SEDIMENTATION, VISCOSITY, AND ELECTROPHORETIC STUDIES ON PURIFIED LEE INFLUENZA VIRUS PREPARATIONS*

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Crude preparations of PR8 influenza virus, obtained by high speed centrifugation of the chorioallantoic fluid of infected chick embryos, contain an impurity which is similar to substances elaborated normally by uninfected embryos. Electron microscope and ultracentrifuge measurements have shown that the impurity is smaller in size than the virus (1-7), while viscosity determinations revealed that it is much more viscous (3, 4). Electrophoretic tests indicate that it is present to the extent of 10 to 20 per cent (8). Removal can be effected by selective adsorption of the virus on chicken red blood cells and subsequent elution therefrom (1, 9), by special methods of high speed centrifugal fractionation (4), or by electrophoretic fractionation (8). Purified virus thus obtained still contains, however, as an integral part of its structure, at least 20 per cent of antigenic groupings characteristic of the impurity (9).

Particles of a size intermediate between those of the normal material and those of the virus occur in centrifugally isolated preparations of Lee influenza virus (2, 10) and also, in some instances, of PR8 virus (2, 11). Evidence has been presented which suggests that such particles may represent degradation products (2, 10) or, possibly, precursors (11) of the virus.

Further studies of Lee virus with the aid of sedimentation, viscosity, and electrophoretic measurements are presented in this paper. Purification of crude preparations was attempted by centrifugal and electrophoretic fractionation procedures and by the adsorption-elution method, and the physical chemical and biological properties of the different fractions were investigated.

Materials and Methods

The Lee strain of influenza virus was supplied by Dr. T. Francis, Jr. Fertile eggs that had been incubated for 10 days at 39° were each inoculated

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with 0.1 ml. of infectious chorioallantoic fluid at a dilution of $10^{-4}$ to $10^{-5}$, incubated for a further 48 hours at 36°, and chilled overnight at 4°. The shells above the air sacs were removed with the aid of a small circular saw, the chorioallantoic membranes were torn, and the fluids were poured through glass wool in a funnel. Good yields of fluid free from suspended material were obtained.

Agglutination titrations of chicken red blood cells were carried out by the method of Hirst and Pickels (12), as modified in this laboratory (13). Infectivity titers were determined with the use of chick embryos (14, 15).

Adsorption of Lee virus on chicken red blood cells and elution therefrom, originally described by Hirst (16), was carried out on a large scale by the procedure described by Sharp and coworkers (10), except that a 1.5 per cent suspension of red cells was used for the adsorption and 0.1 M phosphate buffer at pH 7.1 for the elution. Also, except where indicated otherwise, a single adsorption and elution were performed. When centrifugally isolated virus was subjected to adsorption, it was first diluted to 0.05 mg. of material per ml. in 0.1 M phosphate buffer, for higher concentrations resulted in losses. After elution, the virus was subjected to two high speed centrifugation cycles as described below to remove low molecular weight materials arising from the blood cells (17). Virus purified by the red blood cell procedure was designated "RBC" virus.

Sedimentation of the virus from infectious chorioallantoic fluid was carried out by two 15 minute cycles at 24,000 R.P.M. (5, 17). In some cases, the Sharples centrifuge was used for the first concentration step (17), followed by a single sedimentation in the quantity ultracentrifuge. The final pellets were dissolved in 0.1 M potassium phosphate buffer at pH 7.1, and the solution was freed from aggregated material by centrifugation for half an hour at 3000 R.P.M. in a Swedish type of angle centrifuge. Nitrogen was determined by a micro-Kjeldahl method (18) and converted to values for virus on the basis of 9.7 per cent nitrogen in the virus (19). For further purification with the aid of high speed centrifugation, certain preparations of the "crude" virus were carried through the special fractionation procedure described for Preparation 7 of Lauffer and Stanley (4). The fractions obtained were designated the "heavy" and "light" fractions.

