STUDIES ON THE NATURE OF THE VIRUS OF INFLUENZA

I. The Dispersion of the Virus of Influenza A in Tissue Emulsions and in Extra-Embryonic Fluids of the Chick

By LESLIE A. CHAMBERS, Ph.D., and WERNER HENLE, M.D.

(From the Department of Pediatrics and the Eldridge Reeves Johnson Foundation for Medical Physics, School of Medicine, University of Pennsylvania, Philadelphia, and the Children's Hospital of Philadelphia)

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While certain properties of the influenza virus have been inferred from numerous immunological and epidemiological studies published in recent years, little definite information as to its physical nature has been obtained. Elford, Andrewes, and Tang (1), as a result of ultrafiltration studies of mouse lung emulsions, concluded that the infectious agent has a diameter of 80 to 120 mμ. Elford and Andrewes obtained a value of 87 to 99 mμ by centrifugation (2). This relatively high order of size was supported by Hoyle and Fairbrother (3) when they were able to sediment most of the infectivity from tissue emulsions by the use of a centrifuge running at a speed of 15,000 R.P.M. They observed particles of the indicated size range by dark field examination of the infectious sediments.

Recently it has been shown that the allantoic and amniotic fluids from 10 to 12 day chick embryos inoculated with porcine strains of influenzal virus gave high infectivity titers in spite of their low protein content (4). The same was observed for the human strains of influenza A simultaneously by several investigators (5–7). Preliminary studies of these fluids have led us to the conclusion that the size of the virus may be much smaller than believed heretofore, a tentative estimate of the diameter being 10 mμ or less (8, 9).

The analytical diffusion method employed by Bourdillon (10) on mouse lung virus indicated that a very small amount of the infectivity is carried by particles having a diffusion constant corresponding with that of a spherical body as small as 6 mμ in diameter. The same technique applied to the infected extra-embryonic fluid showed a very large proportion, as much as half, of the pathogenic material to be associated with 6 mμ particles.

Additional studies have been undertaken both on the virus present in tissue emulsions, and on that in the egg fluids. These have confirmed our original surmise that the size of the infectious unit is only a small fraction of that indicated by former studies (1–3), and have led to certain successful purification procedures.

This paper contains the results of a study of the dispersion of the influenza

virus in filtrates from infected lung tissue, and in the extra-embryonic fluids of infected chick embryos. An explanation of the apparent wide discrepancy in the sizes of the infectious units in the two types of preparation has been sought.

Methods and Materials

Source of Virus.—Mice were infected under light ether anesthesia by intranasal instillation of 0.05 ml. of a 10 per cent suspension of infected mouse lung. After 3 to 4 days when the mice had become moribund, the lungs were harvested and stored at −10°C. until used. The period of storage was usually not longer than 24 hours. The lungs were ground with sterile sand and pyrex powder and suspended in 5 to 10 times their weight of sterile broth. After removal of the larger particles in the horizontal centrifuge, the supernatant liquid was passed through a Berkefeld N filter and the filtrate used as starting material.

Egg-adapted influenza virus was propagated in the following manner (6):—Suitable dilutions of infected allantoic fluid were injected (0.5 ml.) through a small opening in the shell above the air sac into the allantoic sac of 9- to 10-day-old embryos. The infected eggs were then incubated at 39°C. for 24 to 48 hours depending on the dilution used as inoculum. When approximately 10 to 15 per cent of the embryos had died of the infection, the surviving eggs were placed in the refrigerator for 1 to 2 hours following which the allantoic fluid was harvested (in earlier experiments the amniotic fluid was also included). The cooling of the eggs prevents bleeding into the allantoic sac during harvest, and the amount of fluid appears to be increased somewhat by this procedure. After centrifugation in the Swedish angle centrifuge at 5000 R.P.M. for 30 minutes, the supernatant fluid was used as starting material for experimentation.

Most of the experiments were conducted with the WS (11) and F-12 strains(12) of influenza A, but some confirmatory evidence was obtained also with the PR-8 (13) and Melbourne strains (14).

