DISRUPTION OF INFLUENZA VIRUS

PROPERTIES OF DEGRADATION PRODUCTS OF THE VIRUS PARTICLE

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PLATE 10

The purpose of this work was to break up the influenza virus particle and to examine the materials released in order to learn more of the structure and composition of the infectious agent. Previous work of this sort on animal viruses is not extensive and is briefly reviewed below.

Two different precipitating antigens have been extracted from vaccinia virus. One, designated the LS antigen (1), is a protein with two antigenically active groups (2, 3); the other, termed the NP antigen, is a nucleoprotein (4). These two antigens apparently comprise a large proportion of the mass of the virus particle. The soluble complement-fixing antigen of influenza virus is usually extracted from infected tissue and is distinct and separable from the virus particle (5, 6). After ultrasonic vibration of purified influenza virus preparations, an increase in complement-fixing antigen was observed (7), and was attributed to disruption of the virus particle.

After treatment of influenza virus particles with ether, hemagglutinating activity can be separated from complement-fixing activity and this has been held to be due to disruption of the virus (8). By this procedure, soluble complement-fixing antigen was released from the particle and small hemagglutinating components were obtained (9).

In the present paper several methods of degrading influenza virus are described. It will be shown that antigenic material from the degraded virus particle can be detected and measured by a technique based on blocking of the hemagglutination-inhibition or virus neutralization reaction. Evidence will be presented that repeatedly frozen and thawed virus preparations show immunologically specific activity associated with material much smaller than the intact virus particle. Finally, an account is given of experiments designed to define the properties and nature of this immunologically active material.

Materials and Methods

Viruses.—The following strains of influenza A virus were used: WS, PR8, FM1, and Wilfong. The Lee strain of influenza B, the Habel strain of mumps virus, and the Hickman strain of Newcastle disease virus were also employed.

To propagate each of the viruses, except mumps, 9- or 10-day-old white Leghorn embryos were inoculated intra-allantoically with 0.2 ml. of a 10^{-3} dilution of infected allantoic fluid. The eggs were incubated at 35°C. for 42 to 48 hours and then were chilled and the allantoic fluids were harvested and pooled. In some instances, the fluids were distributed in volumes of 1 to 6 ml. in nitrocellulose tubes and stored at −65°C. in the absence of gaseous CO_2 (10). In others, they were dialyzed at 4°C. against 100 volumes of 0.1 M phosphate buffer at pH 7.2 and stored at 4°C.

Mumps virus was propagated by intra-allantoic inoculation of 6- to 7-day-old embryos. The allantoic fluids were harvested after incubation at 35°C. for 5 days and were stored at −65°C. as described above.

Red Blood Cells.—Venous blood was drawn aseptically from normal roosters and men. It was collected in acid citrate dextrose solution (11) and stored at 4°C. RBC were washed 3 times in saline and suspensions were prepared in buffered saline. The concentration was calculated from the packed cell volume. Chicken RBC were used within 5 days of drawing and human RBC within 3 days.

Saline.—For most purposes, 0.85 per cent sodium chloride solution was used, buffered to pH 7.2 with 0.01 M phosphate. In complement-fixation tests, a veronal buffered saline solution containing optimum amounts of calcium and magnesium was used (12).

Immune Serum.—Immune sera were prepared in rabbits by intravenous injection of 10 ml. of infected allantoic fluid, followed by intraperitoneal injections of 10 ml. of similar fluid at intervals of 2 to 3 weeks. Serum was collected at various periods after the 2nd week of immunization. Sera from ferrets convalescent from infection with WS or PR8 were provided by Dr. M. Theiler, and similar ferret serum against FM1 as well as normal ferret serum were supplied by Dr. E. H. Lennette. The sera were stored at 4°C. without preservative and inactivated at 56°C. for 30 minutes immediately before use.

Hemagglutination Titrations.—Serial twofold dilutions of infected allantoic fluid were prepared in buffered saline and a half volume of 0.5 per cent RBC was added to each dilution. The RBC were allowed to settle for 1 hour and the highest dilution in which strong (3+) hemagglutination occurred was considered to represent one hemagglutinating unit.

Hemagglutination-Inhibition Titrations.—Serial twofold dilutions of serum were prepared in buffered saline in volumes of 0.4 ml. or greater. To 0.4 ml. of each dilution were added 0.2 ml. of infected allantoic fluid (diluted so as to yield 4 hemagglutinating units in the final mixture) and 0.2 ml. of 0.5 per cent suspension of chicken RBC. The RBC were allowed to settle for 45 to 60 minutes and the final serum dilution in the last tube in which hemagglutination was completely inhibited was taken as the inhibition titer. In some instances, when increased precision was required, the fractional dilution method described previously was used (13).

Infectivity Titrations.—Serial dilutions of infected material were made in chilled buffered saline containing 1,000 units of penicillin and 1 mg. of streptomycin per ml. The dilutions were either in tenfold or 3.16-fold steps and were kept in an ice bath. For titrations in the chick embryo, 0.2 ml. from each dilution was inoculated into the allantoic cavity of each of a group of 3 to 6 eggs which were incubated at 35°C. for 42 hours. The eggs were then chilled, 0.1 ml. of allantoic fluid was removed and mixed with 1.0 ml. of 0.5 per cent suspension of chicken RBC. If hemagglutination occurred, the embryo was considered to be infected. For titrations in mice, dilutions were made in broth-saline and groups of 4 to 6 Swiss mice, 3 to 4 weeks old, were inoculated intranasally under ether anesthesia. Each mouse received 0.05 ml. Deaths were recorded daily and the survivors were killed after 10 days' observation. The lung lesions were scored as described previously (14) and the 50 per cent maximum score end point (MS_{50}) was calculated.

Neutralization Titrations.—Serum was heated at 56°C. for 30 minutes before dilution. Twofold dilutions were prepared in buffered saline or broth-saline and an equal volume of a

virus containing 10 to 100 EID_{50} or MS_{50} was added. After about 20 minutes at room temperature the mixtures were inoculated into chick embryos or mice as described above. 50 per cent end points were calculated in the usual manner.

Antigenic Extinction Titrations.—Serial tenfold dilutions of a virus preparation were made in sterile saline and groups of 4 mice, 5 weeks old, were injected intraperitoneally with 0.5 ml. 7 days later the injection was repeated. 10 days after the second injection, each mouse was given intranasally, 0.05 ml. of a dilution of PR8-infected mouse lung containing about 400 MS_{50}. Mice dying with lung consolidation thereafter were recorded. 10 days after the intranasal inoculation, the survivors were killed and scored for lung lesions as described above.

