CENTRIFUGATION AND ULTRAFILTRATION STUDIES ON ALLANTOIC FLUID PREPARATIONS OF INFLUENZA VIRUS

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The property of influenza virus suspensions to agglutinate red blood cells appears to be closely associated with the infective activity of the virus (1). The complement-fixing antigen, on the other hand, has been shown to consist of two distinct fractions: one is intimately associated with the hemagglutinin and infective virus particle, while the other has a smaller size and is readily separable from the virus particle by centrifugation or adsorption with red blood cells (2). A preliminary report (3) showed that the infectivity and hemagglutinin of the PR8 and Lee strains of influenza virus were associated with particles which were at least 60 mμ in diameter. Subsequent papers by other workers (4, 5) have confirmed this latter finding and have set the size of the PR8 virus particle at 80 mμ and that of the Lee virus particle at 100 mμ.

The present paper presents a further study of the various properties of influenza virus and their interrelationships, by means of experiments in the angle centrifuge and the optical ultracentrifuge, and by filtration with graded collodion membranes. It will be shown that the application of a synthetic density gradient, already described (6), to influenza virus suspensions minimizes convective disturbances in the angle centrifuge and permits the demonstration of sedimenting virus boundaries which can be identified by infectivity, agglutination, and complement fixation tests.

Methods

Preparation of Virus Suspension.—The PR8 strain of influenza A virus (7) and the Lee strain of influenza B virus (8) were used in these experiments. Allantoic fluid suspensions of each virus strain were prepared by a technique already described (9). Briefly, it consisted of inoculating the allantoic sac of 11 day old white Leghorn embryos with 0.2 cc. of a 10^{-3} dilution of allantoic fluid known to contain virus in high titer. After incubation at 37°C. for 48 hours, the eggs were chilled overnight at about 4°C. The blood-free allantoic fluids were then removed, pooled, and cleared by centrifugation at about 1800 R.P.M. for 5 minutes. Without further treatment the fresh allantoic fluids were subjected to the experimental procedures to be described, and all tests were made on the same day.

Infectivity Tests.—The virus suspensions were diluted in steps of $10^{-0.5}$, and 0.05 cc. samples were instilled intranasally into albino Swiss mice under light ether anesthesia.

All dilutions were made in 10 per cent horse serum broth, and a group of 6 mice was inoculated with each dilution. The 50 per cent mortality titer was calculated from the number of mice which died within 10 days after inoculation.

Agglutination Tests.—The virus suspensions were tested for capacity to agglutinate chicken red blood cells with the aid of a photoelectric densitometer by a standard method previously described (10). The titers are expressed as the dilution of virus which agglutinated 50 per cent of the red cells under the standard conditions of the test.

Complement Fixation Tests.—Various dilutions of the virus preparations were mixed with one and a half 50 per cent hemolytic units of complement (titrated in the presence of the immune serum used in the test) and an optimal dilution of a human immune serum, according to a standard technique, already fully described, which utilizes a photoelectric densitometer for estimating the hemolytic reaction (2).

Angle Centrifuge.—Allantoic fluid suspensions of influenza virus were spun in an air-driven centrifuge of the vacuum type (11). To counteract convective disturbances a synthetic density gradient of sucrose was added to the virus fluids, as previously described (6). The method consists of mixing 0.9 cc. samples of the allantoic fluid in separate tubes with equal volumes of physiological saline containing respectively 0, 8, 16, and 24 per cent sucrose. The virus samples in the above mentioned order were then allowed to feed by gravity through a long syringe needle reaching almost to the bottom of a celluloid centrifuge tube, ½ by 3½ inches. When all samples had been added to the tube, a few bubbles of air were gently forced through the needle to cause a slight mixing between adjacent layers of differing density. Thus, although a uniform concentration of virus was obtained throughout the fluid column (7.0 to 7.2 cm. high), the concentration of sucrose increased from a negligible amount at the top to nearly 12 per cent at the bottom of the tube. The virus fluids were then spun at 12,600 R.P.M. for 14, 28, or 56 minutes (equivalent time, including allowances made for sedimentation during the acceleration and deceleration stages). The meniscus of fluid was 6 cm. from the axis of rotation, and the tubes were inclined at an angle of 35° to the axis. After centrifugation 7 equal samples were removed from each tube by means of a sampling apparatus already described (12), the pellet of sediment being resuspended in the lowest sample. Usually samples from several tubes were pooled. Control tubes which had been prepared in identical manner but not centrifuged, and had been allowed to stand for a comparable time, were also sampled. All samples were tested on the same day by infectivity, agglutination, and complement fixation tests.

Ultracentrifuge.—Concentrated influenza virus preparations were also studied in the optical ultracentrifuge (13, 14), in which a sedimenting boundary can be observed or photographed during centrifugation. The ultracentrifuge cell accommodating the virus preparation is so shaped and oriented that its four side walls do not interfere with the sedimentation of the particles, and consequently true sedimentation constants can be determined by the well known Svedberg method. Unfortunately, the ultracentrifuge cell holds only a very small quantity of fluid, so for this and other even more important technical reasons sampling the centrifuged material for biological testing is generally not feasible. A rough correlation can be made, however, between a photographically recorded boundary in the ultracentrifuge and a boundary identified by biological tests in the angle centrifuge (6).

