PRECIPITIN REACTIONS OF HIGHLY PURIFIED INFLUENZA VIRUSES AND RELATED MATERIALS*

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The purification of influenza virus and the correlation of physical and chemical properties with infectious particles have been aided immeasurably by the fact that influenza virus appears to have, in addition to its infectious quality, a specific capacity to agglutinate red blood cells and to be adsorbed on and eluted therefrom (1–5). Studies on influenza virus have also gained a certain advantage in precision by exploitation of the quantitative aspects of the hemagglutination reaction (4, 6), and by establishment of the statistical significance of results obtained in infectivity titrations (7, 8). Therefore, this animal virus has appeared to be a very favorable material on which to make certain fundamental investigations, particularly if preparations can be obtained which are demonstrably pure in the sense that they are essentially free of non-viral material.

From infectious allantoic fluid of chick embryos, highly purified preparations of PR8 influenza virus have recently been obtained which are homogeneous in the analytical ultracentrifuge and in the Tiselius electrophoresis apparatus and which consist of particles about 100 mμ in diameter (9–13). When these particles are sedimented it can be shown that the units carrying infectivity and chick cell agglutinating activity sediment at the same rate (14). Therefore, from the association of biological activity with particles of uniform size and electrochemical behavior, it can be concluded, although possibly prematurely, that such particles represent influenza virus. This conclusion is greatly strengthened by the isolation from mouse lungs infected with PR8 influenza virus of particles possessing very similar chemical, physical, and biological properties (15, 16). However, lacking the proof which could be furnished with simpler organic substances by synthesis, the final test of the identity of the virus must rest, as others have noted (17, 18), upon the inability to demonstrate the presence of more than a single substance by as many independent methods as possible.

The principles of serological specificity so well developed by the researches of Landsteiner and his associates (19) and used effectively in demonstrating

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the presence of non-viral material in purified preparations of the agent of fowl leukosis and sarcoma (20, 21) appeared to offer a useful basis for testing the purity of preparations of influenza virus. When the most highly purified and apparently homogeneous preparations of influenza virus described above were subjected to precipitin tests, using antiserum to the sedimentable protein of normal allantoic fluid (10), they were found to precipitate strongly. This suggested that the best preparations of influenza virus were grossly contaminated with non-viral material and made it appear advisable to examine further the serological reactions of such preparations with the hope of resolving the apparent contradiction between these results and those of the physicochemical tests. The data obtained from various quantitative precipitin tests and an evaluation of their bearing on the nature of influenza virus are given in the present communication.

EXPERIMENTAL

Preparation of Material.—Purified preparations of the sedimentable protein[1] from normal allantoic fluid were obtained by differential centrifugation of allantoic fluid of 14-day-old white Leghorn chick embryos as previously described (10).

The particles from normal mouse lungs were obtained from clarified 10 per cent suspensions of perfused lungs by 2 to 3 cycles of differential centrifugation employing 15 minute periods in the high speed runs at 24,000 R.P.M. and 5 minute periods in the low speed runs at 5000 R.P.M. (16).

The virus preparations were obtained, except as otherwise noted, by adsorption of the virus on and elution from chicken red cells followed by 2 or 3 cycles of differential centrifugation using the conditions recently described (16). The starting material for the preparation of highly purified PR8 virus from allantoic fluid was the concentrate obtained after passage of infectious allantoic fluid through the Sharples centrifuge by suspension of the bowl contents in 0.1 M phosphate buffer (13). This concentrate was spun in an angle centrifuge at 5000 R.P.M. for 5 minutes and the supernatant fluid was diluted with 0.1 M phosphate buffer at pH 7 until it contained about 400 to 500 standard CCA units (6) per ml. Enough packed chicken red cells were then added to the virus solution at 4°C. to give a final concentration of about 4 per cent. The elution of virus from the red cells and the further purification by differential centrifugation were accomplished as recently described (16).

Preparations of Lee virus were obtained by the same method used for the PR8 strain.

Preparations 1 and 5 (16) of mouse lung PR8 virus were employed for most of the tests in which this type of virus was used.

