ELECTROPHORESIS OF THE COMPLEMENT-FIXING ANTIGEN OF HUMAN INFLUENZA VIRUS

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The presence of antigens capable of fixing complement has been demonstrated in several virus diseases. In yellow fever (1), vaccinia (2), myxomatosis (3), and lymphocytic choriomeningitis (4, 5), for example, this antigen has been shown to be soluble and apparently can be freed from the virus itself; in others such as rabbit papilloma (6), it is so intimately associated with the virus that no distinction can be made between the two. The occurrence of a specific complement-fixing antigen in mouse lungs infected with human influenza virus was reported by Smith (7) in 1936, and the work of Hoyle and Fairbrother (8) subsequently suggested that this antigen is soluble and separable from the virus.

Electrophoresis has contributed much to the newer knowledge of proteins and was therefore employed in the investigation reported here. It was applied to the study of the electrical mobility of the complement-fixing antigen present in infected lung suspension, and also to the study of normal and influenza mouse serum. It was thus possible to give greater significance to the pH-mobility curve of the antigen by comparing it with the curves obtained for the various components of mouse serum.

Material and Methods

Normal Mouse Serum.—Normal albino Swiss mice, about 1 month of age, were bled from the heart under ether anesthesia. After being allowed to clot, the blood was stored overnight at 4°C. and on the following day the serum was removed after centrifugation. It was then dialyzed against the appropriate buffer for 2 or 3 days in the cold room and finally diluted 1:2 or 1:3 with the same buffer before being placed in the electrophoresis cell.

Influenza Mouse Serum, Acute Phase.—Mice of the same age and breed were inoculated with the PR8 strain of virus, each receiving 0.05 cc. of a 0.1 per cent suspension of infected mouse lung intranasally under light ether anesthesia. Three to 4 days later, when severe pulmonary consolidation was known to have developed, the animals were bled by cardiac puncture under ether anesthesia, and the serum specimens were pooled. Dilution and dialysis were performed in the same manner as with normal serum.

Mouse Lung Suspension.—Mice inoculated as just described were killed 3 to 4 days after inoculation. The lungs were removed, weighed, ground with alundum, and enough salt solution was added to make a 10 per cent suspension. Salt solution at different pH's was tried, but it was found that the amount of protein dissolved did not vary greatly. Therefore, in the experiments reported below either 0.85 per cent NaCl or a borate buffer of pH 9.8 was generally used.

After grinding, the suspension was centrifuged at 2500 R.P.M. for 30 minutes and the supernatant drawn off and stored at −76°C. Just before use, further clarification was effected either by spinning the suspension in the open-air centrifuge of Bauer and Pickels (9) at 13,000 R.P.M. for 30 minutes, or by filtration through asbestos. The final product, with a protein concentration of about 1.0 per cent, was perfectly clear. This clarity was retained during subsequent dialysis against alkaline buffers; dialysis against acid buffers, however, resulted in irreversible precipitation, which reached a maximum at pH 4.5 and below. (At pH 4.5 in acetate buffer, for example, the precipitate represented about one-third of the proteins present.)

Buffers.—Serum and mouse lung suspensions were dialyzed in the cold room against various buffers. Acetic acid and sodium acetate buffers at pH 4.6; disodium phosphate and sodium acid phosphate buffers at pH 5.4, 6.0, 6.8, and 7.9; as well as boric acid and sodium hydroxide buffers at pH 9.8 were used. All buffers, except the last, had an ionic strength of 0.1. The borate buffer had an ionic strength of 0.04, as it was observed that in stronger concentration (0.1) this buffer caused splitting of the albumin peak.

Electrophoresis.—In these experiments the apparatus originally described by Tiselius (10) with the medium size cell accommodating 11 cc. of solution was used. Photographs of the migrating boundaries were taken by the "schlieren scanning method" of Longsworth *et al.* (11). In order to compensate for the migration of the boundaries during the passage of the current, the arrangement described by Longsworth and MacInnes (12) was employed.

