ELECTROPHORETIC STUDIES ON PR8 INFLUENZA VIRUS*

By GAIL LORENZ MILLER, Ph.D., MAX A. LAUFFER, Ph.D., and W. M. STANLEY, Ph.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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The electrophoresis of PR8 influenza virus present in a suspension of infected mouse lungs has been carried out by Bourdillon with the aid of the Tiselius apparatus and a special sampling technique for measuring biological activities at different levels in the electrophoresis cells (1). The mobilities of the virus at a limited number of pH values were found to agree with the mobilities previously observed for the influenza complement-fixing antigen under the same conditions (2). Friedewald and Pickels have demonstrated recently that the infective unit of influenza virus prepared from chick embryos carries the activity of a complement-fixing antigen (3). It appeared, therefore, that the virus might possess the same isoelectric point as that found earlier for the antigen, namely about pH 5. The loss in solubility of influenza virus at pH 4 and 5, observed in pH stability studies (4), also indicated that the isoelectric point might be in the neighborhood of pH 5.

The isolation of influenza virus by means of differential centrifugation (5–8) made possible, however, direct measurements of the electrophoretic properties of the virus. These measurements have been found to require the use of both the moving boundary method and the microscopic method. The microscopic technique was necessary for the determination of isoelectric point since the virus protein was too insoluble at pH 4–6 to be studied by the moving boundary method under these conditions. On the other hand, the moving boundary method was necessary for establishing the relative degrees of electrochemical homogeneity of different preparations of the virus. Detailed exploratory as well as final measurements were made on the PR8 strain of influenza virus and are described in the present report.

Materials and Methods

Source of Virus.—Samples of purified PR8 influenza virus protein were prepared by differential centrifugation of allantoic fluid harvested from infected chick embryos (8, 9). The pellets of virus obtained from the first high-speed centrifugation were dissolved in 0.1 M phosphate buffer at pH 7, the resulting suspension was clarified in the Swedish angle centrifuge, and the supernatant fluid was subjected to a second high-speed centrifugation. The centrifu-

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gations were usually carried out for 15 to 30 minute periods at 24,000 R.P.M. The final pellets were dissolved in a small volume of phosphate buffer, and the protein content was determined by a micro-Nessler method (9).

Preparations of high molecular weight protein from the allantoic fluids of normal, uninfected embryos (10) were obtained by high-speed centrifugation under the same conditions as those described above for the isolation of the virus protein.

Micro-Electrophoresis Methods.—The Northrop-Kunitz micro-electrophoresis cell (11, 12) was cleaned with acid dichromate, thoroughly rinsed with distilled water, then filled with 1 per cent aqueous gelatin, again rinsed with distilled water, and finally rinsed with 40 cc. of the particular buffer employed in the mobility measurement to be carried out. The use of the gelatin solution insured a uniform coating of the walls of the cell and permitted a saving of valuable virus solution which ordinarily would have been required.

In order to remove phosphate buffer, which caused complications under some circumstances, a suitable aliquot of a solution of centrifugally purified virus protein was recentrifuged at 24,000 R.P.M. and the resulting pellet was taken up in 0.25 cc. of 0.02 M veronal solution at pH 7.4 Collodion particles, prepared by the method of Cannon and Marshall (13), were mixed with water to give a 20 per cent suspension and 0.03 cc. of this suspension was added to the 0.25 cc. of virus solution. A 0.03 cc. aliquot of the mixture was then added to 10 cc. of buffer, and the resulting suspension was used for measurements of mobilities in the micro-electrophoresis cell.

The directions of Moyer were employed as a guide in making the micro-electrophoresis measurements (14). Velocities were measured for five particles in each direction and at both theoretical levels in the cell. The twenty readings which were obtained were then averaged. The final mobilities were calculated from the measurements of current, conductivities of the buffers, and average velocities of movement of the observed collodion particles. The tedium of a great many conductivity and pH measurements was avoided by the preparation of large stocks of 0.02 M buffers, the pH and conductivity of which remained constant for long periods of time.

