DENSITIES AND SIZES OF THE INFLUENZA VIRUSES A (PR8 STRAIN) AND B (LEE STRAIN) AND THE SWINE INFLUENZA VIRUS*

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Studies with the electron microscope have shown that the influenza viruses A (PR8 strain) (1) and B (Lee strain) (2) and the swine influenza virus (3) consist of rounded or bean-shaped particles of variable size and internal structure. The distribution of the sizes of the particles of a given type of the virus has been substantiated qualitatively by means of sedimentation velocity studies (4). Chemically the particles consist of protein, lipid, and carbohydrate with which there is associated nucleic acid of the desoxypentose type (5).

Estimates of the average size of the influenza virus particles have been made by means of the electron microscope and from sedimentation velocity data. In the initial work with the electron microscope, calibration was based on measurements of the widths of tobacco mosaic virus rods (1–3). Subsequent recalibration of the instrument has resulted in higher values (4) which are believed to be more nearly correct. Estimations of size were made (1–4) also from sedimentation velocity data in conjunction with values of the reciprocals of the partial specific volumes of the viruses determined with the pycnometer. It was recognized, however, that accurate knowledge of particle density in a medium compatible with the existence of the particle in its native state must be available if true sizes are to be calculated from sedimentation data.

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Probably the most direct method of determining the density of small particles in suspension is that of varying the density of the dispersion medium and noting the change in the sedimentation rate of the particles in a suitable centrifugal force field. Attempts have been made to use this method for studying the density of virus particles, materials such as sucrose, glycerol, urea, and sodium chloride being employed to vary the density of the suspending medium. It has been found (6–8) that under these conditions alterations occur in the behavior of the virus particles. In a recent report from this laboratory, however, a system was briefly described (9) in which influenza virus A (PR8 strain) was sedimented in bovine albumin solutions of various densities and the relationship obtained over the range of density available for study indicated an ideal behavior of the particles. In the present paper the method will be described in more detail, and the results obtained with influenza virus B (Lee strain) and the swine influenza virus will be given. These findings, together with data on the partial specific volume of these types of virus obtained in previous work and in the present study, will be discussed in connection with existing chemical and physical information to the end of obtaining a more accurate concept of the elementary influenza virus particle.

Materials and Methods

The three types of virus used were influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus, which were studied previously with respect to purification (1–3), chemical constitution (5), and physical properties (4).

Purified virus was obtained by concentration of the respective types of the virus from large pools of virus-infected chorioallantoic fluid from chick embryos by means of (1) adsorption on and elution from chicken red blood cells, followed by ultracentrifugation, or (2) preliminary concentration in the Sharples supercentrifuge, followed by ultracentrifugation.

Details of the procedures for preliminary concentration of the influenza viruses by adsorption on and elution from chicken red blood cells (1–3) and by large scale centrifugation in the Sharples centrifuge (10) have been reported. Further purification was then effected by treatment in the air-driven ultracentrifuge. Eluates or centrifugates in Ringer's solution (1) were spun in the angle centrifuge at 2000 g. The supernatants were then parceled into 15 ml. collodion tubes and spun for 1 hour at 20,000 g in the ultracentrifuge. The resulting clear, gel-like pellets were resuspended in Ringer's solution and spun at 5000 g in the ultracentrifuge for 5 minutes.

The concentrates exhibited a single, slightly diffuse boundary in the analytical ultracentrifuge (4) and a high degree of uniformity of particle kind in electron micrographs. In practically all instances such concentrates

of influenza virus A and the swine influenza virus gave values for the partial specific volume which did not change with further treatment of the virus. To obtain constant values with influenza virus B, however, further purifying ultracentrifugation was often necessary, particularly when preliminary concentration had been effected by centrifugation. The concentrates containing 100 to 400 mg. of virus were diluted to 120 ml. with Ringer's solution, parceled into 15 ml. ultracentrifuge tubes, and spun in the quantity ultracentrifuge head quickly up to and down from 6000 *g*. The virus was sedimented again at 20,000 *g* for 1 hour, resuspended at high concentration, and spun at 5000 *g* for 5 minutes.