Enzymic Activity of Influenza Virus.—Serial dilutions of virus were made in saline. To each was added about 4 inhibiting units of egg white suspension (15). The mixtures were held at 37°C. for 30 minutes and then at 65°C. for 30 minutes. A quantity of inactivated Lee virus (heated at 56°C. for 30 minutes) sufficient to yield 4 units in the final mixture was added in 0.2 ml. The mixture was held at room temperature for 30 minutes and then 0.2 ml. of 0.5 per cent suspension of inhibitor-sensitive chicken RBC was added. The highest dilution showing detectable hemagglutination was considered to represent the enzymic titer of the material.

Complement-Fixation Tests.—A 1 per cent suspension of sheep RBC sensitized with 2 to 3 units of commercial amboceptor and two and one-half 100 per cent hemolysis units of complement were used. In the fixation mixture there were 0.1 ml. of serum, 0.1 ml. of complement, 0.1 ml. of antigen. Fixation was carried out at 37°C. for 1 hour and the usual controls were included. After addition of 0.2 ml. of 1 per cent sensitized sheep RBC and incubation at 37°C. for a further 30 minutes, the mixtures were held at 4°C. overnight and read the next morning. The highest dilution showing no hemolysis was taken as the end point.

Freezing and Thawing Procedure.—As a routine, infected allantoic fluid was dialyzed, as described above. 10 to 20 ml. of fluid was then placed in a nitrocellulose tube of 40 ml. capacity. The tube was immersed in a solid CO_2-alcohol mixture until solidly frozen. It was then transferred to a 37°C. water bath and held there until complete thawing had occurred. This cycle was repeated 50 times.

In a few early experiments 5 ml. of fluid was frozen in an Erlenmeyer flask of 50 ml. capacity in a mechanical refrigerator maintained at −26°C.

EXPERIMENTAL

Degradation of Influenza Virus.—Incubation with strong urea solution destroys the infectivity of influenza virus (16) and releases desoxyribosenucleic acid from bacteriophage (17). It was found that the hemagglutinating activity of influenza virus was destroyed by incubation with 5 M urea at 37°C. for a few hours. The time required varied from one strain to another. By contrast, so called osmotic shock, which ruptures bacteriophage particles (18, 19) was found to have no detectable effect on the infectivity of influenza virus. As an example, 1 volume of PR8 allantoic fluid was mixed with 3 volumes of 4 M NaCl and held at 4°C. for 8 hours. The mixture was poured drop by drop with vigorous stirring into a large volume of cold sterile saline. At the same time, some of the NaCl-treated fluid was diluted slowly by adding water in drops so as to lower the osmotic pressure gradually. The infectivity titers of the two mixtures were identical with the titer of the original fluid showing

that high concentrations of NaCl and sudden extreme changes in osmotic pressure do not demonstrably affect influenza virus.

Since repeated freezing and thawing is known to disrupt bacterial and other cells, this procedure was tried with influenza virus. Infected allantoic fluid, in a thin layer was frozen at $-26°C$. and then thawed under a running tap or in a 37°C. water bath. The results shown in Table I indicate that there was only a small reduction in the infectivity and hemagglutination titers after repeatedly freezing and thawing of undialyzed PR8 allantoic fluid. Because allantoic fluids develop thick precipitates after a few cycles of freezing and thawing, the experiment was repeated with dialyzed allantoic fluid and then a striking reduction in infectivity and hemagglutination titers was found.

TABLE I

Effect of Repeated Freezing and Thawing on Influenza Virus, PR8

| PR8 allantoic fluid	No. of freeze-thaw cycles	Virus titers	
		Infectivity*	Hemagglutination
		$-log$	$-log$
Not dialyzed	0	7.6	3.2
	10	7.0	2.9
	0	7.6	3.8
	55	5.2	3.2
Dialyzed vs. phosphate buffer ‡	0	7.7	3.5
	52	<2.0	1.0

* In ovo.

‡ 0.1 M, pH 7.2.

The effect of repeated freezing and thawing of dialyzed PR8 allantoic fluid was studied in more detail. Aliquots of 5 ml. from a dialyzed allantoic fluid pool were simultaneously frozen and thawed. The freezing and thawing was stopped at 2, 5, 10, and 20 cycles, respectively, and the aliquots were titrated in parallel for infectivity, hemagglutination, and enzymic activity. The results are shown in Text-fig. 1 and indicate that each of the above properties progressively decreased as the number of freezings and thawings was increased. Dialyzed fluids frozen and thawed 50 times were generally used in later experiments as they were usually non-infective and had little or no hemagglutinating activity.

Detection and Measurement of Blocking Antigen:

1. Antibody Absorption by Degraded Virus.—Infectivity, enzymic activity, and hemagglutination are properties of intact influenza virus particles and each is readily inactivated. It was thought that virus which had lost these prop-

erties through urea treatment or multiple freezing and thawing might still
be immunologically active since it has been shown that influenza virus, sim-
ilarly inactive as a result of heating at 65°C. for 30 minutes, will still absorb
specific antibody (20).
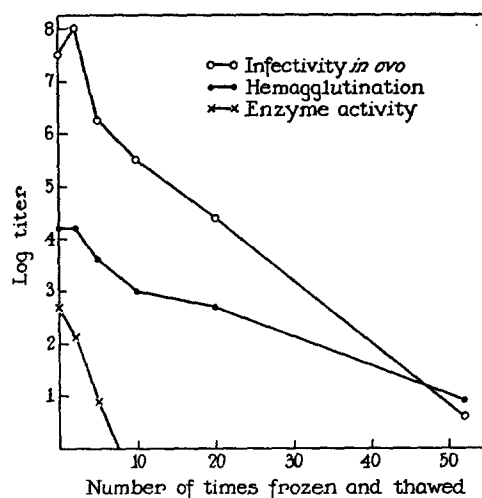
TEXT-FIG. 1. The effect of repeated freezing and thawing of dialyzed PR8 allantoic fluid
on infectivity and hemagglutination titer and on enzymic activity.

TABLE II

Absorption of Antibody by Frozen and Thawed Influenza Virus, PR8

PR8 immune rabbit serum	Antibody titer* vs. PR8	
	Neutralization‡	Hemagglutination-inhibition
Not absorbed	640	2560
Absorbed with PR8 (control)	<20	320
Absorbed with frozen and thawed PR8§	<20	<40

* Expressed as the reciprocal.
‡ *In ovo.*
§ Dialyzed allantoic fluid, 52 freeze-thaw cycles.

To demonstrate such absorption, 2 ml. of a dialyzed PR8 allantoic fluid
pool, which had been frozen and thawed 52 times, was mixed with 0.2 ml. of
PR8 immune rabbit serum. The mixture was held at 4°C. for 11 hours and then
centrifuged at 30,000 g for 30 minutes. The supernatant fluid was titrated
for hemagglutination-inhibiting and neutralizing antibodies. An aliquot of
the allantoic fluid which had not been frozen and thawed was used as a control.
The results shown in Table II indicate that after repeated freezing and thawing

the virus material still combined with homologous antibody. In fact, the titer of hemagglutination-inhibiting antibody was reduced to a greater extent by absorption with frozen and thawed virus than with the intact control virus.