Since the variations with temperature of the mobilities of simple ions and of colloidal ions are both approximately inversely proportional to the variations of the viscosity, the observed rates of electrophoretic migration, at constant current, are essentially independent of temperature. Thus, the migration experiments can be carried out at any temperature. The conductivity measurements were corrected to 0°C. Mobilities at 0°C. were then calculated according to the simplified formula, $v_0^\circ = \frac{d}{T \times I \times R_0^\circ}$, where v_0° represents the mobility in cm. per second per volt per cm. at 0°C.; d, the calibrated distance in centimeters which the particle moves; T, the time in seconds required for that movement; I, the current in amperes; R_0°, the resistance per cm. at 0°C. of the buffer in the micro-electrophoresis cell, calculated from the conductance at 0°C.

The buffers employed were 0.02 M veronal buffers at pH 6.6–9.3, 0.02 M acetate buffers at pH 4.1–5.6, 0.02 M glycine-NaCl-HCl buffer at pH 3.0 and 0.02 M chloroacetate buffer at pH 2.8. A pH of about 1.77 was obtained with 0.02 M hydrochloric acid.

Moving Boundary Electrophoresis Methods.—The Tiselius electrophoresis apparatus with reversible copper-copper chloride electrodes was used for the moving boundary studies. Because of the sensitivity of the virus proteins to copper ions (15), the copper chloride (40 per cent) was introduced around the electrodes through glass capillary tubes sealed to the bottoms of the electrode chambers. The introduction of the copper chloride was preceded by that of 20 per cent potassium chloride which provided a layer between the copper chloride and the buffer. In order to obtain mobility data directly comparable with those obtained by the microscopic method, 0.02 M buffers were employed throughout.

Schlieren boundaries of the migrating proteins were recorded by means of Longsworth's scanning method (16). Because of the strong opalescence of solutions of influenza virus, it was necessary to employ infrared light and infrared sensitive photographic plates as recommended by Treffers and Moore (17). A marked tendency towards streaming of the protein during electrophoresis, similar to that encountered with vaccinia virus (18) and with derivatives of tobacco mosaic virus (19), was diminished by allowing the boundaries to move at a very slow rate of 0.3 cm. per hour. The concentrations of virus were limited to 3 to 4 mg. per cc. because of the difficulty of getting sufficient light through more concentrated solutions.

Anomalies were observed quite commonly in the descending boundaries, which appeared abnormally sharp on the side toward the buffer, and excessively spread out in the direction of migration. The ascending boundaries, on the other hand, were usually quite normal in appearance. However, no differences were observed between the mobilities calculated from the displacements of the ascending and those of the descending boundaries. This result differs from that obtained by Longsworth and MacInnes (20), who observed that the linear displacement of the ascending boundary exceeded that of the descending one. The difference between the two was shown to be associated with the formation of "δ" and "ϵ" boundaries and to be a function of the colloidal concentration. The results observed in the present study are probably due to the facts that the colloid concentration was rather low and that conspicuous "δ" and "ϵ" boundaries were not observed.

Measurements of Virus Activity.—Measurements of the virus activities of different electrophoretic fractions were made by means of tests for chicken red blood cell agglutinating (CCA) activity (21, 22). Estimates of protein concentration were made by nephelometric comparison with a known virus preparation or by isolation of the protein by high-speed contrifugation followed by nitrogen determinations on the resuspended pellets.