The bovine albumin was a 25 per cent solution in 0.9 per cent NaCl solution of a crystalline fraction which Dr. Hans Neurath obtained from the Armour Laboratories, Chicago, Illinois, through the courtesy of Dr. E. J. Cohn, Harvard Medical School, and Dr. H. B. Vickery. To this albumin solution were added the calculated quantities of the salts requisite to make the solvent medium equivalent to Ringer's solution (11), consideration being taken of the volume actually occupied by the albumin itself. Dilutions of the stock albumin solution were made with Ringer's fluid. Virus in Ringer's solution at pH 7.3 to 7.5 was added to the bovine albumin of various concentrations, and the mixtures were brought to room temperature. To obtain virus in the highest concentration of albumin, 25 per cent, the appropriate amount of virus was sedimented in the ultracentrifuge and resuspended in the albumin solution. The concentration of virus in the albumin solution was 2.0 mg. per ml. of influenza virus A, 1.76 mg. per ml. of influenza virus B, and 3.0 mg. per ml. of the swine influenza virus. The time elapsing between addition of albumin and recording of the sedimentation diagrams was never greater than an hour and was generally about 20 minutes. The rotor temperature was recorded for each run.

Viscosity measurements on the albumin in Ringer's solution at 25° were made by Dr. John Erickson. It has been assumed that the change in viscosity of the albumin solution with temperature was the same as that for water. Any error introduced by this simplification would be small, because all centrifuge runs were made near 25° and the error would be random, for some runs were below and some above 25°.

Density of the albumin solutions was measured with the pycnometer in a thermostat at 25°.

For the studies with sucrose, a 64 per cent solution of sucrose (Pfanstiehl) in Ringer's solution was prepared. By appropriate mixture of this with Ringer's solution and concentrated virus, samples containing 3.0 mg. of virus per ml. with the required sucrose content were made immediately before the sedimentation run. Sucrose viscosity corrections were taken from the data of Bingham and Jackson (12), and the density values from

standard tables. Because of the presence of the salts of Ringer's solution, the viscosity and density values given in the tables are not precise in relation to the conditions of this experiment.

An air-driven ultracentrifuge (13) was used for the sedimentation velocity determinations. The mean rotor radius was 6.5 cm., and the cell height was 1.2 cm. The progress of sedimentation in the region of 25° was recorded by the Lamm scale method, and sedimentation rates were calculated as previously described (4).

For estimation of partial specific volume, the ultracentrifuge concentrates containing 14.0 to 23.0 mg. of virus per ml. were brought to room temperature (about 25°); air was removed by placing the vial containing the preparation in a small suction flask and evacuating three successive times to the point where the concentrate began to boil. A sample of the Ringer's solution was treated at the same time in order to control the slight concentration effect due to the evacuations. Virus concentration was then calculated by conversion of micro-Kjeldahl nitrogen determinations made in duplicate, with the appropriate factor for each type of virus (5). The calibrated 2 ml. capped pycnometer was then filled with chilled virus suspension and equilibrated in a water bath at 25° ± 0.05°. Weighings were made on a micro balance with calibrated weights, a duplicate pycnometer being used as a counterbalance. Solvent density determinations were taken from the average of at least three individual equilibrations and weighings. The partial specific volume was calculated by means of the equation given by Kraemer (14).

Results

The experiments have consisted of studies on (1) the sedimentation velocity of influenza viruses A (PR8 strain) and B (Lee strain) and swine influenza virus in bovine albumin solutions of different densities; (2) the sedimentation velocity of the swine influenza virus in sucrose solutions of different densities; and (3) the partial specific volumes of the three types of influenza virus estimated with the pycnometer. The studies with the pycnometer were made to obtain data to supplement those previously reported (4).

Sedimentation Velocity of Swine Influenza Virus in Sucrose Solutions—The results of the experiments with sucrose are given in Fig. 1, which shows the relation of the virus sedimentation rate, S, to the density, ρ_s, of the suspending medium. The ordinate, ηS, is the product of the observed sedimentation rate and the viscosity (relative to water at 25°) of the suspending medium. The abscissa is the density of the sucrose solution at 25°. The experimental points obviously are not distributed in a linear fashion, and through them the curve was drawn as described below. Extrapolation of

this curve would result in an intercept with the abscissa at about the value $\rho_s = 1.19$ for the density of the suspending medium.