All of the purified preparations were carried in 0.1 M phosphate buffer at pH 7 and were freshly prepared when used in the precipitin tests. The identities of the A and B strains of influenza virus used were checked by agglutination inhibition tests (3, 4, 6). The CCA activities (6) of the PR8 virus preparations were in the neighborhood of 40,000 and 30,000 units per mg. of nitrogen for allantoic fluid and mouse lung preparations, respectively. The Lee virus preparations titered about 30,000 units per mg. of nitrogen. Representative preparations of the viruses were found to be homogeneous in the ultracentrifuge and one of the

[1] For convenience the product obtained from normal allantoic fluid by means of differential centrifugation is referred to throughout this paper as the normal allantoic protein or by some similar term. In each case is meant the inhomogeneous material described in (10) which was found to be composed not only of protein but also of lipid, carbohydrate, and nucleic acid.

preparations of purified PR8 virus obtained from allantoic fluid was tested and found homogeneous in the Tiselius electrophoresis apparatus (22). The preparations of normal particles were tested for red cell agglutinating activity and infectivity and were found to be devoid of these capacities.

Preparation of Antisera.—Antisera to the various materials were obtained by subjecting rabbits to a course of 14 to 18 spaced intravenous injections. A total of 50 to 60 mg. of each substance was injected in the appropriate rabbit except in the case of the normal mouse lung material, which did not yield as good antisera as did the other substances. In this case, about 80 mg. of the normal lung particles were injected. Five to 6 days after the last injection, the rabbits were bled by cardiac puncture and the sera were separated from the clotted blood.

Quantitative Precipitin Tests.—The techniques employed were essentially those of Heidelberger (23) with a slight modification in the method of analyzing the antigen-antibody precipitates as described below. The precipitin tests were all made at 4°C. in sterile 12 ml. conical glass centrifuge tubes. Increments of antigen in 1 ml. volumes of 0.1 M phosphate buffer at pH 7 were added to a series of tubes. To each tube was then added 0.5 ml. of undiluted serum. After mixing, the tubes were allowed to remain at 4° for 48 hours after which they were spun in an International clinical centrifuge for 5 minutes at full speed. The supernatant fluids were poured off and the precipitates were washed by suspending in cold physiological saline. The latter was removed after centrifugation by decantation. Washing was continued 2 to 3 times until the supernatant fluids came off clear.[2] The precipitates were then dissolved in 0.5 ml. of 0.02 N sodium hydroxide and aliquots were taken for nitrogen determinations by the micro-Kjeldahl method using direct nesslerization (24).

Untreated serum was employed in most of the tests although, in two series with the antiserum to normal allantoic protein, the effect of non-specific antibodies of the Forssman type was evaluated by comparing the amounts of precipitate from treated serum with those from untreated serum. The treatment consisted in heating 10 ml. of the undiluted serum at 56°C. for 30 minutes followed by incubation at 37°C. for 15 minutes with 3 ml. of packed, washed sheep erythrocytes. The latter were removed by centrifugation at the end of the treatment. Serum thus treated no longer hemolyzed sheep cells in the presence of added complement and thus was free of Forssman-like action.

Results of Precipitin Tests

Results typical of several series of precipitin tests are summarized graphically in Figs. 1 through 3. Other tests, not described in detail here, followed the same trends illustrated in the figures but differed quantitatively from the latter and from each other, presumably by virtue of differences in antibody titers. When comparable but different preparations of antigen, particularly of the purified viruses, were treated with the same serum, the precipitin response observed was strikingly uniform.

Precipitin Reactions with Antiserum to the Sedimentable Protein of Normal

[2] This washing process gives the quantitative technique a specificity which may be lacking in some of the qualitative precipitin tests, particularly those performed with untreated sera. For example, highly purified preparations of influenza virus frequently give precipitates with normal rabbit serum. Such precipitates, in sharp contrast to those obtained with specific antisera, dissolve when washed with cold saline. It should be mentioned that the non-specific precipitation just described can be eliminated by heating the serum at 56 to 65°C. for 30 minutes before use in the tests.

