BIOPHYSICAL PROPERTIES OF PREPARATIONS OF PR8 INFLUENZA VIRUS*

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During the past decade several studies on the physical properties of various strains of influenza virus have been reported. Elford, Andrewes, and Tang (1) obtained a filtration end-point of 160 mμ for the W.S. strain of human influenza and the Shope strain of swine influenza virus. From this they inferred that the infectious particles of influenza virus fall in the size range of 80 to 120 mμ. Elford and Andrewes (2) studied the sedimentation of the infectious principle and inferred, from the observation that there was no sedimentation in a sugar solution of density 1.2, that the density of the influenza particle is 1.2. From this value and the rate of sedimentation in the absence of sucrose, they estimated that the influenza particles could be represented by spheres with diameters between 87 and 99 mμ.

More recently, Bourdillon (3) and Chambers and Henle (4) came to the conclusion that the infectious unit of influenza virus is a much smaller particle of the order of magnitude of 10 mμ in diameter. This conclusion was based principally upon the observation that a small amount of the infectivity seemed to diffuse more rapidly and sediment more slowly than particles with a diameter of the order of 100 mμ. The possibility that these results were due to minor disturbances in the diffusion and sedimentation processes was never rigorously excluded. Nevertheless, the assumption that the infectious principle might be associated with small particles seemed for a time to be strengthened by the observation of Chambers, Henle, Lauffer, and Anderson (5) that particles about 11 mμ in diameter could be demonstrated by the ultracentrifuge and the electron microscope in influenza virus preparations obtained by differential centrifugation of the allantoic fluid of infected chick embryos. However, Stanley (6) has since shown that the specific infectivity of reasonably highly purified preparations of the small material is only a small fraction of that of the larger material. Thus there is reason to suspect that none of these particles are infective and that the residual infectivity of preparations of such small particles is due to contamination.

Friedewald and Pickels (7) studied the angle sedimentation in sugar gradients of allantoic fluids from infected chick embryos. Both A and B strains were investigated. Sedimentation was followed by analyzing successive fractions of the centrifuged tubes of fluid for mouse infectivity and for chicken red cell agglutination. They came to the conclusion that, if the particle density is assumed to be 1.33, the bulk of the infectivity is associated with particles at least 60 mμ in diameter. In a continuation of

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this study they observed that the infectious principles of the A and B strains have filtration end-points of 163 and 180 mμ, respectively, indicating that the infectious particles have diameters within the range 80 to 135 mμ (8).

Taylor, Sharp, Beard, Beard, Dingle, and Feller (9) reported that the particles in purified preparations of PR8 influenza virus gave kidney-shaped images in the electron microscope with an average diameter of 77.6 mμ. The sedimentation constant was reported to be 724 Svedberg units, corresponding to spheres of density 1.2 with an average diameter of 80 mμ. Friedewald and Pickels (8) reported a sedimentation rate of about 700 S for this same strain and a rate of 800 S for the Lee strain. Sharp, Taylor, McLean, Beard, Beard, Feller, and Dingle (10) and Taylor *et al.* (11) studied preparations of the Lee and swine strains of influenza virus in the electron microscope and the ultracentrifuge.

In the present report somewhat more extensive studies on the biophysical characteristics of purified preparations of PR8 influenza virus are presented.

Materials

The PR8 influenza virus was obtained from Dr. Thomas Francis, Jr., of the University of Michigan, and its passage history has been described earlier (12). The purified virus preparations used in these studies were obtained from the allantoic and amniotic fluids of infected chick embryos by means of centrifugation in a high speed quantity centrifuge (13), essentially in the manner described by Stanley (12) as method A. However, the centrifugations at 24,000 R.P.M. were carried out for 5 to 30 minutes, instead of for 2 hours. Nine different preparations were used.

Preparation 1 was obtained by two centrifugation cycles involving high speed runs for 30 minutes. The final concentration was 18.4 mg. of protein per cc., and the preparation possessed 3,800 chicken red cell agglutinating (CCA) units per mg. of protein.

After the studies on preparation 1 were completed, the remnants were pooled with two similar freshly isolated preparations and brought up to 24,000 R.P.M. and down in the quantity centrifuge. The supernatant fluid was centrifuged for 5 minutes at 24,000 R.P.M. The pellets of sedimented protein were taken into solution and the process was repeated. The final precipitate, dissolved in 0.1 M phosphate buffer at pH 7, was used as preparation 1a. The final concentration was 12.5 mg. of protein per cc. and the material possessed 4,000 CCA units per mg. of protein and a 50 per cent infectivity end-point in chick embryos at $10^{-13.5}$ gm.

Preparation 2 was obtained by two centrifugation cycles. The first high speed run was for 5 minutes and the second for 23 minutes. The preparation was dialyzed against distilled water for 48 hours for use in density determinations.

Various preparations isolated during December, 1943, were pooled and concentrated by high speed centrifugation to 27 mg. per cc. to form preparation 3.

Preparation 4 was obtained in a similar manner by pooling three fresh PR8 samples totaling 322 mg. of protein and concentrating to 45 mg. per cc. by high speed centrifugation for 20 minutes.

Preparation 5 was isolated by two centrifugation cycles. The high speed runs were continued for 15 minutes and the preparation was then fractionated. First the material was centrifuged for 5 minutes at 24,000 R.P.M. The precipitate was resuspended in 0.1 M phosphate buffer and the centrifugation was repeated. The supernatant fluids from the last two centrifugations were combined and concentrated by centrifugation for 30 minutes at 24,000 R.P.M. The resulting precipitate was dissolved in 7 cc. of 0.1 M phosphate buffer to give fraction 5a. The precipitate from the second 5 minute high speed centrifugation was dissolved in 0.1 M

phosphate and brought up to 18,500 r.p.m. and down. The precipitate was redissolved and the operation was repeated. The supernatant fluids from the last two operations were combined and concentrated by centrifugation for 15 minutes at 24,000 r.p.m. The material was finally suspended in 4.8 cc. of 0.1 m phosphate buffer to give fraction 5b. This fraction contained 19 mg. protein per cc., and possessed 4,840 CCA units per mg. of protein and a weighted 50 per cent mouse end-point at $10^{-9.3}$ gm. The precipitate from the second 18,500 r.p.m. run was dissolved in 0.1 m phosphate buffer to give fraction 5c.