Titration.—Serial tenfold dilutions of the virus preparation were made in broth and 0.05 ml. of the various dilutions instilled intranasally under light ether anesthesia into each of 4 mice. Animals found dead were autopsied for typical pulmonary involvement and recorded as D_3 = died on the 3rd day, etc. Surviving mice were killed by ether on the 10th or 11th day and examined for lung lesions which were graded in the usual manner as 4 = lung totally consolidated; 3 = ¾ ; 2 = ½; 1 = ¼; and ± less than ¼ consolidated.

Electron Microscopy.—Aqueous suspensions of the particles sedimented by ultracentrifugation were prepared for electron micrographic examination in the following manner. A small drop of the suspension was placed on a very thin collodion membrane supported by a 3 mm. disc of fine mesh screen. After about 1 minute of contact the bulk of excess fluid was removed with a pipette or filter paper, and the mount was permitted to dry in air.

Initial studies were made with the RCA type A electron microscope and one of the pictures taken with this instrument is reproduced in this paper. Subsequent studies were made with the RCA type B microscope in the laboratories of the RCA Manufacturing Company, Camden, New Jersey.

High Speed Centrifugation.—A mechanically driven, high speed centrifuge was used in these experiments, the construction of which has been reported elsewhere (15). For the concentration of the influenza virus from mouse lung preparations 30 minutes at 25,000 R.P.M., *i.e.*, a gravitational force of 50,000 *g*, was found sufficient. Further details, particularly in connection with the allantoic fluid virus are given in the text. The preparation of particulate components from normal mammalian organs has been described (16, 17).

TABLE I

Sedimentation of Influenza Virus from Mouse Lung Suspension and Extra-Embryonic Fluid

Virus	Dilution	Original*	30 min. 25,000 R.P.M.	
			Supernatant fluid	Sediment
WS-101 mouse lung	10^{-2}	D_3 D_3 D_4 D_4	4 4 4 1	D_3 D_3 D_4 D_4
	10^{-3}	D_4 D_4 D_7 0	3 2 2 2	D_4 D_4 D_6 D_7
	10^{-4}	D_5 D_6 D_6 D_6	1 0 0 0	D_4 D_4 D_5 3
	10^{-5}	D_9 D_9 3 0	0 0 0 0	D_7 D_9 3 2
	10^{-6}	4 2 2 2		D_{10} 2 1 1
	10^{-7}	D_8 2 0 0		3 0 0 0
	10^{-8}	0 0 0 0		0 0 0 0
F-12-33 allantoic fluid	10^{-4}	D_5 D_5 D_5 D_8	D_6 D_8 D_8 D_8	D_5 D_5 D_6 D_9
	10^{-5}	D_6 D_6 D_7 D_7	D_6 D_7 D_8 D_8	D_5 D_6 D_7 3
	10^{-6}	D_8 D_9 D_{10} 3	D_8 D_9 3 3	3 3 3 2
	10^{-7}	4 3 3 0	3 2 1 ±	3 1 1 ±
	10^{-8}	2 2 ± 0	D_{10} 1 ± 0	1 0 0 0
	10^{-9}	1 1 0 0	0 0 0 0	0 0 0 0

* Original of mouse lung suspension = Berkefeld filtrate.
Original of allantoic fluid preparation = supernatant fluid after 30 minutes 5,000 R.P.M.

EXPERIMENTAL

Relative Sedimentation in the Concentration Centrifuge

When Berkefeld or Mandler filtrates of infected mouse lungs were run in the concentration centrifuge at 25,000 R.P.M. (50,000 times gravity) for 30 minutes, sedimentation of the infectious agent was usually about 99.9 per cent complete as indicated by subsequent mouse titrations. Berkefeld filtrates from 10 to 20 per cent lung emulsions were used in all cases and the experiment has been repeated frequently using the WS,PR-8, Melbourne, an F-12 strains of influenza A. Since the results were approximately the same in all cases, Table I will serve to illustrate the completeness of the concentration for all the strains under the arbitrarily selected gravitational force.

