THE IMMUNOLOGICAL RESPONSE TO INFLUENZA VIRUS INFECTION AS MEASURED BY THE COMPLEMENT FIXATION TEST

Relation of the Complement-Fixing Antigen to the Virus Particle

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Influenza virus suspensions exhibit two distinct properties *in vitro* which can be readily detected—the fixation of complement in mixture with specific immune serum (1–4) and the agglutination of red blood cells (5, 6). The capacity of the virus suspensions to agglutinate red cells appears to be closely associated, if not identical, with the infective activity of the virus. The complement-fixing antigen, on the other hand, has been found to be considerably smaller than the infective virus particle and separable from it (2, 3). In general, it resembles the well known soluble antigens of other viruses (7), and it has been assumed that this antigen and not the virus particle *per se* is responsible for the complement fixation reaction.

The present paper reports a detailed study of the complement fixation test with influenza virus suspensions and immune sera, and the development of a sensitive and accurate method of determination of the reaction. Evidence will be presented which indicates that an antigen, distinct from the soluble antigen, is intimately associated with the virus particle and is highly specific in its reactions with immune sera.

Methods

Virus Preparation.—The PR8 (8) and W.S. (9) strains of influenza A virus, the Lee (10) strain of influenza B virus, and a swine influenza strain (No. 1976) were used as sources of virus material. The allantoic sacs of 11 day old white Leghorn embryos were inoculated with 0.2 cc. of a 10^{-3} or 10^{-4} dilution of the virus strains. After 48 hours at 37°C. the eggs were chilled overnight at 4°C., and the blood-free allantoic fluids removed. The allantoic fluids were then cleared by centrifugation at about 2000 R.P.M. for 10 minutes. Mouse lung extracts were obtained by instilling 0.05 cc. of the above virus strains into mice under ether anesthesia. After 2 to 3 days the lungs were removed with aseptic precaution and 10 per cent extracts prepared by grinding weighed portions with alundum and suspending in saline or 10 per cent horse serum broth. The extracts were then spun at about 2000 R.P.M. for 10 minutes. All virus preparations were stored at −76°C.

Sera.—Serum specimens were obtained from patients with epidemic influenza during acute and convalescent stages of the disease. Ferret sera were taken 12 to 14

days after intranasal inoculation of the above virus strains. All were stored at 4°C. and inactivated at 56°C. for 30 minutes before use in the tests.

Agglutination Titrations.—The capacity of virus preparations to agglutinate chicken red blood cells and the degree of inhibition of the reaction by specific immune sera (5) were measured by the technique described by Hirst and Pickels (11).

Complement Fixation Test.—A quantitative complement fixation test was devised with the aid of a photoelectric densitometer[1] for estimating the amount of hemolysis. The densitometer was similar to the one already described for measuring the agglutination reaction (10) except that a blue filter was inserted in the light path. This in-

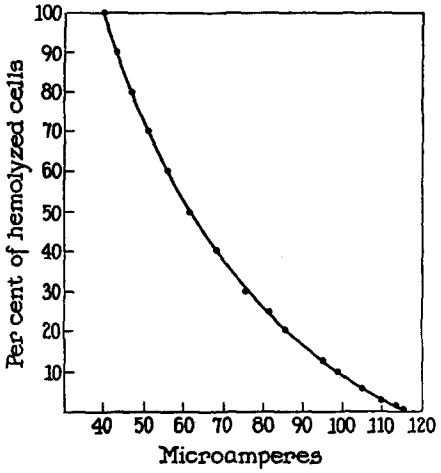


FIG. 1. Calibration of the densitometer in terms of hemolyzed red cell concentration.

100 per cent hemolyzed cells = 0.3 cc. standardized sheep red cells (about 3 per cent).
0.3 " hemolysin.
0.4 " saline (0.9 per cent).
0.2 " complement, sufficient to hemolyze completely the cells.

creased the sensitivity of the method since a greater light absorption by a solution of hemolyzed red cells occurred in the blue region of the spectrum. Clear test tubes having a length of 7 cm. and an internal diameter of 0.8 cm. were used throughout.

The *hemolytic system* consisted of rabbit serum containing immune sheep hemolysin in high titer and thrice-washed sheep cells. The hemolysin was titrated under the conditions of the test to be employed and diluted so that 0.3 cc. contained 4 hemolytic units. The concentration of the washed sheep cells was standardized by means of the photoelectric densitometer as follows: 0.3 cc. of several concentrations of sheep cells (usually from 2 to 4 per cent by volume of packed cells) was mixed with 0.3 cc.

[1] Designed by E. G. Pickels of this laboratory.

of hemolysin (containing 4 hemolytic units), 0.4 cc. of saline, and 0.2 cc. of complement (guinea pig serum in dilution of 1:10), which was sufficient to hemolyze the cells completely. The mixtures were incubated at 37°C. until all of the cells were hemolyzed. The photoelectric densitometer was adjusted to an initial reading of 130 microamperes by means of a rheostat, and the hemolyzed solution producing a reading of 40 microamperes (usually about a 3 per cent cell suspension) was selected as the standard for use in the test. Fig. 1 graphically illustrates the readings in microamperes plotted against decreasing concentrations of hemolyzed red cell solution. It can be seen that relatively small differences in the concentration of hemolyzed red cell solution produce fairly large changes in the microammeter reading. Furthermore, the microammeter reading can be readily converted into terms of hemolysis; 62 microamperes, for example, represents hemolysis of 50 per cent of the cells.