2. *Blocking Antigen Titration.*—Because repeatedly frozen and thawed virus preparations combined with homologous antibody it was possible to devise a more rapid and simple method for measuring the immunologic activity of non-infective and non-hemagglutinating influenza virus material.

Serial twofold dilutions of immune rabbit serum were prepared. To one series was added one volume of the antigen material to be tested and to another an equal volume of saline. The mixtures were shaken and held at 4°C. for 1 hour. Then 2 or 4 hemagglutinating units of virus (in the final mixture), homologous with the immune serum used, and 0.5 per cent RBC suspension were added, each in unit volume, usually 0.2 ml. The cells were allowed to settle for 30 to 45 minutes and the hemagglutination pattern was read. Alternatively, on removing the mixtures of serum and antigen from the refrigerator, 100 EID$_{50}$ of homologous

TABLE III

Blocking of Hemagglutination-Inhibition by Frozen and Thawed Influenza Virus, PR8

Antiserum* dilutions held at 4°C. for 1 hr. with	Hemagglutinating virus (PR8) added	Antiserum dilution						Blocking antigen titer
		160	320	640	1280	2560	5120	
	units							*units*
Saline	4	0‡	0	0	0	0	3	0
PR8 dialyzed fluid, heated 65°C.	4	0	0	3	3	3	3	16
PR8 dialyzed fluid, 52 freeze-thaw cycles	4	0	0	3	3	3	3	16

* PR8 immune rabbit serum.

‡ Degree of hemagglutination.

virus was added to each tube and the mixtures were inoculated into groups of eggs which were incubated and then tested for hemagglutination as described above. The results can be recorded in terms of the titer of the serum alone and of the serum in the presence of the antigen.

Representative results shown in Table III include a titration carried out with one of the dialyzed allantoic fluid pools used in the experiments recorded in Text-fig. 1 after it had been absorbed at 4°C. with RBC. The results are expressed as units of blocking antigen which are calculated in the following way: It is considered that the number of units of antibody in the last negative tube of the control titration, *i.e.* 1:2560, is equivalent to the number of hemagglutinating units of virus added. The number of units of blocking antigen activity is defined as equal to the number of units of antibody present in the lowest dilution of the titration with antigen which shows hemagglutination. Therefore, the titer of blocking antigen with the preparations indicated in Table III was taken as 16 units. Closely similar results were obtained when the

titrations of blocking antigen were done by neutralization *in ovo* as is shown in Table IV.

3. Quantitative Features of Blocking Antigen Titration.—Before using this technique in further studies, its quantitative aspects were examined in hemagglutination-inhibition experiments. Although it was desirable to hold the serum-antigen mixtures at 4°C. in the preliminary stage of the reaction, it was found that it was preferable to perform the remainder of the reaction at room temperature in order to avoid occasional difficulties due to cold hemagglutinins in the immune rabbit serum. Increasing the amount of hemagglutinating virus added decreased the difference between the titers in the antigen and the control series. Dilution of the antigen led to a decrease in the amount of blocking, but the exact relation between the amount of antigen added and the amount of antibody blocked depended on the immune serum used; in particular, on the extent to which the rabbit had been immunized before the serum was

TABLE IV

Blocking of Neutralization in Ovo by Frozen and Thawed Influenza Virus, PR8

Antiserum* dilutions held at 4°C. for 1 hr. with	Infected virus (PR8) added	Antiserum dilution						
		20	40	80	160	320	640	1280
	EID_{50}							
Saline	200	—	—	—	0/3‡	1/3	0/3	3/3
PR8 dialyzed fluid, heated 65°C.	200	0/3	1/3	3/3	3/3	3/3	3/3	3/3
PR8 dialyzed fluid, 52 freeze-thaw cycles	200	0/3	2/3	3/3	3/3	3/3	3/3	3/3

* PR8 immune rabbit serum.

‡ Numerator, No. embryos infected; denominator, No. embryos inoculated.

collected. Slight blocking was obtained with a heated hemagglutinating virus preparation when egg white inhibitor was used instead of antiserum.

Various types of titrations were performed with early and late immune serum specimens from the same rabbit.

One rabbit was given 10 ml. of PR8 allantoic fluid intravenously and a second injection of 10 ml. intraperitoneally 15 days later. Sera collected on the 15th and 56th days after the first injection were titrated against homologous virus by hemagglutination-inhibition and *in ovo* neutralization procedures. Blocking antigen titrations were performed with a preparation of PR8 dialyzed allantoic fluid which had been heated at 65°C. for 60 minutes.

The results are presented in Table V. Those secured with the late as compared with the early immune serum, show that although the hemagglutination-inhibition titer decreased, the titer of the blocking antigen appeared to increase. At the same time, the ratio between the neutralizing and hemagglutination-inhibiting antibody titers increased as was shown previously (18). Similar results were obtained with early and late immune sera from the three other

rabbits, one immunized with PR8 and two with WS. As identical antigens were used with both early and late immune sera, the rise in blocking antigen titer indicates a change in the properties of the antibodies. Both the altered capacity of antibody to combine with heated virus antigen and the ability of the serum to neutralize relatively more virus per hemagglutination-inhibiting unit indicate a change in the antibody with increasing time after immunization. The late antibody appears to combine more irreversibly with heated virus antigen than the early antibody, and this seems parallel to the change in avidity of antibody observed in the course of immunization against diphtheria (21). The alteration might also be due to the development of reactivity with minor antigenic components of the virus, but the late immune sera used in these experiments reacted in a strain-specific manner in blocking antigen titrations as is shown below.

TABLE V

Blocking Antigen Titers of Heated Influenza Virus with Different Immune Sera

Antiserum,* days after start of immunization	Hemagglutination inhibition titer *vs.* 8 units	Neutralization titer *vs.* 100 EID₅₀	Ratio neutralization to hemagglutination-inhibition titer	Blocking antigen titer‡
				units
15	4096	1505	0.37	4
56	1024	1130	1.1	20

* Antiserum specimens from one rabbit immunized with PR8.
‡ Determined with 2 units of hemagglutinating virus.

Further experiments were done with two other anti-PR8 sera to learn more about quantitative differences in the blocking reaction obtained with sera of similar hemagglutination-inhibiting titer.