In comparison with the almost complete sedimentation of the virus from tissue emulsions Table I also includes a selected example of the results obtained using the extra-embryonic fluid of infected chicks under identical conditions.

Concentration of the virus was usually less complete than was the case with lung emulsions. Frequently the washed sediment and the supernatant liquid were infectious in about the same dilution, indicating that a large part of the virus remained in suspension. In a few instances the titer of the resuspended sediment was higher than that of the supernate and occasionally the reverse was true. Such data are summarized in Table II. This varia-

TABLE II

Distribution of Virus Resulting from Ultracentrifugation of Infected Allantoic Fluids and Mouse Lung Filtrates

Strain	Passage	Inoculum	Hour of harvest	50 per cent mortality end point		
				Original	30 min. 25,000 R.P.M.	
					Supernatant fluid	Sediment 2 times washed
WS allantoic fluid	36	10^{-1}	24 + 48	$10^{-5.3}$	$10^{-3.5}$ or more	—
	38	10^{-3}	48	$10^{-6.0}$	$10^{-5.5}$ or more	$10^{-3.8}$
	38	10^{-2}	48	$10^{-4.5}$	$10^{-4.5}$	$10^{-4.8}$
	44	10^{-1}	24	$10^{-6.3}$	$10^{-5.0}$	$10^{-5.7}$
	45	10^{-1}	48	$10^{-5.5}$	$10^{-5.0}$	—
	45	10^{-3}	48	$10^{-5.6}$	$10^{-4.5}$	—
F-12 allantoic fluid	28	10^{-1}	24	$10^{-6.5}$	$10^{-5.5}$	—
	28	10^{-3}	48	$10^{-7.2}$	$10^{-5.3}$	$10^{-7.5}$
	28	10^{-4}	72	$10^{-7.0}$	$10^{-4.8}$	—
	33	10^{-3}	48	$10^{-6.0}$	$10^{-5.5}$	—
	33	10^{-2}	36	$10^{-5.5}$	$10^{-3.2}$	$10^{-4.9}$
	33	10^{-1}	18	$10^{-7.5}$	$10^{-5.5}$	$10^{-5.5}$
	33	10^{-1}	24	$10^{-6.3}$	$10^{-6.0}$	$10^{-4.6}$
WS mouse lung suspension	83		72	$10^{-4.3}$	$10^{-1.5}$	$10^{-4.4}$
	85		72	$10^{-4.0}$	$<10^{-2.0}$	$10^{-3.8}$
	101		72	$10^{-5.2}$	$<10^{-2.0}$	$10^{-5.0}$

tion from sample to sample was probably due to variation in the degree of virus aggregation as will be shown in a subsequent section of this paper.

The examples given are illustrative of the fact that the infectious property in the extra-embryonic fluids is less readily concentrated by centrifugation than is the same agent when present in tissue extracts. Therefore, it appears probable, that at least part of the virus in the former case is associated with particles of a smaller size than those with which it occurs in tissue emulsions.

Comparison of Sedimentable Material from Infected and Normal Mouse Lung

Because of the apparently high concentration of the virus in material centrifuged from lung filtrates, it was expected that qualitative differences could be

found between it and material recovered in a similar manner from the lungs of uninfected mice.

Three large batches of lungs from mice infected with the WS strain of influenza A and two from normal animals were emulsified in 0.85 per cent salt solution, and, after brief low speed centrifugation, passed through Berkefeld or Mandler filters. The filtrates were subjected to a centrifugal force of about 50,000 *g* (25,000 R.P.M.) for 30 minutes and the supernatant fluids discarded. Translucent, gelatinous pellets appeared in all the centrifuge tubes. These were resuspended in salt solution, run in the Swedish angle centrifuge for about 10 minutes to remove large aggregates, and returned to the ultracentrifuge. After washing the sediments twice in this manner, they were finally resuspended in water, dialysed thoroughly in distilled water, and dried from the frozen state. When the dry preparations were weighed, it was found that the yield per lung was about the same in the two cases.