EXPERIMENTAL

Comparative Behavior of PR8 and Lee Viruses in the Angle Centrifuge.—Previous studies (6) with hemocyanin on the process by which sedimentation of protein particles takes place in the angle centrifuge have shown that sedimenting boundaries can be demonstrated with sufficiently concentrated materials but not with dilute protein preparations because of convective disturbances. To counteract these disturbances, which occur during deceleration or as a result of thermal convection, a satisfactory method was developed for supplying the solution with a synthetic density gradient of sucrose which tended to keep each elemental volume of fluid at its initial level and permitted the formation of a sedimenting boundary. The method was found equally applicable to preparations of influenza virus. Analyses of virus suspensions removed from the centrifuge and sampled at different levels of the tube indicated the presence of true sedimenting boundaries, the displacement from the meniscus of which increased with time and centrifugal force, as illustrated in the following experiments.

Fresh allantoic suspensions of PR8 or Lee influenza virus were mixed with a density gradient of sucrose and spun at 12,600 R.P.M. for 28 minutes in the angle centrifuge; 7 samples were removed from each tube, as described under Methods. The samples were then tested by means of the standard infectivity and agglutination tests.

Fig. 1 shows the results of a typical experiment with PR8 allantoic fluid. The 50 per cent mortality titer of the uncentrifuged control allantoic fluid was $10^{-6.2}$, and the agglutination titer was 1:400. After centrifugation at 12,600 R.P.M. for 28 minutes there was a marked drop in virus concentration in the upper cc. of the fluid column to less than 1 per cent of the original concentration, followed by a sharp rise in concentration about one-third the way down the tube for the hemagglutinin and nearly half the way down for the infective agent. The virus concentration in the lower half of the tube was more uniform, although gradually increasing toward the bottom, and the lowest sample showed an increase of several times the original concentration due to deposition of the virus. The control samples showed that the sucrose solutions had no detectable effect on the virus, as tested by either infectivity or agglutination.

The sharp increase in the concentration of either the hemagglutinin or the infective particles near the middle of the tube is in the nature of a *sedimentation boundary* and indicates a large group of particles which are sedimenting at nearly the same rate and are therefore of nearly the same size. It will be convenient to refer to the particles forming these boundaries as the primary infective and agglutinative particles to distinguish them from any other particles possessing similar biological properties but sedimenting at a greatly different rate, as do large aggregates, for example. Analogous results were obtained with

6 other preparations of PR8 allantoic fluid centrifuged in a similar manner. The boundary position for estimating the average sedimentation rate of the primary group of particles as measured either by infectivity or agglutination tests was taken at the mid-point of the sharp rise in virus concentration, as shown in Fig. 1. Several experiments with Lee virus have also shown bound-

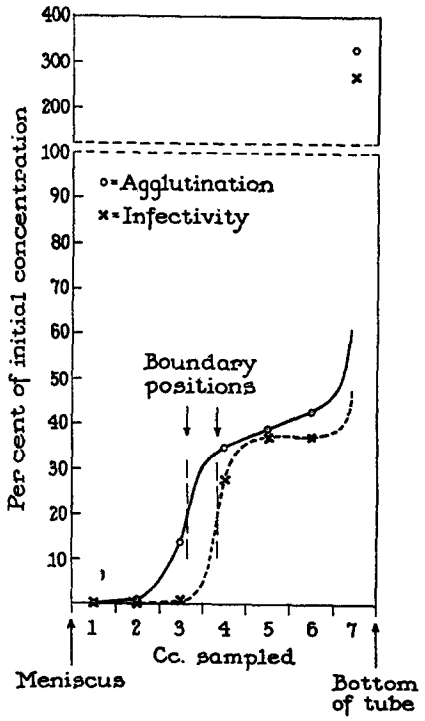

FIG. 1. Sedimentation of infective particles and hemagglutinin of PR8 allantoic fluid (A virus) in the angle centrifuge. Centrifugation 12,600 R.P.M. for 28 minutes (equivalent time).

aries of closely comparable displacement after the 28 minute centrifugation period (as illustrated later in Figs. 2 and 3 and also in a previous publication (3) which describes results obtained by both infectivity and agglutination tests). Concurrent experiments with both PR8 and Lee viruses performed without the protective action of a sucrose gradient showed no indication of a sedimentation boundary. Instead, the virus concentration amounted to several per cent in the upper sample and increased smoothly to the bottom of the tube.

The spread of the boundary (Fig. 1), *i.e.*, lack of extreme sharpness, is due in part to the coarseness of sampling and to the small amounts of remixing which characterize

sedimentation in inclined tubes (6) and which also are necessarily encountered in sampling. In addition, at least in the case of the hemagglutinin, which shows a wider dispersion in sedimentation rate than does the infective property, inhomogeneity of rate is undoubtedly another factor. It should be noted further that a progressive decrease of the average concentration in the plateau region (Samples 4, 5, and 6 in Fig. 1, for example) below the boundary is to be expected because of the radial and hence divergent migration of the particles. Ideally, the concentration should vary inversely as the square of the boundary displacement from the axis of rotation, *e.g.*, should be about 50 per cent in Fig. 1. Although with the angle centrifuge allowance could not be made for this effect with the same exactness (6) as with the uninclined cell of the optical ultracentrifuge, it appeared that the concentration drop in the plateau region was usually more than one would expect with a perfectly homogeneous preparation. Probably a certain number of the particles were in some state of aggregation and settled very rapidly to the bottom of the tube. It was repeatedly observed, as is well illustrated by Figs. 1 and 2, that the concentration drop of hemagglutinin in the plateau region with B virus preparations was significantly greater than with A virus suspensions. This finding suggested that proportionately more active hemagglutinin exists in the form of large aggregates in the case of Lee virus than in the case of PR8 virus.