Allantoic Fluid.—The curves of Fig. 1 illustrate the trends observed in 4 series of tests made over the course of a year with different antisera to the normal allantoic protein. Heavy precipitates were obtained in each instance with highly purified preparations of influenza A (PR8) and B (Lee) viruses obtained

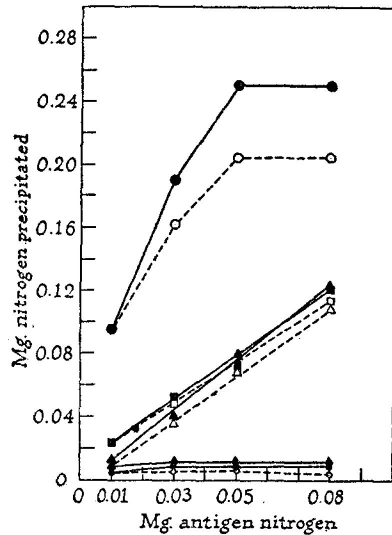

FIG. 1

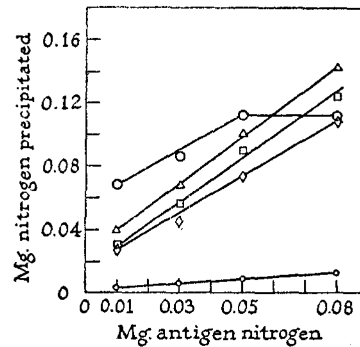

FIG. 2

FIG. 1. Precipitation of influenza viruses and related materials by antiserum to the sedimentable protein of normal allantoic fluid. The solid black symbols represent the values obtained with untreated serum and the open symbols represent the values obtained with serum which, before use, had been heated at 56°C. for 30 minutes and absorbed with sheep cells. ○ normal allantoic protein. □ purified Lee influenza virus obtained from infectious allantoic fluid. Δ purified PR8 influenza virus obtained from infectious allantoic fluid. ◇ purified PR8 influenza virus obtained from infected mouse lungs. o purified particles from normal mouse lungs.

FIG. 2. Precipitation of influenza viruses and related materials by antiserum to purified PR8 influenza virus obtained from infectious allantoic fluid. Untreated serum was employed. ○ normal allantoic protein. Δ purified PR8 influenza virus obtained from infectious allantoic fluid. □ purified Lee influenza virus obtained from infectious allantoic fluid. ◇ purified PR8 influenza virus obtained from infected mouse lungs. o purified particles from normal mouse lungs.

from infectious allantoic fluid. On the other hand, the purified PR8 virus obtained from infected mouse lungs and the particles from normal mouse lungs failed to precipitate with antiserum to the normal allantoic protein.

As shown in Fig. 1, the relationships indicated by the precipitin tests were only slightly altered by treatment of the sera with sheep cells. One can therefore conclude that the hemolyzing antibodies of such sera are of minor importance in the precipitin reactions.

Some data pertaining to the quantitative relationship of the normal allantoic protein to influenza viruses isolated from allantoic fluid were obtained from plots of antigen nitrogen against precipitated antibody nitrogen and are presented in Table I. The method of calculation, which is somewhat less direct than that usually employed (18, 25, 26), was based on the observation that the initial portions of the antibody precipitation curves approximated straight lines and on the assumption that the amount of antigen was directly proportional to the slope of these curves. Such calculations indicate that highly purified preparations of PR8 and Lee influenza viruses contain about 20 per cent and 30 per cent, respectively, of antigenic structures characteristic of the normal allantoic protein. In other tests both higher and lower values were

TABLE I

Quantitative Precipitin Data from the Reaction of Increasing Amounts of Normal Allantoic Protein, and of Highly Purified Preparations of PR8 and Lee Strains of Influenza Virus with Antiserum to Normal Allantoic Protein in the Region of Antibody Excess

