STUDIES ON EPIDEMIC INFLUENZA VIRUS

THE NATURE AND PROPERTIES OF THE COMPLEMENT-FIXING ANTIGEN

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It is now well known that in certain virus diseases, for example, yellow fever (1), vaccinia (2), psittacosis (3), myxoma (4), and lymphocytic choriomeningitis (5), the causal agent is associated with a specific soluble antigen. This antigen, separable from the active virus, appears to be responsible for the *in vitro* immune reactions previously attributed to the virus itself.

Smith (6) first demonstrated that mouse lungs infected with epidemic influenza virus could be used as an antigen in the complement fixation test for the diagnosis of this disease. Subsequently Hoyle and Fairbrother (7) adduced evidence indicating that a soluble antigen was present in suspensions of epidemic influenza virus and that by relatively simple means the virus could be entirely freed of antigen. No further investigations on this problem appear to have been conducted, although more exact information on the nature and properties of the antigen and its relation to the virus is highly desirable.

A detailed study of the specific complement-fixing antigen present in the lungs of mice infected with influenza virus was undertaken in this laboratory. It is the purpose of this report to present evidence concerning the nature and the properties of this antigen, as well as its relationship to the virus.

Material and Methods

Source of Antigen.—The complement-fixing antigen was obtained from mouse lungs infected with the PR8 strain of influenza virus. Albino Swiss mice were inoculated intranasally with 0.05 cc. of a 0.1 per cent suspension of infected mouse lung. Three days after inoculation the animals were killed with chloroform and the lungs removed; the pooled lungs were frozen and stored in a CO_2 cabinet at $-76°C.$ until required.

Preparation of the Antigen.—Mouse lungs containing influenza virus were weighed, ground with alundum, and made into a 10 per cent suspension with saline. The suspension was centrifuged at 2000 R.P.M. for 30 minutes in a horizontal centrifuge. The

markedly turbid supernate was distributed into celluloid tubes and then centrifuged at 13,000 R.P.M. for 30 minutes in the open air angle centrifuge described by Bauer and Pickels (8).

This second centrifugation resulted in the sedimentation of most of the turbid material and yielded a relatively clear, reddish colored supernate. It also caused the suspended fat to form a thin layer on the surface of the suspension. The floating fat was removed with cotton pledgets and the clarified supernate was poured off. It was then redistributed in celluloid tubes and centrifuged for 45 minutes at 27,000 R.P.M. in the high speed vacuum centrifuge (9). The supernate obtained after this third centrifugation constituted the final antigen preparation and, unless otherwise stated, all antigens used in this study were prepared in this manner.

At each step in the preparation of a given lot of antigen an aliquot portion was set aside and held at room temperature until the final antigen preparation was obtained. These specimens were later included as controls in the assays of the complement-fixing capacity of antigens which had been treated in various ways.

Serum.—Two lots of pooled human serum were used throughout these experiments to determine the presence and the concentration of the specific complement-fixing antigen. One lot was obtained from persons recently convalescent from influenza and fixed complement in a dilution of 1:64 when mixed with influenza antigen. The other lot was obtained from normal human beings and possessed no capacity to fix complement with this antigen. These sera, in celluloid tubes, were stored at −76°C.; the contents of a tube were always completely thawed in warm water and stirred before the requisite amount was withdrawn. A 1:10 dilution of serum inactivated in a water bath at 56°C. for 30 minutes was used as a routine.

Complement Fixation.—Guinea pig serum for complement was stored at −76°C. It was titrated before each test and then diluted so that 0.2 cc. represented two exact units. Sensitized sheep cells were prepared by mixing equal parts of 5 per cent cells in saline with an amboceptor dilution containing two units of hemolysin per 0.25 cc.

The test was conducted as follows: Serial twofold dilutions were made of each antigen specimen. To 0.2 cc. amounts of each dilution were added two units of complement followed by 0.2 cc. of pooled human serum, normal or convalescent, inactivated at 56°C. for 30 minutes and diluted 1:10. After incubation in a water bath for 1 hour at 37°C., 0.5 cc. of sensitized sheep cell suspension was added. The extent of fixation was recorded after secondary incubation at 37°C. for 30 minutes; complete fixation of complement was recorded as ++++, failure to do so as −, and intermediate degrees as +, ++, or +++. The end point was taken as the highest dilution of antigen which gave at least +++ fixation. In the tables to be presented below only the initial antigen dilution, which takes no account of the further dilution resulting from the addition of serum and the hemolytic system, will be given. The final dilution of antigen in the whole mixture would thus be 5.5 times that given. Appropriate controls on anticomplementary action of the several reagents and on the hemolytic system were included in every test.