Analytical ultracentrifuge measurements were carried out in a Bauer and Pickels centrifuge (20) equipped with a Svensson optical system (21). Observed sedimentation rates were corrected to standard conditions of water at 20° as the theoretical medium. The value, 0.803 ml. per gm., for the partial specific volume of anhydrous Lee virus preparations (22) was used in the correction. Tracings of sedimentation diagrams were made for comparative purposes and for calculations of standard deviations of sedimentation rates within individual virus preparations. These were drawn midway between the top and bottom edges of the Svensson curves.
Viscosity measurements were made in 0.1 M potassium phosphate buffer at pH 7.1 with a 1 ml. Ostwald viscometer, as described by Lauffer and Stanley (4).

Electrophoretic methods were applied essentially as described previously in studies of PR8 virus (8). Pellets of Lee virus preparations to be used for micro electrophoresis experiments were suspended in 0.02 M potassium phosphate buffer at pH 7.1 instead of 0.02 M veronal buffer at pH 7.4, since, unlike the PR8 strain, the Lee strain was not readily dispersed in the latter medium. Further, because of the anomalous behavior of the Lee virus preparations in the veronal buffer, moving boundary electrophoretic studies were carried out only in 0.1 M potassium phosphate buffer at pH 7.1. The field strength in the moving boundary studies was maintained at 1.35 volts per cm. or lower.

Results

Yield and Chicken Red Blood Cell Agglutinating Activity of Starting Material—Infectious Lee chorioallantoic fluids tested in four different experiments possessed chicken red blood cell agglutination titers of 54, 82, 82, and 95 units per ml. The average value, 78 units per ml., was roughly one-fourth to one-half that of PR8 chorioallantoic fluid (18). Yields of five preparations of crude virus obtained by high speed centrifugation were 0.029, 0.031, 0.034, 0.043, and 0.050 mg. per ml. of chorioallantoic fluid, averaging 0.037 mg. per ml. The average chicken red blood cell agglutinating activity of five crude virus preparations was 1860 units per mg., based on individual values of 1460, 1640, 1900, 2080, and 2210 units per mg. The yields of the crude Lee virus were approximately two-thirds and the chicken red blood cell agglutination titers approximately one-half those obtained under optimum conditions for PR8 virus (18).

Sedimentation Studies—Sedimentation diagrams of different fractions of Lee virus preparations are shown in Fig. 1. The crude preparation employed as starting material reveals two principal components and appears similar to that of Friedewald and Pickels (2). The light fraction contains an increased proportion of the more slowly sedimenting component, while the corresponding heavy fraction is practically free from it. The RBC preparation also appears free from the slowly sedimenting component. The sedimentation constants of the rapidly sedimenting components of the crude, light, heavy, and RBC preparations were 703, 648, 723, and 746 Svedberg units, respectively, the variation in which will be referred to later.

The relationship between concentration of virus and reciprocal of the sedimentation rate is shown graphically in Fig. 2, in which a straight line was fitted to the experimental data by the method of least squares. RBC virus was used for these measurements since, from the above studies together with others yet to be presented, it appeared the purest of the various frac-
Fig. 1. Tracings of Svensson sedimentation diagrams of Lee influenza virus fractions. Positions of boundaries were chosen at which the relative homogeneities of the different fractions were best illustrated, and are not indicative of differences in sedimentation rates. Concentrations of virus were approximately 4 mg. per ml. The arrows represent the positions of the meniscus.

Fig. 2. Relationship between concentration and the reciprocal of the sedimentation rate of a preparation of Lee influenza virus. The sedimentation constant at infinite dilution, determined from the graph, was 802 Svedberg units.
Particles which sediment at a rate as high as 800 Svedberg units would be expected to diffuse extremely slowly. The spread in the boundaries during sedimentation is, therefore, indicative of the existence of a family of particles of slightly variable size or density. The standard deviations of the distributions of the sedimentation rates for the families of particles represented by the heavy fraction and the RBC preparation were calculated to be 7.0 and 6.7 per cent, respectively, of the mean rate. In terms of particle diameters, these figures corresponded to standard deviations of 3.4 and 3.3 per cent. The degree of homogeneity of the purified Lee virus preparations is thus slightly higher than that of centrifugally purified PR8 virus preparations (4).

