THE SEDIMENTATION RATE OF THE BIOLOGICAL ACTIVITIES OF INFLUENZA A VIRUS*

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In studies carried out in several different laboratories, essentially spherical particles with diameters of the order of magnitude of 100 mμ and sedimentation rates of 612 to 832 S have been obtained from the allantoic fluids of chick embryos infected with various strains of influenza virus (1–6). Infectivity seemed in general to be associated with fractions containing these particles. Nevertheless, a most important question confronting those interested in the isolation of pure influenza virus is whether the infectivity resides in the predominating particles in virus preparations or in somewhat different particles present in too low a concentration to be detected by other than biological means. Evidence bearing upon this problem can be obtained by comparing the physical properties of the infectious principle with those of the isolated spherical particles.

From a filtration end-point of 160 mμ, Elford, Andrewes, and Tang (7) inferred that the infectious principles in mouse lung suspensions of the W.S. strain of human influenza virus and a strain of swine influenza virus obtained from Shope were associated with particles between 80 and 120 mμ in diameter. By means of sedimentation studies involving the high-speed centrifugation of influenza virus in capillary tubes, Elford and Andrewes (8) found the infectious principles of both viruses to be represented by spherical particles with diameters between 87 and 99 mμ. From these data, it can be calculated that the sedimentation constants of the infectious principles lie between 840 and 1090 Svedberg units. Friedewald and Pickels (3), using the high-speed angle centrifuge, studied the sedimentation of the PR8 strain of influenza A and the Lee strain of influenza B in the allantoic fluids from infected chick embryos. The sedimentations were carried out in sugar gradients in order to minimize convection. The sedimentation rate of the infectious principle of each virus was estimated to be between 600 and 1000 Svedberg units. In filtration studies on the same strains, end-points of 163 and 180 mμ, respectively, were obtained for the PR8 and Lee strains, indicating that the infectious particles had diameters in the range of 80 to 135 mμ.

It is apparent that certain physical properties of the mouse infectivities of PR8, Lee, W.S., and swine strains of influenza virus are in rough agreement with the corresponding properties of the predominating particles in purified

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preparations. However, the precision of the correlations is not high and is not objectively evaluated. In the present investigation, a somewhat better correlation has been obtained through the use of an ultracentrifuge equipped with a separation cell.

PR8 and F12 strains of influenza A virus were studied. The latter strain was of particular interest because both large- (100 mμ) and small- (10 mμ) sized particles have been found in centrifugally concentrated preparations (6, 9). For a time it was thought that the smaller particles were the infectious units, but considerable evidence has recently been presented (3, 10) to demonstrate that the infectivity of the virus is associated with the larger particles. In order to avoid possible complications which might arise from the use of crude preparations of virus, the samples of influenza virus to be studied were fractionated by differential centrifugation and the heavy fractions were saved as starting materials for the investigation.

The discovery by Hirst (11) and by McClelland and Hare (12) that influenza virus preparations possess chicken red blood cell agglutinating activity as well as infectivity provided a second biological test which could be correlated with the physical properties of the virus. In several separation cell experiments, therefore, measurements were made of red blood cell agglutinating activity, infectivity, and nitrogen content, and comparisons were made of the sedimentation rates of the different factors.

Materials and Methods

Virus Preparations.—Fresh preparations of PR8 or F12 influenza virus were used in each of the separation cell experiments described below. The virus stocks were those described by Stanley (10). Ten-day-old chick embryos were inoculated with either of the two strains and incubated for about 48 hours. The virus was then isolated from the allantoic fluids by two half-hour centrifugation cycles in the manner described previously (10). It has been shown elsewhere (5) that preparations of influenza virus obtained in this manner consist of a principal component with a sedimentation rate of about 700 Svedberg units corresponding to spherical particles about 100 mμ in diameter and of a minor polymer-like constituent with a sedimentation rate of about half that value. Traces of smaller particles are probably also present.