Virus Titrations.—Serial tenfold dilutions of a specimen were made in broth. Each of a group of six Swiss mice was inoculated intranasally under ether anesthesia with 0.05 cc. of the desired dilution. All mice which died during the observation period were autopsied and their lungs were examined for the presence of consolidation. Animals which survived were killed 10 days after inoculation and examined for lung lesions.

The virus titer was determined by the 50 per cent end point calculation method of Reed and Muench (10). Throughout this paper the virus titer will be expressed as the 50 per cent mortality end point.

EXPERIMENTAL

Separation of the Antigen from the Virus

A soluble antigen, distinct from the virus, if present in suspensions of mouse lungs infected with influenza virus, should be separable from the virus by physical methods. Since influenza virus appears to have a relatively large particle size (11), it is rapidly sedimented in centrifugal fields 60,000 times gravity or greater. If the antigen had a smaller particle size than the virus and was not bound to it, there was reason to expect that the antigen would sediment less rapidly than the virus in the high speed centrifuge.

TABLE I

Separation of Complement-Fixing Antigen from Influenza Virus by Serial Centrifugation and Filtration

Experimental procedure	Virus		Complement-fixing antigen			
	Titer 50 per cent mortality	Decrease in titer	Titer			Decrease in titer
			Antigen No.			
	1		1	2	3	Average
A. 2000 R.P.M.; 30 min.	$10^{-6.13}$	—	1:32	1:32	1:64	—
B. A + 13,000 R.P.M.; 30 min.	$10^{-5.25}$	7 times	1:16	1:32	1:32	2 times
C. B + 27,000 R.P.M.; 45 min.	$10^{-3.63}$	316 "	1:16	1:16	1:32	2 "
D. C + Seitz filtration	$10^{-1.25}$	75,000 "	1:8	1:4	1:16	4 "

A number of suspensions of infected mouse lungs were centrifuged serially at increasing rotational velocities, as described above. After the final centrifugation the supernate was filtered through a Seitz EK pad which had been saturated previously by the passage of 25 cc. of 2 per cent inactivated normal rabbit serum in saline. Aliquot portions of the serial supernates were removed after each centrifugation. These and the Seitz filtrate were titered in the complement fixation test in order to determine the concentration of the antigen in them. In certain experiments the infectious titer of the same specimens was determined also so that a comparison could be made of the relative concentrations of both the virus and the antigen.

The results of typical experiments are presented in Table I. It will be observed that the virus titer of the suspension decreased about seven times after centrifugation at 13,000 R.P.M. and more than 300 times after centrifu-

gation at 27,000 R.P.M., whereas the complement-fixing titer decreased only two times. Even more striking was the dissimilarity between the concentrations of the virus and the antigen in the Seitz filtrate. After the completion of all the steps in the procedure it was found that the virus titer had decreased approximately 75,000 times, while the antigen titer had been reduced by only about four times.

It was not expected that the virus would be present in Seitz filtrates since agents of the size of influenza virus are generally thought to be retained by these filters. This experiment was repeated seven times with serially centrifuged suspensions of infected mouse lungs, and it was found that under these conditions the virus was present in three Seitz filtrates but was not demonstrable in the other four.

The results of these experiments indicated that the concentration of the antigen was unrelated to the infectious titer of a suspension and therefore that the antigen was separable from the virus. Had the virus *per se* been responsible for the *in vitro* serological reaction, as has been shown to be the case with the rabbit papilloma (12, 13) and tobacco mosaic (14) viruses, the removal of increasing quantities of the virus from the suspensions should have caused a corresponding decrease in the concentration of the complement-fixing antigen.

Attempted Separation of the Virus from the Antigen

Although the preceding experiments demonstrated that the antigen could be separated from influenza virus, it was of importance to determine whether the virus could be entirely freed of complement-fixing activity. Since the evidence indicated that the antigen remained in only slightly reduced concentration in a suspension from which more than 99.99 per cent of the virus had been removed, attempts were made to free the virus of antigen by repeated washings in the high speed centrifuge.