EXPERIMENTAL

Exploratory Micro-Electrophoresis Experiments.—A preliminary measurement of the isoelectric point of PR8 virus yielded a value of pH 5.0. Measurements on six other samples, however, yielded values of pH 4.3–4.5. It appeared that an incomplete coating of the collodion particles might have been responsible for the observed variation, since no particluar attention had been paid to the concentration of virus in the solutions used to coat the collodion particles in the earlier experiments. In subsequent experiments, the effect of differences in concentration of protein was, therefore, determined as is shown in Figs. 1 and 2. From Fig. 1 it can be concluded that either 5 or 10 mg. of protein per cc. sufficed to ensure a complete coating of the collodion particles near the isoelectric point of the virus. The results shown in Fig. 2 reveal, however, that when mobilities were measured at pH 7.4, there existed a significant dependence on concentration even above the level of 5 mg. of protein per cc. The experiment was repeated and the results were confirmed. This behavior might have been due to the presence of a contaminant which will be described later.

In view of the findings of the above tests, further measurements of isoelectric point were undertaken. The values obtained for eight different, freshly prepared samples of PR8 virus were pH 4.9–5.2, thus significantly higher than those obtained in the first set of experiments, but still showing appreciable variation. It appeared that this particular variability might be due to the presence of

impurities, the most probable of which would be the high molecular weight substance demonstrated by Knight (10) to be present in normal allantoic fluid and which might be expected to occur as well in infectious allantoic fluid. It has previously been shown by indirect evidence that this normal material could comprise 10 per cent or more of centrifugally purified virus preparations (9, 10).

A total of six different samples of the high molecular weight "normal" material, some of which were obtained from Dr. Knight and some of which were

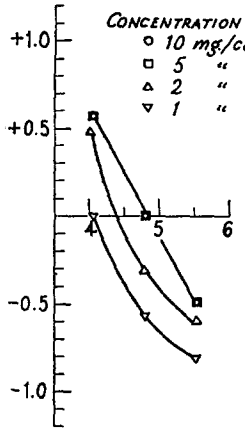

FIG. 1

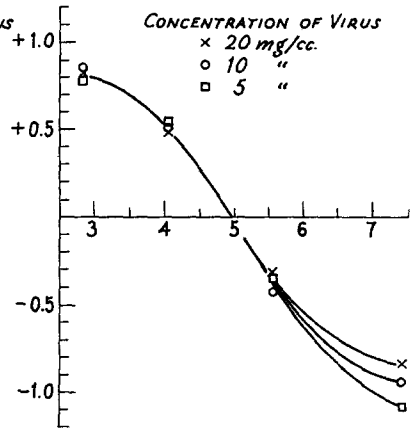

FIG. 2

FIG. 1. Effect of concentration of crude PR8 virus on mobility of collodion particles within range of pH 4.06–5.55. The abscissa scale represents pH; the ordinate scale represents mobilities in cm. per second per volt per cm. × 10^4.

FIG. 2. Effect of concentration of crude PR8 virus on mobility of collodion particles within range of pH 2.84–7.40. The abscissa scale represents pH; the ordinate scale represents mobilities in cm. per second per volt per cm. × 10^4.

prepared by the authors, were examined for isoelectric point. The curve of mobility *versus* pH, shown in Fig. 3, yielded an isoelectric point of pH 2.3. The results showed little if any dependence on the age of embryonated eggs from which the normal heavy material was prepared. It was found in separate experiments that a complete coating of the collodion particles was obtained with concentrations of the normal protein varying from 0.05 to 3.0 mg. per cc., deviations first being detected at the level of 0.02 mg. per cc. Concentrations of normal material much lower than those of virus material thus sufficed to coat the collodion particles.

It seemed quite likely that the contamination of influenza virus preparations with variable amounts of a material as acidic as the normal protein might cause a variable depression in the apparent isoelectric point. This possibility was

tested by determining the mobility curves of samples of influenza virus containing 5 mg. of the virus protein per cc. and varying proportions of added normal material. The results, shown in Fig. 4, reveal a marked effect of the normal material on the apparent isoelectric point of the virus and provide at least a partial explanation for variations in electrophoretic properties of different preparations of influenza virus.