Titration of Complement.—Pooled guinea pig serum from 10 to 15 animals served as the source of complement. The serum was stored at −76°C. The complement was titrated before each experiment under the conditions of the test to be employed. If the test to be done involved dilutions of serum in mixture with complement and a constant amount of antigen, the complement was titrated in the presence of the antigen (usually allantoic fluid containing influenza virus in dilution of 1:10), or, conversely, in tests for complement-fixing antigen the complement was titrated in the presence of the immune serum employed in the test. This procedure has proved to be highly significant because of the enhancement of the hemolytic activity of complement by serum (12) or by allantoic fluid. This effect will be described later. Falling dilutions of complement in 0.2 cc. amounts were mixed with 0.2 cc. of saline and 0.2 cc. of the serum or allantoic fluid in the dilution to be used in the test. The mixtures were incubated 1 hour at 37°C. (the period of primary fixation employed in the test proper); then 0.6 cc. of sensitized red blood cells standardized as described above was added after preliminary incubation at 37°C. for 15 minutes. The mixtures were incubated at 37°C. for 30 minutes and then centrifuged briefly to pack the unhemolyzed cells. The amount of hemolysis in the supernatant fluid was recorded as described above. The amount of complement required to hemolyze 50 per cent of the sensitized cells was taken as the end point, *one unit*, for it has been shown that the 50 per cent end point is more accurate than the end point of complete hemolysis (12, 13).

The serum, complement, and test antigen were mixed so that complement was present when the other two reagents came together. The mixtures containing 0.2 cc. of each reagent were incubated 1 hour at 37°C.; then 0.6 cc. of sensitized red cells was added. The various reagents, except serum or antigen dilutions, were added with an automatic pipetting machine. The tubes were further incubated at 37°C. for 30 minutes and kept in a refrigerator overnight to allow the cells to sediment. The amount of hemolysis in the supernatant fluid in each tube was then recorded on the photoelectric densitometer. The end point was taken as the dilution of serum or antigen producing hemolysis of 50 per cent of the standard red cell suspension (a reading of 62 microamperes). Control tests for anticomplementary effect were made in every experiment. Fig. 2 shows the results of a typical titration of an immune serum obtained from a ferret infected with the PR8 strain of influenza virus. The titration was done in duplicate. The 50 per cent end point usually falls between two measured dilutions, but since the curve over this range is a fairly straight line, the point of 50

per cent hemolysis can be easily calculated from the galvanometer readings by interpolation (10). In the titration shown in Fig. 2 the calculated end point is 1:1780.

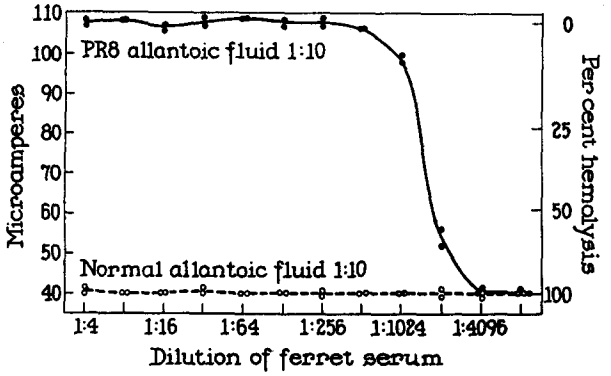


FIG. 2. Titration of influenza complement-fixing antibodies. Two 50 per cent hemolytic units of complement as measured by a preliminary titration in the presence of the allantoic fluid antigens were used in the test (see text).

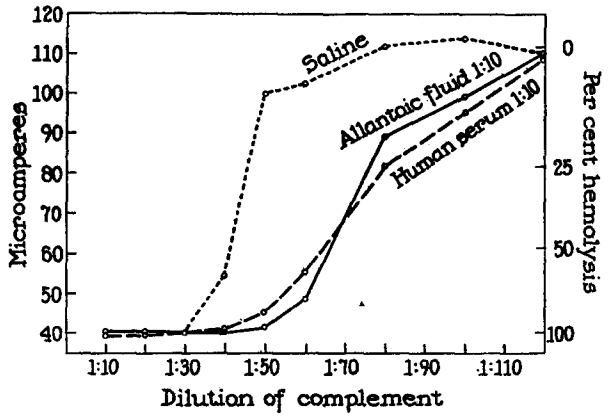


FIG. 3. Titration of complement in the presence of saline, allantoic fluid, and serum.

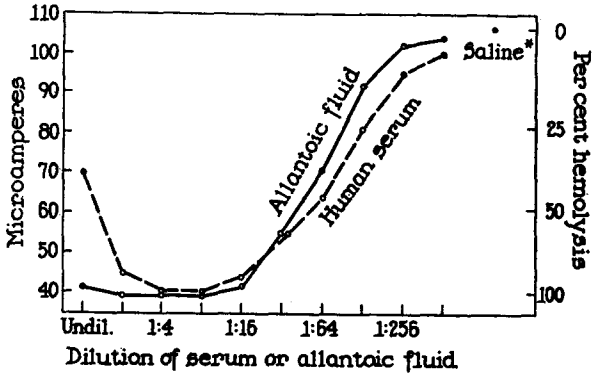
EXPERIMENTAL

Enhanced Hemolytic Activity of Complement in the Presence of Serum or Allantoic Fluid.—Wadsworth, Maltaner, and Maltaner (12) have shown that the hemolytic activity of complement is increased when titrated in the presence of serum or an antigen prepared from tubercle bacilli. Recently Hazen (14) showed that the activating effect of sera was not abolished by heating at 70°C. for 30 minutes. Fig. 3 shows the results of a comparative titration of complement in the presence of 0.9 per cent saline, serum, and a PR8 allantoic

fluid. It is apparent that the serum and the allantoic fluid greatly enhanced the activity of the complement.

Experiments were next done to learn more about the increased activity of complement in the presence of the reagents used in the test. For this purpose falling dilutions of serum or allantoic fluid were mixed with sensitized red blood cells and a dilution of complement which failed to hemolyze the cells in 0.9 per cent saline.

Experiment 1.—Complement was titrated in 0.9 per cent saline as shown in Fig. 3, and a dilution of 1:76 was found to be the largest amount of the complement which



* Mean of 10 points varying from 105 to 109 microamperes.