From these findings, it is seen that the change in the sedimentation rate of the virus particles was not related wholly to the increase in the density of the medium. On the contrary, the curved nature of the relation of ηS to ρ_s shows that with increase in ρ_s, the values of ηS remained higher than would have been the case if the increase in the density of the suspending medium had been the sole influencing factor. It is thus evident that changes occurred in the physical properties of the virus particles which resulted in findings compatible with the assumption that particle density

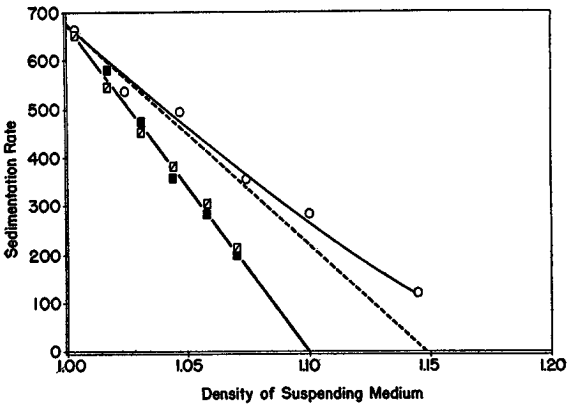

FIG. 1. Swine influenza virus. The sedimentation rates given in Svedberg units corrected for relative viscosity but not for density are plotted against the density of the suspending medium. Two different virus preparations were sedimented in bovine albumin solution, and the data are represented by the open and closed squares, respectively. One of these preparations (closed squares) was sedimented also in sucrose and the resulting data are shown by the open circles and the curve drawn through them. The tangent drawn to the curve at the density 0.9982 of the suspending medium is shown as a dotted line.

increased in relation to, though not in proportion to, the concentration of sucrose.

On further investigation of these changes in the sedimentation characters of the swine influenza virus, it was found that the extent of the alterations of the virus particles is dependent on the time of contact of the particles with the suspending medium. The results were qualitatively similar to those which were found with influenza virus A (PR8 strain), as illustrated in a previous report (9). Influenza virus A suspended in Ringer's solution containing 11 per cent sucrose exhibited at first a rapid and significant increase in sedimentation rate ((9) Fig. 1). With time, in this instance about 2.5 hours, the maximum value was obtained, and thereafter a gradual de-

crease in the sedimentation rate occurred. At the end of 28 hours, an interval not described in the previous report (9), the declining rate of sedimentation had reached a level close to that observed when the virus particles had been in contact with sucrose the shortest time possible for study.

Results qualitatively similar to these with the influenza viruses have been observed by Smadel, Pickels, and Shedlovsky (8) in studies on the elementary bodies of vaccinia. These authors, citing a number of possible explanations of the findings, concluded that the various phenomena were subject to elucidation on the assumption that the changes in particle behavior were due to osmotic withdrawal of water from the particles by the sucrose with consequent increase in particle density. It should be noted here for subsequent discussion that this hypothesis, while accounting for the increase in the sedimentation rate of the particles in a sucrose solution of given concentration, does not suffice to explain why there is a subsequent decrease with time.

The meaning of the curvilinear relations of Fig. 1 with respect to their bearing on the density of the virus particles in their native state is of uncertain significance. Interpretations have been made of such results by constructing a tangent to the curve (8) at the point corresponding to $\rho_s = 1.00$. The tangent is then extended to intercept the abscissa, and the value of the solvent density, ρ_s, at this intercept has been taken (8) to represent the density, ρ_v, of the virus particle in the absence of sucrose.

The observed data for the swine influenza virus shown in Fig. 1 are closely approximated by the relationship

$$\eta S = K_1 - K_2(\rho_s - 1) + K_3(\rho_s - 1)^2$$

which was used with appropriate constants to draw the curve. The slope of a tangent drawn at any point on the curve is then given by

$$\frac{d(\eta S)}{d(\rho_s - 1)} = -K_2 + 2K_3(\rho_s - 1)$$

which for small values of $(\rho_s - 1)$ should be nearly constant. The tangent drawn by this means through the point corresponding to the density of water at 20° ($\rho_s = 0.9982$) intercepts the axis at 1.148. Interpreted in the manner of Smadel, Pickels, and Shedlovsky, this result would indicate a density of 1.148 for the swine influenza virus in the absence of sucrose.