Preparation 6 was obtained by pooling and concentrating several very old centrifuge preparations of PR8 influenza virus.

Preparation 7 was obtained by two centrifugation cycles. It was then fractionated according to the following procedure. Four successive runs to 18,000 r.p.m. and down were made upon the original virus solution. The precipitates were removed after each run and finally combined and redissolved in 0.1 m phosphate buffer. This solution was then subjected to three successive runs as described above, and the precipitates were again combined and redissolved. This third solution was subjected to exactly the same treatment as that which had been accorded the second. The precipitates were combined and dissolved in 0.1 m phosphate buffer to give fraction 7a. The supernatant fluids from all of the runs to 18,000 r.p.m. and down were combined and concentrated by centrifugation at 24,000 r.p.m. into a volume of 7 cc. This was labeled preparation 7b. The CCA activities of 7a and 7b were determined to be 3,960 and 2,700 units per mg. of protein, respectively.

Preparation 8 was obtained in a manner identical with that used to obtain preparation 7. It was separated in the same manner into a heavy, 8a, and a light, 8b, fraction. 8a was subjected to a repetition of the fractionation procedure and the heavier portion was designated 8c.

Preparation 9 was obtained in the same manner as 7 and 8 and was fractionated by the same manner into 9a and 9b. 9a was subjected to a repetition of the fractionation and a fraction 9c comparable to 8c was obtained.

EXPERIMENTAL RESULTS

Electron Microscopy

Electron micrographs of preparations 1, 5b, and 9c were made for the authors by Dr. James Hillier and Dr. R. F. Baker of the RCA Laboratories at Princeton. An RCA type B electron microscope (14) was used. Mounts were made after diluting the virus to a concentration of 10^{-4} gm. per cc. with distilled water.

Fig. 1 represents a section from one field, obtained with preparation 5b reproduced at a total magnification of 28,200. It can be observed that the particles are essentially spherical in shape, not very much different from those shown by the micrographs of Taylor et al. (9). Similar images were obtained with preparation 1, but those obtained with preparation 9c were somewhat more irregular. The diameters of 100 particles found in a large field including that reproduced in Fig. 1 were measured from the micrograph using a vernier calipers. The average diameter of the enlarged images was found to be 3.261 mm. with a total variance of 0.2734. Next, that portion of the total variance due to errors of measurement was calculated from twenty successive measurements on a single particle to be 0.0265. Thus the residual variance due principally to actual variation in the size of the images was 0.2469. The square root of this value, 0.498 mm., is the standard deviation of the distribution of particle

diameters. It is 15 per cent of the mean value. This distribution is almost identical with that found by Taylor *et al.* (9). The mean diameter of the
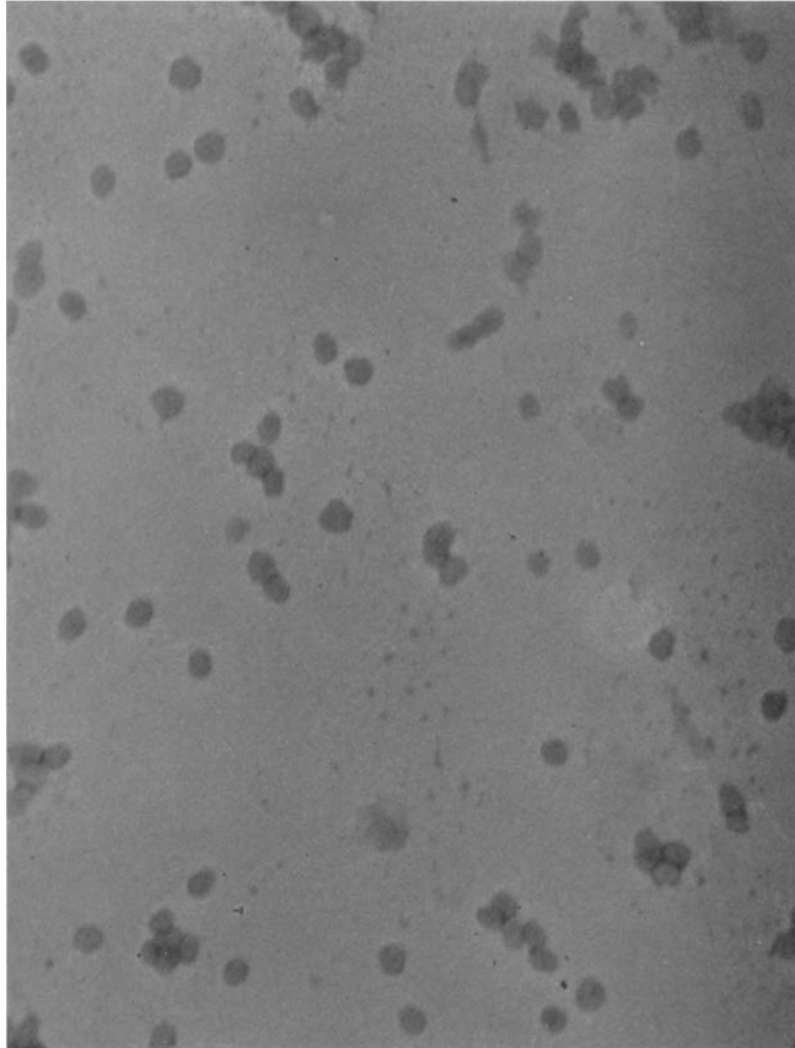

FIG. 1. Electron micrograph of PR8 influenza virus preparation 5*b* mounted at a concentration of 10^{-4} gm. per cc. in distilled water. $\times$ 28,200.

particles after correction for magnification is 115.5 mμ and the standard deviation of the mean is 1.4 per cent of the mean. The mean value obtained with preparation 9*c* was 109.7 mμ, with a standard deviation of the mean of 2.75 per cent.

