. of antigen at various dilutions was added to tubes containing 0.3 ml. of antiserum diluted 1:2. All dilutions were made with 0.85 per cent sodium chloride. The solutions were mixed, then incubated at 37° for 3 to 4 hours, placed in a refrigerator overnight, and examined for precipitates. Quantitative precipitin tests were made employing techniques described by Heidelberger (27). In these tests, all components were cooled to 4°C. before mixing and were stored at 4° for 24 to 48 hours after mixing. The precipitates were removed by centrifugation in the cold, washed twice with cold 0.85 per cent saline, and finally dissolved in 0.002 N to 0.004 N sodium hydroxide. The amount of nitrogen in the precipitates was determined by the Nessler method (17). The antigens used in all precipitin tests were preparations which had been obtained by 2 to 3 cycles of centrifugation, with the exception of

TABLE II

*Precipitation of Normal Protein and of Strains of Influenza Virus with Normal Protein Antiserum*

| Antigen | Dilution of antigen, 1:1 = 1 mg. per ml. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
| Normal protein.................... | ++++* | +++ | ++ | ++ | + | + | ± |
| Lee (centrifuge preparation).......... | +++ | ++ | + | + | ± | 0 | 0 |
| PR8    "       "    .......... | +++ | ++ | + | + | 0 | 0 | 0 |
| PR8 (red cell preparation)............. | +++ | ++ | + | + | 0 | 0 | 0 |

* In Tables II to IV signs indicate degree of precipitation.

TABLE III

*Precipitation of Normal Protein and of a Purified Preparation of PR8 Influenza Virus with Antiserum to a Purified Preparation of PR8 Virus*

| Antigen | Dilution of antigen, 1:1 = 1 mg. per ml. | | | | | |
|---|---|---|---|---|---|---|
| | 1:3 | 1:6 | 1:12 | 1:24 | 1:48 | 1:96 |
| Normal protein................. | +++ | +++ | ++ | + | + | ± |
| Purified PR8 virus.............. | ++++ | +++ | ++ | + | ± | 0 |

one preparation of PR8 virus which was obtained by two adsorptions on and elutions from chicken red cells, followed by 2 cycles of centrifugation. The precipitates obtained were in all cases of a flocculent nature and could be graded easily in the semiquantitative tests and sedimented readily in the quantitative reactions. Negligible amounts of precipitate were obtained with normal rabbit serum.[3]

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[2] Most of the tests were made with sera obtained after injection of 12 to 15 mg. of material because it was found that these sera had about twice the precipitating power of sera obtained after injection of 1 to 2 mg. of normal protein or of purified PR8 virus.

[3] The amounts of precipitate obtained by treatment of normal protein or of PR8 preparations with normal serum ranged from nothing to about 15 per cent of those obtained with anti-normal protein or anti-PR8 sera. The latter amount represents an extreme and appeared to be more characteristic of the PR8 preparations than of the normal protein. Precipitation of this sort could be eliminated by heating the normal sera at 65° for 30 minutes and, therefore, appeared to involve so called natural antibodies (31).

The results shown in Tables II and III indicate, first, that the normal protein is a good antigen; second, that preparations of both A and B types of influenza virus obtained from allantoic fluids by differential centrifugation show a strong serological relationship to the normal protein;[4] and, third, that the presumably specific adsorption of influenza virus activity on red cells does not appreciably diminish the reaction of these preparations with anti-normal protein serum. The results of the qualitative serological tests illustrated in Table II were also confirmed by quantitative methods.

In a typical experiment, equal volumes of antigens and of anti-normal protein serum were mixed in conical glass centrifuge tubes, incubated at 37° for 4 hours, and stored in a refrigerator overnight. The precipitates were separated by centrifugation, washed twice with 0.85 per cent sodium chloride, and suspended in a small amount of 0.002 N sodium hydroxide. Nitrogen analyses were made on these solutions by the Nessler method. From original solutions of normal protein, Lee virus, and PR8 virus, each containing 0.04 mg. of nitrogen, precipitates containing 0.28 mg., 0.21 mg., and 0.16 mg. of nitrogen, respectively, were obtained.