FIG. 4. Enhancement of the hemolytic activity of complement in the presence of various dilutions of serum or allantoic fluid. The complement dilution used was 1:76, which was the lowest dilution that failed to cause detectable hemolysis in the presence of saline.

failed to cause detectable hemolysis. 0.4 cc. of twofold dilutions of a PR8 allantoic fluid was then mixed with 0.2 cc. of a 1:76 dilution of the complement and 0.6 cc. of sheep cells sensitized with 4 units of hemolysin. Comparable mixtures were made in which 0.4 cc. of 0.9 per cent saline or twofold dilutions of a human serum were substituted for the allantoic fluid. (The allantoic fluid and serum were inactivated at 56°C. for 2 hours before use in the test.) The mixtures were incubated at 37°C. for 30 minutes and kept at 4°C. overnight. The amount of hemolysis in the supernatant fluids was then recorded on the photoelectric densitometer.

The results of a typical experiment are shown in Fig. 4. No hemolysis of the sensitized cells was produced by the complement in the presence of saline, yet the addition of serum or allantoic fluid in dilutions up to 1:8 caused complete hemolysis of the cells, and a partial hemolysis was evident in dilutions up to 1:128. The rate of hemolysis was also greatly increased by the serum or

allantoic fluid; complete hemolysis occurred within 10 minutes in the tubes containing serum or allantoic fluid in dilution of 1:4 or 1:8. It will be noted also that the serum undiluted or in dilution of 1:2 was not as effective as higher dilutions of the serum. In fact, sera in low dilutions often fail to show any enhancement of the complement activity or may be anticomplementary. The inhibitory properties of sera appear to be due to relatively heat-labile substances (14).

The nature of the substance (or substances) responsible for the enhanced hemolytic effect of complement has not been ascertained, but the findings thus far obtained which bear on the problem can be briefly summarized:—

Specific hemolysin and complement are necessary for the effect. The addition of serum or allantoic fluid to sheep red blood cells with or without complement does not cause hemolysis.

The substance is dialyzable and heat-stable. Dialysis in a cellophane bag against 0.9 per cent saline at 4°C. for about 20 hours completely removed it from allantoic fluids and most of it from human or ferret sera. The dialysate obtained from serum or allantoic fluid contains the substance in high titer. The active material in serum or allantoic fluid is unaffected by heating at 100°C. for 2 hours.

Normal allantoic fluids, as well as allantoic fluids containing influenza virus, enhance the hemolytic activity of complement but the latter are usually effective in slightly higher titer. Human, ferret, rabbit, horse, sheep, and guinea pig sera have all been found to contain the substance, though some are anticomplementary undiluted and the activating effect may not be manifest until the sera are diluted 1:4 or more with saline or buffer solution.

Sensitized red blood cells exposed to allantoic fluid or serum for 1 hour at 37°C. and then removed by centrifugation and resuspended in physiological saline or buffer solution pH 6.9 do not differ in their susceptibility to hemolysis by complement from cells exposed to saline or buffer for the same period of time.

Influence of Amount of Complement on Sensitivity of Test for Influenza Antibodies and Complement-Fixing Antigen.—The preceding experiments have clearly shown that the preliminary titration of complement in saline, as ordinarily done, gives no true indication of its activity in the complement fixation test in which serum and allantoic fluid are present. It is essential to titrate the complement under conditions as nearly comparable to the actual test as possible. Experiments were next done to see how the amount of complement would influence the test for influenza serum antibodies.

Experiment 2.—The standard test described under Methods was employed. A preliminary titration of complement was made in 0.9 per cent saline and in PR8 allantoic fluid diluted 1:10 in saline (the antigen to be used in the test). The highest dilution of complement causing hemolysis of 50 per cent of the standardized sheep cell suspension was considered as one unit. A series of twofold dilutions of the serum of a ferret convalescent from PR8 virus infection and a human convalescent serum was

made in 0.9 per cent saline. Four 40 per cent hemolytic units of complement as measured by the titration in saline were added to one set of dilutions, and 4, 2, and 1 units as measured by the titration in the presence of the antigen were added to the other sets of dilutions. The PR8 allantoic fluid antigen diluted 1:10 was then added to each group, and for control normal allantoic fluid diluted 1:10 was added to a comparable series of dilutions of each group. One hour at 37°C. was allowed for fixation of complement before the addition of the standardized sheep cell suspension and hemolysin. After further incubation at 37°C. for 30 minutes the tubes were kept at 4°C. overnight. The amount of hemolysis was then determined with the aid of the photoelectric densitometer and recorded as follows: ++++ = no hemolysis (complete fixation), +++ = 25 per cent hemolysis, ++ = 50 per cent hemolysis, + = 75 per cent, 0 = complete hemolysis (no fixation).

Table I shows the results of the experiment. The ferret serum failed to show any detectable fixation when four 50 per cent hemolytic units of complement[2] were used as measured by the preliminary titration in saline. When less complement was used, one to four units, as measured by the titration in the presence of the allantoic fluid used in the test, specific fixation was obtained in dilutions from 1:256 to 1:2048. Similar results were obtained with the human serum. The most sensitive test was obtained when one unit of complement (50 per cent hemolysis) was used. This can be accurately measured with the densitometer, but non-specific summation effects were frequently encountered in the lower dilutions. We have used as routine, therefore, two 50 per cent hemolytic units of complement as measured by a preliminary titration with the antigen in tests for influenza complement-fixing antibodies. Only occasional sera are anticomplementary in low dilutions when this amount of complement is used in the test as described.

Similar tests for complement-fixing antigen in allantoic fluid also revealed that relatively slight excesses of complement largely masked specific fixation. The optimal amount of complement for antigen titrations was found to be one and a half 50 per cent hemolytic units as measured by a preliminary titration in the presence of the immune serum used in the test.