The mean values obtained in these studies differ considerably from the value found by Taylor *et al.*, of 77.6 mμ (9). The chances are that this discrepancy is due to uncertainty in the magnification factor of one of the electron microscopes. The electron microscope at the RCA Laboratories was standardized by measuring the size of the same object with the electron microscope at low magnification and also with an optical microscope. It was then calibrated by measuring the image of a small object at various projector lens currents. The microscope was restandardized immediately before the micrographs of preparation 9c were obtained. In the study reported by Taylor *et al.* (9), the magnification factor of the microscope was determined by micrographing tobacco mosaic virus particles, and then measuring the thickness of the images. The thickness of the virus particles is known from x-ray diffraction studies to be 15.2 mμ. However, under the conditions of contrast afforded by a virus particle on a collodion film, a point in the object appears as a circle in the image, with a radius of from 3 to 5 mμ. Thus, there is a zone of indeterminacy of the order of magnitude of 6 to 10 mμ encountered in attempting to measure the size of an object with contrast such as that encountered in these studies. It is possible that a tobacco mosaic virus particle 15.2 mμ in thickness would appear in the image to be as much as 25 mμ in thickness. Therefore, a magnification factor based on the thickness of tobacco mosaic virus particles could be too low by as much as 70 per cent.

In order to investigate this possibility, an electron micrograph was made of a preparation of the aucuba strain of tobacco mosaic virus on the same day as the micrograph of preparation 9c of PR8 influenza virus was made. The widths of several of the negative images were measured with a microphotometer by Dr. Hillier. The microphotometer curves were found to resemble somewhat flattened Gaussian distribution curves. The widths of five of these were measured at an altitude of half the maximum distance from the base to the level of maximum displacement. An average value of 25 mμ was obtained. The thickness of the rods of this strain of tobacco mosaic virus is known from x-ray diffraction studies to be the same as that of the ordinary strain, 15.2 mμ. Thus it has been shown that the electron microscope overestimates the thickness of tobacco mosaic virus rods and that the use of this material to standardize the microscope will lead to a magnification factor which is too low by a considerable amount. It is thus apparent that the value of 77.6 mμ for the diameter of particles of PR8 influenza virus reported by Taylor *et al.* (9) is probably much too small. The values obtained in the present study, 110 to 115 mμ, are probably much more nearly correct.

Specific Volume Determinations

Specific volumes were usually calculated from determinations of the densities of suspensions obtained by dialyzing virus preparations against distilled water. The total solids present in each suspension were determined by drying a weighed amount of the suspension to constant weight. Two procedures were used. In the first the water was evaporated at just under 100°

C. and then the material was brought to constant weight *in vacuo* over P_2O_5 at 110°. In the second the suspensions were first dried in the frozen state and then brought to constant weight *in vacuo* over P_2O_5 at 110° in an Abderhalden dryer from which the oxygen had first been swept out with nitrogen. This procedure was used because of an observation by Dr. Knight that purified influenza virus preparations sometimes increase in weight when heated in the presence of air, due presumably to the addition of oxygen.

Preparations 1a, 2, 5b, 6, 7a, and 8a were studied. The second method was used for estimating the solid content in the case of preparation 1a and more than 50 mg. of virus were used. Method 2 was also used in the case of preparation 2. About 125 mg. of virus were used and duplicate determinations were made. In the case of preparation 5b, method 2 was used with the exception that the Abderhalden dryer was not first swept out with nitrogen. Upon drying over P_2O_5 *in vacuo* at 110° the material turned brown, indicating that some oxygen may have been taken up. Only about 15 mg. of virus were available. In the case of preparation 6, method 1 was used for determining the solid content. About 90 mg. of virus were used for a single determination. Preparation 7a was dried over P_2O_5 *in vacuo* at 110° in a drying tube first swept free of oxygen. The dry density of the resulting material, consisting of about 55 mg., was determined by the toluene displacement method. Preparation 8a was brought into equilibrium with a solution 0.1 M with respect to sodium chloride and 0.01 M with respect to phosphate buffer at pH 7, by repeated sedimentation and resuspension in the solution. The salt solution was used instead of water in order to avoid possible lysis of the material. The electrolyte compositions of the salt solution and of the virus-salt solution were checked analytically. The total solids content of the virus-salt solution was determined by method 2 and the virus concentration was calculated by difference. The partial specific volume was calculated from five replicate measurements, using a total of about 90 mg. of virus.

The results of the specific volume measurements are listed in Table I. Four of the values, representing the most carefully investigated samples, have a weighted average of about 0.79. The other two values are 0.84 and 0.85, but each of these represents a single determination made under adverse conditions. The higher figures are in agreement with values reported for the swine (11) and Lee strains (10) of influenza virus. However, since the higher values were obtained with limited amounts of material and the concentrations were estimated by an unsatisfactory method, they may be subject to error. On the basis of all available evidence the most probable value for the specific volume of centrifuge preparations of PR8 influenza virus is regarded to be 0.79.