The chicken red blood cell agglutination titers of the crude, light, heavy, and RBC fractions shown in Fig. 1 were 2080, 2010, 2620, and 3010 units per mg., respectively. The data for the titers show, first of all, the efficacy of centrifugal fractionation in increasing the agglutinating activity of crude Lee virus preparations. This was confirmed in another experiment in which the activities of the crude, light, and heavy fractions were 1900, 1280, and 2600 units per mg., respectively. The data also indicate that the RBC virus possessed a titer even higher than that of the heavy fractions. This finding was checked by a further experiment in which an RBC virus preparation containing 2900 units per mg. was obtained directly from a heavy fraction containing only 2520 units per mg. In other experiments, RBC virus of 3020 units per mg. was obtained from crude virus of 2210 units per mg., and RBC virus of 2770 units per mg. was prepared directly from infectious chorioallantoic fluid. The agglutination tests as a whole indicate that the slowly sedimenting material possesses a lower biological activity than the rapidly sedimenting component. Furthermore, the higher activity of the RBC preparations, compared to those of the heavy fractions, suggests the presence of heavy, inactive material in the latter. This can be explained by the fact that an impurity of about the same particle weight as the virus would not be removed by the centrifugal fractionation but would be removed by the more specific adsorption-elution method.

If it is assumed that the chicken red blood cell agglutination titer of the RBC preparations represents that of pure virus and, further, that the presence of impurity does not affect the titers, it can be estimated from the activities of the different preparations that the crude virus contained somewhat over 30 per cent impurity, of which two-thirds was of small particle size and one-third was of a size comparable to that of the virus.

**Viscosity Studies**—The intrinsic viscosities of the crude, light, heavy, and RBC preparations shown in Fig. 1 were found to be 36.4, 46.4, 33.4, and 8.64, respectively. It may be seen that preparations with lowest sedimentation rates possess the highest viscosities, and vice versa. The results
suggest that the slowly sedimenting component contributes more to the viscosity than the rapidly sedimenting one. Furthermore, since the RBC preparation possessed by far the lowest viscosity, it is probable that the more viscous material represents impurities, as was the case with the PR8 virus (4). In another experiment, a crude preparation of Lee virus gave an intrinsic viscosity of 42.1, the RBC preparation only 9.86. To determine whether the viscosity of this RBC preparation might be decreased further, it was subjected to a second adsorption and elution with red blood cells, followed by high speed centrifugation. The final product showed,

However, practically no change, for it possessed an intrinsic viscosity of 9.44.

The relationship between concentration and the specific viscosity of the RBC preparation used in the sedimentation studies of Fig. 2 is shown graphically in Fig. 3. The intrinsic viscosity, calculated from the slope of the straight line drawn through the experimental points, was 9.50. The differences in sedimentation rates at varying concentrations of virus, shown by the data of Fig. 2, can be accounted for very closely by corresponding differences in relative viscosity provided by the data of Fig. 3. Multiplication of sedimentation rate and relative viscosity yields nearly constant values over the range of concentration of virus studied. This relationship is in accord with that previously established with other viruses (4, 23, 24). The sedimentation and viscosity data for the virus fractions of Fig. 1 do
not, however, yield constant results when dealt with as above, indicating effects of other, unknown factors in these instances.