The significance of the tangent to the curvilinear relation is dependent on the validity of the assumption that the relation of ηS to $(\rho_s - 1)$ approaches a straight line in the region of $\rho_s = 1.00$ and, further, that such a linear relation would describe the behavior of the particles in a medium in which no change occurred in the character of the particles. In the investi-

gations with the swine influenza virus and, as well, with the elementary bodies of vaccinia (8), there were insufficient data either to show that the relation became linear in this region or for judging the *extent* of variation of the observed results from those expected under ideal conditions. Furthermore, a tangent drawn to the curve, ηS *versus* $(\rho_s - 1)$, may be used to calculate the density of the sedimenting unit at a particular value of ρ_s chosen only if ηS depends on ρ_s alone. On the contrary, however, it has been observed that ηS is not constant for a given ρ_s of the sucrose solutions but varies significantly with time. It is thus clear that measurements of ηS used to plot the curve ηS *versus* $(\rho_s - 1)$ will be different at different times and, consequently, such a calculated density of the sedimenting unit is likewise dependent on time. The sedimentation studies on the swine influenza virus, like those on the vaccinial elementary bodies (8), were made quickly after mixture of virus with sucrose solution, a period of time in which change in the virus particle is most rapid (8, 9). From these considerations and the results obtained with the bovine albumin experiments described below, then, it would appear unlikely that density values obtained from use of the tangent necessarily provide any information relative to the density of the particles in the native state.

Sedimentation of Influenza Viruses in Bovine Albumin Solution—Utilization of bovine albumin to vary the density of the suspending medium in the present experiments was prompted by the possibility that the change in the behavior of the virus particle in sucrose solutions may be due, as suggested, to the withdrawal of water from the virus particle caused by the osmotic pressure of the medium outside the particle. Addition of bovine albumin to Ringer's solution to the extent of 25 per cent increases the osmotic pressure of the solution by an amount approximately equivalent to the osmotic pressure of 0.002 M NaCl. In contrast, the osmotic pressure of a 25 per cent solution of sucrose corresponds approximately to that of 0.366 M (2.14 per cent) NaCl. The ratio of the osmotic pressure of bovine albumin to that of sucrose of the same percentage concentration is given by the inverse ratio of the respective molecular weights; namely, 342/70,000.

Experiments were done on the sedimentation of two different batches of swine influenza virus in a series of bovine albumin solutions of different densities. The results are shown in Fig. 1. The findings with two batches of influenza virus B are shown with similar symbols in Fig. 2. Data obtained with two batches of influenza virus A have been published elsewhere (9).

From inspection of these data as a whole, there is no consistent evidence of curvature of the relations between sedimentation rates, ηS, and the densities, ρ_s, of the suspending medium. Instead, the observed points appear to be arranged in an essentially linear fashion. For the group of data of

each type of the virus, a straight line was drawn through the points by the method of least squares. The various points are seen to lie close to the respective lines. The apparent goodness of fit is substantiated by the results of statistical analysis of the findings, which show that the deviation of the observed points from the straight line is well within the limits of error of

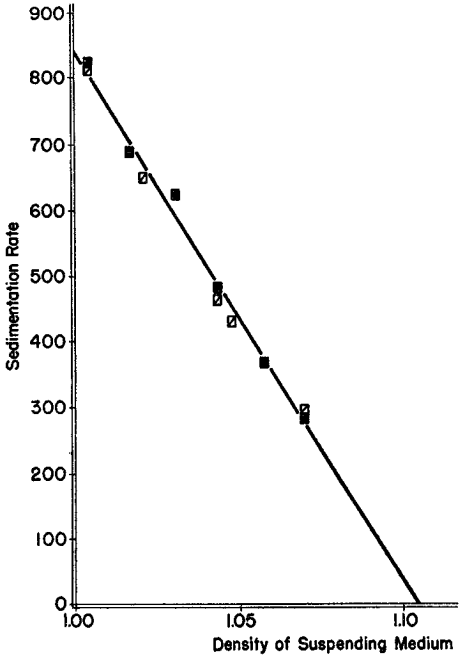

FIG. 2

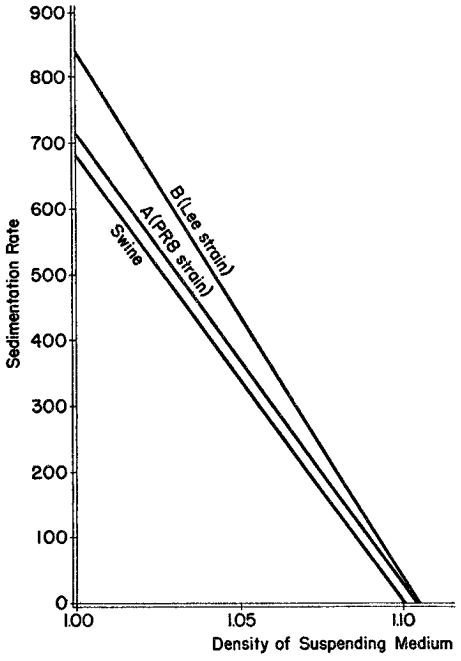

FIG. 3

FIG. 2. Influenza virus B (Lee strain). The sedimentation rates given in Svedberg units corrected for relative viscosity but not for density are plotted against the density of the albumin solution. Two different virus preparations were sedimented in bovine albumin solution and the data are represented by the open and closed squares.