Throughout this paper the electrophoretic mobility (U cm.^{2}, volt^{-1}, sec.$^{-1}$) will be referred to simply as mobility. The mobility of the visible boundaries was determined by timing their displacement along a scale placed in the focal plane of the camera, or by measuring the distance separating them in the schlieren diagram from the location of the initial boundary before passage of the current. The mobility of the antigen was determined by sampling the fluid in the cell at various levels after the completion of a run, and testing the samples for specific activity. In detail, the latter procedure was as follows:—

The cell was closed by sliding aside the two middle sections, the electrode vessels were disconnected and removed, and the cell was elevated in the water bath until one-half the top section was out of the water. The buffer in the top section was syphoned off, and the top and two middle sections were moved entirely to one side, leaving the bottom section to close the U tube. Then a long metal needle fitted to a 5 cc. syringe was slowly lowered by hand into the tube, and in general four samples per section, about 1.15 cm. in length and 0.9 cc. in volume, were successively removed. With a little care this could be accomplished without undue mixing.

Complement Fixation Test.—The amount of antigen present in each sample removed after electrophoresis was determined by the use of the complement fixation test, original material which had not been subjected to electrophoresis serving as a standard for comparison. The test was conducted as follows: Serial twofold dilutions were made of each

specimen. To 0.2 cc. amounts of each dilution were added 2 units of guinea pig complement followed by 0.2 cc. of a 1:10 dilution of inactivated pooled human influenza convalescent serum. After incubation at 37°C. for 1 hour, 0.5 cc. of sensitized sheep cells, consisting of a mixture of equal parts of a 5 per cent sheep cell suspension in saline and 2 units of amboceptor, was added. Results were read after secondary incubation at 37°C. for 30 minutes.

This procedure was applied to lung suspension after electrophoresis at pH between 5 and 10. Electrophoresis of the antigen was not studied at pH below 5, since in that region it was found that the buffer was anticomplementary.

EXPERIMENTAL

Electrophoresis of Normal Mouse Serum.—Since schlieren patterns obtained from the ascending side of the U tube are usually easier to read than those from the descending side, only the former have been reproduced here. Diagrams A and B of Fig. 1 give patterns obtained for normal serum at pH 6.8 and 9.8. Reading from left to right one goes from the top toward the bottom of the ascending side in the U tube. Each peak represents one protein fraction. On the basis of data obtained by Tiselius (13) for horse serum and by Stenhagen (14) for human serum, the principal peaks have been identified as albumin and as α and β globulins. A small fraction, close to the albumin, has been labeled x. The last peak, expressing a fraction which remained almost immobile, has been called $\gamma + \delta$, since it presumably includes Tiselius' γ globulin, and the "δ boundary disturbance;" the latter is an anomaly, the origin of which has been analyzed by Longsworth and MacInnes (12), and is not due to an actual protein fraction. This nomenclature has been used here as a matter of simple convenience. There is little doubt that the fraction called albumin is nearly the same as the albumin fraction isolated by the usual precipitation procedures. But no claim is made, however, as to the absolute identity of the α and β globulin fractions with the same fractions described by Tiselius or Stenhagen. Diagrams A and B of Fig. 1 show complete similarity, except for the fact that the concentration in Diagram B is slightly greater, so that all the peaks are higher. The mobilities of the various fractions, calculated from these diagrams, are given in Table I, which includes also the mobility of normal mouse serum albumin at pH 7.9.

Electrophoresis of Serum from Mice Infected with Influenza Virus.—Diagrams C, D, and E of Fig. 1 show the results obtained on a single specimen of pooled serum. Diagrams C (pH 6.8, ionic strength 0.1) and D (pH 9.8, ionic strength 0.04) appear similar, the only difference being the appearance, at the higher pH, of a small new fraction labeled x'. These patterns are similar to those obtained with normal serum under the same conditions.