Non-viral components of F12 and Lee influenza virus preparations, separated by means of differential centrifugation (23, 24), were found in micro-electro-

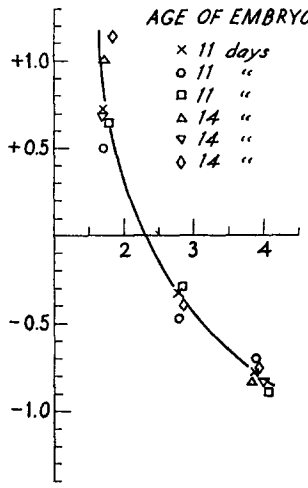

FIG. 3

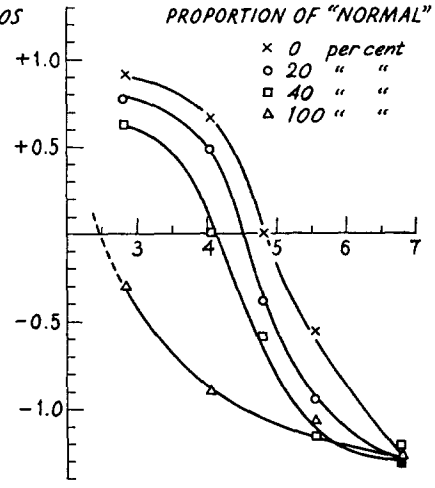

FIG. 4

FIG. 3. Mobilities and isoelectric point of high molecular weight "normal" material. The abscissa scale represents pH; the ordinate scale represents mobilities in cm. per second per volt per cm. × 10^4.

FIG. 4. Mobilities and isoelectric points of mixtures of "normal" material and crude PR8 influenza virus. The abscissa scale represents pH; the ordinate scale represents mobilities in cm. per second per volt per cm. × 10^4.

phoresis tests to be very similar in electrochemical properties to the normal protein described above. Isoelectric points of 2.2 and 2.4, respectively, were obtained. Although these components were isolated from crude preparations of strains of viruses other than PR8, it is to be expected that similar material would also be present in the latter strain.

Exploratory Moving Boundary Experiments.—In order to obtain direct evidence for the degree of homogeneity of preparations of influenza virus, it appeared necessary at this point to carry out moving boundary studies on preparations of both the influenza virus and the normal heavy material. From scanning diagrams of each it was evident that the normal material was quite homogeneous electrophoretically, whereas the virus preparation contained two

separate components. The normal material possessed a mobility of -1.03×10^{-4} cm. per second per volt per cm. in 0.02 M veronal buffer at pH 7.4. The slow component of the virus preparation moved at a rate of -0.61 units under the same conditions. The mobility of the faster component of the virus, -1.11 units, corresponded to that obtained for the normal heavy material, and indicated that the two materials might be identical. Further electrophoresis measurements on four other preparations of crude influenza virus yielded essentially the same results with mobilities of 0.65, 0.62, 0.61, and 0.60 units for the slow component, and 1.01, 1.08, 0.96, and 1.00 units for the faster component. On the basis of the sizes of the scanning peaks, there was 10 to 20 per cent as much fast component as slow component present in the crude virus preparations. These amounts corresponded to the proportions anticipated (9, 10).

A solution of crude virus containing 10 mg. of protein per cc. was next subjected to electrophoresis with mechanical compensation (25) for a period of time which, on the basis of the differences in rates of migration, should have placed pure slow component in the uppermost compartment of the descending limb of the U-tube and the pure fast component in the uppermost compartment of the ascending limb. The V-shaped bottom compartment of the U-tube should have contained essentially the original mixture. Due to the occurrence of streaming in the direction of migration, the fast component was expected to contain slow component as an impurity. On the other hand, the slow component should have been quite pure. The isoelectric points of the different fractions measured by the micro-electrophoresis method were found to be 4.6, 4.9, and 5.4, respectively, for the fast fraction, the original fraction, and the slow fraction. The low isoelectric point of the fast fraction is in harmony with the hypothesis that this material would contain normal heavy component contaminated with virus. The high isoelectric point of the slow fraction was to be expected to result from the removal of acidic normal material. The CCA activities of the fast, the original, and the slow fraction were found to be 1,430, 2,670, and 3,280 CCA units per mg. of protein, respectively. These results may be interpreted on the same basis employed to explain the results of the corresponding measurements of isoelectric points.