FIG. 3. Summary of the sedimentation characteristics of the three types of influenza virus in bovine albumin solutions of different densities. The sedimentation rates, expressed in Svedberg units, were corrected for the relative viscosity but not for the density of the bovine albumin solution.

the method of centrifugal study. The lines obtained with the respective types of influenza virus are compared in Fig. 3.

In order to learn whether or not any change in ηS occurred with time of contact of the virus with the bovine albumin, the sample of influenza virus B (Lee strain) was studied at various intervals of time after mixing with the

highest concentration of bovine albumin used. The total elapsed times from mixing the virus with the 25 per cent albumin solution until sedimentation pictures were made were 48, 138, 226, and 313 minutes, for which the following corrected sedimentation rates, ηS, resulted: 309, 303, 323, and 313 $\times$ 10^{-13}, respectively. Although a possible increase was seen within the longer periods, it was thought likely that studies made within an hour would give values unaffected by possible changes due to the influence of the albumin. These are the same conclusions as those reached from data on influenza virus A (PR8 strain) at albumin density 1.037, as previously reported.

The degree of success of obtaining an estimate of the actual density of the virus particle in its native state by a method of this nature would be expected to be dependent on a number of factors. Electron micrographs show that the particles of the three types of influenza virus are approximately spherical. The sedimentation behavior of the particles would then be closely approximated by that of a sphere for which the equation of dynamic equilibrium for the centrifuge is as follows:

$$\frac{4}{3}\pi r^3(\rho_v - \rho_s)\omega^2 R = 6\pi\eta_1 r\frac{dR}{dt} \quad (1)$$

In this equation R is the distance at time t from a particle of radius r to the center of the centrifuge rotor. The angular velocity of the rotor is ω; the difference between the density of the virus and that of the suspending medium is $\rho_v - \rho_s$; and η_1 is the absolute viscosity of the suspending medium. Substitution of the sedimentation constant, which is by definition $S = (1/\omega^2 R)\ (dR/dt)$, in Equation 1 results in the simplification

$$\frac{2}{3}r^2(\rho_v - \rho_s) = 3\eta_1 S \quad (2)$$

From Equation 2 it is seen that under ideal conditions a linear relationship should exist between density of the suspending medium and the product $\eta_1 S$ for any given particle density, ρ_v, and that $\eta_1 S \to 0$ as $(\rho_v - \rho_s) \to 0$, indicating the limiting density of the virus particle.

The applicability of the theoretical considerations to estimation of the density of the virus particles is dependent chiefly on the constancy of both the size and the density of the particles despite the changes in the density of the dispersing medium. As noted above, the influenza virus particles are close enough to the spherical shape to fulfil the conditions of Stokes' law. Direct evidence of whether the size and density of the virus particles did or did not change is not available. However, the relation of the corrected sedimentation rate to the density of the albumin solution is essentially linear. If the relation is actually linear, it may be concluded, as

indicated in Equation 1, that no change occurred in the size of the particle. A possible change in particle density is highly improbable, since such a change would have had to be a linear function of the density of the dispersing medium. Further evidence for the absence of change within the conditions of the experiments was obtained in studies on both influenza virus A (PR8 strain) as previously reported and on the influenza virus B (Lee strain) in the present work.

Assuming that the relation is linear, for which the data provide good evidence, calculations were made of the particle density of the three types of influenza virus. For this Equation 2 was used, and the resulting values are shown in Table I. These values, 1.104, 1.104, and 1.100, represent the densities of the sedimenting units of influenza viruses A and B and the swine influenza virus, respectively, including water moving with the particles. The sizes of the particles under these conditions may be calculated from their densities as given in Table I and the respective sedimentation constants obtained by extrapolation to infinite virus dilution as previously reported (4). The average sizes obtained in this way (Table I) were 116, 124, and 117 mμ, respectively. A close approximation to the particle sizes may be obtained directly from the data on sedimentation in albumin solution from the slopes of the lines of Fig. 3. The slopes of the respective lines are given by the first derivative of Equation 2