Methods.—A Bauer and Pickels (13) air-driven ultracentrifuge was used in these studies. The virus preparations were allowed to sediment at a speed of 11,100 R.P.M. in a separation cell similar to that described by Tiselius, Pedersen, and Svedberg (14). The sedimentation of the material was followed optically by the Svensson (15) schlieren method. The fraction of the material remaining above the barrier at the end of the sedimentation run was calculated from the position of the meniscus and the final position of the boundary with respect to that of the barrier, in the same manner as was used in the case of tobacco mosaic virus (16). The residual material was removed from the top compartment and subjected to chemical or biological analyses. Similar analyses were made on the initial fluids. Mouse infectivity measurements were carried out in the manner outlined by Lauffer and Miller (17). Chick embryo infectivities were measured according to the procedure described by Knight (18). Red cell agglutinating activity was measured by the method of Hirst as modified by Miller and Stanley (19). Protein analyses were carried out by a micro-Nessler method (20). The ratios of the various residual biological activities in the top compartment to those in the initial fluids were compared with the ratios determined from the final position of the sedimenting boundary.

PRESENTATION AND DISCUSSION OF RESULTS

Correlation of Mouse Infectivity with Sedimenting Boundary.—Five successive experiments were carried out as outlined on PR8 virus preparations and five on F12 virus preparations in order to correlate the sedimentation rate of the visible boundary with that of mouse infectivity. The results are presented in Table I. The logarithms to the base 10 of the ratio of initial to final concentration of the sedimenting material, presented in the 6th column, were computed from the data given in the preceding three columns. For the purpose of these calculations, the assumption was made that the virus preparations

TABLE I

Correlation of Mouse Infectivity with Sedimenting Boundary

Strain	Effective time of sedimentation	Distance from axis of rotation			Log_{10} initial/final concentration	Log_{10} initial/final mouse infectivity
		Meniscus	Final boundary position	Barrier		
	sec.	*cm.*	*cm.*	*cm.*		
PR8	560	6.07	6.44	6.66	0.48	−0.15
PR8	850	5.93	6.30	6.66	0.34	+0.65
PR8	985	5.84	6.34	6.66	0.45	+1.12
PR8	1160	5.80	6.45	6.66	0.70	+1.96
PR8	915	5.82	6.28	6.66	0.40	+0.55
F12	780	5.94	6.42	6.66	0.54	+1.61
F12	855	5.94	6.44	6.66	0.54	+0.49
F12	920	5.96	6.36	6.66	0.41	−0.06
F12	765	5.80	6.38	6.66	0.55	+0.81
F12	810	5.82	6.27	6.66	0.39	+0.78

were essentially homogeneous with respect to sedimentation rate. It can be observed that the ratios of initial to final concentrations are not directly correlated with effective times of sedimentation. This is because the cell was not filled to the same level every time, as indicated by the variation in the position of the meniscus, and also because the temperature, and therefore the absolute rate of sedimentation, varied from run to run. The logarithms of the ratios of initial to final infectivity of the material above the barrier, presented in the 7th column, were calculated from the mouse infectivity titers of the original material and of that remaining in the upper compartment.

If the sedimentation rate of the infectious principle is the same as that of the isolated material which can be observed visually, the data of columns 6 and 7 should agree within experimental error. For the case of the PR8 strain, the logarithm of the infectivity ratio was found to be on the average 0.35 unit greater than the logarithm of the concentration ratio. The standard error and the probable error of the mean of the differences between the logarithms of the

infectivity ratios and the concentration ratios were calculated to be 0.31 and 0.21 unit, respectively. For the case of the F12 strain, the logarithm of the infectivity ratio was found to be on the average 0.23 unit greater than the logarithm of the concentration ratio. The standard error and the probable error of the mean in this case were determined to be 0.26 and 0.17 unit, respectively. It is obvious that the observed average differences between the ratios are too small in both cases to be regarded as significant evidence of real differences between sedimentation rates of the mouse infectivities and the isolated components. As will be discussed later, however, the correlation of the bulk of the infectivity with the principal sedimenting component can be improved for both strains by recalculating the results on the basis of the assumption that a certain portion of the infectivity is associated with aggregated particles.