TABLE III

Comparative Compositions of Particles from Normal and Infected Lungs

	Origin of preparations	
	Normal lungs	Infected lungs
Total N, *per cent*	8.38	8.58
Phosphorus, *per cent*	1.21	1.22
Purine N, *per cent*	0.13	0.08
Ether extractable, *per cent*	14.3	15.4
Total lipids, *per cent*	42.0	48.0
Ash, *per cent*	5.9	5.1
Ribose	Present	Present
Desoxyribose	Absent	Absent
Density	1.22	1.21

Weighed samples of the various preparations were analyzed for total nitrogen. In addition, qualitative color tests for carbohydrates were carried out. The amounts of ether and alcohol-ether extractable substances were determined. Densities were estimated pycnometrically on samples resuspended in water. Table III summarizes the results obtained.

The infectious and normal materials were not significantly different in any particular of composition within the scope of analysis. Both appear to contain protein, nucleic acid, mono- and diamino-phosphatides, and some ash. The nucleic acid is apparently of the ribose type since the Feulgen and diphenylamine tests were negative while the Bial test was positive. Spectral absorption curves showed no well defined bands in the visible region although the suspensions appeared distinctly yellowish.

The analytical results are strikingly similar to those obtained by Claude (18) from particles 50 to 150 mμ in diameter isolated from chick embryos, chick tumors, mouse embryos, and mouse sarcomas, by a centrifugation procedure similar to that which we employed. He has suggested that the particles

may be mitochondria. We have shown elsewhere that the normal particles possess a considerable degree of organ specificity (16, 17).

Freshly sedimented undried mouse lung concentrates from either normal or infected tissue are visible in the dark field microscope as highly refracting bodies without marked asymmetry. Their staining properties determined by centrifugal packing of the particles after addition of the dyes to the suspensions, lend support to Claude's inference that they are mitochondria. They do not stain typically with either cytoplasmic or nuclear stains, but are blackened by osmic acid and absorb strongly Janus green B, a stain reputed to be specific for mitochondria.

No particles resolvable under the light microscope were present in any of the preparations. Electron micrographs were made of both normal and infectious particles through the courtesy of Dr. T. F. Anderson in the laboratories of the RCA Manufacturing Company in Camden, New Jersey. The materials were thereby shown to consist of roughly spherical, sharply defined particles of uniform density, ranging in diameter between 50 and 150 mμ (Fig. 1). No differences between the infectious and non-infectious preparations could be demonstrated.

While it cannot be stated that the normal and infectious particles from mouse lungs are identical, it is apparent that they are alike within the accuracy of the techniques used and the scope of the analytical comparison. This being the case three possibilities present themselves: (*a*) that some relatively minor change in a component of normal lung cells results in the acquisition of the infective property by the particle, or (*b*) that we are dealing with a mixture of infectious and non-infectious units, the former comprising so small a fraction of the total as to produce only a minor effect on the over-all composition, or (*c*) that the infectious unit is much smaller than the normal lung particles to which it is strongly adsorbed.

Since infectivity is known to be associated with less easily sedimented particles in the case of the egg fluids, the view that the mitochondria from lung cells adsorb a much smaller virus particle and thereby become infective units seems to be the most plausible.

Absorption of Virus from Extra-Embryonic Fluids with Normal Lung Particles

That the virus *can* be absorbed by normal lung particles was demonstrated by an experiment the results of which are given in Table IV.

A sample of extra-embryonic fluid containing the virus in a titer of $10^{6.3}$ was mixed with a concentrated suspension of the washed normal particles. Washed particles derived from about 5 gm. of wet tissue were resuspended in 8 ml. of the fluid. After 30 minutes at room temperature, the mixture was centrifuged at 25,000 R.P.M. for 30 minutes together with a tube containing untreated fluid. The sedimented particles were resuspended, washed, and diluted in broth to the original volume of the mixture.