One serum (early immune) was secured from a rabbit which had received a single intravenous injection of PR8 allantoic fluid. The other serum (hyperimmune) was a pool from a number of rabbits which had received multiple intravenous injections over a period of months of PR8 purified by adsorption and elution on red cells. Dialyzed PR8 allantoic fluid heated at 65°C. for 30 minutes was used as blocking antigen. Serial dilutions of both antigen and serum were prepared and each dilution of serum was tested against each dilution of antigen in triplicate or quadruplicate. The mixtures were held at 4°C. for 1 hour and then the hemagglutinating virus and RBC were added as described above.

Representative results are recorded in Table VI. The findings were also analyzed by calculating linear regression equations between the logarithm of the antigen dilution used and the logarithm of the apparent serum titer obtained. The regression coefficient in the experiment with the early serum was 0.56 (s.d. ± 0.07) and in that with the hyperimmune serum it was 0.93 (s.d. ± 0.06). The results show that the amount of blocking was greater when

hyperimmune serum was used. They also show that there was a marked difference in the quantitative relations between blocking antigen and early immune serum on one hand and hyperimmune serum on the other. With the former, the amount of blocking which occurred was roughly a function of the square root of the antigen concentration; with the latter, it was almost directly proportional to the antigen concentration. Thus, blocking antigen titers determined in experiments with hyperimmune sera were higher and more consistent than those measured with early immune serum.

TABLE VI

Extent of Blocking of Hemagglutination-Inhibition Obtained with Heated Influenza Virus, PR8, and Immune Rabbit Sera

Antiserum*	Dilution of blocking antigen‡	Dilution of serum							Blocking antigen titer
		64	128	256	512	1024	2048	4096	
									units
Early immune	2	—	—	0§	0	3	3	3	16
	4	—	—	0	0	3	3	3	32
	8	—	—	0	0	0	3	3	32
	16	—	—	0	0	0	3	3	64
	32	—	—	0	0	0	0	3	0
	Saline	—	—	0	0	0	0	3	—
Hyperimmune	2	0	3	3	3	3	3	3	128
	4	0	0	3	3	3	3	3	128
	8	0	0	0	3	3	3	3	128
	16	0	0	0	3	3	3	3	256
	32	0	0	0	0	3	3	3	256
	Saline	0	0	0	0	0	0	3	—

* PR8 rabbit serum.
‡ Dialyzed PR8, heated 65°C. for 30 minutes.
§ Degree of hemagglutination.

Disruption of the Virus Particle by Repeated Freezing and Thawing.—As was shown above, repeated freezing and thawing degraded influenza virus. Because a technique was available to measure degradation products of the virus in the form of blocking antigen, experiments were performed to determine whether the blocking antigen was released in a soluble form after freezing and thawing.

Dialyzed PR8 allantoic fluids which had been frozen and thawed 50 times or treated with 5 M urea as described above were used. As controls, dialyzed PR8 allantoic fluids without further treatment or heated at 65°C. for 1 hour were employed. In addition, a chloroform-treated extract of PR8 chorioallantoic membrane was included. All the fluids and the extract were centrifuged at 60,000 g for 30 minutes. The supernatants were collected and the sediments were resuspended in phosphate buffer at pH 7.2. The supernatants and resuspended

sediments were then tested separately in the following titrations: hemagglutination, blocking antigen with hyperimmune rabbit serum, and complement-fixation with serum from a patient convalescent from influenza A. In the latter test serial dilutions of both serum and antigen were used.

The results are shown in Table VII. Blocking antigen was present in the supernatant from the repeatedly frozen and thawed preparation though the gravitational field used sedimented over 99 per cent of the hemagglutinating virus in the untreated control material. In addition, there was slight com-

TABLE VII

Effects of a High Gravitational Field on the Properties of Various PR8 Preparations

Treatment of dialyzed PR8 allantoic fluid	After centrifugation at 60,000 g for 30 min.	Hemag-glutination titer	Complement-fixing titer*	Blocking antigen titer
				units
None	S‡	8	0	—§
	R	4096	4	—
Heated at 65°C. for 1 hour	S	‖	0	<4
	R		2	128
Urea 5 M at 37°C. for 8 hours	S	‖	0	<4
	R		1	16
Frozen and thawed 50 times	S	<4	2	8
	R	>4	2	—§
Chloroform treated extract of PR8 chorioallantoic membrane	S	‖	80	8
	R		4	8

* With human influenza A convalescent serum.

‡ S, supernatant; R, sediment resuspended to original volume.

§ Not done because preparation contained hemagglutinating activity.

‖ No hemagglutinating activity before centrifugation.

plement-fixing activity in the supernatant of the frozen and thawed preparation but none in that of the untreated or heated control materials. These results indicated that immunologically active material was released from the virus particle by the freezing and thawing process. In contrast, the supernatant from the urea-treated preparation had neither blocking antigen nor complement-fixing activity. Whereas, the supernatant from the preparation of soluble complement-fixing antigen extracted from the infected chorioallantoic membrane had a complement-fixing titer of 80 and a blocking antigen titer of 8.

Further experiments were done to determine whether, after repeated freezing and thawing, PR8 allantoic fluid contained non-sedimentable blocking and

complement-fixing antigens which were not present in the untreated fluid. A dialyzed fluid which had been frozen and thawed 65 times and an aliquot of the original allantoic fluid were absorbed with 5 to 10 per cent washed RBC and the supernatants were centrifuged twice at 60,000 g for 30 minutes. Aliquots of the supernatants obtained at each step in the procedure were tested for hemagglutinating, complement-fixing, and blocking antigen activity. The results are shown in Table VIII and indicate that the supernatant from the original allantoic fluid, after absorption with RBC and 2 cycles of high speed centrifugation, contained no detectable complement-fixing or blocking antigen activity, whereas, the supernatant from the 65 times frozen and thawed fluid contained both. The titers obtained with the latter material

TABLE VIII

Soluble Blocking and Complement-Fixing Antigens from Influenza Virus, PR8, after Freezing and Thawing

Treatment of PR8 allantoic fluid	Hemagglutina-tion titer	Complement-fixing titer*	Blocking antigen titer‡
			units
A. None	4096	—	—
B. (A) + absorbed with RBC§	4	0	—
C. (B) + 2 cycles centrifugation at 60,000 g§	0	0	0
D. Dialyzed, frozen and thawed 65 times	0	4	16
E. (D) + absorbed with RBC§	0	4	8
F. (E) + 2 cycles centrifugation at 60,000 g§	0	2	8

* Determined with human influenza A convalescent serum.
‡ "   "   PR8 hyperimmune rabbit serum.
§ Supernatant.

were about ¼ those shown by the resuspended sediment from a PR8 allantoic fluid which had been heated at 65°C. for 30 minutes and then centrifuged at 60,000 g for 30 minutes. The evidence indicated that the freezing and thawing procedure had released antigenic material from influenza virus in substantial amount and the properties of this material were examined.