A 10 per cent suspension of infected mouse lungs in saline was centrifuged at 2000 R.P.M. and then at 13,000 R.P.M., as described above. The rather bulky sediments obtained from the second centrifugation were resuspended in one-tenth the original volume of saline. Resuspension was accomplished by rubbing up the sediments with a glass rod and adding saline drop by drop until a thick, uniform suspension was obtained. Further gradual additions of saline were made until the requisite amount was added; this produced a very turbid suspension which contained an appreciable number of small aggregates which could not be broken up, even by the use of a rubber plunger with a diameter slightly less than the bore of the celluloid tubes used. This resuspended sediment was then centrifuged at 13,000 R.P.M. for 30 minutes and the supernatant liquid poured off. Washing by resuspension of the sediment in one-tenth the original volume of saline and centrifugation at 13,000 R.P.M. was repeated three times, and the final washed sediment was taken up in one-tenth the original volume of saline.

Another series of virus sediments was prepared from a suspension of infected mouse lungs by centrifugation at 2000, 13,000, and finally at 27,000 R.P.M., as described above. The small gel-like sediments obtained from the third centrifugation were treated in two ways. In one the sediments were resuspended in one-tenth the original volume of saline, washed three times by sedimentation at 27,000 R.P.M. for 45 minutes with resuspension of the sediment in one-tenth volume of saline, and finally taken up in one-tenth the

TABLE II

Results of Attempts to Remove Complement-Fixing Antigen from Influenza Virus by Repeated Washing in the High Speed Centrifuge

Specimen	Treatment	Sample tested	Virus titer	Complement-fixing titer					
				1:2	1:4	1:8	1:16	1:32	1:64
Sediment from 13,000 R.P.M. centrifugation	Washed 3 times by resuspension in 1/10 original volume of saline and centrifugation at 13,000 R.P.M. for 30 min.	Control*	†	++++	++++	++++	++++	++++	−
		1st wash water		++++	++++	++	±	−	
		2nd wash water		+	−	−	−	−	
		3rd wash water		+	−	−	−	−	
		Washed sediment (1/10 volume)	†	++++	++++	++++	++++	++	
Sediment from 27,000 R.P.M. centrifugation	Washed 3 times by resuspension in 1/10 original volume of saline and centrifugation at 27,000 R.P.M. for 45 min.	Control‡	$10^{-5.25}$	++++	++++	++++	++++	++++	−
		1st wash water		++++	++++	++++	++++	++++	−
		2nd wash water		++++	++++	+++	+	−	
		3rd wash water		++++	++	+	−	−	
		Washed sediment (1/10 volume)	$10^{-5.5}$	++++	++++	++++	++		
	Washed 3 times by resuspension in original volume of saline and centrifugation at 27,000 R.P.M. for 45 min. Brief preliminary and concluding centrifugations at 13,000 R.P.M.	Control‡	†	++++	++++	++++	++++	++++	++
		1st wash water		++++	++++	++	±	−	
		2nd wash water		++++	+++	±	−	−	
		3rd wash water		±	−	−	−	−	
		Washed sediment (original volume)	$10^{-4.6}$	++++	+	−	−	−	

* Supernate from centrifugation at 2000 R.P.M.

† Not done.

‡ Supernate from centrifugation at 13,000 R.P.M.

original volume. In the other the sediments were resuspended in the original volume of saline and centrifuged for 1 to 2 minutes at 13,000 R.P.M. to throw down large particles, and the supernate was then centrifuged at 27,000 R.P.M. for 45 minutes. The resulting sediment was washed twice more by resuspension in the original volume of saline and centrifugation at the same speed for the same time and finally centrifuged for 1 to 2 minutes at 13,000 R.P.M. to remove aggregated material.

Aliquots of the original suspensions, from which these various washed sediments were prepared, and from the supernatant liquids obtained from each serial washing were saved. These and the washed sediments were titered in the complement fixation test to determine the concentration of antigen in each specimen. The results of these tests are shown in Table II. It was found that successive wash waters contained progressively

less and less of the antigen. When present, the complement-fixing power of the third wash water usually was slight.