The above findings did not rule out the possibility that the fast moving component might actually possess an appreciable amount of CCA activity. It appeared desirable, therefore, to repeat the electrophoretic fractionation experiment and to obtain, if possible, a more clean cut separation of the components. In order to minimize still further the streaming, the boundaries were allowed to move at an average rate of only 0.1 cm. per hour. After 4 days of migration, part of which time compensation in the opposite direction was applied, the uppermost compartment of the descending limb appeared to contain pure slow component, and the uppermost compartment of the ascending

limb, pure fast component. The CCA activities of the fast and slow components were found to be 570 and 3,880 units per mg., respectively. Sufficient material was not available for complete micro-electrophoresis measurements; however, an isoelectric point in the neighborhood of pH 2.8 was obtained for the fast fraction. The improved separation of fast and slow components which was obtained provided strong evidence for the hypothesis that the fast moving component represented normal material lacking CCA activity, and also that the slow component represented the active influenza virus protein.

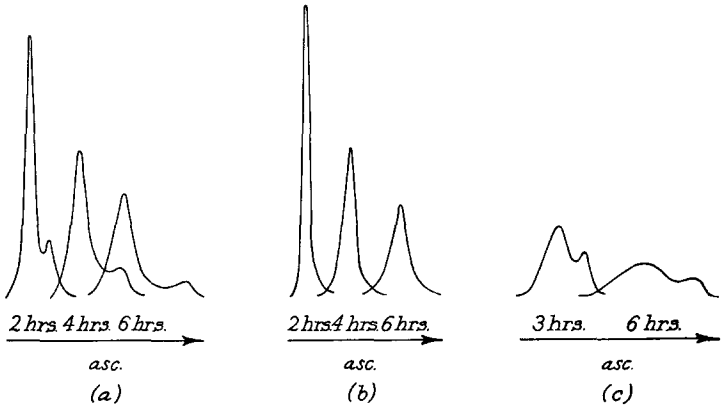

FIG. 5. Tracings of Longsworth scanning diagrams of ascending boundaries of PR8 influenza virus fractions. Field strength, 0.7 volts per cm. (*a*) Crude, unfractionated virus. Mobilities, 0.61 × 10^{-4} and 0.96 × 10^{-4} cm. per second per volt per cm., for slow and fast components, respectively. (*b*) "Heavy" centrifuge fraction. Mobility, 0.66 × 10^{-4} cm. per second per volt per cm. (*c*) "Light" centrifuge fraction. Mobilities, 0.68 × 10^{-4} and 0.96 × 10^{-4} cm. per second per volt per cm., for slow and fast components, respectively.

Final Electrophoresis Experiments.—Since normal heavy material was found to occur as a contaminant in preparations of PR8 virus, and further, since it is known that the normal heavy material sediments more slowly than influenza virus (8, 10), it appeared that repeated fractionation by centrifugation at high speed should effect a removal of the impurity from the virus. Fractionation by this method was therefore carried out as described by Lauffer and Stanley (26). The "heavy" fraction, presumably representing quite pure virus, and the "light" fraction, expected to contain normal material contaminated with virus, were subjected to tests of homogeneity both in the Tiselius electrophoresis apparatus and in the analytical centrifuge. The diagrams of electrophoresis scanning boundaries which were obtained at pH 7.4 in 0.02 M veronal buffer are presented in Fig. 5, from which the high degree of homogeneity of the heavy fraction compared with the double boundary characteristic of the light fraction and of the crude, unfractionated virus can readily be seen. The

higher proportion of fast component in the light fraction than in the crude virus also is apparent. In order to confirm these findings the entire fractionation experiment was repeated several times with fresh preparations of crude virus. The electrophoresis diagrams which were obtained for the heavy and light fractions were similar to those obtained in the original experiment. Moving boundary measurements also were carried out with the heavy fraction in veronal buffers at pH 9.3 and 8.8, in glycine-NaCl-HCl buffer at pH 3.0, and