It has been reported that at least 95 per cent of normal human sera fail to show complement-fixing antibodies against influenza A virus (29), although neutralizing antibodies are usually demonstrable. Table II shows the results of complement fixation tests with fourteen human sera taken at random from adults who had no recent history of influenza. Specific antibodies were detectable in only one serum in low titer (1:4) when 4 units of complement were employed as measured by a preliminary titration in saline. When the same sera were tested concurrently with 2 units of complement as measured by a preliminary titration in the presence of the antigen used in the

[2] This amount of complement was equivalent to 2 complete hemolytic units as ordinarily used.

TABLE I

Influence of Amount of Complement on Titer of Influenza Serum Antibodies

Convalescent serum	Dilution of complement used in test	Complement fixation test: Antigen, PR8 allantoic fluid diluted 1:10; Dilution of serum 1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Ferret	1:18 = 4 units* titrated in saline	0	0	0	0	0	0	0	0	0	0	0
	1:32 = 4 units } Titrated in	±	+	++	+++	+++	+++	++±	0	0	0	0
	1:64 = 2 " } presence	++++	++++	++++	++++	++++	++++	++++	++++	++±	0	0
	1:128 = 1 unit } of antigen	ac	++++	++++	++++	++++	++++	++++	++++	++++	+++±	++
Human	1:18 = As above	++	++±	++	+±	±	0	0	0	0	0	0
	1:32 " "	++++	++++	++++	+++±	+++	±	0	0	0	0	0
	1:64 " "	++++	++++	++++	++++	++++	++++	+	0	0	0	0
	1:128 " "	ac	ac	++++	++++	++++	++++	++++	+++	++	++	++

++++ = complete fixation (no hemolysis).
+++ = 75 per cent fixation.
++ = 50 " " "
+ = 25 " " "
0 = 0 fixation (complete hemolysis).
ac = anticomplementary.

* One unit of complement = highest dilution causing hemolysis of 50 per cent of standard red blood cell suspension (see text).

TABLE II

Complement Fixation Tests with Normal Human Sera

Human serum No.	Serum antibody titer*: 4 units‡ of complement as measured by titration in saline; Antigen: PR8 allantoic fluid 1:10	Antigen: Normal allantoic fluid 1:10	2 units‡ of complement as measured by titration in presence of antigen; Antigen: PR8 allantoic fluid 1:10	Antigen: Normal allantoic fluid 1:10
1	–	–	28	–
2	–	–	24	–
3	–	–	15	–
4	–	–	13	–
5	–	–	–	–
6	4	–	60	–
7	–	–	30	–
8	–	–	5	–
9	–	–	6	–
10	–	–	15	–
11	–	–	37	–
12	–	–	52	12
13	–	–	13	–
14	–	–	13	6

– = titer less than 1:4.

* Expressed as reciprocal of highest dilution causing hemolysis of 50 per cent of standardized red cell suspension as measured by photoelectric densitometer (see text).

‡ 50 per cent hemolytic units.

test (PR8 allantoic fluid 1:10), only one serum failed to show complement-fixing antibodies. Two of the sera (12 and 14) were anticomplementary in low dilution. The experiment illustrates further the sensitivity of the test for influenza complement-fixing antibodies and its applicability to the study of sera with low antibody titers.

Strain Specificity of Ferret Serum Antibodies.—In the course of complement fixation tests with ferret antisera and allantoic fluids containing various virus strains, it was noted that a given antiserum invariably reacted in much higher titer with its homologous virus strain than with other virus strains.

Neutralization tests have shown that many strains of influenza A virus elicit antibodies in ferrets or rabbits (15) which react best with the homologous strains but may also show cross neutralization in lesser degree with heterologous strains. The strain differences between PR8, W.S., Lee, and swine influenza viruses are demonstrable also by the agglutination inhibition test (5). Francis (16) showed that influenza A and influenza B virus strains could be readily differentiated by the complement fixation test. Considerably more difficulty has been encountered, however, in demonstrating strain differences within the influenza A group and in differentiating the swine virus by the complement fixation test. Lennette and Horsfall (3) could demonstrate little or no difference in antibody titer in cross tests with PR8, W.S., and swine ferret antisera and their respective mouse lung antigens. Lush and Burnet (17) reported slight strain specificity with ferret antiserum using antigens prepared on the chorioallantoic membrane of the developing egg; and Eaton (18) found that the sera of mice immunized with human and swine strains exhibited strain specificity by complement fixation using mouse lung antigens, but the specificity was less definite than that found by neutralization tests.

Experiment 3.—Antisera were obtained from ferrets 14 days after intranasal inoculation of the PR8 or W.S. strain of influenza A virus, the Lee strain of influenza B virus, or a strain of swine influenza virus. Allantoic fluids containing the various strains in high titer were obtained as described under Methods. Cross complement fixation tests were set up using falling dilutions of the sera in mixture with two 50 per cent hemolytic units of complement and allantoic fluid containing the various virus strains in dilution of 1:10.

The results of several tests using different sera and antigens are summarized in Table III. It will be seen that in every instance the serum antibody titer was considerably higher with the homologous virus strains than with the other strains. PR8 antiserum No. 1, for instance, reacted in highest titer with PR8 allantoic fluid (1:2306) and in relatively low titer with W.S. and with swine virus strain (1:24 and 1:28 respectively). There was no detectable crossing with the Lee virus strain or with normal allantoic fluid. The Lee antisera reacted only with the Lee virus strains. Manifestly the complement-fixing antibodies of ferret sera show the same order of specificity for the virus strains tested as is noted on neutralization or agglutination tests (5, 15).

Complement Fixation Tests with Human Sera and Different Virus Strains.—The preceding experiment showed that the immunological response of ferrets

to infection with influenza virus as measured by the complement fixation test was highly specific for the virus strain inoculated. The next step was to compare the strain specificity of the serum antibody response in ferrets and in human beings.