The washed sediments obtained after centrifugation at 13,000 R.P.M. invariably possessed a higher concentration of antigen than did those obtained from the same mouse lung suspension after centrifugation at 27,000 R.P.M. Since the volume of the former sediments was always much greater than that of the latter, this difference in titer may be explained on the assumption that the larger sediments contained a greater number of particles to which the antigen was adsorbed, or of which it formed an integral part. Although little or no antigen was present in the supernatant after the third washing of the sediment, it will be observed that all the washed sediments contained an appreciable amount of antigen. Even in the case of the sediment obtained by centrifugation at 27,000 R.P.M., after preliminary clarification at 2000 and 13,000 R.P.M., the sediment still contained antigen after three successive washings in the original volume of saline. The concentration of antigen remaining in this sediment was about sixteen times less than in the initial supernatant from which the sediment was obtained. If the antigen had been entirely unassociated with the sedimenting material and freely soluble in the saline used for washing, it can be shown that its concentration in the washed sediment should have been reduced by more than 100,000 times.

It appears from these findings that repeated washing of influenza virus does not entirely free the virus from complement-fixing antigen. It seems probable that some of the antigen is intimately associated with the virus; either it is "bound" to the virus particles or constitutes an integral part of their structure. The discrepancy between these results and those obtained by Hoyle and Fairbrother (7), which indicated that sediments did not contain antigen, is readily explicable since the initial starting material used by these authors contained relatively little antigen or virus, and the mere dilution of the sediment incident to resuspension was sufficient to abolish the reaction.

Sedimentation of the Antigen in the Centrifuge.—It will be seen from the results presented in Table I that the concentration of antigen in the pooled supernates after high speed centrifugation for short intervals was only slightly reduced. In order to gain information regarding the approximate size of the antigen, a number of experiments were done in which suspensions were centrifuged at high speed for varying intervals and the concentration of the antigen was determined at different levels in the tubes.

Suspensions of infected mouse lungs were centrifuged serially at 2000, 13,000, and 27,000 R.P.M., as described above. The supernates after the final centrifugation were pooled from several tubes as usual, but from two other tubes specimens were removed at different levels in the fluid column by the method described by Hughes, Pickels, and Horsfall (15). All specimens were then titrated in Swiss mice for their virus content and assayed for their complement-fixing capacity.

The results of these experiments are presented in Table III. It will be seen that the concentration of antigen in sample 1 had decreased by about four times, as had that in sample 2. This decrease in antigen at these levels was almost exactly paralleled by the increase in antigen observed in sample 3. It will also be observed that the degree of sedimentation of the antigen did not parallel the sedimentation of the virus since in samples 1 and 2 the virus titer was reduced by approximately 300 times.

TABLE III

Sedimentation of Influenza Virus and of Complement-Fixing Antigen by Centrifugation at 27,000 R.P.M. *for 45 Minutes*

Lot	Sample	Distance from meniscus	Virus titer	Complement-fixing titer						
				1:2	1:4	1:8	1:16	1:32	1:64	1:128
		cm.								
1	A*	–	$10^{-5.25}$	++++	++++	++++	++++	++	–	–
	B†	–	$10^{-3.63}$	++++	++++	++++	+++	+		
	1	0.0–1.6	$10^{-3.37}$	++++	++++	++	±	–		
	2	2.4–4.0	$10^{-3.0}$	++++	++++	++++	+	±		
	3 + sed.	4.8–6.7	$10^{-5.67}$	++++	++++	++++	++++	++++	++++	–
2	A*	–	$10^{-5.25}$	++++	++++	++++	++++	+++	–	–
	B†	–	$10^{-3.16}$	++++	++++	++++	+++	+		
	1	0.0–1.6	$10^{-2.2}$	++++	++++	++++	±	–		
	2	2.4–4.0	$10^{-2.5}$	++++	++++	++++	++	±		
	3 + sed.	4.8–6.7	$10^{-5.5}$	++++	++++	++++	++++	++++	++++	–

* Control; pooled supernates from centrifugation at 13,000 R.P.M.

† Control; pooled supernates from A centrifuged at 27,000 R.P.M.

In order to determine the extent of sedimentation of the antigen after prolonged centrifugation at high speed, the following experiment was done.

A serially centrifuged suspension of infected mouse lungs was filtered through a Seitz pad previously prepared with 2 per cent inactivated normal rabbit serum in saline. The filtrate was then centrifuged at 27,000 R.P.M. for 3 hours. On removal from the centrifuge, a striking feature was the almost complete sedimentation of hemoglobin from about the uppermost 0.5 cc. of fluid and its gradual increase in amount towards the bottom of the tube.

The fluid column was divided into samples as before except that the last one was subdivided in order to secure separately the sediment and the fluid immediately overlying it. The sediment, consisting of a blob of clear, gel-like material, was resuspended in one-tenth the original volume of saline.