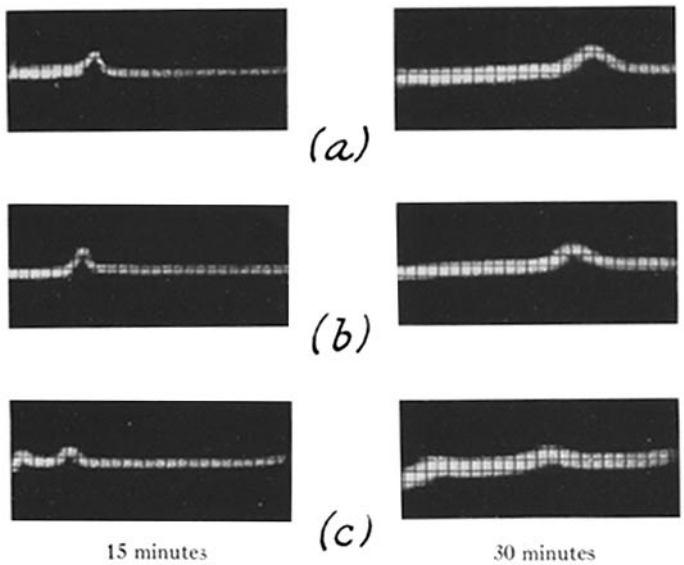

FIG. 6. Svensson-Philpot sedimentation diagrams of PR8 influenza virus fractions. Centrifugal field, 8,800 times gravity. (*a*) Crude, unfractionated virus. s_{20} = 674S at 5.0 mg. per cc. (*b*) "Heavy" centrifuge fraction. s_{20} = 654S at 5.0 mg. per cc. (*c*) "Light" centrifuge fraction. s_{20} = 134S and 542S, respectively, at 11.4 mg. per cc. The sedimentation constants are not corrected for viscosity and concentration (26).

in chloroacetate buffer at pH 2.8. Single boundaries again were obtained in each case. The mobilities which were calculated were −0.67, −0.64, +0.87, and +0.94 units, respectively. The electrochemical homogeneity exhibited by the heavy fraction under widely varying conditions of pH may be taken as further evidence for the purity of the material.

The sedimentation boundaries, shown in Fig. 6, reveal a single boundary in the heavy fraction, and, similar to the electrophoresis results, a double boundary in the light fraction. The slowly sedimenting component in the latter case represents the normal material. It is interesting to note, however, that the slowly sedimenting material is not readily detectable in the diagram for the crude starting material. In repeat experiments with heavy and light fractions

obtained from fresh preparations of crude virus similar results have been obtained except that, in the case of the light fraction, the slowly sedimenting component was found to exhibit widely varying sedimentation rates, varying from 200 to 300 S. This anomaly, together with the failure of the slowly sedimenting component to be detectable readily in sedimentation diagrams of crude unfractionated virus, is similar to anomalies in the sedimentation behavior of the normal material observed by Knight (10) and by Stanley and Lauffer (24).