TABLE I

Sedimentation Constant, Density, Mean Diameter, Partial Specific Volume, and Water Content of Influenza Viruses

	Influenza virus A (PR8 strain)	Influenza virus B (Lee strain)	Swine influenza virus
Sedimentation constant*	742×10^{-13}	840×10^{-13}	727×10^{-13}
Density in aqueous suspension	1.104	1.104	1.100
Diameter, from sedimentation velocity data, mμ	116	124	117
Diameter, from electron micrographs; direct calibration, mμ*	101	123	96.5
Partial specific volume	0.822	0.863	0.850
Water content, % *by volume*	52.0	34.5	43.3

* Data previously published (4).

$$\frac{d(\eta_1 S)}{d\rho_s} = -\frac{2}{9}r^2 \tag{3}$$

from which it is seen that

$$r = 3\sqrt{\frac{-\text{slope}}{2}} \tag{4}$$

Although Equation 4[1] is attractively simple, it gives values in these experiments which are 3 to 5 per cent lower than those calculated as described above by using values of S obtained from a separate experiment extrapolated to zero concentration of virus and the densities calculated from the straight lines of Fig. 3. The reason for this probably lies in the dependence of S on virus concentration, which tends to reduce the slope of the line. This should, however, have no influence on the determination of limiting density, for its influence diminishes with S and approaches zero with it.

Partial Specific Volumes of Influenza Viruses—The values obtained for the partial specific volumes of the three types of virus are given in Table II. Each tabulated value is the average of two or three independent replicate

TABLE II

Partial Specific Volumes of Influenza Viruses

Influenza virus A (PR8 strain)				Influenza virus B (Lee strain)				Swine influenza virus			
Preparation No.	Ultracentrifugal cycle	Virus concentration	Partial specific volume	Preparation No.	Ultracentrifugal cycle	Virus concentration	Partial specific volume	Preparation No.	Ultracentrifugal cycle	Virus concentration	Partial specific volume
		mg. per ml.				*mg. per ml.*				*mg. per ml.*	
21	1	10.85	0.823	30	1	14.43	0.814	26	1	16.85	0.850
21	2	5.60	0.822	30	2	13.00	0.862	26	1	12.25	0.852
23	1	22.3	0.820	30	3	13.30	0.861	26	1	7.98	0.850
23	2	20.3	0.822	30	3	13.30	0.862				
				30	3	9.07	0.862				
				32	1	18.06	0.800				
				32	2	16.66	0.840				
				32	3	7.72	0.863				
				30–32*	4	9.05	0.862				

* Preparations 30 and 32 were combined for the fourth ultracentrifugal cycle as explained in the text.

determinations at the indicated virus concentration and ultracentrifugal cycle. The results were essentially identical with those previously reported (4). With the influenza virus A no difficulty was encountered in obtaining ultracentrifugal concentrates from either red blood cell eluates or centrifugates which gave constant values. The same was generally true with the virus of swine influenza. In the instance of influenza virus B, however, the concentrates required at least two and often three cycles of ultracentrifugation before a constant value for the partial specific volume was reached.

[1] The negative sign under the radical will disappear when the observed negative slope is introduced in the equation. Relative viscosity (η) must be changed to the absolute value (η_1) for this calculation.

Preparation 30 gave a value of 0.862 after the second ultracentrifugal sedimentation, and this was not changed by additional sedimentation. Preparation 32 gave the value 0.863 only after three ultracentrifugal sedimentations. Since there was insufficient material remaining of either Preparation 30 or 32 to permit further ultracentrifugation, the two preparations, both having had three ultracentrifugal cycles and both giving essentially identical values for partial specific volume, were combined for a fourth ultracentrifugal cycle. As shown in Table II, no significant change in specific volume was observed following the fourth cycle.

Water Associated with Virus Particles—By combining the results of the measurements on partial specific volume with the density of the virus particles determined from the studies with bovine albumin, the amounts of water associated with the virus particles were calculated. The values, Table I, were for influenza virus A (PR8 strain) 52 per cent, influenza virus B (Lee strain) 34.5 per cent, and the swine virus 43.3 per cent by volume. The value, 66 per cent, previously reported (9) for influenza virus A (PR8 strain), was calculated from essentially the same data that are presented here. The difference was due to an error in the method of calculation. The present value for this virus, 52 per cent, is likewise somewhat lower than the value, 60 per cent, found by Lauffer and Stanley (15).