Additional serological tests were made to ascertain whether or not selective centrifugation might reduce the amount of normal protein in virus preparations. Three preparations of PR8 virus were employed, all of which were obtained from the same lot of infectious allantoic fluid by 2 cycles of differential centrifugation. The preparations differed only in the period of time used in the high-speed centrifugation. The period for one was limited to the time required to bring the centrifuge to speed and down again and the other two preparations were obtained by 30 and 90 minute runs, respectively, at speed. From a consideration of sedimentation principles, the preparations thus obtained should contain amounts of normal protein in proportion to the length of time employed in the high-speed centrifugation if the normal protein is present in such preparations as an autonomous impurity. However, quantitative precipitin tests revealed no significant differences in the three preparations, all of which reacted strongly with anti-normal protein serum. 0.5 mg. samples of each preparation were treated with 0.5 ml. of anti-normal protein serum and the amounts of nitrogen found in the washed precipitates were 0.091 mg., 0.082 mg., and 0.097 mg. for the 0, 30, and 90 minute preparations, respectively.

[4] After many of the serological tests described in this paper had been made, it was discovered that antisera to normal protein and to PR8 virus preparations strongly hemolyzed sheep erythrocytes and hence presumably contain Forssman type antibodies. When these antibodies were removed from antiserum to the normal protein by heating at 65° for 30 minutes and absorbing with sheep cells, the ability of such serum to precipitate normal protein or the material in preparations of PR8 virus was not abolished but only reduced by 10 to 60 per cent. Therefore, it can be concluded that the use of the sera herein described containing the Forssman antibodies, increased the total amounts of precipitates obtained but did not significantly alter the relative amounts of precipitates and hence the relationships deduced therefrom. It is particularly important to note that treated anti-normal protein serum, which no longer hemolyzed sheep cells, still reacted strongly with a PR8 virus preparation obtained by two adsorptions on and elutions from chicken red cells.

Further evidence regarding the relationship of normal protein to purified virus was obtained by means of cross-absorptions followed by precipitin tests.

One mg. of normal protein in 1 ml. of solution was mixed with an equal volume of anti-PR8 virus serum and 4 mg. of purified PR8 virus in 1 ml. of solution were mixed with 1 ml. of anti-normal protein serum. All materials were chilled to 4° before mixing. Flocculent precipitates appeared in both tubes a few moments after mixing. The mixtures were stored at 4° for 48 hours after which the precipitates were separated by centrifugation, washed twice with cold saline, and analyzed for nitrogen.

The precipitate obtained by treating the anti-normal protein serum with purified PR8 virus contained 0.63 mg. of nitrogen and that from the reverse treatment, 0.16 mg. The supernatant fluids, which contained the absorbed sera, were tested against fresh antigens at various dilutions. The results obtained at optimal dilutions are summarized in Table IV. It is apparent that the absorbed anti-normal protein serum had almost completely lost its original

TABLE IV

*Precipitation of Normal Protein and of PR8 Virus with Absorbed Antisera at Optimum Concentrations*

| Antiserum absorbed | Antigen used for absorption | Antigen | |
|---|---|---|---|
| | | Normal protein | PR8 virus |
| Normal protein............... | PR8 Virus | ± | ± |
| PR8 virus..................... | Normal protein | 0 | +++ |

ability to precipitate either normal protein or purified PR8 virus. On the other hand, the absorbed PR8 antiserum no longer reacted with normal protein but still reacted strongly with purified PR8 virus. These results clearly demonstrate the dual antigenic nature of centrifugally purified preparations of PR8 virus.

### Agglutination Inhibition and Neutralization Tests

The relationship of the normal protein to influenza viruses was further investigated by means of agglutination inhibition and neutralization tests.

The rabbit sera previously described were employed. The PR8 virus used was a centrifugal preparation which gave a 50 per cent infectivity endpoint in chick embryos of $10^{-14}$ gm. (28). Infective allantoic fluid containing Lee virus was used for this strain. The technique used for the agglutination inhibition tests was essentially that described by Hirst (15, 16), with the exception that about 6, rather than 4 agglutinative units of virus were employed. Agglutination endpoints were determined by the method of Hirst and Pickels (16) as modified in this laboratory (19). Since normal rabbit serum was found to inhibit the agglutination of red cells, all serum dilutions were started at 1:64, a point at which this effect was diluted out.