Antigen N	Antibody N precipitated			Normal antigen in	
	Normal allantoic protein	PR8	Lee	PR8	Lee
mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*
0.030	0.131	0.024	0.036	18.3	27.5
0.035	0.141	0.026	0.040	18.5	28.4
0.040	0.150	0.029	0.043	19.3	28.7
0.045	0.156	0.032	0.046	20.5	29.5
0.050	0.162	0.034	0.049	21.0	30.2
0.055	0.167	0.036	0.052	21.6	31.1
0.060	0.172	0.039	0.055	22.7	32.0
Average				20.3	29.6

obtained. In each case the figures must be regarded as a crude approximation but valuable in that they give a more precise estimate than can be gathered from qualitative data. The variability of the values obtained with different sera and the lack of close agreement in the data given in Table I are not readily explained, although the following factors probably contribute. The normal protein of allantoic fluid is difficult to obtain in large amounts and thus the amount of antiserum to this material which can be procured is sharply limited. This in turn reduces the quantity of data obtainable and in particular curtails the number of replicate determinations made. Another significant factor is the large size of the antigens involved which reduces the ratio of antibody nitrogen to antigen nitrogen in the precipitates with a consequent reduction in the accuracy readily attainable in analyses on such precipitates. The heterogeneity of the normal protein antigen (10) is perhaps also of importance.

There are at least two kinds of particles in the sedimentable material from normal allantoic fluid. These differ in size and undoubtedly in antigenic nature. Moreover there is reason to believe that the composition of preparations of the normal material varies more than does that of preparations of highly purified virus. This could account for part of the discrepancy between results obtained with different lots of sera.

The quantitative precipitin tests also yielded information regarding the proportion of components of the normal protein system which were involved

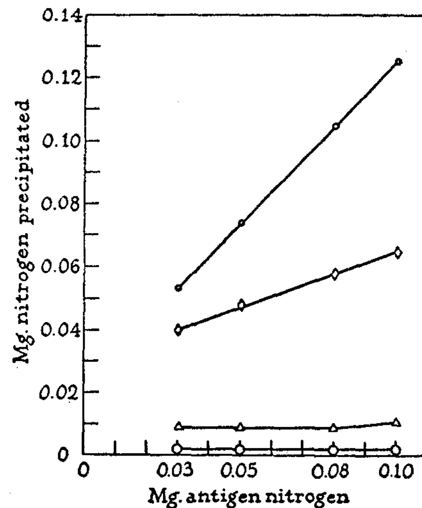

FIG. 3. Precipitation of influenza viruses and related materials by antiserum to purified particles from normal mouse lungs. Untreated serum was employed. ○ purified particles from normal mouse lungs. ◇ purified PR8 influenza virus obtained from mouse lungs. Δ purified PR8 influenza virus obtained from infectious allantoic fluid. ○ normal allantoic protein.

in the virus preparations. As was expected from size and composition relationships, the homologous reaction with antiserum to normal allantoic protein reached a state of antigen excess at a level of antigen nitrogen at which there was still antibody excess in the case of the purified preparations of virus. In one series of tests in which the heterologous reactions were carried considerably beyond the points shown in Fig. 1, it was found when the point of antigen excess was reached, that PR8 virus had removed 76 and Lee virus 87 per cent as much antibody nitrogen as the maximum removed by normal material. These figures suggest that the bulk of the material comprising the normal allantoic protein is also present in the virus preparations.

Precipitin Reactions with Antiserum to Highly Purified PR8 Virus Obtained from Allantoic Fluid.—With antiserum to highly purified PR8 virus obtained

from infectious allantoic fluid, precipitates were obtained, as shown in Fig. 2, with the protein of normal allantoic fluid, with highly purified preparations of Lee virus and of mouse lung PR8 virus as well as with the homologous antigen. Only the particles from normal mouse lungs failed to give significant precipitates.

Although abundant precipitates were always obtained when the most highly purified preparations of Lee virus were treated with antiserum to PR8 virus, the antibodies responsible for these reactions are without neutralizing capacity and lack ability to inhibit the agglutination of red cells to a significant extent compared with the homologous system. On the other hand, the antibodies involved in the precipitation of mouse lung PR8 virus are neutralizing antibodies and inhibit the agglutination of red cells by 8 units of the mouse virus to a serum dilution of about 40,000 (16).