DISCUSSION

The behavior of the influenza viruses centrifuged in bovine albumin solutions revealed no consistent evidence of change in the sedimentation characters of the particles within the conditions of the experiments. This is in marked contrast to the pronounced changes occurring in the presence of sucrose, which have been interpreted (6–8) to be due to an increase in density and a possible decrease in particle size as the result of the osmotic influence of the suspending medium. The results of the experiments with bovine albumin solutions of high density but of low osmotic pressure provide strong presumptive evidence of the verity of the hypothesis. Within the limits that the data indicate an ideal behavior of the particles, it is possible, without further assumption, to employ the findings directly for the calculation of the density of the particles in their native state.

Use of bovine albumin for virus density determinations presents certain limitations of both theoretical and practical nature. It is possible that the relation of sedimentation rate to the density of the albumin solutions is not wholly linear. It is conceivable that the virus particles become coated with albumin and thus increase in size and density. Further, the density of the dispersing medium feasible for study is somewhat limited, since bovine albumin solutions of densities greater than 1.07 are of high viscosity. The high centrifugal fields necessary to sediment the virus through the denser

albumin solutions tend to cause sedimentation of the albumin. For this reason it is possible that the lower virus sedimentation values (ρ_s near 1.07) may be somewhat higher than their true level, yielding densities of the virus particles slightly greater than the true values.

The densities observed were 1.104, 1.104, and 1.100 for the influenza viruses A and B and the swine influenza virus, respectively. These values obtained in the studies with bovine albumin were far lower than the value suggested by the results with sucrose solutions for the swine influenza virus. The tangent drawn mathematically to the curve of the experimental data, Fig. 1, intercepted the abscissa at the point corresponding to the density 1.148 of the suspending medium. The experimental data observed for influenza virus A with sucrose by Lauffer and Stanley (15) were similar to those found for the swine virus with sucrose in the present work. The curve of the latter, on extrapolation, intercepted the abscissa at solvent density 1.19, whereas that described by Lauffer and Stanley intercepted at 1.18. In interpreting their results, however, these authors stated: "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, 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." On the basis of their results, which are shown by the points and the curve reproduced in part in Fig. 4 from an enlarged photograph of their Fig. 3 (15), it is not clear how such an inference could be drawn. The findings of these authors have been analyzed by deriving the equation of the curve from the observed points as was done in the instance of the swine influenza virus. From the value of the first derivative of the equation at the point of the density of water at 20°, 0.9982, the tangent was drawn as shown in the broken line of Fig. 4. The value of the intercept with the abscissa, 1.143, is greatly different from that "inferred" by Lauffer and Stanley, but, incidentally, is nearly identical with the value obtained in the present work with sucrose for the swine influenza virus.

The values of the partial specific volumes observed are of an order of magnitude in accord with the composition of the dehydrated virus particles (5). The selection of an accurate method for the determination of the partial specific volume of chemical complexes such as the influenza viruses warrants careful consideration. The removal of salts from the suspending medium by dialysis, as carried out by Lauffer and Stanley (15), results in precipitation of the influenza viruses. Owing to the difficulty of maintaining uniform dispersion, such precipitated material is not suitable for accurate pycnometric determinations. The classical toluene displacement

method is likewise inapplicable, since the virus contains lipid as an integral part of the complex. For these reasons the present studies were made on concentrated virus preparations in saline medium (Ringer's solution). With virus concentrates containing 8 to 25 mg. of virus per ml., the differences between the weights of the suspending medium and those of the virus concentrates were of the order of 1000 to 8000 γ on the micro balance, and the replicate weights of a given preparation were reproducible to within 10 γ. The greatest source of error is in the Kjeldahl nitrogen determination and the conversion of nitrogen to virus concentration. Constant boiling HCl and calibrated pipettes and burettes were used, and the duplicate nitrogen determinations showed better than 1 per cent agreement. This