Antiserum to the normal protein was found to inhibit the agglutination of red cells by preparations of PR8 virus and of Lee virus. The calculated 50 per

cent agglutination endpoints were at serum dilutions of 636 and 767 for the PR8 and Lee virus solutions, respectively. While these values are large when compared to the inhibition shown by normal rabbit serum alone, they are rather small in comparison to those obtained with antiserum to centrifugally isolated PR8 virus. In the latter case a serum dilution of 112,000 times was required before a 50 per cent agglutination endpoint was obtained with 6 agglutinative units of PR8 virus. The demonstration that the antiserum to normal protein can inhibit red cell agglutination at considerably higher dilutions than normal sera is a fact which should be considered in interpretations of results obtained by this method.

Neutralization tests were made in chick embryos by inoculation with mixtures containing 1:100 serum and approximately 1, 10, and 100 infective doses of the PR8 virus preparation used in the agglutination inhibition tests. Ten embryos were inoculated with each test dilution. The antiserum to the normal protein failed to prevent infection at any of the three dosage levels tested, whereas the homologous serum gave complete protection in all cases. In similar tests employing mice, final mixtures containing about 4 and 400 fifty per cent mortality doses of the PR8 virus preparation and 1:100 serum were instilled intranasally. Although the antiserum to the normal protein appeared to delay death as compared with normal rabbit serum, it failed to prevent death. within the 10 day test period. On the other hand, the homologous serum gave complete protection to all mice including those which had received 400 fifty per cent mortality doses. The lungs of the latter mice were examined after 10 days, and no lesions were found.

*Relation of the Small Components of Strains of Influenza Virus to the Sedimentable Protein of Normal Allantoic Fluid*

In current preparations of various strains of influenza virus obtained from extra-embryonic fluids, essentially all of the virus activity appears to be associated with 600 to 800 S components (6–8, 10, 11). However, the presence of a 30 S component in centrifugally purified preparations of F12 influenza virus has been well established (10, 18) and it has recently been shown that this component, contrary to earlier conclusions, is not the virus (10). In addition, a non-viral small component has been observed in purified preparations of Lee virus (29) and components having sedimentation constants ranging from 75 S to 450 S have been reported for preparations of PR8 virus (12, 30). The small components in purified preparations of influenza virus are demonstrable by means of the ultracentrifuge mainly in concentrated solutions; *i.e.*, 10 to 30 mg. per ml. Except for serological evidence of the type presented in this report, one might frequently conclude that preparations of virus containing 5 mg. per ml. or less contain essentially no material other than the characteristic 600 S component with which virus activity is at present associated, despite

the facts that concentration of such preparations may reveal as much as 50 per cent of small component and that good boundaries may be obtained with such separated small components at 2 mg. per ml. Moreover, it would seem logical to assume that in the case of these virus preparations, the various small components are strain-specific products of obscure function arising as a result of the influenzal infection. However, the sedimentation behavior and the high viscosity of the normal protein herein described made it appear plausible that such components, despite their apparent variation in sedimentation constant, were one and the same and were identical with the sedimentable protein of normal allantoic fluid. This hypothesis was tested by isolating small amounts of the small components from purified preparations of F12 and Lee viruses and comparing their precipitability with anti-normal protein serum to that of the normal protein itself. The method employed for isolation of the small components was essentially that described by Stanley (10). Examination in the ultracentrifuge showed that there was no measurable amount of 600 S material (*i.e.* less than 5 per cent) in the preparations of the small components and that the bulk of the material in each case could be classified as 30 S component.[5] The electrophoretic behaviors (25) of the small components were found to be indistinguishable from those of preparations of normal protein. The small component from the F12 virus preparation was found by infectivity tests in chick embryos (28) to possess less than 1 per cent of the activity of the large component from which it was separated and the 30 S component separated from the Lee virus, less than 0.3 per cent.