It was noted that the dialysis of PR8 virus preparations against distilled water resulted in marked decrease in CCA and virus activities. For example, before dialysis, preparation 1a possessed 4,000 CCA units per mg. of protein and a 50 per cent infectivity end-point in chick embryos at $10^{-13.5}$ gm. After dialysis for 3 days at 4° against three successive 1 liter portions of distilled water, this preparation possessed less than 5 CCA units per mg. of protein and the 50 per cent infectivity end-point in chick embryos was $10^{-8.9}$ gm. Following the specific volume determination, the dried sample of preparation 1a was analyzed by Dr. A. Elek of The Rockefeller Institute and 55.11 per cent carbon, 7.86 per cent hydrogen, 10.35 and 10.36 per cent nitrogen, 1.13 per cent phosphorus, and 3.26 per cent ash were found. Electron micrographs of dialyzed preparations of PR8 influenza virus have provided no evidence that the particles

are extensively disrupted by plasmolysis. However, it did seem possible that dialysis might result in the loss of material. In order to gain some information on this point one portion of preparation 4 was dialyzed against 0.1 M phosphate buffer at pH 7 and another portion was dialyzed against distilled water and then frozen and dried. On analysis by Dr. Elek the latter was found to contain 55.98 and 55.87 per cent carbon, 8.16 and 8.08 per cent hydrogen, 11.01 and 10.92 per cent nitrogen, 1.07 per cent phosphorus, 0.97 per cent sulfur, and 3.13 per cent ash. A value of 1.06 per cent phosphorus was obtained by Dr. Knight on a portion of the dialyzed solution by the King method (15). In the case of the portion dialyzed against 0.1 M buffer, analysis for phosphorus by Dr. Knight by the King method on the solvent and protein solution indicated that the protein contained 1.29 per cent phosphorus. This result indicates

TABLE I

Apparent Specific Volume of Preparations of PR8 Influenza Virus

Preparation	Solvent	No. of replications	Apparent specific volume
1a	H_2O	1	0.78
2	H_2O	2	0.78 ± 0.008
5b	H_2O	1	0.84
6	H_2O	1	0.85
7a	Toluene	3	0.775* ± 0.012
8a	Electrolyte solution	5	0.80 ± 0.008

* Reciprocal of dry density.

that dialysis against water does not result in an appreciable loss of phosphorus from the virus material. The results just described as well as the results obtained with preparation 8a in buffer solution provide ample reason for regarding the value of 0.79 as the most probable value for the true specific volume of PR8 influenza virus preparations obtained by differential centrifugation. It can be noted that the composition of this material, as described above and by Knight (25), is not inconsistent with this value for the specific volume.

Concentration Dependence of Sedimentation Velocity

It has been observed that the sedimentation constant of PR8 influenza virus preparations varied considerably from preparation to preparation. It appeared necessary therefore to determine the nature of some of the variables responsible for this lack of constancy.

Preparation 1 was studied at several concentrations in a Bauer and Pickels (16) type air-driven ultracentrifuge equipped with a Svensson optical system (17). It was found that the reciprocal of the sedimentation rate of the principal constituent increased approximately in a linear manner with increasing concentration. The results are presented graphically in Fig. 2, curve A. This behavior is analogous to that of tobacco mosaic virus preparations (18).

The material was then fractionated to form preparation 1a. The results of several centrifugation runs at different concentrations are also presented in curve A of Fig. 2. The effect of concentration upon sedimentation rate was also determined for preparation 5b. The results for the principal component are shown in curve B of Fig. 2.

It can be observed that curve B for preparation 5b extrapolates at zero concentration to a value of $\frac{1}{S}$ equal to the reciprocal of 722 Svedberg units. Curve A for preparations 1 and 1a extrapolates to a value of 658 Svedberg units.
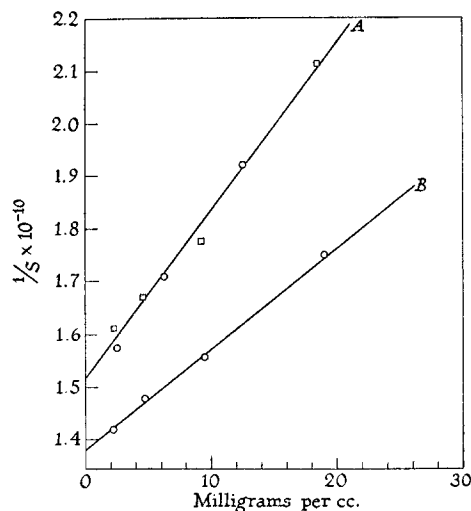

Fig. 2. Reciprocal of the sedimentation rate in Svedberg units plotted as a function of the concentration of PR8 influenza virus. Curve A, □, preparation 1, O, preparation 1a. Curve B, preparation 5b.

This would seem to indicate that there can be a real difference between the sedimentation rates of various preparations of the same strain of influenza virus.

Sedimentation in Sucrose Solutions of Different Densities

Sedimentation rates of preparations 3, 5b, and 8a were determined in solutions containing 0.1 M phosphate buffer at pH 7 and amounts of sucrose varying between about 10 and about 50 per cent by weight. The determinations were made immediately following the preparation of the mixtures of virus and sucrose. The rates were corrected for sucrose viscosity by using the data available in the International Critical Tables.

The sedimentation rates for preparation 5b at constant virus concentration, not corrected for the density of the medium, are plotted as a function of density of the medium in Fig. 3. It can be observed that the particles do not sediment at all in a solvent with a density of about 1.18. In more dense solutions the

direction of sedimentation is reversed. The same point of inversion was found for the other two preparations. This result is in general agreement with that obtained by Elford and Andrewes (2) in a similar experiment designed to measure the sedimentation rate of the infectious principle. It means that in a sucrose solution with a density of about 1.18 the density of the particles of the influenza preparation in the present experiment and that of the infectious unit in Elford and Andrewes' case are equal to the density of the solvent.