Experiment 4.—Four virus strains were obtained from patients acutely ill with influenza A by direct isolation in chick embryos (19). The strains were inoculated

TABLE III

Complement Fixation Tests with Four Virus Strains and Their Respective Ferret Antisera

Virus strain inoculated into ferrets	Ferret serum No.	Serum antibody titer*				
		Antigens‡				
		PR8	W.S.	Swine	Lee	Normal
PR8	1	**2306**	24	28	—	—
	2	**1780**	56	49	—	—
	3	**1670**	60	28	—	—
W.S.	4	208	**1024**	182	—	—
	5	112	**447**	30	—	—
	6	60	**479**	26	—	—
Swine	7	10	23	**588**	—	—
	8	4	147	**1175**	—	—
Lee	9	—	—	—	**416**	—
	10	—	—	—	**388**	—
	11	—	—	—	**158**	—
Normal ferrets	12	—	—	—	—	—
	13	—	—	—	—	—
	14	—	—	—	—	—
	15	—	—	—	—	—

Complement, two 50 per cent hemolytic units.

— = less than 1:4.

* Titer expressed as reciprocal of highest dilution of serum causing hemolysis of 50 per cent of red blood cells.

‡ Allantoic fluid containing respective virus strains, diluted 1:10 in saline.

intranasally into ferrets, and sera were obtained 14 days later. Acute and convalescent sera from the patients yielding the virus strains were available for test. The complement-fixing antibody titer of the ferret sera against the homologous and heterologous virus strains was compared with the titer of the human sera tested against the same virus strains.

The results of the experiment are shown in Table IV. The ferret sera reacted in highest titer with the homologous virus strain (736, 811) or with the PR8 strain (778, 816). Much lower titers were obtained with the W.S.

and swine strains. The findings indicate that the strains used (736, 811, 778, 816) were closely related to the PR8 strain but were quite different from the W.S. and swine strains. The antibody response of the sera from patients to these virus strains was wholly different. The sera from only one patient, No. 4, reacted in highest titer against the homologous or PR8 strain. The sera from two patients, Nos. 1 and 3, showed antibody rises of about the same

TABLE IV

Comparative Strain Specificity Tests with Human and Ferret Antisera

Patient No.	Virus strain isolated from throat washing	Antigens*	Serum antibody titer‡ as determined by complement fixation tests		
			Ferret sera	Human sera	
			Convalescent	Acute	Convalescent
1	736	736	1450	14	120
		PR8	362	14	158
		W.S.	32	20	128
		Swine	26	18	169
2	811	811	1350	26	22
		PR8	835	22	45
		W.S.	49	28	37
		Swine	39	21	28
3	778	778	84	34	128
		PR8	256	30	275
		W.S.	4	37	120
		Swine	6	49	362
4	816	816	294	23	1270
		PR8	632	24	1560
		W.S.	10	30	315
		Swine	9	23	169

Titers expressed as reciprocal of dilution of serum causing 50 per cent hemolysis. None of the sera were anticomplementary when tested concurrently with normal allantoic fluid as antigen.

* Allantoic fluid containing respective virus strains diluted 1:10 in saline.

‡ Complement, two 50 per cent hemolytic units.

order against all of the virus strains tested. The serum from the remaining patient, No. 2, showed a twofold rise in antibody when tested with the PR8 strain and a slight rise with the W.S. and swine strains but no increase when the homologous virus strain was used as antigen.[3]

[3] Agglutination tests with the sera from this patient revealed a threefold rise in titer when tested with the homologous virus strain (811) and a ninefold rise against the PR8 virus. The sera showed no rise against the W.S. or the swine virus strains. Agglutination tests with the ferret serum (811), however, showed antibody responses

It is apparent from this limited experiment that the antibody response as measured by the complement fixation test shows less strain specificity in human beings than in ferrets. Neutralization and agglutination tests with human sera also show less specificity for strains of influenza A virus than ferret sera (20).

Relation of the Complement-Fixing Antigen to the Virus Particle.—The complement-fixing antigen obtained from mouse lungs infected with influenza virus has been studied by various workers (2, 3). They found that the antigen could be readily separated from the virus particle by differential centrifugation, and, in general, it was similar to the well known soluble antigens of other viruses. Lennette and Horsfall (3), however, were unable to wash the virus completely free of the antigen.

In the next experiment an attempt was made to separate the complement-fixing antigen from the hemagglutinin and infective virus particle obtained from allantoic fluid by differential centrifugation. It has been shown that the property of influenza virus preparations to agglutinate red blood cells is closely associated, if not identical, with the infective virus particle (5, 21). Furthermore, the hemagglutinin and infective particle in allantoic fluid sediment at the same rate in the high speed centrifuge, having a particle size of at least 60 mμ (22).

Experiment 5.—PR8, W.S., and swine allantoic fluids were obtained as described under Methods. A portion of each allantoic fluid was spun at 27,000 R.P.M. for 45 minutes in a vacuum centrifuge (23), all of the supernatant fluid was removed, and the pellets of sediment were gently resuspended in the original volume of dilute phosphate buffer solution pH 6.9. The resuspended sediments were subjected to two further centrifugations, each at 27,000 R.P.M. for 45 minutes with subsequent resuspension in the original volume of fresh buffer solution. A test sample was removed following each centrifugation and resuspension of the pellets of sediment. The materials thus obtained were tested for capacity to agglutinate chicken red blood cells and to react in the complement fixation test in mixture with one and a half 50 per cent hemolytic units of complement and a human serum in dilution of 1:30. In addition, some of the samples were tested for infectivity in mice.