If the Lee virus particles are assumed to be spherical, and the reported values of 0.863 ml. per gm. and 1.104 gm. per ml. are assumed for the anhydrous partial specific volume and the hydrated density, respectively, of the particles (22), the theoretical intrinsic viscosity, calculated from the Einstein equation (25), \( \eta / \eta_0 - 1 = 2.50\phi \), where \( \phi \) represents the volume fraction of the solute, becomes 3.46. The discrepancy between this value and the value of 9.50 reported above indicates the presence of small amounts of either highly viscous impurity or non-spherical virus structures such as the filamentous and branched forms reported by Mosley and Wyckoff (26). Similar calculations for the PR8 strain from available data (22) give a value of 4.73 for the theoretical intrinsic viscosity, whereas the minimal experimental value reported has been 11.3 (4).

Electrophoretic Studies—Moving boundary electrophoretic diagrams obtained for crude, heavy, and RBC fractions of Lee virus preparations are shown in Fig. 4. The light fractions were not studied by the moving boundary method. The crude virus exhibits three major components whose mobilities are 0.33, 0.52, and 0.65 \( \times 10^{-4} \) cm. per second per volt per cm., respectively. The heavy fraction also reveals these same components. This indicates that the light material which was removed by centrifugal fractionation possessed an electrophoretic mobility approximately the same as that of one or more of the heavy components, and thus

\[ \text{FIG. 4. Tracings of Longsworth scanning diagrams of ascending boundaries of Lee influenza virus fractions. The arrows indicate the positions of starting boundaries. Intervals of time are based on a field strength of 1.35 volts per cm. (a) Crude unfractionated virus; mobilities, 0.33, 0.52, and 0.65 \( \times 10^{-4} \) cm. per second per volt per cm., respectively. (b) Heavy centrifuge fraction; mobilities, 0.31, 0.47, and 0.65 \( \times 10^{-4} \) cm. per second per volt per cm., respectively. (c) RBC preparation; mobilities, 0.35 and 0.52 \( \times 10^{-4} \) cm. per second per volt per cm., respectively.} \]
its removal was not readily detectable. Diagrams for the RBC preparation, on the other hand, reveal only two major components, the more rapidly migrating one in the crude and heavy preparations having been removed. Other RBC preparations also showed only two components and differed from the preparation shown in Fig. 4 in exhibiting greater or lesser spread of the boundaries during electrophoretic migration.

It is evident from the data just described that the rapidly migrating component of the crude and heavy preparations represents an impurity and, further, that the apparent proportion of the impurity, as indicated by the areas under the peaks, is around 50 per cent. To substantiate this evidence, electrophoretic fractionation was carried out. A solution of crude virus containing 4 mg. of material per ml. was subjected to electrophoresis with mechanical compensation (27) for a period of time which, from the differences in rates of migration, should have placed the pure fast component in the uppermost compartment of the ascending limb of the U-tube and the slower components in the uppermost compartment of the descending limb. The slow and fast fractions thus obtained possessed chicken red blood cell agglutination titers of 2770 and 108 units per mg., respectively. Corresponding infectivity measurements in chick embryos yielded 50 per cent end-points at dilutions of $10^{-11.22}$ and $10^{-10.15}$ gm. per ml. By the agglutination test, the slow fraction was, therefore, 26 times as active as the fast; by the infectivity measurements, 12 times as active. The results obtained by the two methods may be considered in fair agreement within their limits of accuracy. Because of the possibility of contamination of the fast fraction with the more slowly migrating material during electrophoresis (8), the rapidly migrating component may actually have been completely inactive.

Micro electrophoresis measurements yielded isoelectric points for the crude, light, heavy, and RBC preparations of 5.0, 4.6, 5.1, and 5.4, respectively. These results show that centrifugal fractionation increased the amount of acidic material in the light fraction and decreased the amount in the heavy fraction. The isoelectric point of the RBC preparation is much higher than that of the heavy fraction, indicating the presence of still less acidic material. This acidic material can nearly certainly be correlated with the most rapidly moving boundaries of the crude and heavy fractions shown in Fig. 4, the absence of which from the RBC preparation explains the higher isoelectric point observed in that case.