When a borate buffer of 0.1 ionic strength was used, as in Diagram E, the albumin was split in two, so that the x and x' fractions became invisible. The rest of the pattern remained unchanged. The splitting of the albumin suggests that this fraction is not as homogeneous as is usually supposed. The same phenomenon, at acid pH, has been observed by Luetscher (15) in horse and in human serum. Between Diagrams D and E the sample was dialyzed against acetate at pH 4.6, the slight precipitate formed was removed, and the sample was redialyzed against borate pH 9.8 and ionic strength 0.04. The pattern obtained then was exactly similar to Diagram D, except for the almost complete disappearance of the α globulin. This shows the slight effect on serum of acidification to pH 4.6, whereas the same procedure applied to lung suspension causes considerable precipitation.

TABLE I

Mobility of Mouse Serum Proteins (Anode)

Serum	Buffer	pH	Ionic strength	Mobility × 10^5				
				Albumin	x	x'	α	β
Normal	Phosphate	6.8	0.1	5.5	5.0	—	3.5	2.3
	"	7.9	0.1	7.0	—	—	—	—
	Borate	9.8	0.04	10.2	9.5	—	6.6	5.2
Influenza	Phosphate	6.8	0.1	6.2	5.6	—	4.1	2.7
	Borate	9.8	0.1	8.7 7.5	—	—	5.9	4.4
	"	9.8	0.04	10.4	9.6	8.5	6.8	5.1
	After acidification to pH 4.6	9.8	0.04	10.0	9.1	8.2	—	5.0

The mobilities of the various serum fractions are given in Table I. As would be expected, the mobilities are much greater in the less concentrated borate. Comparison with the figures obtained for normal mouse serum shows that at pH 6.8 the various fractions in influenza serum had a slightly greater mobility. At pH 9.8 the mobilities are the same.

Electrophoresis of Lung Suspension. Optical Results.—Diagram F of Fig. 1 gives the schlieren pattern obtained at pH 9.8 after the original solution had been concentrated twice by pressure dialysis. The smaller boundary showed the same mobility as serum albumin and has therefore, for convenience' sake, been called albumin. The other boundary migrated like the hemoglobin which could be seen with the naked eye and has therefore been labeled hemoglobin. Never more than two boundaries were observed, and at other pH's they always showed the mobility that could

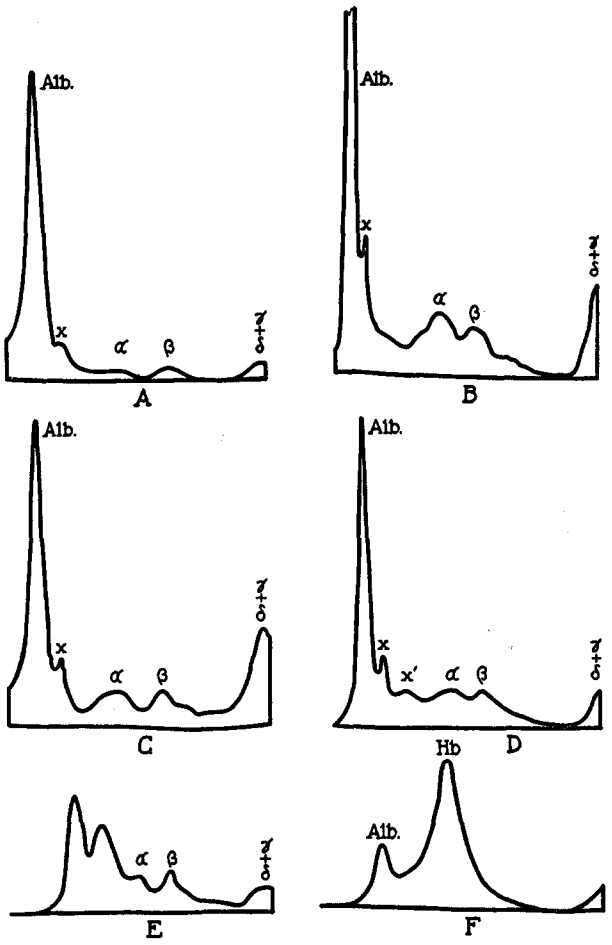

FIG. 1. Electrophoretic diagrams of mouse serum and mouse lung suspension (anode). Abscissae: distances measured from top of cell. Ordinates: concentration gradients.