There was another significant difference in the behavior of PR8 and Lee hemagglutinins. As illustrated by the results shown in Figs. 2 and 3, resuspended sediments of Lee virus showed sufficient concentration of hemagglutinin almost to account for the total amount of starting material minus that remaining in the other 6 samples. As illustrated by Fig. 1, this was never the case for PR8 preparations; 25 per cent or more of the total hemagglutinin was frequently lost through the centrifugation and resuspension of the sediment. Usually, proportionately less of the total infectivity than of the hemagglutinin was recovered after centrifugation, as shown for PR8 virus in Fig. 1, and for Lee virus in a previous publication (3). However, there was one instance with Lee virus in which results by agglutination and infectivity paralleled each other closely and an experiment with PR8 virus in which the final quantity of the infective agent actually exceeded the starting amount.

With PR8 virus preparations striking differences were consistently observed between the behavior of the primary particles of the hemagglutinin and those of the infective principle. These differences are illustrated in Fig. 1. First, there was always an appreciable difference in the average sedimentation rates of the hemagglutinin and the infective particles, the latter migrating more rapidly. Second, the infectivity measurements indicated a sharper boundary, *i.e.*, a more homogeneous group of particles. Both differences were very pronounced in a few cases where, although the infectivity boundary was sharp and normally displaced, the agglutination boundary was markedly polydisperse and showed considerable material settling at a significantly lower rate, the concentration of agglutinin being as high as 7 per cent in sample 2. Third, the concentration of the hemagglutinin in the plateau region was always lower than that of the infective principle. As might be anticipated, the divergence

here was greatest when the difference in average rates was most pronounced. It should be pointed out that the dual testing of each sample in these experiments was performed on aliquots of exactly the same material.

Such consistent differences between the behavior of the agglutinin and the infective principle were not observed with Lee virus, as results given in a previous article (3) demonstrate. There were usually very small differences of the same nature as those described above, notably in samples 2 and 3. However, further and more elaborate experimentation would be required to evaluate the significance of these minor differences.

Effect of Centrifugation Time on Sedimentation of Virus in the Angle Centrifuge.—To substantiate the assumption that the sharp rise in virus concentration represented a true sedimenting boundary, it was shown that displacements of the boundary were approximately proportional to the amount of applied centrifugation, as illustrated in the following experiment.

A series of tubes containing an allantoic fluid suspension of Lee virus and a sucrose gradient was prepared as described in the preceding experiment. Three of the tubes were spun at 12,600 R.P.M. for 14 minutes and then sampled immediately as already described. The corresponding samples of each tube were pooled. Three other tubes were spun at the same speed for 28 minutes, and a third group for 56 minutes. Sampling and pooling were done as before. Uncentrifuged control tubes containing the virus fluid and sucrose gradient were allowed to stand for a comparable period of time. All samples were then tested by means of the standard agglutination test.

The results of the experiment are summarized in Fig. 2. The sedimenting virus boundary as indicated by the sharp rise in hemagglutinin concentration moved only about one-sixth of the tube length when spun at 12,600 R.P.M. for 14 minutes. After centrifugation at the same speed for 28 minutes the boundary had moved more than one-third of the total distance, and when the centrifugation time was increased to 56 minutes the migration was more than three-quarters complete.[1] In the latter instance careful sampling revealed that less than 0.1 per cent of the original concentration of virus remained in the upper cc. of the fluid column, as measured by infectivity tests. On the other hand, virus preparations spun at 12,600 R.P.M. for 56 minutes without a sucrose gradient showed about 5 per cent of the original virus concentration in the upper sample. The gradient technique thus afforded a fiftyfold improvement in the degree of sedimentation of virus from the supernatant fluid.

Centrifugation of the Complement-Fixing Antigen.—To compare the sedimentation behavior of the complement-fixing antigen with that of the hemagglutinin and the infective particle, runs were made for various lengths of time in the angle centrifuge and the sample material was tested by the various

[1] It should be remembered that the centrifugal force, and hence the sedimentation rate, increases with the distance from the axis of rotation.

methods. Gradients of sucrose were employed as already described. Uncentrifuged control tubes showed that the exposure of the antigen to the sucrose for a comparable time had no detectable effect.