The effects of fractionation by differential centrifugation were also revealed in measurements of CCA activities of the heavy and light fractions. The results obtained in three different experiments are summarized in Table I. The lower CCA activities of the light fractions are due to the presence of inactive, slowly sedimenting contaminants. The proportions of inactive materials present, as calculated from the CCA data, agree well with those indicated by

TABLE I

CCA Activities of Heavy and Light Fractions Obtained by Fractional Centrifugation

Experiment No.	CCA activity	
	Heavy fraction	Light fraction
1	3960*	2700
2	2740	2010
3	2880	2220

* Units of CCA activity per mg. of protein.

electrophoresis and sedimentation diagrams. The differences in specific CCA activities of the various heavy fractions probably are not indicative of differences in purity since it has been observed in a great many tests (27) that preparations of crude virus of apparently equal purity as far as proportion of normal material present is concerned, may vary in CCA activity from 1,800 to 4,000 units per mg. Further study is being carried out at the present time on the question of the specific CCA activity of purified preparations of PR8 virus.

When measured in the micro-electrophoresis apparatus at virus concentrations of 10 mg. per cc. samples of two different heavy fractions were found to possess an isoelectric point at pH 5.3. The corresponding light fractions were isoelectric at pH 4.8. The differences between the heavy and light fractions are attributable to the presence of acidic normal material in the latter. The curves of mobility *versus* pH for two preparations of "heavy" fractions obtained by centrifugation and also for one preparation of "slow" fraction obtained by electrophoretic separation are shown in Fig. 7. Points obtained from moving boundary measurements also are shown. The agreement between the results obtained by the microscopic and moving boundary methods is quite good except

at high pH values. This is possibly due to the effect of a residual trace of normal material, which could cause the mobilities by the microscopic method to be slightly high in alkaline media. It has been found, for example, that, when measured by the microscopic method, the mobilities of the normal material alone, or of virus preparations known to contain normal material, are much higher in the alkaline range than that of the purified virus. On the other hand, differences between the mobilities obtained by the moving boundary and the micro-electrophoresis methods might be due to hydrodynamic causes or to slight electrochemical changes incident to adsorption on a collodion surface. The value of pH 5.3 for the isoelectric point of PR8 influenza virus may be consid-

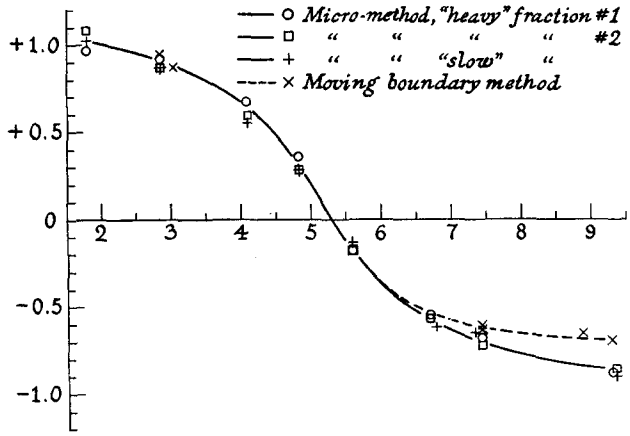

FIG. 7. Mobilities and isoelectric point of purified PR8 influenza virus preparations. The abscissa scale represents pH; the ordinate scale represents mobilities in cm. per second per volt per cm. $\times 10^4$.

ered quite nearly correct since, as can be seen from Fig. 7, preparations of virus purified either by fractional centrifugation or by electrophoretic fractionation yielded essentially the same results.

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SUMMARY

Crude preparations of PR8 influenza virus, obtained by high-speed centrifugation, contain two electrophoretically distinct components. One of these, present to the extent of 10 to 20 per cent, was identified by electrophoresis, centrifuge, and activity tests, as an impurity similar to or identical with a high molecular weight acidic substance shown by Knight to be elaborated by normal uninfected embryos. The other component, present to the extent of 80 to 90 per cent, appeared to represent the active virus. The virus fraction was sep-

arated from the impurity by repeated fractional centrifugation. It then appeared homogeneous in the analytical centrifuge and in the Tiselius apparatus, and possessed an isoelectric point at pH 5.3 as measured by the micro-electrophoresis method.

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