According to the fundamental sedimentation equation, the rate of sedimentation of a suspended particle should be directly proportional to the difference between the density of the particle and that of the medium. Thus, if the density or the specific volume of the particle remains constant, the sedimenta-
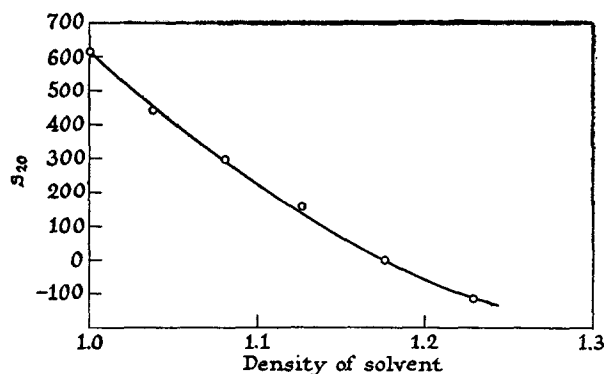

FIG. 3. Sedimentation rate in Svedberg units of preparation 5b of PR8 influenza virus in sucrose solutions plotted as a function of the density of the suspension medium.

tion rate should be a linear function of the density of the medium. It can be observed that the data on PR8 influenza virus preparations do not fall on a straight line. This behavior is identical with that observed by Smadel, Pickels, and Shedlovsky (19) for elementary bodies of vaccinia. It can be interpreted most readily as meaning that the density of the suspended particles increases as the sugar concentration is increased. This means that the density in dilute electrolyte solutions must be considerably less than that in concentrated sugar solutions. A tangent to the experimental curve at the point corresponding to the density of water, drawn in the manner outlined by Smadel, Pickels, and Shedlovsky (19), will describe the sedimentation behavior which the particles would exhibit if they did not change density with increasing sucrose concentration. Such particles would just float in a solvent of density equal to about 1.1, hence it can be inferred that the density of PR8 influenza virus particles in the absence of sucrose is about 1.1.

In conjunction with these experiments, the stability of influenza virus infectivity in sucrose solutions was determined. Solutions containing 1 mg. of

influenza virus preparation 5b per cc. in 0.1 M phosphate buffer at pH 7 and containing 0, 20, 30, and 50 per cent, respectively, by weight of sucrose were allowed to stand at 4° C. A duplicate virus solution in 20 per cent sucrose was allowed to stand at 25°. Virus infectivity measurements were made in 4-week old mice at the end of 10 and 100 hours by the method described previously (20). The results are shown in Table II. It can be observed that there was no demonstrable change in infectivity in 20 per cent sucrose at 4°. In 20 per cent sucrose at 25°, and in 30 and 50 per cent sucrose at 4°, the infectivity decreased about 1.5 logarithmic units in 100 hours. The error of the infectivity titration is such that this would correspond to a loss of between 90 and 99 per cent of the infectivity in 100 hours. These results indicate that no great

TABLE II

Effect of Sucrose on Infectivity of PR8 Influenza Virus

Temperature	Sucrose content per cent by weight	Weighted 50 per cent end-points in mice expressed in gm. of protein	
		After 10 hrs.	After 100 hrs.
°C.			
4	0	—	$\begin{cases}10^{-8.9}\\10^{-9.4}\end{cases}$
4	20	$10^{-8.7}$	$10^{-9.3}$
25	20	$10^{-7.7}$	$10^{-7.7}$
4	30	$10^{-8.6}$	$10^{-7.8}$
4	50	$10^{-7.7}$	$10^{-7.7}$

changes took place in the infectious particles during the short time required for the sedimentation experiments, particularly in the cases involving the lower sucrose concentrations.

Homogeneity of PR8 Virus Preparations with Respect to Rate of Sedimentation

Fig. 4 is a tracing of a sedimentation diagram obtained by the Svensson schlieren method on preparation 5b at a concentration of 4.75 mg. per cc. By making the assumption that diffusion is negligible, one can estimate the distribution of sedimentation rates from the progressive broadening of the peaks. In this manner it was estimated that the standard deviation of the distribution of sedimentation rates is about 8 per cent of the mean rate. Since the sedimentation rate of a sphere is a function of the square of its radius, this corresponds to a standard deviation of particle diameters of only 4 per cent of the mean diameter. This result is in marked contrast with the much broader distribution obtained by means of electron microscopy on the same preparation. The probable meaning of this observation will be discussed later.

Additional Component in PR8 Virus Preparations Obtained by Differential Centrifugation

In connection with the experiments on the effect of concentration on sedimentation rate, it was observed that, when each of the preparations used in that study was examined at its highest concentration, an additional, more slowly sedimenting component could be observed. Friedewald and Pickels (8) also observed smaller components in preparations of PR8 and Lee strains of influenza material. The rates calculated for the slower component were 75 S for preparation 1, 240 S for 1*a*, and 370 for 5*b*. In the case of preparation 1 and 1*a*, the more slowly sedimenting material seemed to constitute about half of the total material, and in the case of preparation 5*b* it seemed to constitute about
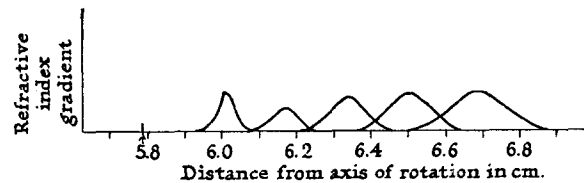

Fig. 4. Tracings of sedimentation diagrams obtained by Svensson schlieren method on PR8 influenza virus preparation 5*b*. Virus concentration = 4.75 mg. per cc.; speed of centrifuge = 11,100 r.p.m.; exposure interval = 5 minutes; angles between fixed and inclined slits = 85°, 80°, 80°, 75°, and 70° for exposures 1, 2, 3, 4, and 5, respectively.

10 to 20 per cent of the total. Fig. 5 is a tracing of a sedimentation diagram obtained with preparation 1*a* at a concentration of 12.5 mg. per cc. after 20 minutes of sedimentation at 11,100 r.p.m., showing the two components. However, when each of these preparations was studied at concentrations of half or less of the maximum concentrations indicated in the data of Fig. 2, the more slowly sedimenting component could no longer be observed. This type of behavior is similar to that observed previously in a study of the blood of normal and jaundice-diseased silkworms (21). The explanation advanced in the case of the silkworm blood was that the smaller material was so inhomogeneous that it appeared as a boundary only when in very high concentration. It may be that such an explanation could also apply to the PR8 virus preparations.