Table V shows the results. The first centrifugation at 27,000 R.P.M. for 30 minutes removed all of the detectable hemagglutinins from the supernatant fluids of the virus preparations. The amount of complement-fixing antigen removed by this amount of centrifugation varied from 44 per cent (swine) to 86 per cent (PR8). Repeated washing of the virus particles by sedimentation and resuspension in buffer solution failed to remove the complement-fixing

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similar to those obtained with the complement fixation test. Other cases of influenza have been encountered in which the convalescent serum showed a significant rise against one or more A virus strains and at the same time failed to show any rise against other well-established A strains.

TABLE V

Sedimentation of Hemagglutinin and Complement-Fixing Antigen of Influenza Virus in the Centrifuge

Allantoic fluid containing virus strain	Centrifugation	Fraction tested		Complement-fixing antigen titer*	Hemagglutinin titer‡	Infective titer§
PR8	R.P.M. 2000	Supernatant		70	275	$10^{-5.6}$
	27,000	Resuspended sediment	1×	60	74	
			2×	39	60	
			3×	30	37	$10^{-4.5}$
		Supernatant (wash water)	1×	10	—	
			2×	3	4	
			3×	—	—	
W.S.	2000	Supernatant		69	315	$10^{-6.3}$
	27,000	Resuspended sediment	1×	39	69	
			2×	9	39	
			3×	6	—	$10^{-2.8}$
		Supernatant (wash water)	1×	37	—	
			2×	6	2	
			3×	—	—	
Swine	2000	Supernatant		120	60	$10^{-3.5}$
	27,000	Resuspended sediment	1×	60	42	
			2×	36	34	
			3×	28	32	$10^{-2.7}$
		Supernatant (wash water)	1×	69	—	
			2×	20	—	
			3×	7	—	

— = titer less than 1:4.

* Reciprocal of highest dilution of antigen producing 50 per cent hemolysis end point in mixture with one and one-half 50 per cent hemolytic units of complement and a pooled human serum diluted 1:30.

‡ Reciprocal of highest dilution producing agglutination of 50 per cent of standard suspension of chicken red cells.

§ 50 per cent mortality titer in mice.

antigen. The complement-fixing antigen titers of the resuspended sediments remained roughly proportional to the hemagglutinin and infective titers although a loss occurred with each centrifugation. In some instances the hemagglutinin and infective titer decreased more than the complement-fixing

antigen titer; for instance, the sediment of the W.S. allantoic fluid resuspended for the third time failed to cause detectable agglutination and the infective titer was about 1000-fold less, yet the complement fixation titer was only about tenfold lower. It is possible that aggregation of the virus particles will explain these discrepancies. No complement-fixing antigen was detected in the third wash water of the PR8 and W.S. virus sediments, and only a small amount remained in the wash water of the swine preparation.

Subsequent experiments have confirmed the above findings. In general, allantoic fluid virus preparations contained much less of the soluble antigen, separable from the virus particle by centrifugation, than mouse lung extracts. Experiments with mouse lung extracts containing PR8 virus, for instance, have shown that most of the hemagglutinin can be sedimented by centrifugation at 12,000 R.P.M. for 56 minutes, yet the complement-fixing antigen titer of the supernatant fluid decreased only about 20 per cent. As obtained above with allantoic fluid virus preparations some of the complement-fixing antigen was intimately associated with the virus particle and could not be removed by repeated washings in the centrifuge.

Adsorption of Complement-Fixing Antigen by Red Blood Cells.—Hirst (21) has shown that the infective agent and the hemagglutinin in influenza virus suspensions are simultaneously adsorbed by and eluted from chicken red cells. Experiments were next undertaken to learn whether the complement-fixing antigen of influenza virus suspensions could be adsorbed by chicken red cells.

Experiment 6.—Equal volumes of PR8 or Lee allantoic fluids and 1.5 per cent suspensions of chicken red blood cells were mixed in a series of tubes. All were kept at 4°C., and the tubes were removed at various times and the cells discarded after light centrifugation. The supernatant fluids were then tested for amount of hemagglutinin and complement-fixing antigen as described in the preceding experiment.

Fig. 5 *a* shows the results of an experiment with PR8 allantoic fluid. As previously reported (21), most of the hemagglutinin was rapidly adsorbed by the red cells. About 50 per cent of the complement-fixing antigen was adsorbed by the red cells within the first 5 minutes and no further decrease was observed.

In a concurrent experiment a portion of the same allantoic fluid was adsorbed with chicken red blood cells for 30 minutes at 4°C., the supernatant fluid discarded after the cells had been sedimented by centrifugation, and the cells were resuspended in dilute phosphate buffer solution pH 7.0 (½ original volume) at 37°C. for 2 hours to permit elution of the virus. The cells were then removed by centrifugation and discarded. The virus in the supernatant fluid was again adsorbed onto fresh red cells and then eluted into buffer solution (⅕ original volume) in the same way. When this preparation of influenza virus was adsorbed with red cells as in the experiment with the untreated allantoic fluid

(Fig. 5 *a*), the hemagglutinin and complement-fixing antigen were rapidly removed together (Fig. 5 *b*). Furthermore, the hemagglutinin and complement-fixing antigen were eluted from the red blood cells at 37°C. at about the same rate (not shown in figure), both returning to the original titer within 3 hours, and repeated adsorptions of the virus particles with red cells failed to remove the capacity of the particles to react in the complement fixation test.