Precipitin Reactions with Antiserum to Particles from Normal Mouse Lungs.— The results obtained with antisera to normal lung particles were analogous to those obtained with antisera to the sedimentable protein of normal allantoic fluid. As shown in Fig. 3, the highly purified virus obtained from mouse lungs gave definite precipitates whereas, significantly, the purified virus from allantoic fluid or the normal protein from allantoic fluid did not.

Precipitin Tests on PR8 Virus Obtained by Centrifugal and Electrophoretic Fractionation

Some quantitative precipitin tests were made on materials, kindly furnished by Dr. G. L. Miller, which had been obtained by centrifugal or electrophoretic fractionation (12) of PR8 virus preparations obtained from infectious allantoic fluid.

Two samples from the centrifugal fractionation were tested. One was the crude, unfractionated virus which gave double boundaries in the analytical centrifuge and in the Tiselius apparatus and the other was the "heavy" fraction which appeared homogeneous in the centrifuge and in the Tiselius apparatus (12). Samples containing 0.05 mg. of nitrogen were treated in the cold with 0.5 ml. of undiluted antiserum to the sedimentable protein of normal allantoic fluid. After 48 hours at 4°, the precipitates were separated from the fluids by centrifugation and were washed and analyzed as described under Quantitative precipitin tests. The precipitates obtained from the crude and "heavy" fractions were found to contain 0.114 and 0.097 mg. of nitrogen, respectively, as compared with 0.126 mg. in the homologous precipitate obtained with the normal allantoic protein.

The electrophoretic samples tested included the inhomogeneous original preparation, and components separated from this which are designated fast and slow with reference to the comparative velocities of their electrical migration (12). The fast component was believed to contain an appreciable amount of the normal allantoic protein and the slow component was assumed to be the virus (12). The precipitates obtained by treating samples containing 0.025 mg. of nitrogen with 0.25 ml. of undiluted antiserum to the normal allantoic protein contained 0.043, 0.046, 0.038, and 0.063 mg. of nitrogen for the original material, the fast component, the slow component, and the normal allantoic protein, respectively.

It is apparent from the results of the tests described above, that the serological reactions follow the same trend as the physicochemical tests to the extent that the crude materials in each case appear to possess the most normal protein antigen and the homogeneous "heavy" material (or slow component) the least. However, the total amounts of normal antigen indicated by the two types of tests are of an entirely different magnitude. For example, in the fractionation procedures one starts with material consisting of two main components, one of which behaves in the centrifuge and in the Tiselius apparatus as though it were normal allantoic protein. After fractionation, one has a product which is homogeneous in the centrifuge and in the Tiselius apparatus and which behaves in these two physical tests as though it were free of normal protein. From the serological viewpoint, one starts with material which reacts strongly with antiserum to normal protein and ends with a product which reacts only 25 to 30 per cent less strongly as judged from the amount of antibody removed.

Relation of Normal Antigen to the Particles Bearing Virus Activity

Preparations of the F12, Lee, and PR8 strains of influenza virus obtained from infectious allantoic fluids by means of differential centrifugation have been shown to contain variable amounts of a component which sediments more slowly than the approximately 100 mμ particles with which virus activity is at present associated (10–12, 27–30). These smaller components have been separated from preparations of F12 and of Lee viruses and have been shown to possess the sedimentation, the serological, and the electrochemical properties of the purified sedimentable protein of normal allantoic fluid and to have only a fraction of 1 per cent of the virus activity of the preparations from which they were derived (10, 27). Since it has been shown that this slight activity is undoubtedly due to contamination with a trace of virus (27), it is apparent that independent particles of normal protein exist in centrifugally isolated preparations of F12 and of Lee influenza viruses. Sedimentation and electrophoretic studies (11, 12) together with the serological tests on centrifuge fractions described in the present report, indicate that the same situation prevails with comparable PR8 virus preparations. However, when preparations of virus are obtained by adsorption on and elution from red cells followed by an appropriate number of cycles of differential centrifugation, or when they are got by electrophoretic fractionation, or by centrifugal fractionation, it can be shown that these preparations are essentially devoid of independent particles of the sedimentable component characteristic of normal allantoic fluid. Nevertheless, as described in another section of this report, such homogeneous preparations react strongly with antiserum to the normal protein. Hence, it must be concluded either that they contain particles of normal protein of size