The pertinent points which were consistently observed with both PR8 and Lee virus after centrifugation at 12,600 R.P.M. for 28 minutes are illustrated by

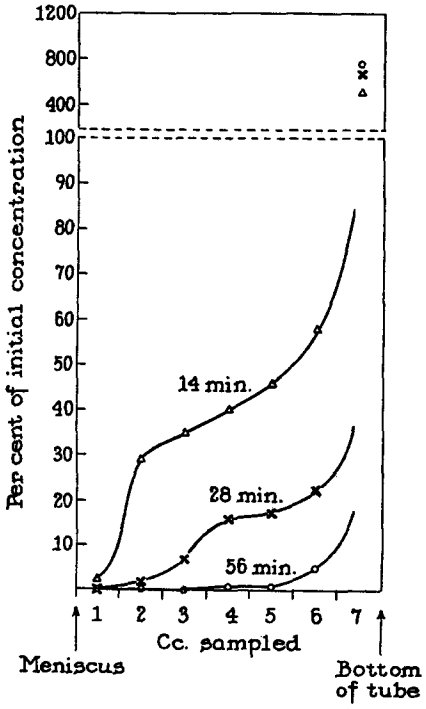

FIG. 2

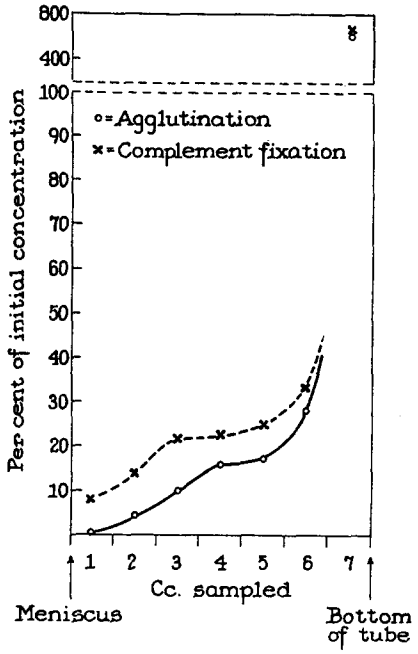

FIG. 3

FIG. 2. Sedimentation of hemagglutinin of Lee allantoic fluid (B virus) in the angle centrifuge. Centrifugation 12,600 R.P.M.

FIG. 3. Sedimentation of hemagglutinin and complement-fixing antigen of Lee allantoic fluid in the angle centrifuge. Centrifugation 12,600 R.P.M. for 28 minutes (equivalent time).

the experiment shown in Fig. 3. It can be seen that much of the complement-fixing antigen sedimented at nearly the same rate as the hemagglutinin. However, in the supernatant fluid (sample 1), where the concentration of hemagglutinin was immeasurably small, there was always found a considerable amount (5 to 10 per cent) of the slowly sedimenting antigen. This divergence may be considered significant since for each sample the individual determinations were made on aliquots of the same material. In the plateau region the

concentration of the hemagglutinin was usually less than that of the complement-fixing antigen. When the centrifugation was prolonged (12,600 R.P.M. for 56 minutes) no hemagglutinin could be detected in the upper half of the tube, yet the concentration of complement-fixing antigen remained almost unaffected. Obviously the complement-fixing antigen comprised at least two general fractions, one sedimenting at nearly the same rate as the primary virus particles, and the other migrating at a considerably lower rate, *i.e.*, behaving as "soluble antigen." Further evidence for the two fractions of the complement-fixing antigen has already been reported (2).

It has been shown in previous studies (6) by one of the authors that from measurements of boundary displacements in the angle centrifuge a rough determination of sedimentation constant can be made. When sucrose gradients were used, dilute protein boundaries always progressed away from the axis a little more rapidly than they did in the ultracentrifuge under comparable conditions. The increase in rate (measured radially) sometimes amounted to as much as 50 per cent. In the present experiments the radial rates (corrected for viscosity) for both PR8 and Lee viruses were found to be of the order of 1000×10^{-13} cm./sec./unit field of force. The average sedimentation constants for the principal particles of these strains of influenza virus, as measured by infectivity and agglutination tests, may therefore be taken as falling within the range 600–1000 Svedberg units. However, in the case of PR8 virus particularly, a portion of the obviously polydisperse hemagglutinin sedimented appreciably more slowly than the infective agent, probably with sedimentation constants somewhat less than 600 S.

Sedimentation of Influenza Virus in the Optical Ultracentrifuge.—The preceding experiments showed that a sedimentation boundary of virus particles as identified by infectivity and agglutination tests could be clearly demonstrated in the angle centrifuge if convective disturbances were counteracted by a density gradient. Studies of allantoic fluid preparations of influenza virus were then undertaken with the optical ultracentrifuge, in which a sedimenting boundary can be observed or photographed during centrifugation.