Similar adsorption experiments with mouse lung extracts showed that most of the complement-fixing antigen (about 95 per cent of the total) was not adsorbed by red cells, whereas the hemagglutinin was rapidly and almost com-

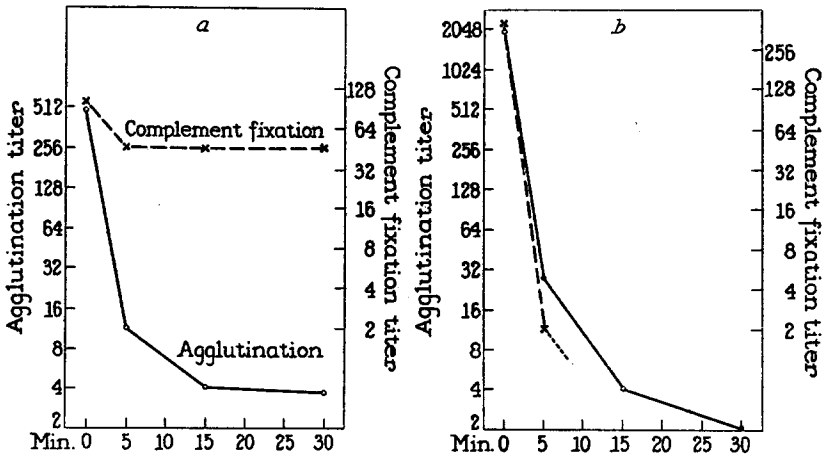

FIG. 5. Comparative agglutination and complement fixation titers on supernatant fluids of a PR8 allantoic fluid after adsorption with 0.75 per cent red cells (final concentration) at 4°C. (*a*) Untreated PR8 allantoic fluid. (*b*) The same allantoic fluid adsorbed with red blood cells and eluted into one-fifth of the original volume of buffer solution (see text).

pletely removed by the red blood cells. When the virus was adsorbed from the mouse lung extracts and eluted into buffer solution as in the experiment shown in Fig. 5 *b*, both the hemagglutinin and complement-fixing antigen were adsorbed and eluted together.

Specificity of Complement Fixation Tests with Different Preparations of Antigen.—The preceding experiments showed that influenza virus preparations contained an antigen which could be readily separated from the virus particle by centrifugation or adsorption with red cells, and also an antigen intimately associated with the virus particle which could not be removed by these procedures. It remained to be seen whether these antigens also differed in their reactions with immune sera.

Experiment 7.—Antigens from PR8, W.S., swine, and normal allantoic fluids were prepared as follows: (*a*) whole allantoic fluids containing each virus strain were obtained as described under Methods: (*b*) 30 cc. of each allantoic fluid was adsorbed with 1 per cent chicken red blood cells and the virus eluted into 10 cc. of normal allantoic fluid as described in the preceding experiment; the adsorption and elution were then repeated with final elution into 5 cc. of normal allantoic fluid to provide a "purified" preparation of virus particles; (*c*) the supernatant fluid of (*b*) which had been adsorbed with red cells was cleared further by centrifugation at 27,000 R.P.M. for 45 minutes to provide a "purified" preparation of the soluble antigen. The antigens were then

TABLE VI

Cross Complement Fixation Tests with Ferret Antisera and Various Antigens Obtained from Allantoic Fluid

Allantoic fluid—antigen			Ferret serum antibody titer			
Virus strain	Preparation	Titer	PR8	W.S.	Swine	Normal
PR8	(*a*) Whole allantoic fluid	128	**1670**	69	60	—
	(*b*) Virus particles of (*a*)*	239	**1024**	49	9	—
	(*c*) Soluble antigen of (*a*)‡	13	**4**	12	15	—
W.S.	(*a*) As above	138	223	**779**	239	—
	(*b*) " "	128	208	**835**	182	—
	(*c*) " "	49	6	**60**	52	—
Swine	(*a*) " "	239	79	147	**1100**	—
	(*b*) " "	256	45	56	**1024**	—
	(*c*) " "	60	4	30	**91**	—
Normal	(*a*) " "	—	—	—	—	—
	(*b*) " "	—	—	—	—	—
	(*c*) " "	—	—	—	—	—

— = titer less than 1:4.

* Obtained by adsorption of virus from the allantoic fluid with chicken red cells and subsequent elution into normal allantoic fluid 1/5 original volume).

‡ Supernatant fluid after removal of virus particles by adsorption with red cells and centrifugation at 27,000 R.P.M. for 30 minutes (see text).

tested in cross complement fixation tests with PR8, W.S., swine, and normal ferret sera. Two 50 per cent hemolytic units of complement were used, and the antigens (*a*) and (*b*) were diluted 1:5, whereas (*c*) was undiluted. The antigens were also tested for capacity to fix one and a half 50 per cent hemolytic units of complement in mixture with a pooled human serum diluted 1:30.

Table VI shows the results of the experiment. The ferret sera reacted in highest titer with the homologous whole allantoic fluid as already described (Table III). Similar results were obtained with the "purified" virus particles, and in some instances the heterologous titer was lower, indicating a somewhat

greater specificity of the reaction with this preparation of antigen. The sera reacted in much lower titers with the "purified" soluble antigens, on the other hand, and, in general, showed less strain specificity, particularly the PR8 serum which reacted in lowest titer with the homologous antigen.

A similar experiment with the same ferret sera and mouse lung antigens prepared as described above is shown in Table VII. The ferret sera showed no difference in their reactions with the homologous and heterologous soluble antigen preparations yet revealed a high degree of specificity when the virus particles from the extracts were used and somewhat less specificity when the whole extracts served as antigens. The fixation obtained with the normal

TABLE VII

Cross Complement Fixation Tests with Ferret Antisera and Various Antigens Obtained from Mouse Lung Extracts

Mouse lung extract—antigen			Ferret serum antibody titer*			
Virus strain	Preparation‡	Titer	PR8	W.S.	Swine	Normal
PR8	(*a*) Whole extract	2560	**512**	128	16	16
	(*b*) Virus particles of (*a*)	160	**512**	8	—	—
	(*c*) Soluble antigen of (*a*)	1280	**8**	8	6	—
W.S.	(*a*) As above	5120	256	**1024**	64	16
	(*b*) " "	160	32	**256**	—	—
	(*c*) " "	2560	48	**32**	32	—

* Dilutions of serum in mixture with two 50 per cent hemolytic units of complement and the antigens diluted 1:30.