In the precipitin tests, 0.5 ml. of F12 small component containing 0.05 mg. of nitrogen was mixed with 0.5 ml. of anti-normal protein serum and similarly 0.5 ml. of Lee 30 S component containing 0.07 mg. of nitrogen was mixed with an equal volume of serum. Simultaneously, 0.5 ml. of anti-normal protein serum was mixed with each of two 0.5 ml. portions of normal protein containing 0.05 and 0.07 mg. of nitrogen, respectively. All materials were cold when mixed and were stored at 4° for 24 hours. At the end of this time, the precipitates were separated by centrifugation and washed twice with cold saline after which they were taken up in 0.002–0.004 N sodium hydroxide and analyzed for nitrogen by the Nessler method.

The precipitate obtained with the F12 small component contained 0.17 mg. of nitrogen compared to 0.19 mg. obtained in the homologous reaction and the precipitate obtained with the Lee 30 S component contained 0.14 mg. of nitrogen as compared to 0.20 mg. in the homologous precipitate. Since the method of preparation of the small components would not be expected to yield as pure a product as obtained in the direct isolation of normal protein from allantoic

[5] The term 30 S is used as a convenient designation for the small non-viral components, since it is a value that was frequently obtained in earlier work with the F12 strain (10, 18). It should be noted, however, that the actual sedimentation value obtained for these small components is undoubtedly dependent upon the concentration and viscosity of the preparations.

capacity to precipitate the material in PR8 virus preparations (Table IV), and by the negligible neutralizing capacity of anti-normal protein serum as compared with the tremendous neutralizing capacity of antiserum to the material in the purified virus preparations. The hypothesis which at present appears to fit the experimental data best is that infection with influenza viruses stimulates the production or release of presumably normal material in 12 day or younger embryos in amounts similar to or greater than those appearing spontaneously on the 13th or 14th days in uninoculated embryos. This normal material becomes associated with the 80 to 120 m$\mu$ particles, with which virus activity is also associated, in varying amounts and in degrees of firmness depending upon the strain of virus. From data obtained in the analytical ultracentrifuge it has been concluded that centrifugally purified preparations of F12 virus can contain as much as 30 per cent and similar preparations of PR8 and of Lee viruses as much as 50 per cent of small material, although the amounts of these components which can actually be isolated are, due to incomplete separation of small and large components and to losses during purification, considerably smaller (10, 29, 30). Serological tests on the residual large material after fractionation to remove 30 S components indicate that appreciable amounts of normal material are still present. Hence estimates based on ultracentrifugal data of the amount of small component present in preparations of these viruses are possibly low. Quantitative serological methods appear to offer a much more precise means of estimating the total amounts of normal protein in purified preparations of virus and such studies are now in progress.

The demonstration by serological methods of considerable amounts of normal protein in apparent close association with the infectious particles of purified influenza virus preparations derived from allantoic fluid has implications important to every phase of investigation employing such virus preparations. Obviously, the presence of this non-viral material in such substantial amounts as indicated above can have an important bearing on the results of chemical and physical analyses. Recent reports have described the isolation from allantoic fluids and characterization of several strains of so called influenza virus (6–8). Actually the materials thus characterized undoubtedly contained variable amounts of normal protein. The presence of normal material can also greatly influence the sedimentation behavior, and hence conclusions regarding size, of the particles bearing virus activity (29). It is not yet known what effect the association of appreciable amounts of normal protein with centrifugally purified virus may have on the use of such preparations as vaccines. It is possible that this circumstance may be of little importance in most parenteral applications but it should be noted that intravenous injection of 0.6 mg. or more of normal protein without a previous conditioning injection

of about 0.01 mg. has been observed to result occasionally in the death of a rabbit within 24 to 48 hours.

While emphasis has been placed throughout this report on the presence of non-viral material in association with the infectious entity in preparations of the latter purified by 2 differential centrifugation cycles, nevertheless it should be recognized that these preparations represent by far the purest and most potent concentrates of influenza virus yet reported. This fact is strikingly illustrated in the potency of the rabbit antiserum to the PR8 preparations. The 50 per cent red cell inhibition titer of the anti-PR8 rabbit serum described in the present report was approximately 18 to 19 times that reported by Hirst for ferret immune serum (15). It seems probable that similar antisera to centrifugally purified influenza A and B viruses would be found superior to current sera when employed in the manner described by Smorodintseff and associates (32).