Graded centrifugation of frozen and thawed influenza virus preparations was carried out and the results are presented in Table IX. They show that although some blocking antigen activity was sedimented at low speeds (4,000 R.P.M. for 30 minutes), much of the antigen remained in the supernatant after centrifugation at 40,000 R.P.M. for 2 hours, a gravitational force that is sufficient to sediment some large protein molecules.

Observations with the Electron Microscope[1].—Efforts were made to visualize with the electron microscope changes in the virus particles which resulted

[1] Dr. K. Porter kindly prepared the electron micrographs.

from repeated freezing and thawing. After some preliminary trials, the following experiment was done:—

Allantoic fluids of eggs inoculated with 1 to 10 EID_{50} of PR8 were harvested after 42 hours of incubation. To 9 ml. of pooled fluid was added 0.5 ml. of fresh, washed, and packed human group A RBC. The mixture was shaken and then held at 4°C. for 3 hours. The RBC were centrifuged down, washed with cold saline, resuspended in 9 ml. of saline, and held at 37°C. with occasional shaking while elution of the virus took place. The RBC were then centrifuged out and the supernatant was centrifuged again at 5,000 g for 4 minutes to remove RBC fragments. The supernatant was next centrifuged at 60,000 g for 30 minutes. The translucent, grayish sediment was resuspended in about 1 ml. of 0.1 M phosphate buffer, pH 7.2. Half of the material was frozen and thawed 50 times. Drops from the control aliquot

TABLE IX

Centrifugation of Blocking Antigen in Dialyzed Allantoic Fluids after Repeated Freezing and Thawing

Dialyzed allantoic fluid*	Centrifugal speed	Time	Gravitational force	Blocking antigen titer of supernatant
	R.P.M.	*min.*	*g min.*	*units*
PR8	0	0	0	8
"	4,000	30	0.03×10^6	6
"	15,000	30	0.5×10^6	6
"	30,000	30	1.8×10^6	4
PR8	30,000	30	1.8×10^6	4
"	40,000	120	14×10^6	3
WS	30,000	30	1.8×10^6	6
"	40,000	120	14×10^6	6

* Frozen and thawed, 50 times.

and the thawed concentrate were then placed on collodion coated screens, dried, washed 3 times with distilled water, shadowed with chromium at an angle of 12.5° and examined under the electron microscope.

Three representative electron microscope fields are shown in Figs. 1 *a*, *b*, and *c*. The upper field (*a*) shows the rounded particles, about 100 mμ in diameter, found in the control virus concentrate, which are considered to represent intact virus particles. The middle field (*b*) shows the appearance of most of the areas visualized in the repeatedly frozen and thawed preparation. Clearly the large virus particles have disappeared. There are, in moderate number, tiny particles about 10 to 20 mμ in diameter. However, similar tiny particles are visible among the virus particles of the control preparation, and they cannot be positively identified as products of virus particle disruption. Here and there, in the otherwise almost empty fields observed with the frozen

and thawed preparation were large aggregates of amorphous material. Part of one such aggregate is shown in the lower field (c). No such aggregates were found in the control virus concentrate.

Preparation of Soluble Blocking Antigen.—Dialyzed allantoic fluids were used which had been obtained from eggs 24 hours after inoculation of a 10^{-2} dilution of infected allantoic fluid. The fluids were frozen and thawed 50 times, absorbed overnight at 4°C. with about 5 per cent washed human RBC, and centrifuged at 60,000 g for 30 minutes. The supernatant after this procedure has been designated soluble blocking antigen. Such preparations were water-clear fluids which did not agglutinate RBC or cause infection when inoculated allantoically in chick embryos. The designation soluble blocking antigen serves to distinguish such preparations from heated or urea-treated virus preparations in both of which the blocking antigen activity is sedimented by centrifugation at 60,000 g for 30 minutes.

It was thought possible that allantoic fluids which had not been frozen and thawed might contain blocking antigen in soluble form released from infected cells. Therefore, PR8 allantoic fluid was treated by three cycles of absorption with 5 per cent RBC and centrifuged at 60,000 g for 30 minutes in order to remove the infective and hemagglutinating virus. After this procedure blocking antigen activity could not be detected in the supernatant and mice inoculated with undiluted material did not develop immunity to the virus. Thus, it appears that there was no soluble blocking antigen demonstrable in PR8 allantoic fluid until it had been treated by freezing and thawing.

Properties of Soluble Blocking Antigen:

1. Strain Specificity.—Experiments were performed with the WS, PR8, and FM1 strains of influenza A virus to determine whether soluble blocking antigens obtained from them were strain-specific. Serological blocking experiments were performed with antigen prepared, as described above, from each strain and serum against each strain. Hemagglutination-inhibiting and neutralizing antibody titrations with each serum were carried out in the presence of each of the antigens. The test virus was homologous with the immune serum used in each case.

The results are shown in Table X and indicate that the soluble blocking antigen was strain-specific. For example, when tested with WS virus, the titer of the WS immune serum was unaffected by PR8 or FM1 soluble blocking antigen, but was definitely diminished when the serum was mixed with WS soluble blocking antigen.

With two closely related influenza A virus strains, it was found that the blocking reaction may be even more strain specific than the hemagglutination-inhibition reaction. The data from one such experiment are shown in Table XI. The blocking titrations were done in exactly the same way as the preceding

experiments, but the data are arranged so as to make comparison of the sensitivities of the two techniques as direct as possible.

2. *Reactivation of Neutralized Virus.*—In the experiments described above, it was shown that soluble blocking antigen can combine with homologous antibody and prevent virus of the same strain added later from being affected by the antibody. Experiments were undertaken to determine whether soluble

TABLE X

Specificity of Soluble Blocking Antigens from Different Influenza A Virus Strains

Antigen derived from strain	Hemagglutination-inhibition titer			Neutralization titer in mice		
	Immune serum			Immune serum		
	WS	PR8	FM1	WS	PR8	FM1
	vs. strain*			*vs.* strain‡		
	WS	PR8	FM1	WS	PR8	FM1
WS	**256**	4096	1024	**200**	10,240	660
PR8	1024	**512**	1024	3600	**4150**	755
FM1	1024	4096	**256**	5800	20,480	**<80**
Saline	1024	4096	1024	3200	13,700	—

* 4 hemagglutinating units.
‡ 10 MS$_{50}$.

TABLE XI

Specificity of Soluble Blocking Antigens from Closely Related Influenza A Virus Strains

Immune serum		Hemagglutination-inhibition titer *vs.*		Blocking titer of soluble blocking antigen prepared from	
		FM1	Wilfong	FM1	Wilfong
				units	*units*
FM1	R4	4096	2048	8	<4
"	R5	4096	2048	8	4
Wilfong	R6	1024	2048	4	8
"	R7	2048	2048	4	16

blocking antigen added to neutral serum-virus mixtures would displace active virus from the antibody.