Infectivity titrations of the unabsorbed fluid, the supernatant fluids, and the washed sediments showed that the particles have absorbed most of the virus from the extra-embryonic fluids while centrifugation alone left a marked amount of virus in suspension. The particles, as a result, were able to function as infective units.

The ability to absorb the virus strongly is not a specific property of lung particles since, as the table indicates, derivatives of other organs, *e.g.* the liver and kidney, also become infectious at the expense of egg fluid virus under similar experimental conditions. Furthermore, red blood cells from the chick combine strongly, although not as completely, with the virus and behave as infectious units when inoculated intranasally into mice (*cf.* Hirst (19) and Hare (20)).

TABLE IV

Absorption of Influenza Virus from Extra-Embryonic Fluid by Normal Organ Particles

Dilution of virus	Original fluid	Supernatant fluid after absorption with			Infectivity of normal particles after adsorption of virus	
		—	Mouse lung particles	Mouse liver particles	Mouse lung*	Mouse liver*
10^{-1}			D_7 D_7 D_8 D_9	D_5 D_9 D_{10} 3		
10^{-2}			D_9 3 3 3	D_9 D_{11} 3 2		
10^{-3}			2 2 1 1	3 2 2 2	D_6 D_6 D_7 D_{10}	D_5 D_5 D_6 0
10^{-4}			± 0 0 0	2 1 0 0	D_6 D_6 D_8 D_{10}	D_6 D_8 D_9 D_{11}
10^{-5}	D_5 D_6 D_7 D_7	D_{10} D_{10} 4 4	± 0 0 0	± 0 0 0	D_9 D_{10} 3 3	D_{10} 3 3 2
10^{-6}	D_7 D_7 D_{11} 3	3 2 1 0	0 0 0 0	0 0 0 0	2 2 2 0	D_{10} 3 2 1
10^{-7}	3 3 2 2	2 2 2 0				
10^{-8}	2 2 0 0	2 0 0 0				

* The untreated organ particles did not produce lesions.

Absorption experiments such as these do not provide absolute proof that the large infectious bodies (100 mμ diameter) derived from infective mouse lungs are passive carriers of a relatively small elementary infectious unit. On the other hand, it is evident that such bodies are present in the tissue emulsions and that they can absorb the virus strongly. Hence, there is strong presumptive evidence that any small virus particles occurring in tissue might become associated with the larger bodies previous to, or at the time of, emulsification. The findings indicate that a part, if not all, of the large infectious bodies from tissue are normal cell constituents acting as passive carriers of the pathogenic agent.

Agglutination and Neutralization of the Infectious Lung Particles

Further evidence that the virus is passively carried on tissue particles not normally infectious was obtained by observing the agglutinative and neutralizing effects of antisera against normal lung particles and against egg fluid virus, on suspensions of infectious lung particles. The first mentioned

serum produced agglutination, but no neutralization of infectivity, and most of the virus was carried down with the agglutinate. The latter serum caused neutralization of the virus but failed to agglutinate the particles with which it was associated (Table V). Sera against the infectious particles produced both agglutination and neutralization. On the other hand, specific flocculation was observed when infected allantoic fluid was mixed with either rabbit serum against PR-8 mouse lung or human convalescent serum (6), while antiserum *vs.* influenzal allantoic fluid gave precipitates with both infected and normal fluids.

These clear-cut differences in serological behavior indicate that the infectious unit from allantoic fluid is less complex in make-up than is the unit derived

TABLE V

Neutralization and Agglutination of Influenza A Virus from Mouse Lung Suspension

Serum	Agglutination		50 per cent infectivity end point	
	Particles from		Agglutinate	Supernatant fluid
	PR-8 lung	Normal lung		
Rabbit anti-PR-8 mouse lung 3....	++++	++++	0	0
Rabbit anti-normal mouse lung 24 .	++++	++++	$10^{-6.0}$	$10^{-4.8}$
Rabbit anti-normal mouse kidney 25.....	++	+	$10^{-5.0}$	$10^{-5.0}$
Normal rabbit serum 24*	−	−	$10^{-4.0}$	$10^{-5.8}$
Rabbit anti-PR-8 allantoic fluid 17.....	−	−	0	0