Correlation of Chicken Red Blood Cell Agglutinating Activity, Chick Embryo Infectivity, and Nitrogen Content with Sedimenting Boundary.—For the purpose of correlating chicken red blood cell agglutinating (CCA) activity, chick embryo infectivity, and nitrogen content with the sedimenting boundary, an original experiment consisting of six sedimentation runs continued for varying lengths of time on the same preparation was carried out. The original and the material remaining in the top compartment were analyzed for CCA activity, chick embryo infectivity, and nitrogen. The results are presented in the upper part of Table II. The data of column 1 were obtained in a manner analogous to those of column 6 of Table I. The percentages remaining of CCA activity, of chick embryo infectivity, and of nitrogen are given in columns 3, 4, and 5, respectively. It can be seen that the agreement between the amounts of the three quantities is excellent, indicating that CCA activity, chick embryo infectivity, and nitrogenous material sediment at the same rate. However, the agreement between these quantities and the amount of residual material as calculated from the position of the sedimenting boundary determined optically is less satisfactory. The small amount of activity and of protein nitrogen remaining after the boundary had passed beyond the barrier is probably due to contamination, as was shown to be the case with tobacco mosaic virus (16). The discrepancies at other levels constitute evidence that the assumption that the amount of material remaining in the top compartment can be calculated from the boundary position is not strictly valid for this particular experiment. This might be due to inhomogeneity of the material. For example, if 42 per cent of the protein, the CCA activity, and the chick embryo infectivity are assumed to be associated with highly aggregated particles which sediment very much faster than the optically obvious component, the data of column 1 can be adjusted to obtain the data of column 2. It is apparent that the data of column 2 are in much better agreement with those of columns 3, 4, and 5.

The postulation of a certain amount of highly aggregated active material in influenza virus preparations is not unreasonable, for it has been observed on

many occasions that the material sedimented by 10 minutes' centrifugation at 5000 R.P.M. in an angle centrifuge contains considerable activity. This postulate was tested in part in a duplicate experiment. A preparation of PR8 influenza virus was first fractionated in the same manner as that described by Lauffer and Stanley (5) for preparation 7 in order to eliminate as much as

TABLE II

Correlation of Chicken Red Blood Cell Agglutinating Activity, Chick Embryo Infectivity, and Nitrogen Content with Sedimenting Boundary

Fraction remaining in top compartment				
From position of sedimenting boundary	Adjusted values from boundary positions	From CCA activity	From infectivity in chick embryos	From nitrogen content
Original experiment				
100	100*	100	100	100
77	45	57	57	65
61	35	35	‡	39
58	34	37	‡	39
43	25	23	20	17
25	15	12	15	§
0	0	2	‡	§
Duplicate experiment				
100	100‖	100	100	100
70	50	58	74	58
58	41	39	102	38
35	25	30	16	17
26	19	15	12	(10)§
0	0	1	2	(3)§

* Calculated on the assumption that 42 per cent of the active material is highly aggregated.
‡ Samples not tested.
‖ Calculated on the assumption that 28 per cent of the active material is highly aggregated.
§ Samples too small for accurate analysis.

possible of any slowly sedimenting constituents which might be present. The preparation was then subjected to a procedure designed to remove some of the highly aggregated material. This was done by rapidly bringing the material up to a speed of 15,000 R.P.M. in the quantity centrifuge and then at once decelerating rapidly. The pellet was removed and the supernatant fluid was subjected again to this treatment. The supernatant liquid from this second centrifugation was used for the duplicate separation cell experiments. The only additional difference between the experimental technique of this duplicate and that of the original experiment was that 1 per cent sucrose was added to the

virus solution below the barrier. This was found to be necessary in order to prevent convection in the separation cell. It was not found necessary in the original experiment, probably because the virus concentration was much higher in that case.

The results for the duplicate experiment are shown in the bottom half of Table II. In spite of the attempted elimination of aggregated material, the results resemble those of the original experiment. The fact, however, that it is necessary to postulate only 28 per cent of aggregated material to bring the CCA activity data into reasonable harmony with the computations from boundary positions may indicate that the fractionation in the angle centrifuge did effect at least a partial removal of aggregated material.

The theory that the discrepancy is due to aggregates was subjected to further test by accelerating the separation cell filled with a PR8 influenza virus preparation to a speed of 11,100 R.P.M. and decelerating immediately. Chick cell agglutination and nitrogen analyses indicated losses of 8 and 18 per cent, respectively. The experiment was repeated, this time holding the cell at 11,100 R.P.M. for 1 minute. Analyses showed losses of 20 and 19 per cent, respectively, of CCA activity and nitrogen. Inasmuch as very little of the 100 mμ component should have sedimented under such mild conditions, these results demonstrate conclusively that 10 to 20 per cent of the CCA activity of this preparation was associated with aggregated material.