In order to determine whether the slower constituent or the principal faster component bears the infectiousness of the PR8 virus, preparation 4, at a concentration of 45 mg. per cc., was centrifuged for an effective time of about 40 minutes at 11,100 r.p.m. in a separation cell of the type designed by Tiselius, Pedersen, and Svedberg (22), and used in studies on tobacco mosaic virus in this laboratory (23). After the centrifuge was stopped the content of the top compartment was removed. It was found to contain 2.1 mg. of protein per cc. and to consist largely of a component with a sedimentation rate of 169 Svedberg

units at a concentration of 1 mg. per cc. In the separation cell, when this component was at a higher concentration and was in the presence of the more rapidly sedimenting component, it had a sedimentation rate of about 90 Svedberg units.   CCA activity and mouse infectivity were determined for both the original and the material isolated from the top compartment.   The original was found to contain 1,800 CCA units per mg. of protein and the material from the top compartment, 900 units per mg. of protein.   The 50 per cent weighted end-points of the mouse infectivity were at $10^{-10.0}$ and $10^{-8.7}$ gm. per cc., respectively, for the original and for the fractionated materials.   Similar results were obtained in more extensive studies of a comparable nature with the Lee strain of influenza.   It is thus apparent that the material in the upper compartment of the separation cell has a lower specific infectivity and a lower specific CCA activity than the original material.   It is probable that the residual
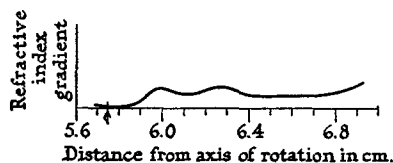

Fig. 5. Tracing of sedimentation diagram obtained by Svensson schlieren method on PR8 influenza virus preparation 1a after 20 minutes of sedimentation.   Concentration of solids = 12.5 mg. per cc.; speed of centrifuge = 11,100 R.P.M.; angle between fixed and inclined slits = 70°.

infectivity is due to an amount of the heavier component not readily demonstrable in the centrifuge.

Viscosity Determinations

Most of the viscosity measurements were made in an Ostwald viscometer requiring a volume of about 4 to 5 cc. The temperature was held at 25.0° in an accurately controlled water bath. Relative viscosity in 0.1 M phosphate buffer at pH 7 was determined for preparations 3 and 5b at various concentrations. In Fig. 6 the natural logarithm of relative viscosity is plotted as a function of concentration in grams per cubic centimeter. Curve A represents the data of preparation 3 and curve B those of 5b. The logarithm of relative viscosity is a linear function of concentration in the range studied.

In very dilute solutions, the relative viscosity of most substances is equal to unity plus a constant times the concentration of solute. $\eta r = 1 + KC$ or $\eta r - 1 = KC$.   The constant K can be evaluated by obtaining the quotient $\dfrac{\eta r - 1}{C}$ when C approaches zero.   As C approaches zero, $\eta r - 1$ approaches zero, and as $\eta r - 1$ approaches zero, it becomes practically equal to the natural logarithm of ηr.   Thus the constant K is equal to the natural logarithm of

ηr divided by concentration, C, over the range in which log ηr is a linear function of C. The constant K is called the intrinsic viscosity. Its value is dependent upon the shape of the solute particles and upon the volume they occupy in solution. The intrinsic viscosities of preparations 3 and 5b, calculated on the basis of solute concentration expressed in grams per cubic centimeter of solution, are listed in Table III. Intrinsic viscosities of preparations 9, 9a, 9b, and 9c
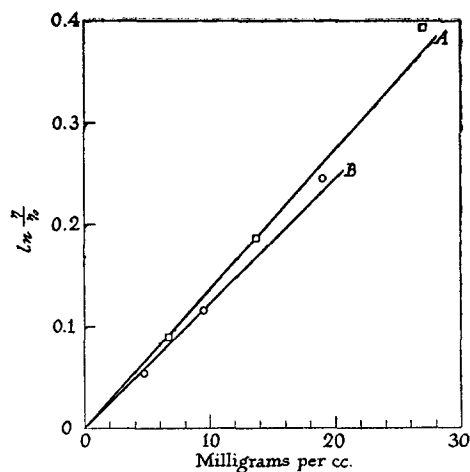

Fig. 6. The natural logarithm of relative viscosity plotted against concentration of PR8 influenza virus. A, preparation 3; B, preparation 5b.

TABLE III

Intrinsic Viscosities of PR8 Influenza Virus Preparations

Preparation	Intrinsic viscosity	Preparation	Intrinsic viscosity
3	13.7	8a	16.5
5a	11.3	8c	13.2
5b	12.2	9	27.4
5c	16.0	9a	13.6
7a	12.9	9b	38.7
7b	36.0	9c	14.1

were determined in the same viscometer, and intrinsic viscosities of preparations 5a, 5c, 7a, 7b, 8a, and 8c were determined in a 1 cc. Ostwald viscometer. These results are also listed in Table III.

DISCUSSION

The Concentration Dependence of the Sedimentation Rate.—The rate at which a suspended particle sediments in a centrifuge is directly proportional to the magnitude of the centrifugal field and the difference between the weight of the

particle and that of the medium it displaces and inversely proportional to the viscosity of the medium and to a characteristic of the particle called the frictional factor. It is usual to assume that a particle sediments through its solvent, and that, thus, it is the viscosity of the solvent and not that of the solution which controls the rate of sedimentation. In reporting sedimentation data, it is customary to correct the observed sedimentation rate to the rate the particle would have in a solvent with the density and viscosity of water at 20°. The viscosity correction is made by multiplying the observed sedimentation rate by the ratio of the viscosity of the solvent at the temperature of the experiment to the viscosity of water at 20°. It has been observed that standard sedimentation rates of this sort are often functions of the concentration of solute. It has been shown for the case of tobacco mosaic virus preparations

TABLE IV

Sedimentation Constants of PR8 Influenza Virus Preparation 5b at Different Concentrations and Following Correction for Solution Viscosity

Concentration	$S_{20°} \times 10^{13}$	Relative viscosity	$S \times \dfrac{\eta}{\eta_0} = S_\eta$
mg. per cc.			
19.0	570	1.268	723
9.5	642	1.125	722
4.7	675	1.061	718
2.4	704	1.030	725

and for numerous other systems that if the sedimentation rate is corrected for the viscosity of the solution instead of the viscosity of the solvent, this variation of sedimentation rate with concentration tends to disappear (24). This is also true for the case of preparations of PR8 influenza virus. In Table IV the sedimentation rate of preparation 5b is shown as a function of concentration, first, when corrected in the usual manner, and finally, when corrected for the viscosity of the solution. This latter correction can be applied by simply multiplying the sedimentation rate calculated in the usual manner by the relative viscosity of the solution at the concentration in question. These results leave little doubt that the apparent variation of sedimentation rate corrected in the usual way with concentration is due to the variation of the viscosity of the solution with solute concentration.