Concentrated suspensions of influenza virus obtained from allantoic fluid were prepared by two different procedures: (*a*) 200 cc. of PR8 or Lee allantoic fluid were spun at 27,000 R.P.M. for 45 minutes in the vacuum centrifuge, and the supernatant fluid was discarded. The pellets of sediment were resuspended in about 5 cc. of isotonic saline solution buffered at pH 6.9 with M/50 phosphate. The resuspended material was cleared of coarse particulate matter by brief centrifugation at 5000 R.P.M. (*b*) The other method consisted of first adsorbing the virus from allantoic fluid onto red blood cells and then eluting the virus into buffered saline solution (9). For this purpose usually 200 cc. or more of PR8 or Lee allantoic fluid were thoroughly mixed with sufficient packed chicken red blood cells to give a 1 per cent suspension, and this material was kept at 4°C. for 20 to 30 minutes. The red cells were then

packed in the bottom of the tube by light centrifugation, and the supernatant fluid was discarded. The cells were then mixed with 200 cc. of the same diluent as was used in procedure (*a*) and the material was kept at 37°C. for 2 hours, with frequent gentle shaking. The red cells were then discarded following light centrifugation. The 200 cc. of supernatant fluid containing the eluted virus were treated exactly as the original allantoic fluid was under procedure (*a*). The centrifugation and resuspension in a small amount of fluid was shown by test to increase the concentration of hemagglutinin about fifteenfold in the case of PR8 and about twenty-fivefold in the case of Lee virus. These determinations were made after the concentrated material had been cleared of very large aggregates by low speed centrifugation.

Fig. 4 shows a series of photographs made on concentrated preparations of influenza virus examined in the ultracentrifuge by a refractive index method which has been described elsewhere (13, 14). In the case of PR8 virus concentrated from allantoic fluid by centrifugation (photograph 1), it will be seen that light-refracting particles of widely different sedimentation rate were present, ranging from very slowly moving ones up to those exhibiting a fairly well defined maximum rate. This maximum rate was approximately the same (700 S) as that at which the hemagglutinin and infective particle of influenza virus sedimented in the angle centrifuge (Fig. 1). When the virus in a quantity of the same allantoic fluid was concentrated by adsorption onto red blood cells and eluted into fresh buffered saline solution as described above, practically all of the slowly sedimenting material was excluded (photograph 2), as evidenced by the return of the curve to the base line near the meniscus. Two small boundaries, with sedimentation constants of approximately 460 and 700 Svedberg units, remained. Area measurements indicated respective concentrations of roughly 0.06 and 0.1 per cent, assuming the particles to consist largely of hydrated protein. Nearly the same amount of the material characterized by these boundaries appears to be represented in photograph 1. Tests showed that the agglutination titer of the concentrated material (photograph 2) was 1:7000. It should be noted that these two groups of particles which showed the ability to be preferentially adsorbed and eluted, just as do the infective and agglutinating properties of influenza virus, are themselves the larger, more rapidly moving particles whose sedimentation constants are similar to those of the infective and agglutinating properties.

The Lee virus preparations prepared only by centrifugation showed a single, well defined boundary, sedimenting at a rate not greatly different from that of the more rapidly migrating material in the case of PR8 virus. Again, particles, in a relatively lower concentration, were observed settling at various rates ranging up to that of the main boundary. When the virus suspension was obtained from the same allantoic fluid but with adsorption of the virus onto red cells and subsequent elution into buffered saline solution, most, if not all, of the material of lower sedimentation rates was excluded and only the single,

well defined boundary remained (photograph 4). The material characterized by this boundary was lowered in concentration from about 0.13 per cent (photograph 3) to about 0.05 per cent (photograph 4) by the adsorption-elution

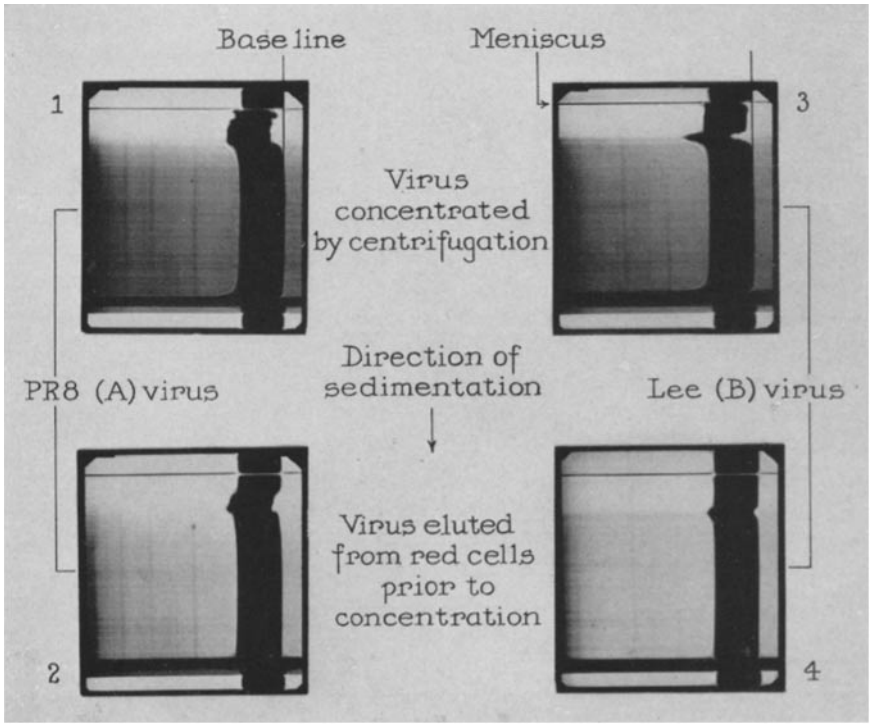

FIG. 4. Refractive index photographs taken after 20 minutes at 6300 R.P.M. in the ultracentrifuge showing sedimenting boundaries of influenza virus particles in buffered saline solution. Material had been concentrated from allantoic fluid about fifteen- and twenty-fivefold in photographs 1 and 2, and the same in photographs 3 and 4, excepting losses of about 40 per cent occurring in red cell adsorption and elution. The presence of sedimenting material, or more exactly a concentration gradient, is indicated by a displacement of the black band to the left of the base line. A peak or hump indicates a sedimenting boundary of nearly homogeneous particles and its area is proportional to their concentration. The sedimentation constant of the boundary in photograph 4 is 800 S; two small boundaries of 460 S and 700 S are apparent in photograph 2.

process. Its sedimentation constant of approximately 800 S is higher than either of those associated with A virus. The agglutination titer of the concentrated material (photograph 4) was 1:3600.