Diagram A. Normal mouse serum. pH 6.8, ionic strength 0.1. Dilution 1:3. Potential gradient 8.8 V/cm. After 111 minutes.

Diagram B. Normal mouse serum. pH 9.8, ionic strength 0.04. Dilution 1:2. Potential gradient 14.8 V/cm. After 39 minutes.

Diagram C. Influenza serum. pH 6.8, ionic strength 0.1. Dilution 1:2.5. Potential gradient 8.7 V/cm. After 100 minutes.

Diagram D. Influenza serum. pH 9.8, ionic strength 0.04. Dilution 1:2.5. Potential gradient 11.3 V/cm. After 48 minutes.

Diagram E. Influenza serum. pH 9.8, ionic strength 0.1. Dilution 1:2.5. Potential gradient 7.4 V/cm. After 72 minutes.

Diagram F. Influenza lung suspension. pH 9.8, ionic strength 0.04. Potential gradient 12.2 V/cm. After 24 minutes.

be expected of albumin and hemoglobin respectively. It is thus probable that albumin and hemoglobin were the two most conspicuous components of the suspension. But that other proteins were present is obvious from the consideration that in pure normal blood the ratio albumin/hemoglobin is about 1:8 (assuming that the values for man can be applied here), whereas Diagram F shows that the areas under the "albumin" and the "hemoglobin" peak are in the ratio 1:4. Besides, as pointed out above, acidification of the solution caused an appreciable fraction to precipitate out, which does not happen with blood alone. These facts are not of prime importance in the present work, and, because of technical complications, a more detailed study of the schlieren diagram of mouse lung suspension did not seem warranted at this point. The mobilities of the albumin and hemoglobin are given in Table II.

TABLE II

Mobility of Proteins and Soluble Antigen in Influenza Mouse Lung Suspension (Anode)

Buffer	pH	Ionic strength	Mobility × 10^5		
			Albumin	Hemoglobin	Complement-fixing antigen
Acetate	4.6	0.1	0.7	−4.9	—
Phosphate	5.4	0.1	—	—	1.1
"	6.0	0.1	4.9	−1.6	3.8
"	6.8	0.1	—	—	5.2
"	7.9	0.1	7.0	2.1	5.4
Borate	9.8	0.1	7.9	—	5.8
"	9.8	0.04	9.9	6.5	7.8

Electrophoresis of Lung Suspension. Analytical Results.—Several preliminary runs were made in order to obtain an approximate idea of the mobility of the antigen and the best experimental conditions. In such a procedure inaccurate sampling, analytical error, or a diffuse boundary will all cause lack of sharpness in the results. In general, when the mobility of a substance is determined by the analytical method, the absolute experimental error will depend in part upon the thickness of the layer taken as a sample when this includes the boundary. Therefore it becomes important to allow the electrophoresis to proceed as long as possible, and to make the samples as small as possible. Limitations, on the other hand, are offered by the total height of the cell and by the mobility of the other constituents of the solution, inasmuch as allowing any of the latter to move out of the bottom section creates severe disturbances due to density differences. In the experiments reported here electrophoresis was allowed to

proceed from 2 to 4 hours, with a potential gradient of about 8 V/cm., and appropriate adjustments were made with the compensation device.

After each run the fluid was sampled in the apparatus and the presence of antigen determined in the samples, as described above. The maximum dilution at which the presence of antigen could be detected in the samples varied with each experiment from 1:4 to over 1:32. Most determinations were made on the ascending side only, and the results suggested, in so far as the experimental error of the procedure permitted, that the boundary of the antigen was quite sharp, as sharp as that of a substance with a clear-cut schlieren band. In some cases, samples from the descending side were