++++ = all particles agglutinated.
+++-+ = various degrees of intermediate agglutination.
− = no agglutination.
0 = virus completely neutralized.
* Serum taken before immunization.

from infected tissue. The infective lung particles possess an antigenic structure comparable to that of particles from normal lungs. On this the antigenic structure of the virus as it occurs in allantoic fluid, appears to be superimposed. Therefore, information concerning the infectious agent can probably best be obtained by study of the unit present in extra-embryonic fluids of the chick.

The Dispersion of the Infectious Unit in Extra-Embryonic Fluids

Mixed allantoic and amniotic fluids of 10 to 12 day chick embryos contain rather low concentrations of protein, usually not more than about 0.4 mg. per ml. When collected with care, the preparations show only very slight turbidity and are almost free from red cells. A relatively large amount of uric acid is always present, but this can be removed completely by dialysis against 0.85 per cent NaCl solution buffered with phosphate at pH 7.0 without appreciable

loss of infectivity (Table VI). Some decrease in activity is produced by dialysis against distilled water.

The degree of physical dispersion of the virus varies from one lot of fluid to another. As pointed out, centrifugation in a field of 50,000 *g* resulted frequently in approximately equal infectivity titers in the supernatant fluid and the resuspended sediment. Such a division suggests a degree of inhomogeneity which could result either from partial aggregation or from association of a part of the virus with larger, easily sedimented bodies or debris.

Sedimentation of the virus was generally more complete from fluids harvested 36 to 72 hours after inoculation of the eggs than in those taken after 12 to 24 hours. Since the virus titer usually attains its maximal level in less than 24 hours, depending upon the dose used as inoculum, it seemed possible that a concentration factor might be involved. Therefore, sedimentation

TABLE VI

Influence of Dialysis on Infectivity of Virus

Dilution of virus	F-12-33 (extra-embryonic fluid)		
	Original	Dialyzed against	
		Water	0.85 per cent NaCl
10^{-5}	D_7 D_7 D_8	D_8 D_9 D_{11}	D_5 D_5 D_6
10^{-6}	D_8 D_8 D_{11}	D_9 D_9 D_9	D_7 D_8 3
10^{-7}	D_{11} 3 3	3 2 1	D_9 3 2
10^{-8}	3 2 2	1 1 0	D_{11} 2 1
10^{-9}	3 2 0	1 0 0	2 2 0

experiments were carried out using older egg fluids diluted with salt solution. The results indicated that some dispersion of virus aggregates was accomplished since a smaller proportion of the total infectivity was sedimented after dilution.

The sediments which were thrown down in a field of 50,000 *g* were never completely sedimentable by recentrifugation at the same speed after suspension in water or salt solution. Indeed the wash water from the third successive precipitation of the material frequently contained as many infectious units per cubic centimeter as did that from the first. Such an occurrence may be explained as a continuing redispersion of large aggregates.

A number of extra-embryonic fluids were vibrated for 10 or 15 minutes in the treatment vessel of a magnetostriction oscillator driven at 9000 cycles per second. This procedure is known to disperse aggregates of bacteria and has been shown to act the same way on impure preparations of pepsin (21). Fluids which showed relatively large concentrations of virus in the centrifuge sediments showed increases of 10- to 30-fold in infectivity when dilutions were

made immediately following vibration (Table VII). On the other hand, fluids which gave relatively small precipitates gave no increase in titer as a result of the sonic treatment.

A similar effect was produced in certain fluids by heating the material for 10 minutes in a 50°C. water bath. The heat treatment increased the infectivity titer of some of the preparations and reduced the proportion of virus sedimentable at 25,000 R.P.M.