A 10^{-4} dilution of WS allantoic fluid was mixed with equal volumes of diluted WS immune rabbit serum. The serum-virus mixtures were held at room temperature for about 20 minutes. Then a half volume of a WS soluble blocking antigen preparation or saline was added and the mixtures were held for 90 minutes at 4°C. The mixtures were then inoculated in 0.2 ml. volume in 11-day eggs.

The results of this experiment are shown in detail in Table XII and show that the addition of homologous soluble blocking antigen changed a neutral

mixture to an infective one. These results indicate that soluble blocking antigen can compete with intact virus particles for antibody.

In Table XIII the results of three further reactivation experiments are shown. The results are expressed in terms of the dilution of the immune serum in the mixture which showed 50 per cent embryo infectivity. They serve to demonstrate the strain specificity of the reactivation obtained with soluble blocking antigens. When the soluble blocking antigen was injected into the

TABLE XII

Reactivation of Influenza Virus in a Neutral Mixture by Soluble Blocking Antigen

Dilutions in mixture		Embryo infectivity after indicated addition to the mixture	
WS virus	WS immune serum	Saline	WS soluble antigen
$-log$	$-log$		
4.0	2.0	0/2*	0/3
4.0	2.5	0/3	3/3
4.0	3.0	3/3	2/2

* The numerator indicates the number of eggs infected and the denominator the number of eggs inoculated.

TABLE XIII

Strain Specificity of Reactivation of Influenza Virus by Soluble Blocking Antigen

Mixture of*		Infectivity endpoint‡ *in ovo*			
		After addition of soluble blocking antigen prepared from			
Virus	Serial dilutions of immune serum	Saline control	WS	PR8	FM1
WS	WS	1780	562	1780	1000
PR8	PR8	1410	1780	562	1780
FM1	FM1	132	234	234	74

* Held for 20 minutes before addition of soluble blocking antigen.

‡ Serum dilution which showed 50 per cent embryo infectivity.

allantoic cavity before a neutral serum-virus mixture was injected no reactivation occurred.

3. Complement-Fixation.—Since the influenza virus particle and the soluble complement-fixing antigen are known to react with different antibodies in complement-fixation tests, numerous experiments were carried out to determine the manner in which soluble blocking antigen preparations behaved in such tests.

Preliminary experiments showed that soluble blocking antigen, prepared as described above, gave non-specific fixation with rabbit immune sera due to

chick protein antibodies which the sera contained. Therefore, before preparing antigen the virus was purified and concentrated by two cycles of adsorption onto and elution from human RBC. The eluates were made in 0.1 M phosphate buffer, pH 7.2, and had hemagglutination titers from 12,800 to 25,600. They were frozen and thawed 50 times and centrifuged at 60,000 g for 30 minutes.

Such purified antigens did not fix complement with homologous or heterologous rabbit immune serum. They also did not fix complement after being held overnight at 4°C. with immune ferret sera which showed high titers of strain-specific fixation with intact virus particles in dialyzed infected allantoic fluids. However, antigens of this sort did show blocking of hemagglutination-inhibition by the same ferret sera when tested as described above. Such antigens prepared from PR8 and FM1 both showed a complement-fixing titer of 1:2 with serum from a patient convalescent from influenza A which contained much antibody against the soluble complement-fixing antigen. These findings indicated that, although soluble blocking antigen combined with antibody, the resulting complex did not fix complement to a detectable extent with ferret or rabbit immune sera. In addition, soluble blocking antigen failed to block the complement-fixing reaction between ferret immune serum and intact virus particles. The low titer obtained with human convalescent serum may have been due to the presence of a small amount of soluble complement-fixing antigen.

4. *Precipitin Reactions.*—With purified and concentrated intact virus preparations, and rabbit immune sera absorbed with chick embryo material, it was not possible to show specific precipitation by the capillary technique. Also, no reaction with precipitins was demonstrable in similar tests with such concentrated materials after they had been frozen and thawed 50 times.

5. *Antigenicity.*—The antigenicity of soluble blocking antigen was determined by measuring the antibody response of rabbits and the amount needed to immunize mice.

Allantoic fluids were collected from 10-day embryos incubated for 24 hours after inoculation with 0.2 ml. of a 10⁻³ dilution of PR8 allantoic fluid. The fluids were pooled, dialyzed, and an aliquot was frozen and thawed 50 times. The frozen and thawed material and the control fluid were then absorbed with 20 per cent human RBC and centrifuged twice at 60,000 g for 30 minutes. Blocking antigen activity was present in the supernatant from the frozen and thawed fluids, but not in that from the control fluid, and neither caused hemagglutination. Ten ml. volumes of the supernates were injected intravenously in rabbits.

The results of titrations on sera obtained 20 days after the injection are shown in Table XIV. It appeared that the soluble blocking antigen had provoked a somewhat greater antibody response than the absorbed and centrifuged control fluid, but this was much less than that obtained after the injection of untreated PR8 allantoic fluid.

To obtain a better measure of the antigenicity of soluble blocking antigen

the minimum amount required to immunize mice against infection with PR8 virus was determined by means of antigenic extinction titrations (22, 23) carried out as described above. A preliminary experiment showed that soluble blocking antigen had an immunizing activity about 30,000-fold less than that of the PR8 allantoic fluid from which it was prepared.

It was thought that, though repeated freezing and thawing released antigenic material from the virus particle, it might also degrade such material, and that antigenic material which had just been released from the virus particle might still immunize. Therefore, an attempt was made to measure the immunizing capacity of soluble blocking antigen prepared by freezing and thawing virus only a few times. A dialyzed allantoic fluid was divided into two portions which

TABLE XIV

Antigenicity in Rabbits of Soluble Blocking Antigen from Influenza Virus

Immunized with	Rabbit No.	Antibody titers vs. PR8	
		Hemagglutina-tion-inhibition	Neutralization*
Soluble blocking antigen (PR8)	8	128	10
	9	256	18
Control material (not frozen and thawed)‡	10	32	<2.5
	11	32	<2.5
Allantoic fluid (PR8)	a§	5,120	—
	b§	10,240	—

* Determined against 50 MS_{50} in mice.

‡ PR8 allantoic fluid absorbed with RBC and centrifuged twice at 60,000 g.

§ Comparable serum collected at 15 days.

were frozen and thawed 2 and 10 times, respectively. They were then adsorbed 3 times with 5 per cent human RBC, and finally centrifuged at 60,000 g for 30 minutes. At various stages of the process the fluids were titrated for egg infectivity, hemagglutinating activity, blocking antigen activity, and capacity to immunize mice.