Curves of mobility versus pH obtained by the micro electrophoresis method for several crude and purified virus preparations are shown in Fig. 5. The lower mobilities of the crude virus on the acid side of the isoelectric point and higher mobilities on the alkaline side, when compared with corresponding mobilities of the purified virus, reflect the influence of the presence
in the former of an acidic impurity. The agreement in results obtained for electrophoretically purified virus with those for RBC virus indicates comparable degrees of purity for the two different types of preparations. It should be pointed out that, in view of the low degree of electrochemical homogeneity of Lee virus preparations as shown by the moving boundary diagrams of Fig. 4, the values of the mobilities shown in Fig. 5 may at best be considered only average, or approximate ones.

Crude Lee virus preparations appear to contain impurities whose over-all characteristics are a wide range in particle sizes, a high viscosity, and acidic electrophoretic properties. The data also show that both the small and the large particles of the impurity are inactive, viscous, and acidic. Except for the range in particle size, these properties correspond in general to those of the normal heavy material elaborated by uninfected embryos. If the large particles represent degradation products or precursors of the virus, they must be much more asymmetric and possess a higher proportion of acidic groupings than the virus itself.

Purified Lee virus preparations show a higher sedimentation constant than corresponding preparations of PR8 virus. This confirms findings of other workers (1, 2, 10, 28). It may be noted, however, that whereas crude Lee virus in the present studies and those of the Duke University investigators (10) appeared less homogeneous and less readily capable of purification
than PR8 virus, the PR8 strain was found the least homogeneous by Friedewald and Pickels (2). The possibility is therefore suggested that single strains of influenza virus may undergo mutation in different laboratories. Salk (29) has recently reported additional evidence of such changes.

The isoelectric point of purified preparations of Lee virus, namely about pH 5.4, was not significantly different from that of the PR8 strain. The Lee virus showed a differently shaped curve of mobility versus pH, however, and was found less homogeneous in moving boundary tests than the PR8 virus. The appearance of a double electrophoretic boundary for the Lee virus was an unusual finding, but seemed reproducible and characteristic of the strain under examination.

The average specific chicken red blood cell agglutinating activity of the purest Lee virus preparations, about 3000 units per mg. of virus, was significantly lower than maximum values in the neighborhood of 4000 units per mg. reported for the PR8 strain (4, 5, 8). Knight has already reported the same findings in agglutination measurements of RBC preparations of both strains (9) and so the distinction appears well established. The differences are of practical interest for purposes of characterization, although from a theoretical standpoint the arbitrary conditions of the test may favor the apparent activity of one strain over the other since, as has been shown by Hirst (16), the two viruses behave somewhat differently in their reactions with red cells.

SUMMARY

Crude Lee virus preparations obtained by high speed centrifugation were found to contain 30 to 50 per cent of impurity, based on measurements of chicken red blood cell agglutinating activity and electrophoretic studies. The impurity was characterized by a wide range of particle sizes, high viscosity, and acidic electrophoretic properties. Removal of small sized particles of impurity was effected by fractional centrifugation, but electrophoretic fractionation or adsorption of the virus on chicken red blood cells and elution therefrom was required to remove both large and small particles of impurity. Large particles of the impurity were similar to the virus in sedimentation rate but more like the normal heavy material elaborated by uninfected embryos in viscosity and electrophoretic properties.

Purified Lee virus preparations exhibited a single sedimentable component representing a family of particles with an average sedimentation constant of 802 Svedberg units at infinite dilution and a standard deviation of 6.7 per cent from the mean sedimentation rate. The intrinsic viscosity was found to be 9.50 compared with the theoretical value of 3.46 for spherical particles calculated from available data with the aid of the Einstein equation, suggesting the presence of small amounts of either highly viscous im-
purity or non-spherical virus structures. Moving boundary experiments revealed the presence of two very diffuse boundaries, both appearing, however, to represent the virus. The isoelectric point, determined by the micro electrophoresis method, was at approximately pH 5.4. The chicken red blood cell agglutinating activity was 3000 units per mg., a value significantly less than that of about 4000 units per mg. reported previously for the PR8 strain.

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