A number of ultracentrifugation studies on different preparations of influenza virus have demonstrated these consistent differences in the PR8 and

Lee virus strains, *i.e.*, the double boundary as contrasted with the single boundary, and the difference in sedimentation rate. In one instance, the virus from PR8 allantoic fluid was eluted into only one-sixtieth the usual amount of fluid and then concentrated an additional tenfold by centrifugation. The ultracentrifugal analysis, as illustrated in Fig. 5, showed that in this more concentrated preparation the ratio of concentrations of the 460 S and 700 S components was radically different, the smaller component predominating. It was not determined definitely whether or not the effect was due to the modified elution technique, which was demonstrated to be only about 50 per cent efficient by agglutination tests. With this material kept at 4°C., it was also found that these boundaries remained unaltered during repeated centrifugation and resuspension into the same medium over a 23 day period.

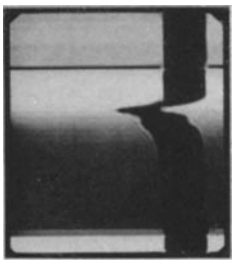

FIG. 5. Refractive index photograph taken after 30 minutes at 6300 R.P.M., showing double boundary of PR8 virus in a preparation more highly concentrated than those shown in Fig. 4.

Photographs taken concurrently with ultraviolet light by the Svedberg absorption method, which confirm the foregoing findings, have been previously reported (3).

Ultrafiltration of Influenza Virus

Elford *et al.* (15) reported that the W.S. strain of influenza virus in mouse lung extracts would not pass through collodion filters having an average calculated pore diameter of less than 160 mμ, which indicated a size range of 80 to 120 mμ after application of the correction formula (16). Ultrafiltration experiments were now done with the PR8 and Lee virus strains obtained from allantoic fluid.

The collodion membranes of graded pore diameter were provided by Dr. J. H. Bauer of this laboratory. PR8 and Lee allantoic virus preparations were obtained as described under Methods. They were further cleared by centrifugation at 5000 R.P.M. for 15 minutes and subsequent passage through collodion membranes with an average pore diameter of 600 mμ. Ten cc. aliquots of the 600 mμ filtrates were then passed through graded collodion membranes which had just been treated in the usual manner by the passage of beef infusion broth. A positive nitrogen pressure of 8 to 10 atmos-

pheres was used. The filtrates thus obtained were immediately tested in mice in the usual way. The infectivity of the virus was enhanced by addition of 0.1 cc. of normal

TABLE I

Ultrafiltration of Allantoic Fluid Suspensions of Influenza Virus

Allantoic fluid virus preparation		Average pore diameter of collodion membrane	Infectivity in mice			Agglutination of red blood cells*
Virus strain	No.		No. inoculated	No. died	No. with pulmonary lesions	
		mμ				
PR8	1	425	6	6	6	+
		205	6	6	6	+
		172	6	0	0	0
		150	6	0	0	0
		132	6	0	0	0
		97	6	0	0	0
	2	239	6	6	6	+
		205	6	6	6	+
		196	6	6	6	+
		188	6	0	5	0
		172	6	6	6	0
		163	6	0	0	0
		150	6	0	0	0
		139	6	0	0	0
Lee	3	425	6	6	6	+
		205	6	6	6	+
		180	6	0	0	0
		150	6	0	0	0
		132	6	0	0	0
	4	205	6	6	6	+
		196	6	6	6	+
		180	6	0	0	0
		172	6	0	0	0
		163	6	0	0	0
		150	6	0	0	0
		139	6	0	0	0

* Visible agglutination recorded as positive (+).

horse serum broth per cc. of filtrate. The filtrates were also tested for capacity to agglutinate chicken red blood cells and to react in the complement fixation test.

The results of four experiments with different virus preparations are summarized in Table I. It will be seen that the PR8 virus in preparations 1 and 2 was held back by membranes with average computed pore diameters of 172 mμ

and 163 mμ, or less, respectively, as determined by infectivity tests. The Lee virus in both preparations failed to pass through membranes with an average pore dimater of 180 mμ or less. The capacity of the filtrates to agglutinate red cells closely paralleled their infectivity, except in the case of the PR8 preparation No. 2, where the 188 mμ and 172 mμ filtrates were infective but showed no detectable agglutination of red cells. The infectivity test for influenza virus, however, is far more sensitive than the agglutination test. A small amount of complement-fixing antigen passed through membranes with an average pore diameter of only 97 mμ. Elford (16) has shown that it is necessary to apply a correction factor of 0.625 ± 0.125 to calibrated pore sizes of this order to determine the effective diameter of pores which are protein-coated. The present results thus indicate that the size (maximum dimension) of the hemagglutinin and infective particle of both PR8 and Lee viruses certainly is within the range 80–135 mμ.