The results are shown in Table XV. The data for the fluid, frozen and thawed twice, indicate that the immunizing potency decreased more or less in parallel with the decrease in the infectivity and hemagglutination titers. After 10 freezings and thawings and 3 adsorptions with RBC, a fluid was obtained which did immunize mice at low dilution, though it had no measurable infectivity or hemagglutination titer. However, all the immunizing activity was sedimented by centrifugation at 60,000 g for 30 minutes, but the blocking antigen titer remained unaffected. These findings indicate that soluble blocking

antigen is not capable of inducing significant immunity to the homologous virus in mice.

6. Blocking Antigens from Other Viruses.—Similar soluble blocking antigen preparations were made from the Lee strain of influenza B, Newcastle disease, and mumps viruses by 50 alternate freezings and thawings of dialyzed infected allantoic fluids. In each case these were absorbed with RBC and centrifuged at 60,000 g as described above. Definite blocking of hemagglutination-inhibition by homologous immune serum was obtained with each preparation. The results of these experiments show that soluble blocking antigen can be obtained from various viruses in the influenza-Newcastle-mumps group.

TABLE XV

Antigenicity in Mice of Soluble Blocking Antigen from Influenza Virus

Dialyzed PR8 allantoic fluid			Virus titers		Antigen titers	
Frozen and thawed	Absorbed with RBC	Centrifuged at 60,000 g	*In ovo* EID$_{50}$	Hemagglut- ination	Blocking antigen*	Antigen extinction‡
cycles	*cycles*	*cycles*	*−log*	*−log*	*units*	*−log*
0	0	0	7.50	3.6	—	4.6
2	0	0	5.75	3.3	—	3.7
2	3	0	4.25	<0.6	2	1.8
2	3	1	1.50	<0.6	<2	0.6
10	0	0	<1	0.9	—	2.4
10	3	0	<1	<0.6	>32	1.6
10	3	1	<1	<0.6	>32	0

* Determined by the hemagglutination-inhibition procedure with homologous immune serum and virus.

‡ Determined in mice with 400 MS$_{50}$ of PR8 virus, after two intraperitoneal injections of diluted antigen.

7. Interference.—One ml. volumes of dialyzed PR8 allantoic fluid which had been frozen and thawed 50 times were injected allantoically into 10-day embryos. From 1 to 24 hours later about 10 EID$_{50}$ of PR8 virus was inoculated. The allantoic fluids were harvested and titrated individually after 28 or 42 hours of incubation. The hemagglutination titers were not different from those obtained with control eggs injected initially with saline. However, profound reductions in hemagglutination titer were demonstrable with eggs injected first with PR8 virus heated at 56°C. for 30 minutes and then inoculated with infective virus as above. This evidence indicated that PR8 blocking antigen preparations did not act as interfering agents in the allantoic sac.

8. Some Physical and Chemical Properties of Blocking Antigen.—The blocking activity of 50 times frozen and thawed, dialyzed PR8 allantoic fluid was

destroyed by heating at 56°C. for 30 minutes and was partly eliminated by holding at 37°C. overnight. Blocking activity was precipitated in half saturated ammonium sulfate solution.

Similar frozen and thawed virus preparations in 0.1 M phosphate buffer, pH 7.2, were incubated with various enzymes and the blocking antigen activity was then determined. The enzymes used were all highly purified, crystalline preparations.[2] Some typical results are shown in Table XVI. Trypsin and chymotrypsin in low concentration destroyed blocking activity while desoxyribonuclease and ribonuclease did not, even when used in high concentration.

These findings are consistent with the idea that the antigen is protein in nature. The fact that chymotrypsin and trypsin were about equally active

TABLE XVI

Effect of Enzymes on Blocking Antigen from Influenza A Virus, PR8

Crystalline enzyme	Concentration of enzyme	Hrs. at 37°C.	Trypsin inhibitor added after incubation	Blocking antigen titer*
	γ/ml.		γ/ml.	units
Trypsin	4‡	19	6	0
"	400	20	600	0
Chymotrypsin	4‡	18	500	0
"	400	18	2,000	0
Ribonuclease	400	18	—	6
Desoxyribonuclease	400§	18	—	6
Trypsin inhibitor	200	18	—	6
Saline control	—	18	—	6

* Determined by the hemagglutination-inhibition procedure with homologous immune serum.

‡ No effect at 4 hours on blocking antigen activity.

§ MgSO₄ added to final concentration, 0.04 M.

in destroying blocking activity suggests that the antigen may be a basic protein.

DISCUSSION

The results of this study indicate that influenza virus particles can be disrupted by repeated freezing and thawing. Such disrupted virus particles are not infective, do not agglutinate chicken or human red blood cells, and have no enzymic activity. In addition, the characteristic spherical structure

[2] The trypsin was crystallized once. The desoxyribonuclease was crystalline and free of proteolytic activity. The trypsin inhibitor was crystallized from soybean. All these were obtained from Worthington Laboratories, New Jersey. The chymotrypsin had been five times recrystallized from ammonium sulfate and once from alcohol and was supplied by Dr. M. Kunitz. The ribonuclease was crystalline and was supplied by Dr. M. McCarty.

of the virus as visualized by means of the electron microscope is lost. The freezing and thawing procedure was effective in causing disruption whether $-26°C$. followed by cold water, or a CO_2-alcohol mixture at $-65°C$. and a $37°C$. water bath were used. The widespread impression that freezing and thawing has little or no effect on influenza virus seems to have arisen because relatively few freeze-thaw cycles were used or because the virus was protected by some component of the suspending medium (24), as is indicated by results secured with undialyzed allantoic fluid in this study. In this connection, freezing and thawing in distilled water or in dilute saline has been found to be an effective method of rupturing bacteriophage T_2 (25).

To detect antigenic material from the disrupted virus particle, it was necessary to devise a special technique for the measurement of antigen concentration. It was found that the antigen was capable of blocking both *in vitro* and *in vivo* reactions between the intact virus particle and specific homologous antibody. A similar procedure was employed earlier to demonstrate blocking of virus neutralization by a soluble substance in bacteriophage filtrates as well as reactivation of a neutral mixture of phage and immune serum (26). In addition, the blocking of antigen-antibody union and of precipitation was used previously to demonstrate the degraded LS antigen of vaccinia virus (27). The present work indicates that blocking of the hemagglutination-inhibition or virus neutralization reaction is useful for detecting and measuring noninfective or degraded antigens from viruses belonging to the influenza-Newcastle-mumps group. The technique may perhaps be applicable also to other viruses against which potent antisera can be made.

Disruption of the influenza virus particle releases antigen in a form which is much less readily sedimented than the intact virus particle. For convenience this has been designated, soluble blocking antigen. The soluble blocking antigen seems to be closely related to, if it is not identical with, antigens in the intact virus particle for it appears to be highly strain-specific and reacts with those antibodies which cause inhibition of hemagglutination and neutralization of virus infectivity. This is in sharp contrast to findings with the soluble complement-fixing antigen, which is not strain-specific and reacts with antibodies distinct from those directed against the intact virus particle. Further, when present in sufficient concentration, the soluble blocking antigen can displace infective virus particles from antibody in a manner comparable to that of inactivated virus and lead to the so called reactivation phenomenon (28). Soluble blocking antigen, like soluble complement-fixing antigen, does not immunize mice or rabbits effectively and so resembles the haptens of classical immunology.