TABLE VII

Influence of Sonic Vibration on 50 Per Cent Mortality Dose in Relation to Sedimentation

Strain and passage	50 per cent mortality end point		
	Original fluid	Vibrated	Supernatant fluid* 30 min. 25,000 R.P.M.
WS-45	$10^{-5.6}$	$10^{-7.0}$	$10^{-4.5}$
F-12-48	$10^{-7.2}$	$10^{-8.5}$	$10^{-5.3}$
F-12-34	$10^{-5.2}$	$10^{-6.5}$	$<10^{-4.0}$
F-12-31	$10^{-6.5}$	$10^{-6.7}$	$10^{-6.2}$
F-12-33	$10^{-6.0}$	$10^{-6.0}$	$10^{-5.5}$

* From original fluid; not vibrated.

DISCUSSION

Since the only source of active influenza virus has, until recently, been infected tissue, the identification, isolation, and chemical characterization of the active agent has been complicated by the presence of cellular debris and intact cytological components. The difficulties introduced by the presence of these adventitious elements in close association with the infectious material are, to a considerable extent, encountered in studies of most other virus diseases, particularly those affecting animals. Several investigations within the past 2 or 3 years have called attention to the presence in normal cells, of particulate material separable from tissue emulsions by the identical procedures adopted for isolation of the infective agents. Thus the viruses of fowl leucosis (Kabat and Furth, (22)) and fowl sarcoma (Claude (18)) have been shown to be associated with a tissue fraction sedimentable in 1 hour in the ultracentrifuge at about 27,000 R.P.M. Publications by Amies and Carr (23), Sharp, Taylor, Finkelstein, and Beard (24), and others have also focussed attention on the relationship between the "heavy" components of normal tissues and the infectious agents of certain neoplastic diseases.

The studies of Claude are of particular interest since he showed that the sedimentable fraction from sarcomatous tissue was practically identical in chemical composition with that of normal tissue and he tentatively identified the particles with mitochondria (18).

The presence of a large proportion of normal cell components in a centrifugally sedimented concentrate of a virus, that of influenza for example, casts doubt on all measurements of the size of the infective unit as long as information is lacking as to whether or not association, or aggregation is involved. Centrifugation, ultrafiltration, diffusion, and titration of infectivity might all indicate that particles of 100 mμ diameter are infectious; however, if particles of this size normally present in the cell should happen to adsorb a smaller infectious unit strongly, the same conclusion might result from such studies. It would not follow that the 100 mμ infectious particles are the elementary infectious units.

It is easy to demonstrate that the infectious unit of influenza A as it occurs in allantoic fluid must be a smaller mass than that represented by a 100 mμ sphere. For example, many such fluids (F-12 or WS strain) have produced death at 10^{-8} and infection at 10^{-9} to 10^{-10} dilution. The total protein content of the fluid is usually about 0.4 mg. per ml. It follows that about 2×10^{-14} to 2×10^{-15} gm. of the *total protein* is infectious for mice while the weight of a 100 mμ particle is of the order of 6×10^{-16} gm. Thus an infectious dose could contain no more than 3 to 30, 100 mμ particles even if all the protein were virus. Quantitative studies to be reported in the following paper have shown that only about 2 per cent of the total protein is associated with influenzal activity so it is obvious that the infectious unit can be only a fraction of the size indicated by studies of the virus obtained from emulsified mouse lung.

Attempts to show differences in the composition of infectious extra-embryonic fluids as contrasted with those from normal chicks of the same age have thus far been largely unsuccessful. A very slight but probably significant increase in purine nitrogen and in total phosphorus results from the infection and this obviously suggests the appearance of nucleic acid or nucleoprotein. In a previous publication (25) we have described briefly the concentration of the influenza A virus from egg fluids by precipitation with protamine in the pH range from 7.0 to 9.0. Protamine does not produce precipitation in carefully collected fluids from normal embryos. The active agent concentrated as a protamine-virus complex is being analyzed in detail, and apparently will give information as to the chemical composition. Enough of the analysis has been completed to show that a large proportion of the material consists of ribose-nucleoprotein. It, therefore, appears that the influenzal activity of extra-embryonic fluids is associated with a nucleoprotein which appears concurrently with the infection.