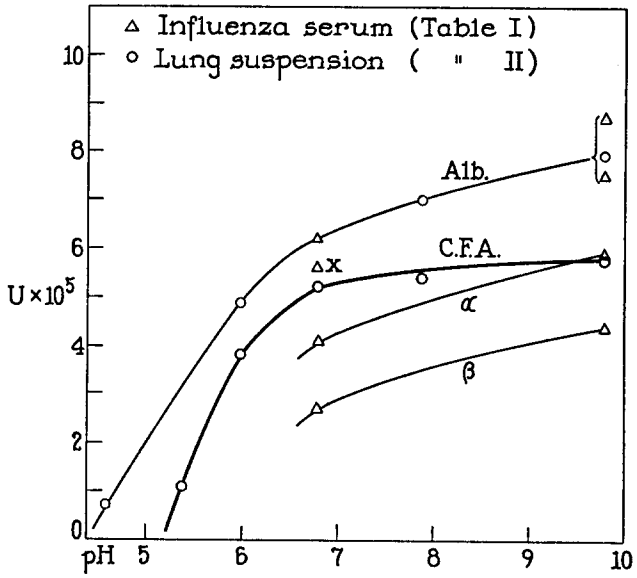

FIG. 2. Mobility (U) of complement-fixing antigen as compared to that of serum proteins.

tested and showed that the descending boundary was symmetrically located. The results also suggested that the descending boundary traveled more slowly by about 10 per cent, which is usually observed with proteins under similar conditions. The titer of the samples between the assumed positions of the boundaries was always quite constant for each experiment, and the titer of the samples above the boundaries was zero. The mobilities thus obtained for the complement-fixing antigen on the ascending side at various pH's are given in Table II. It is obvious that the antigen cannot be identified with either of the two main components detected optically.

The results from mouse serum and mouse lung suspension have been

summarized in Fig. 2, in which the mobilities at 0.1 ionic strength have been plotted against pH. It is obvious that the complement-fixing antigen has a mobility between that of albumin and α globulin, or between the x fraction and α globulin, with an isoelectric point probably slightly above pH 5. At an ionic strength of 0.04 the relative position of the antigen is the same, as can be gathered from the mobilities in Tables I and II.

DISCUSSION

Presentation of the mobilities of serum protein fractions and complement-fixing antigen on the same chart (Fig. 2) is obviously only a convenient device which makes the results more easily visualized. But we do not know whether the antigen is present at all in serum. It may be entirely absent from it, or present in amounts too small to be detected. It is probably not present in serum in large amounts, for, if some inhibiting factor made its detection there impossible, it would not be detected in the mouse lung suspension either, since the lung suspension contains so much blood.

The mobility of the complement-fixing antigen shows that it is electrophoretically similar to a serum globulin. Its mobility would be near that of α globulin on the alkaline side, but greater than α globulin at neutrality. More accurate conclusions are not warranted because of the crudeness of the sampling device and because of the experimental error in the complement fixation test, which make impossible an accurate determination of mobility.

Little is known of the physicochemical properties of the complement-fixing antigens of viruses. Hughes (1) found that the antigen present in yellow fever serum precipitated like an albumin with ammonium sulfate. Those present in vaccinia (16) and myxomatosis (17) precipitate with the globulins. In lymphocytic choriomeningitis the complement-fixing antigen is found both in the albumin and in the globulin fraction (5). One should bear in mind nevertheless that the present classification of proteins into "albumins" and "globulins," according to whether they precipitate at full saturation or half saturation with ammonium sulfate, has been derived from the study of normal serum. It may be extended to pathological sera or tissue proteins, as a matter of convenience, but only so long as its importance is not overestimated. That the soluble antigen of influenza virus stands in close relationship to the infectious moiety of the virus is suggested by preliminary experiments which indicate that the infectious agent has about the same electrical mobility as the soluble antigen, and also by the observation, which is at variance with Hoyle and Fairbrother's

conclusions (8), that it has not been possible to prepare a washed virus suspension entirely free from complement-fixing properties. These points will be dealt with in later publications.

SUMMARY

1. An electrophoretic study has been made of normal mouse serum, influenza mouse serum, and influenza mouse lung suspension. The mobilities of the protein fractions present have been determined at various pH's by the optical method.

2. The pH-mobility curve of the soluble (complement-fixing) antigen present in the lung suspension has been determined analytically by sampling and application of the complement fixation test. The results show that the complement-fixing antigen has a mobility definitely smaller than that of serum albumin and close to that of α globulin, with an isoelectric point close to pH 5.

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