DISCUSSION

The addition of a synthetic density gradient of sucrose to influenza virus preparations has proved to be a satisfactory method for counteracting disturbances of convection in the angle centrifuge and permits the demonstration of sedimenting virus boundaries. The virus boundaries can be clearly identified by sharp changes in virus concentration in the fluid column after centrifugation, as shown by infectivity or agglutination tests (Fig. 1). The outstanding advantage of the method is that it permits sampling of large aliquots of fluid for biological tests without greatly altering a sedimenting boundary. Furthermore, allantoic fluid suspensions of influenza virus can be studied in their native state without preliminary concentration of virus or other procedures which possibly alter the state of the virus, and, as in the present instance, direct correlation can be made between the sedimentation of various properties of the virus. The sedimentation rates thus obtained can be roughly correlated with those obtained optically in the ultracentrifuge.

In a preliminary report (3) on the present experiments, it was shown that the sedimentation rates observed for the primary boundaries of influenza virus, both the PR8 and the Lee strain, indicated a particle size of at least 60 mμ. These experiments have failed to indicate the presence in any significant amount of infective particles having a size near the value of 11 mμ reported by other investigators (17, 18). A sedimentation boundary formed by particles of such a size could have migrated in the angle centrifuge only a fractional part of the distance represented by the top sample under the present conditions of experiment. Actually, virus found in this sample amounted to only a few per cent of the original concentration when untreated allantoic fluid was used; and when convection was reduced by the imposition of a density gradient formed with sucrose, the value dropped to less than 0.1 per cent. It should be emphasized

that the gradient technique as used in the present experiments should not be expected to counteract completely the disturbances of convection in the angle centrifuge. Although remixing can be greatly reduced, traces of virus can be always expected to remain in the supernatant fluid. Without proof to the contrary, it can be assumed that its presence represents an inherent deficiency of the centrifugation technique (6).

The results obtained with the optical ultracentrifuge clearly show that Lee virus preparations contain a group of relatively discrete and monodisperse particles which are adsorbed and eluted by red blood cells and which have an approximate sedimentation constant of 800 S. PR8 virus preparations, on the other hand, show two groups of particles, with approximate average sedimentation constants of 460 S and 700 S, which are adsorbed and eluted by red cells. The sedimentation rates observed in the ultracentrifuge were entirely consistent with the rates of boundaries obtained in the angle centrifuge and detected by actual agglutination tests on sampled material. In the case of PR8 virus two distinct boundaries of hemagglutinin were not differentiated in the angle centrifuge, but this was hardly to be expected in view of the necessarily coarse data and the poor resolution observed with the boundaries in the optical ultracentrifuge. The spread of the hemagglutinin boundary in the angle centrifuge was sufficient to account for the two groups of particles. From correlations established in the angle centrifuge by agglutination and infectivity tests, it appears that the hemagglutinin and the infective particles of the Lee virus have almost the same sedimentation constant and hence presumably represent a common entity, at least to a large extent. There was some inconclusive evidence that a small amount of hemagglutinin settled at a slightly lower rate. In the case of PR8 virus, there was strong though not entirely conclusive evidence that only the more rapid (700 S) of the two hemagglutinin boundaries was associated to a great extent with the infective activity of the virus. Throughout the experiments in the angle centrifuge the sharper, more nearly homogeneous boundaries were recorded by infectivity measurements. It may well be that the particles associated with the 460 S boundary represent inactive virus particles which are in some state of disintegration. It may even be speculated that these inactive particles are roughly equivalent to halves of the primary particle.

If the density of influenza virus is taken as 1.20 gm./cc. (19), the diameter (or length, if not spherical) of an infective particle of Lee virus is computed by the Stokes formula to be at least 85 mμ. For PR8 virus the corresponding value is 80 mμ, on the basis of a sedimentation constant of 700 S. These values are in close agreement with those recently reported by other workers (4, 5). Even if some infective material should be shown to be associated with the optically observed component of 460 S, the value would still be at least 65 mμ. The actual values may be somewhat higher by reason of lower density or non-spherical shape.

The foregoing results, which indicate only minimum particle sizes, are consistent with the data obtained from the ultrafiltration experiments. These experiments, with both the Lee and the PR8 strain of influenza virus, indicated that the infective particle and the hemagglutinin in allantoic fluid have sizes within the range 80–135 mμ. These findings are in close agreement with the results of the filtration experiments of Elford *et al.* (15) with the W.S. strain of influenza virus obtained from mouse lung extracts (80–120 mμ).