and electrochemical properties uncommon to normal allantoic fluid or that virus activity and normal antigen are associated in the same particle. The former possibility seems unlikely in the case of the preparations obtained by adsorption on and elution from red cells, for at present the experimental data indicate that this procedure is highly specific. Moreover, appropriate tests show that a major portion of the infectivity of the most highly purified preparations of virus can be specifically precipitated by antiserum to the normal allantoic protein. The following is a typical experiment of this sort.

A preparation of PR8 influenza virus was obtained from infectious allantoic fluid by the procedure involving adsorption on and elution from red cells described under Preparation of material. Samples containing 0.1 mg. of nitrogen in 1 ml. were placed in each of 3 tubes and treated at 4°C. with 0.5 ml. of undiluted antiserum to purified PR8 virus, 0.5 ml. of antiserum to purified normal allantoic protein, and 0.5 ml. of normal rabbit serum, respectively. Before use all sera were heated to 56° for 30 minutes to minimize non-specific precipitation. After 48 hours the tubes were spun in a clinical centrifuge and the supernatant fluids were poured off. The precipitates were washed twice with cold saline and each time the washings were added to the original supernatant fluids. The washed precipitates were dissolved in 0.02 N sodium hydroxide and analyzed for nitrogen and the supernatant fluid and washings for each sample were tested for infectivity in 10-day chick embryos, using 10 embryos for each dilution tested. The precipitates were found to contain 0.172, 0.140, and 0.008 mg. of nitrogen for the homologous reaction, the reaction with antiserum to normal protein, and the reaction with normal rabbit serum, respectively.

The 50 per cent infectivity endpoints in chick embryos (8), calculated on the basis of the 10^{-4} gm. of virus nitrogen in the original samples, were $<10^{-6}$ gm. of nitrogen for the supernatant fluid and washings from the homologous reaction, $10^{-12.8}$ gm. of nitrogen for the supernatant fluid and washings from the reaction with antiserum to normal protein, and $10^{-14.2}$ gm. of nitrogen for the comparable fluid from the treatment with normal rabbit serum.

From the above data and on the assumption that the supernatant fluid and washings from the treatment with normal rabbit serum represent the full infectivity of the amount of virus used, it can be calculated that treatment of the purified virus with antiserum to normal protein specifically precipitated about 96 per cent of the infectivity. This sort of result has been obtained repeatedly and combined with other observations can be considered evidence that in the case of highly purified PR8 virus, the capacity to precipitate when treated with antiserum to normal allantoic protein is a property of the same particles which are infectious and which cause agglutination of red cells.

Precipitin Reactions of Mouse PR8 Virus after One Passage in Chick Embryos

With the discovery that highly purified preparations of mouse lung virus and of allantoic fluid virus contained distinctive antigens related to a normal material characteristic of the source from which each was derived, it was of considerable interest to test the product obtained by a single passage of mouse lung virus in chick embryos.

240 10-day embryos were inoculated with a 10^{-4} dilution of a 10 per cent suspension of infected mouse lung. After 48 hours at 37°, the eggs were chilled and the allantoic fluid was harvested. Partially purified virus was obtained from this fluid by 1 cycle of differential centrifugation and highly purified virus was next obtained by adsorption on and elution from red cells followed by 2 cycles of differential centrifugation. One ml. samples of the purified virus containing 0.08 mg. of antigen nitrogen were treated with 0.5 ml. portions of each of the sera and under the same conditions employed in the tests illustrated in Figs. 1 to 3. The washed precipitates obtained from the treatments with antisera to purified PR8 virus from allantoic fluid, to normal allantoic protein, and to particles from normal mouse lungs contained 0.147, 0.123, and 0.008 mg. of nitrogen, respectively.