The ratio between antigen activity and complement-fixing activity with human influenza A convalescent serum varies widely in different preparations, and it appears that the two activities are attributable to different substances.

The low and inconstant complement-fixing activity of soluble blocking antigen preparations may be due to the fact that freezing and thawing releases some soluble complement-fixing antigen which is closely associated with the virus particle (7).

Treatment of influenza A virus with ether (8) seems to release soluble complement-fixing antigen; nevertheless, the virus particle is not greatly affected since it will still agglutinate red blood cells and fix complement with ferret and rabbit immune serum and is enzymically active (29). Electron micrographs indicate that after ether treatment the virus particle is somewhat reduced in size (9). One interpretation of the findings is, that ether treatment releases from the virus particle materials containing soluble complement-fixing antigen, but leaves the particle otherwise intact, whereas, repeated freezing and thawing disrupts the particle and releases a different antigenic material. The soluble blocking antigen released by freezing and thawing is probably protein in nature and probably forms part of the surface and possibly also the deeper structure of the intact virus particle.

SUMMARY

1. The hemagglutinating capacity, enzymic activity, and infectivity of several influenza viruses were destroyed by repeated freezing and thawing of dialyzed allantoic fluids containing them.

2. Influenza virus degraded by freezing and thawing, by treatment with 5 M urea, or by heating at 65°C. still combined with homologous antibody and was demonstrable by blocking of the hemagglutination-inhibition and virus neutralization reactions.

3. After 50 cycles of freezing and thawing, much of the blocking antigen activity was not sedimented by centrifugation at 120,000 g for 2 hours, and electron microscopy showed complete disruption of the virus particles. So called soluble blocking antigen was obtained from four strains of influenza A, the Lee strain of influenza B, mumps, and Newcastle disease viruses.

4. Soluble blocking antigens from influenza A viruses were highly strain-specific; gave little or no reaction in complement-fixation tests; stimulated but little antibody production in rabbits and did not induce immunity in mice; caused reactivation of infective virus in neutral mixtures of homologous virus and immune serum.

5. Repeatedly frozen and thawed influenza virus preparations did not interfere with the propagation of infective virus in the allantoic sac. The blocking antigen activity they contained was precipitated by half saturated ammonium sulfate, destroyed by trypsin, chymotrypsin, or heating at 56°C. for 30 minutes, but was unaffected by desoxyribonuclease or ribonuclease.

6. These findings are in accord with the view that soluble blocking antigen obtained from influenza virus particles on disruption by repeated freezing and

thawing is protein in nature and represents the essential antigenic material of the intact virus.

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EXPLANATION OF PLATE 10

FIG. 1. The effect of repeated freezing and thawing on PR8 influenza virus particles. Electron micrographs of chromium-shadowed preparations.

(a) Partly purified influenza virus.

(b) Preparation of same virus as shown in part (a) after 50 freeze-thaw cycles.

(c) Same preparation shown in part (b). Part of one of the large masses seen here and there.
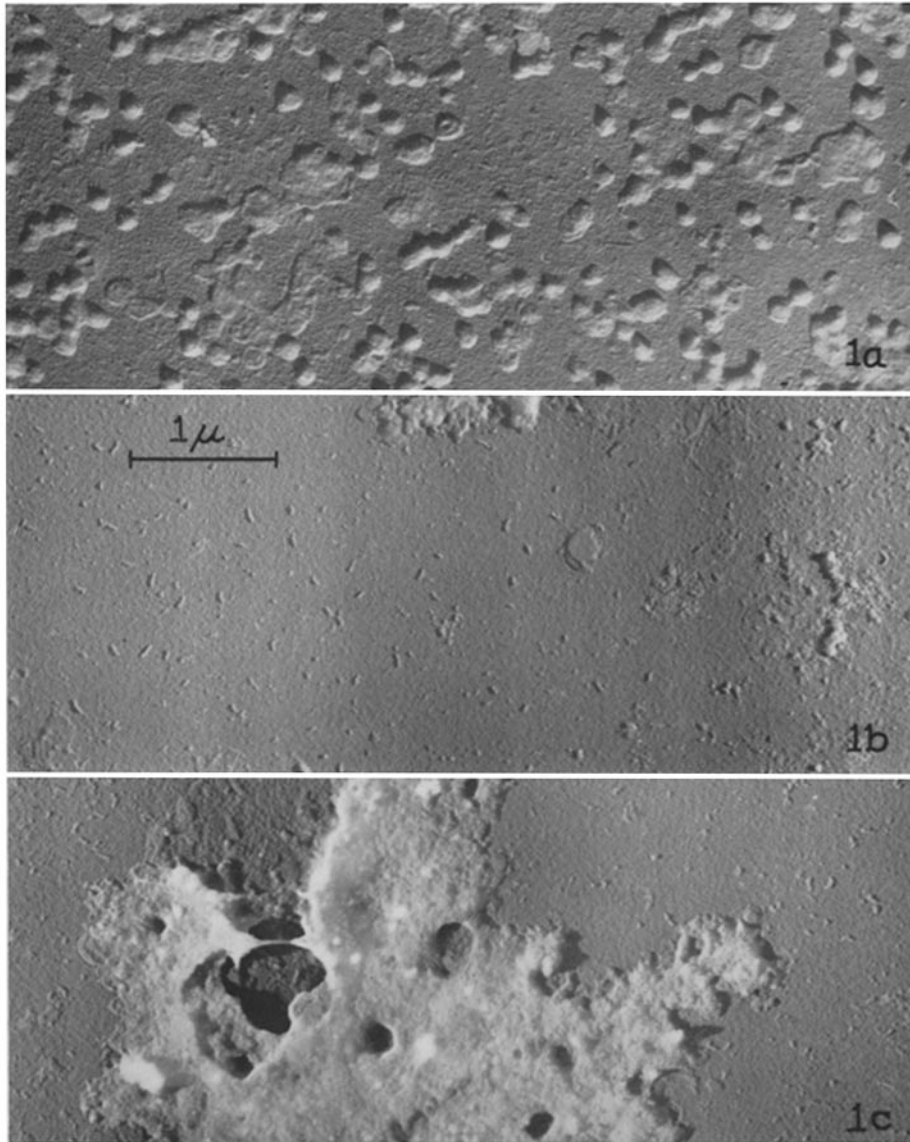
(Tyrrell and Horsfall: Disruption of influenza virus)