The results obtained with the angle centrifuge indicate that most of the complement-fixing antigen of allantoic fluid suspensions of influenza virus is intimately associated with the primary infective virus particles, while an appreciable amount (several per cent) is associated with smaller, more slowly sedimenting particles and hence constitutes a "soluble antigen." Other studies (2) have shown that the soluble and centrifugally separable antigen is not adsorbed and eluted by red blood cells, whereas the more rapidly sedimenting antigen is closely associated with the hemagglutinin and infective particle as indicated by centrifugation and red cell adsorption experiments. It is conconsidered significant in this connection that experiments in the ultracentrifuge showed a considerable amount of material which sedimented more slowly than the primary virus boundaries and which, in contrast to the primary particles, was not adsorbed and eluted by red blood cells. Furthermore, the character of the refractive index photographs (Fig. 4), which show the extraneous material sedimenting at varying rates up to but not above that of the primary particles, strongly suggests that the extraneous material represents virus particles which have undergone various stages of disorganization and disintegration. It seems highly probable that the soluble antigen is closely associated with this debris of inactive virus particles. Also, it seems possible that the particles of 11 mμ, previously reported by others as the size of the primary virus particle (17, 18), may have been associated with this debris.

It is interesting to estimate the number of virus particles required to produce various effects. If the primary hemagglutinin particles of influenza are assumed to have a shape which is not greatly different from spherical, the approximate particle weight can be computed from the average diameter which has been obtained from the sedimentation rate. Then, from the concentration values determined in the ultracentrifuge, the number of primary particles per cubic centimeter of suspension can be calculated. Thus, for PR8 preparations the particle weights of the 460 S and 700 S components were computed to be approximately 14×10^{-16} and 26×10^{-16} gram respectively, and the concentration of each was approximately 4×10^{11} particles per cubic centimeter in the case of the purified, concentrated preparation shown in photograph 2, Fig. 4. For Lee preparations the weight was computed to be 31×10^{-16} gram and the number of particles per cubic centimeter in the purified preparation (photograph 4, Fig. 4) to be 1.6×10^{11}. In this latter case, for example, the agglutination titer was known to be 1:3600; hence in the dilution which produced a

50 per cent agglutination of red blood cells, there were evidently about 4.5×10^7 primary hemagglutinin particles per cubic centimeter. This correlates surprisingly well with the concentration of red blood cells used to determine the 50 per cent agglutination end point in the standard test. Actual counts have shown about 9.2×10^7 red cells per cubic centimeter. Thus the ratio of hemagglutinin to blood cells, in terms of number of particles, is approximately 0.5; in the case of PR8 preparations, the ratio is computed to be approximately 1.2, assuming the 460 S component as well as the 700 S one to represent agglutinin. There may be some error in the determination of these ratios because of aggregates which were not eliminated by the preliminary centrifugal clarification or because of erroneous assumptions regarding particle density and shape.

It is also possible to make a rough estimate of the minimum number of virus particles required to produce infection in mice and chick embryos. With untreated PR8 allantoic fluid suspensions, lung lesions have been repeatedly demonstrated after intranasal instillation of 0.05 cc. of dilutions ranging from $10^{-7.5}$ to $10^{-8.5}$. From the previous discussion relating to concentrated preparations, it can be stated that in untreated PR8 allantoic fluid, there are of the order of 2×10^{10} particles per cubic centimeter which sediment at the same rate as the infective agent and hence presumably represent it, possibly in an inactive as well as active form. Thus in 0.05 cc. of a 10^{-8} dilution, there are of the order of 10 virus particles. A comparable number of particles is required to produce infection of chick embryos with either PR8 or Lee virus, since titers and virus concentrations do not differ greatly. However, the titer of Lee preparations in mice is only 10^4 to 10^5; hence about 10,000 particles would be required to produce demonstrable infection.

SUMMARY

A synthetic density gradient technique has been applied to the study of the PR8 and Lee strains of influenza virus in the angle centrifuge. The method counteracted convective disturbances and permitted about a fiftyfold improvement in clearing supernatant fluids of virus. Sedimenting boundaries of infective virus particles, hemagglutinin, and complement-fixing antigen were obtained in the angle centrifuge and correlated with boundaries observed optically in the ultracentrifuge. The sedimentation constant of infective Lee virus particles is approximately 800 Svedberg units, while that of PR8 virus is only about 700. On the assumption of spherical shape, these values correspond to approximate diameters of 85 and 80 mμ respectively. These values agree with those obtained by filtration with graded collodion membranes. The concentration of primary virus particles in untreated allantoic fluid preparations of PR8 or Lee virus is of the order of 0.01 per cent.

The primary infective particles are identical with the hemagglutinin and the complement-fixing antigen to a large extent. However, allantoic fluid prepa-

rations of PR8 virus also show a slightly inhomogeneous group of particles with an average sedimentation constant of 460 S, which are adsorbed by and eluted from red blood cells yet appear to be non-infective. In addition the virus preparations contain a small amount of "soluble antigen" which sediments more slowly than the virus and is not adsorbed by red blood cells. This soluble antigen is probably associated with material which was observed optically in the ultracentrifuge to sediment at rates ranging from very low values up to that characteristic of the primary virus boundary. This distribution of rate makes it seem likely that the material represents disintegrated virus particles.

Calculations based on the experimental results obtained indicate that of the order of 10 influenza virus particles are required to produce infection of chick embryos or mice with the PR8 virus. While a comparable number is required with Lee virus for infection of chick embryos, about 10,000 particles are necessary for infection of mice. The ratio of hemagglutinin to red blood cells required to produce 50 per cent agglutination with dilute virus suspensions in the standard test is roughly 1.

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