The figures for nitrogen obtained in this experiment can be compared directly with the values for purified egg-adapted virus and shown in Figs. 1 to 3, which are 0.142, 0.124, and 0.009 mg. of nitrogen, respectively. It is apparent from this comparison that after only one passage in embryos, the mouse lung virus becomes indistinguishable from the egg-adapted virus with regard to behavior in quantitative precipitin tests.[3]

Precipitin Reactions with Concentrated Human Antibodies

Concentrated gamma globulin from fractionated, pooled human blood was kindly furnished by Dr. E. J. Cohn of Harvard University. This concentrate of antibodies, which contained 200 mg. of protein per ml., was used undiluted, and diluted 2 and 4 times in qualitative precipitin tests employing various dilutions of antigens. The undiluted globulin was very viscous and apparently prevented formation of precipitates, for no precipitates were obtained with the undiluted material. However, at dilutions of 2 and 4 times, positive tests were obtained with highly purified preparations of PR8 virus from infectious allantoic fluid and from infected mouse lungs. No precipitates were obtained at any dilution with normal allantoic protein or with the particles from normal mouse lungs.

Since the concentrate of human antibodies used represented a conglomerate of unknown serological composition, it was considered desirable to test the specificity of the precipitin tests observed. This was done by correlating the removal of antibodies from the globulin mixture with the ability of the mixture to inhibit specifically the agglutination of chicken red cells by influenza virus. The globulin mixture, diluted 1 to 4 with saline, was carefully absorbed with

[3] However, there appears to be an important difference in the products for in four tests that have been made thus far, the purified preparations obtained after inoculating embryos with mouse lung virus have had CCA activities in the neighborhood of 30,000 units per mg. of nitrogen whereas the preparations of egg-adapted virus obtained by identical procedures have had CCA activities of 40,000 units or more. This suggests that some quality of the mouse virus is maintained through one passage in chick embryos, for it has been observed that purified preparations of mouse-adapted PR8 virus have a lower CCA activity than comparable preparations of egg-adapted PR8 virus (16).

purified PR8 virus until neither an excess of antigen nor of influenzal antibodies remained as shown by the inability of aliquots of the mixture to agglutinate red cells or to give further precipitates upon addition of more of the virus preparation. Before the absorption, the globulin mixture had a 50 per cent agglutination inhibition endpoint of 1024 and after absorption the value was less than 32. This demonstrates that the antibodies removed in the precipitin tests were of the influenza-neutralizing type and hence that the precipitin tests described above are indicative of a relationship between the influenza virus which once infected certain blood donors and the purified influenza virus preparations under study in this laboratory.

DISCUSSION

If one has antiserum to a purified antigen, it is then possible from precipitin tests and from appropriate calibration curves to estimate accurately the amount of that substance present in a mixture of antigens (18). However, the relationship between normal allantoic protein and highly purified preparations of influenza virus does not present a clear-cut system of that sort. The main complicating facts are that the normal allantoic protein consists of more than one component and that, rather than existing as a physical mixture with influenza virus particles, an antigenic structure characteristic of the normal protein appears to make up part of the influenza virus particles. The intimate association of normal protein with virus particles and the resultant decrease in active groupings together with the fact that the virus-normal protein particles possess a diameter more than double that of the majority of particles in preparations of normal protein, cause the best estimates from quantitative precipitin data of the amount of normal protein antigen coupled with the virus to be minimal.[4]

Whether the normal antigen is incorporated into the virus particles in the form of the nucleoprotein-lipid-carbohydrate complex as it is isolated from normal allantoic fluid, or as one of the major components of the complex, is not yet known. In any case the significant point appears to be the incorporation of normal antigen from the infected host into the virus particles. This is in marked contrast with the situation applying to tobacco mosaic virus, for the most highly purified preparations of the latter give no indication by serological

[4] From a consideration of some quantitative precipitin data, Cohen reported that a preparation of PR8 influenza virus similar to those studied in detail in the present report contained about 29 per cent of particles characteristic of chorioallantoic membranes of normal embryos (31). In a qualitative test, particles of this normal material, kindly furnished by Dr. Cohen, were found to precipitate strongly with antiserum to normal allantoic protein. Hence, it can be assumed that these particles, as might be expected, are closely related to the material previously isolated from allantoic fluid (10) and employed in the experiments described herein. Insufficient data are presented in the paper by Cohen to permit an extensive comparison of his results with those obtained in the present study.

means of the presence of normal antigens, even when tested by the extremely sensitive anaphylactic test (32).