The Viscosity of Preparations of PR8 Virus.—Knight (25) has shown that influenza virus preparations of the type produced in this laboratory contain, in addition to the units presumed to be the virus, considerable amounts of a component indistinguishable by serological means from material isolated from the extra-embryonic fluid of normal chick embryos. The slower component found in preparations 1, 1a, and 5b at the highest concentrations studied is presumably

such a normal constituent. The intrinsic viscosity of one preparation of normal material in phosphate buffer, kindly supplied by Dr. Knight, was found to be 197 at 25°. In agreement with this result, sedimentation data obtained in this laboratory on another preparation and reported by Knight (25) show that such normal material has a sedimentation rate which varies strongly with concentration. On the basis of these facts, it would seem natural to conclude that the relatively high intrinsic viscosities of the preparations of PR8 influenza virus are due at least in part to the presence of normal constituents. In fact, preparation 7b, which had an intrinsic viscosity of 36, showed a boundary in addition to that of the usual component in both the ultracentrifuge and the Tiselius apparatus (26) corresponding to the normal component. Preparation 7a, which had an intrinsic viscosity of 12.9, showed only the single boundary of the usual component. Furthermore, a preparation was separated in the Tiselius apparatus by Miller, Lauffer, and Stanley (26) into three fractions, anodic, cathodic, and central with intrinsic viscosities of 44.2, 16.7, and 22.1, respectively. The anodic fraction should contain a higher percentage, and the cathodic a lower percentage of normal component, for the normal component is known to have a higher anodic electrophoretic velocity than the usual virus component at the pH used. The results obtained during the fractionation of preparation 9 lend further support to the conclusion that a considerable portion of the viscosity of PR8 influenza virus preparations could be due to a slower sedimenting, highly viscous component. As shown in Table III, crude preparation 9 had an intrinsic viscosity of 27.4. The heavy and light components obtained upon fractionation had viscosities of 13.6 and 38.7, respectively, thus showing that material which sediments more slowly than the principal component contributes much to the viscosity. All of these facts constitute evidence that the normal component can contribute greatly to the intrinsic viscosity of PR8 influenza virus preparations.

It is difficult to decide whether or not the normal component can be regarded as contributing part of the viscosity to preparations showing the minimum viscosity of 11.3 to 16.5. In some of these preparations it was not possible to observe a contaminant by electrophoretic and ultracentrifugal analyses. Furthermore, in the case of preparations 8a and 9a, further fractionation by centrifugal means failed to change the intrinsic viscosity appreciably, for the data of Table III show that the viscosities of preparations 8c and 9c do not differ very much from those of 8a and 9a, respectively. Thus, this range seems to represent a practical minimum. If the virus particles have the composition described in the next paragraph, the intrinsic viscosity ought to be about 8. However, as little as 3 per cent on a dry weight basis of the highly viscous contaminant could raise the intrinsic viscosity from about 8 to about 14. Such a small amount of contaminant could easily escape detection.

The Nature of the Particles.—The electron micrographs show the principal

particles in the most carefully studied preparation of PR8 virus as essentially round images with an average diameter of 115 mμ. The sedimentation constant of this component corrected to water at 20° is 722 Svedberg units. Determinations of the anhydrous specific volume are somewhat less satisfactory. However, the best value obtained thus far is 0.79 ± 0.01. Since the particles can be presumed to be spherical, the diameter they would have if they were anhydrous can be calculated from the sedimentation constant and the specific volume. The value so calculated is 70 mμ. This value is quite different from the average diameter obtained from the electron micrographs. Even allowing for a possible error of 10 per cent in the magnification factor of the microscope, agreement cannot be forced.

The difference between the particle diameter calculated from sedimentation and specific volume data and that indicated by electron microscopy provides a most important clue to the true nature of the influenza virus particle in solution. Certain other facts have been observed in the course of this study which also bear on the nature of the particle in solution. First, rates of sedimentation in sucrose solutions suggest that sedimentation velocity is not a linear function of solvent density, and that, therefore, the density of the particles is a function of the sucrose content of the solvent. The results indicate that the density in the absence of sucrose is about 1.1. The density in 40 per cent sucrose is 1.18. Contrasted with this, the anhydrous density or the reciprocal of the specific volume is 1.27. Second, the apparent distribution of particle diameters observed with the electron microscope has a standard deviation of 15 per cent, while the distribution of diameters as calculated from the boundary spread in the ultracentrifuge is only 4 per cent. Coupled with this is the fact that many of the particles in the electron micrograph are somewhat irregular in appearance. Third, it has been observed previously (27) that influenza virus activity is destroyed when the electrolyte concentration of the solvent is reduced by dilution. In the present study dialysis against distilled water was found to cause loss of virus and CCA activity. All of these facts are consistent with the assumption that the particles in preparations of PR8 influenza virus are spherical bodies composed not only of protein, carbohydrate, and fat, as shown by chemical analysis, but also of considerable water, of the order of 60 per cent of the net weight. This assumption would explain the sedimentation data in sucrose solutions, for the sucrose would be expected to dehydrate the particles by virtue of its osmotic pressure. The results indicate that in 40 per cent sucrose enough water has been removed to increase the density to 1.18. Since the anhydrous density of the virus particles is about 1.27, this means that considerable water still remains in the particles at this sugar concentration. This assumption can explain the wider distribution of particle diameters, irregularities in shape, and the greater mean diameter observed in the electron microscope. If the particles in suspension contain water, that water must be removed when the electron microscope mounts are allowed to dry. This would

cause the particles to shrink. If they shrink more in the dimension perpendicular to the film than in the opposite dimension, they will dry into flattened ellipsoids. The apparent diameter of these ellipsoids will obviously be greater than the diameters of the hypothetical anhydrous spheres. If the particles do not all shrink in quite the same way, the apparent distribution of diameters will be increased.