‡ Titers expressed as reciprocal of highest dilution producing ++, or better, fixation. The dilutions of antigen are in terms of weight of lung tissue extracted, in mixture with two 50 per cent hemolytic units of complement and a pooled serum diluted 1:30. Antigens *a*, *b*, and *c* prepared as described in experiment shown in Table VI (see text).

ferret serum and the whole mouse lung extracts is probably due to heterophile antibody (3). In general, the results obtained are similar to those shown in Table VI with the antigens from allantoic fluid.

DISCUSSION

The complement-fixing antigen of influenza virus in allantoic fluid or mouse lung extracts consists of two distinct fractions. One is intimately associated with the virus particle and sediments at the same rate as the hemagglutinin and infective particle in the centrifuge. This fraction, like the hemagglutinin and infective particle, is rapidly adsorbed by red blood cells and eluted from the cells on standing at room temperature or 37°C. It has not been possible to dissociate this antigen from the virus particles by repeated washings in the

centrifuge or by repeated adsorptions with red blood cells. A second fraction has a smaller size, remaining in the supernatant fluid following centrifugation sufficient to sediment most of the hemagglutinin and infective particle. It is not adsorbed by fowl red blood cells. The two types of complement-fixing antigen also differ in the specificity of their reactions with ferret antisera. The antigens associated with the virus particles of the PR8, W.S., and swine viruses can be readily differentiated in cross complement fixation tests with ferret antisera in that the serum antibody titer is always much higher with the homologous antigen. The degree of specificity of the complement fixation test with these antigens is comparable to that obtained with neutralization or agglutination tests. The soluble antigens from these virus strains, on the other hand, show less specificity in cross complement fixation tests, and the antisera react in relatively low titer.

Further study of these antigens will be necessary to ascertain their rôle in the activity of the virus. The findings thus far obtained suggest that the serological response to influenza virus infection as measured by the complement fixation test is due not only to the soluble antigen, but also to an antigen intimately associated with the virus particle itself. The latter antigen is probably an integral part of the virus, whereas the soluble antigen might represent a wholly different antigen elaborated by the virus or merely disintegrated virus particles as suggested by Burnet (24). The complement-fixing antigens of certain other viruses (vaccinia, psittacosis, lymphocytic choriomeningitis (7)) might be similar to the antigen of influenza virus, for it has not been possible to free completely the virus particles of antigen, although the soluble antigen has been dominant in all. The rabbit papilloma virus (25) and the tobacco mosaic virus (26), on the other hand, have shown no evidence of a soluble antigen, and the serological response to these viruses appears to be due entirely to the virus particle *per se*.

The enhancement of the hemolytic activity of complement in the presence of serum or allantoic fluid has proved to be an important factor in the complement fixation test for influenza virus antigens and antibodies. It is essential to determine accurately the activity of the complement in a preliminary titration in the presence of the reagents used in the test and under conditions comparable to the actual test. It is then possible to select the optimal amount of complement and to render the test considerably more sensitive, for relatively small excesses of complement may largely mask specific fixation (Tables II and III). The photoelectric densitometer provides a simple, objective, and accurate method for estimating the hemolytic reaction, and the microammeter readings can be readily converted into terms of percentage of red blood cells which have been hemolyzed (Fig. 1).

Sera from man, ferret, rabbit, horse, sheep, and guinea pig have all increased the hemolytic activity of complement, but the effect may not be manifest until the sera are diluted 1:4 or more with physiological saline or buffer solution, for it

is well known that undiluted sera are often anticomplementary. As well as sera, the presence of allantoic fluid obtained from the developing chick embryo (Fig. 3) increases the activity of complement. The substance (or substances) in serum or allantoic fluid responsible for this effect resists heating at 100°C. for 2 hours and is small enough to pass through a cellophane membrane ordinarily used for dialysis. These findings indicate that the active material is probably not a protein. The substance appears to differ from the agents known to cause lysis of red blood corpuscles in the presence of serum, such as silicic acid (27) or brilliant green (28), for it requires the presence of both complement and hemolysin. It seems likely that the substance acts either by rendering the red blood corpuscles more susceptible to the action of complement and hemolysin or by directly influencing the activity of the complement itself.

SUMMARY

A quantitative complement fixation test with influenza immune sera and virus antigens obtained from allantoic fluid is described. The method utilizes a photoelectric densitometer which provides a simple, objective, and accurate determination of the hemolytic reaction. The enhancement of the hemolytic activity of complement in the presence of serum or allantoic fluid necessitates a preliminary titration of complement in the presence of these agents. An accurate appraisal of the activity of the complement under the conditions of the actual test permits the selection of an optimal amount of complement and greatly increases the sensitivity of the test. The substance (or substances) responsible for the enhanced hemolytic activity of complement has been found in human and many animal sera and in allantoic fluids obtained from the developing chick embryo. It requires the presence of both complement and hemolysin, resists heating at 100°C. for 2 hours, and is dialyzable.

Allantoic fluid or mouse lung preparations of influenza virus contain a complement-fixing antigen which is intimately associated with the virus particle. It sediments in the high speed centrifuge at the same rate as the hemagglutinin and infective particle and, like the latter, is adsorbed by fowl red blood cells and eluted from the cells on standing at room temperature or 37°C. It cannot be separated from the virus particle by repeated washings in the centrifuge or repeated adsorptions with red blood cells; the hemagglutinin and complement-fixing antigen titers remain roughly proportional. This antigen shows a high degree of strain specificity in cross complement fixation tests with PR8, W.S., and swine ferret antisera, and, as found with the neutralization test, it shows little or no strain specificity with human sera. A soluble antigen is also present in influenza virus preparations which can be readily separated from the virus particle by centrifugation. It is not adsorbed by red blood cells. Furthermore, it reacts in lower titer with ferret antisera and usually shows less strain specificity in cross complement fixation tests. In general, allantoic fluid virus preparations contain much less of the soluble antigen than mouse lung extracts.

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