The mode of combination and the function, if any, of normal antigen in influenza virus particles are both obscure. The fact that the normal antigens from two hosts have isoelectric points distinctly more acid than that of the influenza particles suggests the possibility of the combination of an acidic normal component with an alkaline viral component to form the infectious particles with an intermediate isoelectric point which are at present isolated from tissues infected with influenza virus. In this connection it is interesting to note that infection of embryos with influenza virus results in the appearance in the allantoic fluid of much greater amounts of normal component than are found in the fluid of uninfected embryos of the same age (10).

In the light of present evidence, the following conception of the nature of influenza virus in the form in which it is isolated by present techniques can be advanced. There is a common denominator of antigenic structure for a given strain of influenza virus throughout its host range. This is demonstrated in the present study in the serological cross-reactions involving the chick embryo, the mouse, and man. Antibodies to this structure neutralize the infectivity and inhibit the agglutinating capacity of the virus from whatever source it is obtained. Combined with the common moiety to an extent presumably governed by chemical affinities, and in such a manner as to result in particles which are homogeneous in physicochemical tests, there is a specific normal component which is characteristic of each individual host from which the virus is obtained. To this extent the influenza virus particles from a given host differ from those obtained from every other host, but with regard to size and shape and to general chemical and biological properties, the virus particles from various hosts are similar. Whether the normal components are functionally essential to the virus particles or whether they are simply coincidental constituents remains to be shown.

In general, the picture of influenza virus just presented is similar to that deduced by Gye and Purdy from experiments with Fujinami tumor virus obtained from two different hosts (33). These workers found that the virus in cell-free tumor extracts from chickens and from ducks was neutralized by antiserum to tumor extracts from either host. In addition, antiserum to normal chick embryo neutralized virus in chicken tumor extracts but not in duck tumor extracts, and antiserum to normal duck embryo neutralized virus in duck tumor extracts but not in chicken tumor extracts. The situation with the tumor virus differs from that of influenza virus to the extent that antiserum to normal material neutralizes the virus from the appropriate host, for in the case of influenza virus no neutralization is observed with antiserum to normal host material. Likewise, in the case of the agent of fowl leukosis and sarcoma, no neutralization of the virus is observed with antiserum to normal host material although such serum causes precipitation of the active agent (20, 21).

Vaccines against influenza have been developed which employ purified virus obtained from the infectious allantoic fluid of chick embryos (5, 34). The use of such vaccines is contraindicated in the cases of individuals who are hypersensitive to egg protein. An obvious application of the serological findings described above would be to prepare a vaccine from the highly purified virus of infected mouse lungs (16) for use in such instances.

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SUMMARY

Antisera to purified PR8 virus, to purified protein from normal allantoic fluid, and to purified normal mouse lung particles were obtained from hyperimmunized rabbits and used in quantitative precipitin tests employing various purified preparations of influenza virus and related materials as antigens. The results of those tests indicated that the most highly purified preparations of PR8 or of Lee influenza virus obtained from infectious allantoic fluid contain an antigen characteristic of normal allantoic fluid and likewise that highly purified mouse lung PR8 virus contains an antigen characteristic of normal mouse lungs. Since the infectivity of virus preparations which were ultracentrifugally and electrochemically homogeneous was precipitated by the appropriate antisera to normal antigens, it was concluded that the normal antigens constitute a part of the 100 mμ particles with which influenza virus activity is at present deemed to be associated. It was estimated from quantitative precipitin data that the most highly purified preparations of PR8 and of Lee influenza viruses obtained from infectious allantoic fluid contain at least about 20 and 30 per cent, respectively, of an antigenic structure characteristic of the sedimentable protein of normal allantoic fluid.

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