The evidence leaves very little reason to doubt that the infectious unit of influenza A in allantoic fluid is smaller than that in tissue suspensions. Apparently the observed distribution of virus in organ filtrates is due to adsorption of the virus by mitochondria or some other tissue components. Almost all the activity is sedimentable from tissue suspensions at a relatively low

centrifuge speed (25,000 R.P.M.) while a much lower percentage is removed from allantoic fluids by the same treatment. The percentage sedimentable at such a speed in the latter case varies with the age of the embryo, history of the fluid subsequent to removal from the allantoic sac, temperature, and other factors which might influence the extent of virus aggregation. A considerable amount of evidence has been presented to indicate that much of the virus sedimentable at 25,000 R.P.M. is reversibly aggregated.

While sedimentation of the virus from the allantoic fluid in the form of large aggregates, or associated with adventitious materials at 25,000 R.P.M. often removes a considerable percentage of the activity, one should not overlook the fact that a large part of the virus may fail to sediment. It is this non-sedimentable fraction which is of prime interest in studies of the elementary virus unit. The mere existence of a fairly large, though variable, fraction of non-sedimentable virus is strong evidence that the true infectious unit is smaller than evidence obtained from tissue suspensions has indicated.

SUMMARY

A considerable fraction of the influenza A virus contained in infected allantoic fluid of the developing chick is not sedimentable under conditions which remove virus activity almost completely from filtrates of emulsified mouse lung. The infectious unit from tissue suspensions is about 100 mμ in diameter and is of the same chemical composition as particles of the same size and abundance separated from normal tissues by an identical procedure. Evidence has been presented showing that the infectivity can be, and probably is, carried on such normal cell components as an adsorbate. Other non-infective particles such as erythrocytes may also become infectious units through adsorption of the virus.

The virus occurs in allantoic fluid in two states of dispersion. A variable percentage is associated with particles considerably less than 100 mμ in diameter, probably more nearly 10 mμ, while the remainder is reversibly aggregated. Reversal to the more disperse state may be effected by dilution, sonic vibration, or moderate heat treatment.

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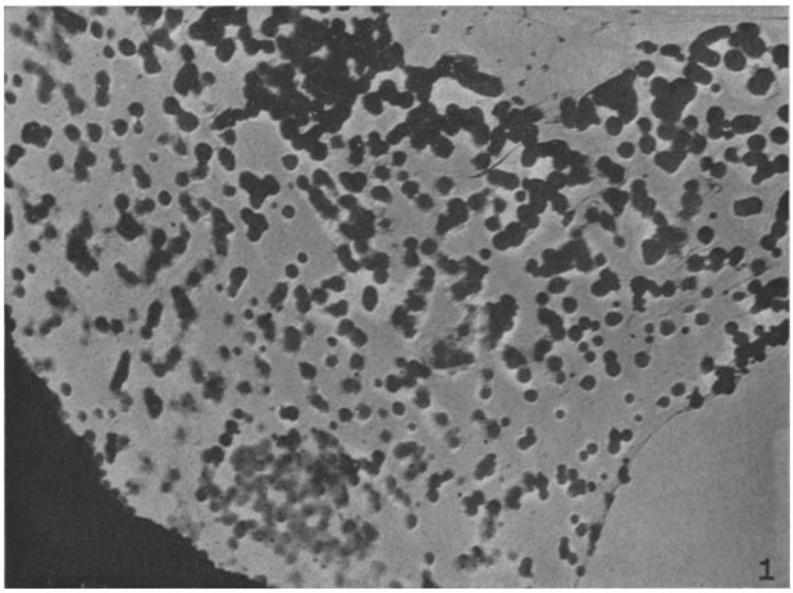
EXPLANATION OF PLATE 13

FIG. 1. Electron micrograph of particles recovered by centrifugation from filtrates of infected mouse lung. Particles of the same shape, size, and appearance are recoverable in essentially equal quantities from lungs of normal, healthy mice. × 19,000: contact print.



(Chambers and Henle: Dispersion of influenza virus)