## SUMMARY

Macromolecular material was isolated from normal allantoic fluid by a centrifugation procedure comparable to that currently employed for the concentration and purification of influenza viruses. The yield of material was found to vary with the age of the embryo, reaching a maximum average value after 14 days of incubation at 39°C. of about 0.02 mg. per ml. of allantoic fluid. The purified material was found to contain protein, carbohydrate, and lipid and to have a general composition similar to purified preparations of PR8 influenza virus. A typical preparation of normal material had an isoelectric point at pH 2.3. Sedimentation studies indicated that the normal material can give a variety of sedimentation constants depending upon the concentration and viscosity of the preparations. The sedimentation constant, corrected for viscosity, of the major component of a fresh preparation was 170 S. The diameters of the predominant particles shown in electron micrographs of the normal material and of preparations of PR8 influenza virus were about 40 and 100 m$\mu$, respectively. Serological tests indicated that the normal material is a good antigen and that preparations of both A and B types of influenza virus obtained from allantoic fluids by centrifugation show a strong serological relationship to the normal material. Freezing and thawing of allantoic fluid, and repeated adsorption of virus on red cells, failed to provide a practical basis for the separation of normal protein from the virus entity in the case of PR8 virus. In the cases of similar preparations of F12 and of Lee viruses, a partial separation of a small component was accomplished by fractional centrifugation and this component and the normal protein were shown to be identical or very closely related. Antiserum to the purified normal material inhibited red cell agglutination by A and B types of influenza virus at serum dilutions of

600 to 700, but failed to show significant neutralizing capacity in chick embryo and in mouse tests at a serum dilution of 100. Rabbit antiserum to purified preparations of PR8 virus gave a 50 per cent red cell agglutination inhibition endpoint at a serum dilution of 112,000. Some of the implications of the findings are discussed.

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EXPLANATION OF PLATE 6

FIG. 1. (a) Electron micrograph of a purified preparation of the sedimentable protein from normal allantoic fluid. Applied at $10^{-3}$ gm. per ml. $\times$ 15,000. (b) Same except applied at $10^{-4}$ gm. per ml. (c) Electron micrograph of a purified preparation of PR8 influenza virus. $\times$ 15,000.
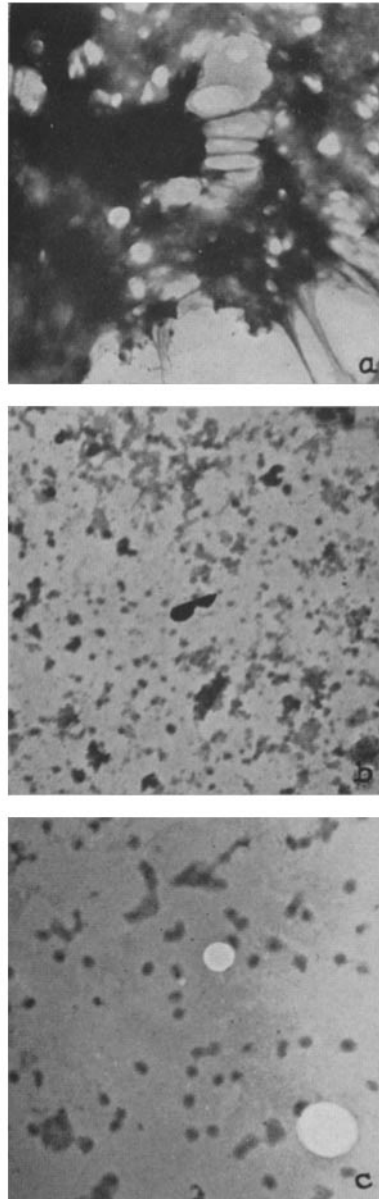
FIG. 1

(Knight: Sedimentable component of allantoic fluid)