The assumption of a water content of about 60 per cent by weight was arrived at by the consideration that the density of the virus particle in the absence of sucrose seems to be about 1.1, and its minimum dry density is around 1.27. The diameter of the hydrated particle can be recalculated from the sedimentation constant and the estimated hydrated specific volume to be 114 $m\mu$. Even though this figure is no more reliable than the estimate of the hydrated specific volume, it seems reasonable to regard the hydrated particle diameter as being approximately equal to 100 $m\mu$. The true anhydrous particle diameter must be somewhat greater than the figure of 70 $m\mu$ calculated from the sedimentation constant and the anhydrous specific volume. It can be calculated from the degree of hydration and the diameter of the hydrated particle to be about 80 $m\mu$.

SUMMARY

The biophysical properties of several preparations of PR8 influenza virus have been studied. Electron micrographs showed slightly irregular, circular particles with an average diameter of 115 $m\mu$ and a standard deviation of the distribution of diameters of 15 per cent. The specific volume was determined with a pycnometer to be 0.79. The sedimentation rate was found to vary inversely with the concentration of virus. The extrapolated value for one preparation was 722 Svedberg units and the value for another preparation was 658 units. Sedimentation studies in sucrose solutions of varying densities showed a non-linear dependence of sedimentation rate upon solvent density, indicating that the density in solution increases with increasing sugar concentration. The virus particles floated in a sugar solution with a density of 1.18. The density in the absence of sucrose was estimated to be about 1.1. The virus was shown not to lose infectivity very rapidly in sucrose solutions. The spreading of the boundary during sedimentation was shown to be represented by a standard deviation of the sedimentation rate equal to 8 per cent of the mean. This corresponds to a distribution of diameters with a standard deviation of 4 per cent of the mean. A more slowly sedimenting component was observed with the ultracentrifuge in the highest concentrations of several of the preparations. The intrinsic viscosities, calculated on the basis of concentrations expressed as grams per cubic centimeter, of several highly purified preparations were determined to be between 11.3 and 16.5. The variation of sedimentation rate with concentration was shown to be due entirely to the variation of solution viscosity with concentration. The viscosity of the virus preparations can be explained

as being due in great part to the presence of the slower sedimenting contaminating components which possess a very high intrinsic viscosity.

The average particle diameter of the anhydrous PR8 virus particle was calculated from sedimentation and specific volume data to be about 80 mμ. The discrepancy between this value and that obtained from the electron micrographs, the greater size distribution from the electron micrographs, the slight irregularities in some of the particles as observed in the electron micrographs, the behavior of the sedimentation process in sucrose solutions of different densities, and the inactivation of the virus by withdrawal of electrolytes can all be explained in a straightforward manner if it is assumed that the virus particles in solution contain, in addition to the constituents shown by chemical analysis, about 60 per cent by weight of water. It is estimated that such hydrated virus particles are about 100 mμ in diameter.

BIBLIOGRAPHY

1. Elford, W. J., Andrewes, C. H., and Tang, F. F., *Brit. J. Exp. Path.*, 1936, **17**, 53.
2. Elford, W. J., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1936, **17**, 422.
3. Bourdillon, J., *J. Gen. Physiol.*, 1941, **25**, 263.
4. Chambers, L. A., and Henle, W., *Am. J. Path.*, 1941, **17**, 422.
5. Chambers, L. A., Henle, W., Lauffer, M. A., and Anderson, T. F., *J. Exp. Med.*, 1943, **77**, 265.
6. Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 267.
7. Friedewald, W. F., and Pickels, E. G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 261.
8. Friedewald, W. F., and Pickels, E. G., *J. Exp. Med.*, 1944, **79**, 301.
9. Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.
10. Sharp, D. G., Taylor, A. R., McLean, I. W., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *Science*, 1943, **98**, 307; *J. Immunol.*, 1944, **48**, 129.
11. Taylor, A. R., Sharp, D. G., McLean, I. W., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *Science*, 1943, **98**, 587; *J. Immunol.*, 1944, **48**, 361.
12. Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 255.
13. Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1936, **64**, 503.
14. Hillier, J., and Vance, A. W., *Proc. Inst. Radio Engineers*, 1941, **29**, 167.
15. King, E. J., *Biochem. J.*, 1932, **26**, 292.
16. Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1937, **65**, 565.
17. Svensson, H., *Kolloid-Z.*, 1939, **87**, 181.
18. Lauffer, M. A., *J. Physic. Chem.*, 1940, **44**, 1137.
19. Smadel, J. E., Pickels, E. G., and Shedlovsky, T., *J. Exp. Med.*, 1938, **68**, 607.
20. Lauffer, M. A., and Miller, G. L., *J. Exp. Med.*, 1944, **79**, 197.
21. Lauffer, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 330.
22. Tiselius, A., Pedersen, K. O., and Svedberg, T., *Nature*, 1937, **140**, 848.
23. Lauffer, M. A., *J. Biol. Chem.*, 1943, **151**, 627.
24. Lauffer, M. A., *J. Am. Chem. Soc.*, 1944, **66**, 1195.
25. Knight, C. A., *J. Exp. Med.*, 1944, **80**, 83.
26. Miller, G. L., Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, 1944, **80**, 549.
27. Knight, C. A., *J. Exp. Med.*, 1944, **79**, 285.