The concentration of antigen in each of these samples was determined, and the results are presented in Table IV.

It is apparent from Table IV that even after centrifugation at 27,000 R.P.M. for 3 hours there was only partial sedimentation of the antigen and the top samples still contained about one-fourth, while the middle samples possessed almost one-half, of the initial concentration. This decrease in antigen in the top and mid samples was roughly paralleled by the increase in the concentration of the antigen in the bottom samples. Repetition of this experiment using both filtered and unfiltered antigens gave similar results as shown in Table IV.

TABLE IV

Determination of the Sedimentation Factor of Complement-Fixing Antigen of Influenza Virus by Centrifugation at 27,000 R.P.M. for 3 Hours

Sample	Distance from meniscus*	Complement-fixing titer (Seitz filtrate)		Sample	Distance from meniscus†	Complement-fixing titer			
						Unfiltered		Seitz filtrate	
		1	2			3	4	3	4
	cm.				*cm.*				
Initial	—	1:32	1:16	Initial	—	1:16	1:16	1:8	1:8
1	0.0 –1.6	1:8	1:4	1	0.0–1.0	1:2	1:2	1:2	1:2
2	2.4 –4.0	1:16	1:8	2	1.0–2.0	1:4	1:8	1:4	1:4
3	4.8 –5.75	1:16	1:16	3	2.0–3.0	1:8	1:8	1:4	1:4
4	5.75–6.5+	1:64	1:64	4	3.0–4.0	1:8	1:8	1:4	1:8
Sed.‡	—	1:64	1:64	5 + sed.	4.0–5.0	1:32	1:16	1:8	1:16
Sedimentation factor..		93.7	106.2	Sedimentation factor....		105.0	95.0	90.0	70.0

* Total height of fluid column = 6.7 cm. (8.0 cc.).

† Total height of fluid column = 5.0 cm. (6.0 cc.).

‡ Sediment resuspended in one-tenth original volume of saline.

It has been shown previously (15) that the so called sedimentation factor (S.F.) obtained under identical conditions was 33.9 for serum globulin and 159.6 for *Limulus polyphemus* hemocyanin. Applying this method to these results, it is possible to calculate that the approximate S.F. for the antigen was 90. Since the molecular weights of both serum globulin and the four components in this hemocyanin are known, it seems reasonable to suggest that the particle weight of the complement-fixing antigen lies somewhere between them and is very probably greater than 180,000 but less than 1,000,000.

Ultrafiltration of the Antigen

As pointed out above, successive centrifugation of a virus suspension in gravitational fields of increasing force led to a marked loss of virus in the

supernatant fluid but to no comparable loss in complement-fixing activity. Prolonged centrifugation at high speed, however, did result in definite sedimentation of the soluble antigen. From these observations it appeared obvious that the antigen possessed a particle size smaller than that usually assigned to influenza virus (80 to 120 mμ (11)).

Although an approximate estimate of the size of the antigen was obtained by the determination of the sedimentation factor, it was of interest to corroborate this estimate by another method. A number of ultrafiltration experiments were therefore carried out.

TABLE V

Ultrafiltration of Complement-Fixing Antigen of Influenza Virus

Average pore diameter of membrane	Complement-fixing activity of filtrate of antigen				
	1	2	3	4	5
mμ					
256				1:32	1:32
225			1:8		
206	1:8		1:8	1:32	1:32
174		1:8	1:8		
154	1:4			1:32	1:16
146		1:8	1:2		
124				1:32	1:16
104	1:2	1:2	1:2	1:16	1:8
72		1:8	1:2	1:4	1:4
50	—	—	—	—	—
34	—				
20	—				

Titer = highest dilution in which ++, or better, fixation was obtained.
— = no fixation demonstrable.

Five serially centrifuged antigen preparations, both unfiltered and filtered through a Seitz pad, were used for ultrafiltration. Collodion membranes (16) of average pore diameter from 256 mμ to 20 mμ were used. 10 cc. amounts of antigen were filtered through each membrane which had previously been saturated by the passage of 10 cc. of 5 per cent inactivated normal rabbit serum in saline. Filtration was done under 30 to 40 pounds pressure of nitrogen. The filtrates were titered in the complement fixation test, and the results are shown in Table V.