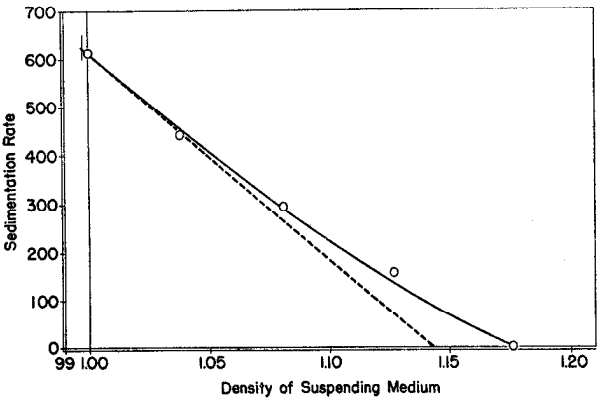

FIG. 4. Influenza virus A (PR8 strain). The sedimentation rates are given in Svedberg units with suitable correction for the viscosity of the suspending medium. The sedimentation rates are plotted against the density of the sucrose suspending medium. The data were taken from those of Lauffer and Stanley (15). The tangent at $\rho_s = 0.9982$, which was not given in the original publication, has been constructed by the present authors as shown in the dotted line.

Kjeldahl nitrogen conversion error should be relatively negligible at the high virus concentrations used.

The specific volume of any pure substance should be constant if the complex is an independent chemical and physical entity of constant constitution. The present results indicate that the influenza virus particles are entities of this nature. The composition of a given type of influenza virus is uniform (5) with respect to the proportions of protein, lipid, and carbohydrate, though these proportions, particularly of the lipid, vary to a slight but probably significant degree with the type of the virus. The values of the partial specific volumes likewise reveal distinct type differences. Moreover, a definite limiting value characteristic for each type was reached which did not change with repeated ultracentrifugation nor with dilution.

Lauffer and Stanley (15) regarded a "value of 0.79 as the most probable value for the true specific volume of PR8 influenza virus preparations obtained by differential centrifugation." Only one of their preparations was examined directly in electrolyte solution, and the value observed, 0.80, approaches the value seen with influenza virus A in the present work. Two other preparations examined by these authors gave values of 0.84 and 0.85, which are higher than any obtained in this laboratory with this type of the virus.

From the present findings certain implications may be drawn relative to the nature of the influenza virus particles. Chemically they are constituted of protein, fat, carbohydrate, and, presumably, inorganic salts with which water is associated. The nature of the association of water with the particles is such that the water is drawn rapidly from the particles in the presence of sucrose in solution. This result is explained simply by the assumption that the withdrawal of water is due to the osmotic influence of the sucrose. Substantiation of this possibility is seen in the finding that comparable changes do not take place in bovine albumin solutions of low osmotic pressure. As noted in a section above, however, this assumption does not explain all of the findings, for it was observed that after a period of increase in the apparent density of the virus particles in sucrose solution there followed a definite and substantial decrease in apparent density. This phenomenon was noted also by Smadel, Pickels, and Shedlovsky (8) in studies on the elementary bodies of vaccinia. In order to explain the findings as a whole, it would appear necessary to postulate a membrane or membrane-like structure enveloping the particle which is permeable to sucrose but impermeable to bovine albumin. Under these conditions the particles placed in sucrose would first lose water and thus increase in density. At the same time sucrose would begin to enter the particle, and after an initial period of water loss the concentration of sucrose inside the particle would reach a level sufficient to reverse the flow of water, which would result in subsequent decrease in density. These possibilities are closely in accord with the actual experimental findings in the present work and with results previously reported (9).

It seems plain that the influenza viruses are not molecular in nature. Instead, there is mounting evidence that they exhibit a behavior and a structure which strongly suggest that these viruses are relatively highly organized cell-like bodies limited by a definite membrane-like structure similar in certain of its properties to the analogous semipermeable cell wall of bacteria.

The authors are greatly indebted to Mr. Aaron Herr for his aid in the analysis of the Lamm scale diagrams.

SUMMARY

The densities of influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus were estimated from the results of sedimentation in bovine albumin to be 1.104, 1.104, and 1.100, respectively. Because of the apparently ideal behavior of the virus particles under the conditions of the experiments, these values are considered to represent the densities of the particles in their native state. The average sizes of the particles, calculated from these data, were 116, 124, and 117 mμ, respectively. The true partial specific volumes determined by pycnometer measurement were 0.822, 0.863, and 0.850. From the density values and the partial specific volumes, the amounts of water associated with the respective types of the virus were 52, 34.5, and 43.3 per cent by volume.

Studies were made also on the sedimentation velocity of the swine influenza virus in sucrose solutions. The results obtained, together with findings with influenza virus A previously reported, are discussed in relation to the results of the studies with bovine albumin solution.

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DENSITIES AND SIZES OF THE INFLUENZA VIRUSES A (PR8 STRAIN) AND B (LEE STRAIN) AND THE SWINE INFLUENZA VIRUS

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