The result just described suggested that the separation cell might be used to separate aggregated material from the unaggregated with a higher degree of efficiency than was realized with the angle centrifuge. Accordingly, a preparation of PR8 influenza virus, from which most of the more slowly sedimenting material had been removed by fractionation in the manner described by Lauffer and Stanley (5) for preparation 7, was centrifuged for 1 minute at 11,100 R.P.M. in the separation cell. Ten such runs were made, and the materials remaining in the top compartment were pooled. The virus concentration as estimated by nitrogen determinations was reduced from 21 mg. per cc. to 16 mg. per cc. by this treatment. The pooled top compartment material was diluted to a concentration of 8 mg. per cc. and subjected to a series of separation cell runs. To minimize the danger of aggregation subsequent to this purification, the runs were completed within 12 hours of the beginning of the fractionation. To prevent convection, 1 per cent of sucrose was dissolved in the virus solution placed below the barrier. Chicken red blood cell agglutinating activity determinations and nitrogen analyses were performed on the material isolated from the upper compartment for each run. The amount of 100 mμ component remaining in each case was calculated from the optical data on the sedimenting boundary. The results are presented in Table III.

It can be seen that the data of Table III show an almost perfect correlation between the sedimentation rates of the 100 mμ component as judged by optical

means and the CCA activity. The correlation between both of these and the nitrogen content is also good. This experiment demonstrates that the sedimentation rate of the CCA activity is the same, or at least very nearly the same, as that of the 100 mμ component. It also demonstrates, with finality, that the failure to obtain perfect agreement in the previous experiments was actually due, as postulated, to the presence of aggregated material bearing activity.

The results presented earlier in Table II show that within the error of the method, chick embryo infectivity sediments at the same rate as CCA activity and nitrogenous material. Since it was possible to conclude that some CCA activity is definitely associated with aggregated material but that the bulk of it is associated with the 100 mμ particles, it is possible to deduce from the results of Table II that most of the chick embryo infectivity likewise is associated with the 100 mμ component and a smaller amount with aggregated matter.

TABLE III

Correlation of Chicken Red Blood Cell Agglutinating Activity and Nitrogen Content with Sedimenting Boundary

Fraction remaining in top compartment		
From position of sedimenting boundary	From CCA activity	From nitrogen content
100	100	100
73	72	66
47	45	43
17	18	11

In the discussion of the data of Table I, it was pointed out that in both the case of the PR8 strain and that of the F12 strain, the ratio of residual infectivity in the upper compartment after centrifugation to the original was on the average less than the comparable ratio for the 100 mμ component as judged by the final boundary positions. It was stated that the error in the mouse infectivity measurements was too great for this result to be significant on the basis of the criteria usually accepted by statisticians. Nevertheless, the agreement between the results obtained by mouse titrations and those computed from boundary positions would be improved greatly by recalculating on the basis of the assumption that a considerable portion, but less than half, of the mouse infectivity in the preparations studied was associated with highly aggregated material and the rest with the 100 mμ component. The results of Table I rule out the possibility that the bulk of the mouse infectivity is associated with particles which sediment much faster or much slower than the 100 mμ particles.

On the whole, the results obtained in the present study indicate that the sedimentation rates of the mouse infectivity, the chick embryo infectivity, and particularly the chicken red blood cell agglutinating activity of the PR8

strain of influenza A virus are in excellent agreement with the sedimentation rate of the conspicuous component obtained upon isolation by differential centrifugation. They also indicate that the mouse infectivity of the F12 strain has about the same sedimentation rate as the isolated component. When these results are considered in conjunction with those of Friedewald and Pickels (3) and with those of Stanley (10) and of Miller, Lauffer, and Stanley (21), which showed that the more slowly sedimenting constituents have low specific activities, it can be concluded with a high degree of probability that the three biological activities are associated physically with the particles which have been isolated and characterized as spheres with a diameter of about 100 mμ.

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SUMMARY

The sedimentation rates of the mouse infectivity principles of the PR8 and the F12 strains of influenza A virus were shown by studies in the separation cell to have values which are not significantly different from sedimentation rates of the principal components of purified preparations. It was shown further that the bulk of the chicken red blood cell agglutinating activity and of the chick embryo infectivity of PR8 influenza virus preparations sediments at the same rate as that of the 100 mμ component. Some activity was shown to be associated with aggregates. These results lend strong support to the assumption that the three biological activities are associated with the particles characterized as spheres with a diameter of about 100 mμ.

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