It will be observed that the antigen regularly passed through all membranes with an average pore diameter of 72 mμ or greater but that it was held back by those with an average pore diameter of 50 mμ or less. This was true even with antigens which had previously been filtered through Seitz pads. The difficulty of obtaining accurate ultrafiltration end points

with substances present in relatively low concentrations is well known. In the present case reduction of 90 per cent or more in the initial concentration of antigen as a result of passage through the smaller pore size membranes would permit so little antigen to pass into the filtrate as to render it undetectable by the method used. Losses to this extent or greater are regularly encountered in ultrafiltration. Despite these limitations, the results indicate that the antigen is definitely smaller than the virus and has a diameter which is certainly no greater than 35 mμ and very probably is considerably less.

Stability of the Antigen

Effect of Temperature.—When stored in the refrigerator at 4°C., the antigen does not retain its potency in an unaltered state for a long enough period to warrant its preparation in large amounts unless they are to be used rapidly; antigens have been found to lose as much as 50 per cent of their titer in 3 months of storage at this temperature. It was found to be preferable to store the infected mouse lungs at −76°C. and to make up small batches of antigen at frequent intervals as required.

Inactivation of the antigen at higher temperatures was investigated, a 30 minute time exposure being used regularly. At 37°C. and 40°C. there was no demonstrable reduction in activity. A small loss in titer occurred on heating at 45°C. and 50°C., and at 56°C. serious impairment of activity occurred; this was so marked that all antigenic power was lost in the weaker preparations. Antigenicity of even the most potent preparations was destroyed completely at 63°C.

Effect of Hydrogen Ion Concentration.—Studies on the electrophoretic mobility of the soluble antigen (17) necessitated acquisition of information on the stability of the antigen in buffers of varying hydrogen ion concentrations. Data derived from experiments on this subject are given below.

Serially centrifuged preparations were filtered through Seitz pads previously saturated with 2 per cent rabbit serum-saline. The filtrates were distributed in 2 cc. amounts in cellophane sacs which were tied off and immersed in flasks of the appropriate buffer. The hydrogen ion concentration of the buffers, which covered a pH range from 1.0 to 12.0, was determined with the glass electrode. After dialysis for 24 hours in the cold room, the sacs were rinsed with distilled water and transferred to phosphate buffer at pH 6.8. Dialysis was allowed to proceed in this medium for an additional 24 hours in the cold, after which the sacs were rinsed in fresh buffer and their contents removed. It was noted that dialysis against alkaline buffers resulted in very slight precipitation; the use of acid buffers, however, was attended with irreversible precipitation of coarse, brown floccules.

The dialyzed samples were centrifuged at 13,000 R.P.M. for 30 minutes, and the clear supernates were drawn off for titration of antigen.

The antigen was stable and retained its ability to fix complement from pH 4.6 to 11.0. At pH values outside this wide range, however, the antigen was inactivated. Precipitates which appeared on dialysis contained no antigen.

Precipitation of the Antigen

Precipitation by Alcohol-Ether Mixtures.—A modification of Hartley's method (18) was employed. A serially centrifuged antigen preparation was added dropwise to 9 volumes of a 2:1 alcohol-ether mixture which had been cooled to −18°C. The bulky precipitate was packed by centrifugation and the supernate discarded. The precipitate was resuspended in 9 volumes of cold anhydrous ether, vigorously shaken, and centrifuged again. This washing was repeated three times. All centrifugations were done at −18°C. The washed precipitate was dried *in vacuo*, and, when all traces of ether had been removed, was resuspended in the original volume of saline. This suspension contained a considerable residue which did not dissolve but was removed by centrifugation. The clear supernate obtained was found to contain antigen in a concentration almost identical with that of the original preparation. The insoluble residue contained no antigen.

Precipitation by Ammonium Sulfate.—To 15 cc. of a serially centrifuged antigen which had been filtered through a Seitz pad were added 15 cc. of a saturated solution of ammonium sulfate. The mixture was kept in the cold room for 4 hours and then centrifuged for 15 minutes at 2000 R.P.M. The supernate was saved and the precipitate, dissolved in 7.5 cc. of distilled water, was dialyzed against running water for 48 hours. The "euglobulin" which was precipitated out during this dialysis was dissolved in 10 cc. of saline; the soluble "pseudoglobulin" fraction was brought to 10 cc. with distilled water and made isotonic with sodium chloride.

The supernate obtained after the initial salting out of the "globulin" fractions was then saturated with ammonium sulfate and placed in the cold room overnight. The precipitated "albumin" fraction was collected on filter paper, washed with saturated ammonium sulfate solution, dissolved in 7.5 cc. of distilled water, and dialyzed for 48 hours against running water. A 5 cc. portion of the filtrate was also dialyzed. Both specimens were finally brought to 10 cc. with distilled water and made isotonic with sodium chloride.

All fractions were tested in the complement fixation test. It was found that the antigen regularly was present solely in the pseudoglobulin fraction; the euglobulin and albumin fractions, as well as the protein-free filtrate, did not contain any detectable antigen.

DISCUSSION

The results of these investigations indicate that epidemic influenza virus, during the process of increase, elaborates an antigen which when mixed with specific immune serum will fix complement. This antigen remained

in suspensions from which the greater part, though not all, of the virus had been sedimented by centrifugal force. It was also present in ultrafiltrates which were not infectious for mice and presumably, therefore, did not contain active virus. On the basis of this evidence it is apparent that the antigen can be separated from the virus. However, it was not possible to separate completely the virus from the antigen even by differential centrifugation and repeated washing of the sedimented virus. The washed virus regularly contained antigen although in lower concentration than in the original suspensions.

Both high speed centrifugation and ultrafiltration experiments afforded clear evidence that the antigen was of a size definitely smaller than that of the virus. Because the antigen is present only in relatively low concentrations, it was manifestly unreasonable to place too much weight on the apparent particle size as determined by ultrafiltration though even with this technique it was shown that the diameter of the antigen was not greater than 35 mμ. The calculation of the sedimentation factor (15) probably gave a fairly close approximation of the particle weight, if it is assumed that the antigen has the density of serum proteins and is spherical; this was found to lie somewhere in the range between 180,000 and 1,000,000.

A number of the properties possessed by the antigen suggest that it is a protein, the specific antigenic structure of which is relatively unstable. The lability of the antigen as shown by heating, its gradual loss of activity on prolonged storage in the ice box, and its inactivation at hydrogen ion concentrations greater than pH 4.5 indicate that it is unstable. The precipitability of the antigen with ammonium sulfate, its presence only in the pseudoglobulin fraction, its relatively large size, and the available data concerning its electrophoretic mobility (17) are consistent with the hypothesis that the antigen is a protein. The fact that it can be precipitated and extracted by alcohol and ether in the cold indicates that lipids are not constituents essential to the activity of the antigen.

It seems most reasonable to think that the antigen is produced by the virus, as seems also to be the case with vaccinia (2), psittacosis (3), myxoma (4), and lymphocytic choriomeningitis (5) viruses, and not that it results from the effects of the virus on infected cells as appears to be true in the case of yellow fever (1). This view is supported by the fact that it was not possible to remove all of the antigen from influenza virus by repeated washing.

Whether the complement-fixing antigens produced by various strains of the virus are identical in all instances remains a subject for further investigation. Cross neutralization (19) and cross immunity (20) tests

have demonstrated that strains of the virus may differ markedly one from another but have yielded no information as to what portion of the virus particle endows it with strain-specific properties. While the complement-fixing antigen is usually considered as being "group-specific," in the sense that such antigens prepared from widely dissimilar strains of the virus will fix complement in the presence of human convalescent sera, such an assumption is not entirely warranted. Human sera possess the capacity to neutralize different strains of the virus to about the same extent and, therefore, would not be expected to reveal any differences in the antigenic structures of antigens prepared from various strains. Although sera from ferrets recovered from infection by different strains reflect the variations in their antigenic structures when used in neutralization tests, attempts to effect a similar assessment of the complement-fixing antigens have been generally unsuccessful owing to the lack of a suitable test employing ferret tissues or sera. Infected ferret lungs, unlike mouse lungs, appear to contain little, if any, of the antigen, while the serum, in addition to possessing heterophile antibodies which interfere with the use of influenza antigens from species possessing heterophile antigens (*e.g.*, the mouse), frequently exerts a marked lytic action on sheep cells. Procedures designed to circumvent these difficulties have not, up to the present, been entirely satisfactory. The importance of the subject, however, warrants further effort directed at its resolution.

SUMMARY

Evidence is presented which indicates that influenza virus elaborates a soluble antigen. The antigen is considerably smaller than the virus and can be separated from it, but the virus has not been washed free of antigen. The properties of the antigen suggest that it is an unstable protein of relatively large size.

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