STUDIES OF TWO KINDS OF VIRUS PARTICLES WHICH COMPRISE INFLUENZA A2 VIRUS STRAINS

III. Morphological Characteristics: Independence of Morphological and Functional Traits*

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Plates 75 to 80

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In 1946 Mosely and Wyckoff (1) reported the presence of filamentous forms in addition to spherical particles (2–4) in preparations of allantoic fluid from chick embryos infected with influenza virus. Chu and coworkers (5) found that filamentous forms were more numerous in newly isolated strains, though some filaments were also present in earlier, laboratory-adapted strains. These authors also presented evidence that the filament was a form of influenza virus.

In tissue cultures (6) and in sections of chick chorioallantoic membrane (7, 8) it was found that both filaments and spheres are produced concurrently by infected cells, and in sections it was seen that the filaments projected from the surface of the infected cell (7, 8). Considerable evidence has now been obtained to show that the filament forms by extrusion from the virus-infected cell (7–13), and there are indications that the filament may contain host cell as well as viral constituents (14, 15). Evidence has been reported that filaments are infective (15–17).

Burnet and Lind (14) found that the filament count/hemagglutinin ratio was a characteristic of influenza A1 virus strains which was retained when the strains were passed at limiting dilutions. However, continued passage of two strains at low dilution resulted in a gradual decrease in the proportion of filaments produced. These authors concluded that the loss of the filamentary character resulted from one or more mutational changes.

Recently, recombination studies involving filamentous influenza A2 (Asian) viruses have been reported which indicate that influenza virus morphology is an exchangeable genetic trait (18).

The present communication describes morphological characteristics of influenza A2 (Asian) virus strains as observed in the electron microscope. The change from prominently filamentous to predominantly spherical morphology on repeated serial passages of strains in the chick embryo is reported. Morpho-

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logical characteristics of the previously described (19–21) "+" and "−" substrains of influenza A2 virus strains are presented, and lack of correlation between spherical or filamentous morphology and the other properties of these substrains is reported.

Materials and Methods

Viruses.—The Japan/305 and RI/1 through RI/6 strains of influenza A2 virus, and the substrains $RI/4^+$, $RI/4^-$, $RI/5^+$, and $RI/5^-$ were employed. The origin and isolation of these strains and substrains have been described (20, 22, 23). Virus preparations for electron microscopy were obtained by inoculation of 0.1 ml. of a 10^{-3} dilution of infected allantoic fluid into 10-day-old chick embryos. Allantoic fluids were harvested after approximately 48 hours of incubation at 35°C. and chilling overnight at 4°C.

Hemagglutination Titrations.—The procedure employed has been described (20, 23).

Neutralization Titrations.—A constant amount of normal horse serum and serial dilutions of virus were used, and the titrations performed by the method previously described (20).

Electron Microscopy.—Freshly harvested infected allantoic fluid was prepared and examined in the electron microscope by the previously described procedure using osmium fixation (7, 18).

EXPERIMENTAL

Effect of Serial Passage on Morphological Characteristics of Influenza A2 Strains.—The RI/1 to RI/6 and Japan/305 strains were serially passed in the chick embryo, and the passages were examined in the electron microscope.

For each passage 4 to 6 embryos were employed and allantoic fluids were pooled after harvest. The amount of virus present was determined by hemagglutination titration. Strains were carried through 11 to 16 serial passages and, with few exceptions, each passage beginning with the second passage was examined and photographed in the electron microscope.

The results of these experiments are summarized in Table I. Those passages in which filaments were very prominent have been designated "PF." It should be emphasized that a small to moderate component of spherical particles was present in such passages. Those later passages which consisted almost entirely of spheres have been designated as spherical, "S." In some passages a large number of filaments were present, but the relative number of spheres had increased, and, therefore, the filaments were less prominent. These passages have been designated "LF." No attempt has been made to quantitate these intermediate stages, and the designation is employed only because there is an obvious transition between the early prominently filamentous passages and the later spherical passages.

As can be seen in Table I, in all 7 strains there was a change toward spherical morphology, and every strain except one eventually became almost entirely spherical. The number of passages required for the transition to spherical morphology varied considerably from complete transition in 7 passages with the RI/4 strain, to incomplete transition in 13 passages with the RI/2 strain.

In a single instance, not shown in Table I, an almost entirely spherical population was found in the fourth passage of the RI/3 strain. In no instance did a change occur from spherical back to filamentous morphology. In general, the titer of virus increased with continued passage.

Figs. 1 to 3 show the prominently filamentous appearance of the earlier passages and the spherical appearance of the later passages of the RI/1, RI/4, and RI/5 strains, respectively.

To investigate the possibility that the number of passages required for a given strain to become spherical might be relatively constant for that strain and to determine whether individual eggs yield virus with different morphological characteristics, multiple serial passages of the RI/4 and RI/5 strains

TABLE I

Morphology of Serial Passages of Influenza A2 Virus Strains

Strain	Passage No.														
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
RI/1	PF*	PF	PF	PF	PF	LF	LF	LF	S	S	S	S			
RI/2	PF	PF	PF	PF	PF	PF	LF	LF	LF	LF	LF	LF			
RI/3	PF	PF	LF	LF	LF	LF	S	S	S	S	S	S			
RI/4		PF	PF	PF	LF	S	S	S	S	S					
RI/5	PF	PF	PF	PF	PF	LF	LF	S	S	S					
RI/6	PF	PF	PF	PF	PF	PF	LF	LF	LF	LF	S	S	S	S	S
Japan/305					PF	LF	LF	LF	S	S					

* PF, prominently filamentous. LF, less prominently filamentous. S, spherical.

were carried out using single eggs for each passage. The RI/4 strain was passed in four lines beginning with 2nd passage virus, and the RI/5 strain was passed in three lines. The lines were carried through the 12th passage. The method employed was otherwise identical with that used above. The inoculum consisted of 0.1 ml. of a 10^{-3} dilution of infected allantoic fluid and the inoculated eggs were incubated for 48 hours.

The results obtained in these experiments were similar to those already described. Transition to spherical morphology occurred with both strains. The number of passages required for a strain to become spherical varied somewhat among the different lines of each strain. However, there was no evidence that passage through any individual egg caused a marked change in the morphology of the virus.

These results indicate that influenza A2 virus strains which were prominently filamentous in early passages became almost entirely spherical after repeated passages at low dilution (10^{-3}) in the chick embryo. With some strains transition to spherical morphology occurred after only 4 to 7 passages, and within two passages the morphology could change markedly. However, in other strains a greater number of passages was required before virus populations assumed an almost entirely spherical morphology.

The term "spherical" has been used to denote all virus particles that are not filamentous. It should be emphasized that the particles that have been called spherical range in shape from true spheres to oblong or "bacillary" forms. In some passages of the influenza A2 strains, the predominant form seen is the oblong one. This finding is illustrated in Fig. 4, which is an electron micrograph of the 10th egg passage of the Japan/305 strain. No evidence is available at present to explain the differences between the appearance of these forms and that of the more nearly spherical particles.

TABLE II

Morphology, Hemagglutination Titer, and Ratio of "+" to "−" Virus Particles of Serial Passages of the RI/5 Strain

Passage No.	Morphology*	Hemagglutination titer	Ratio of "+"/"−" virus particles
1			2.8×10^4
3	PF	256	9.9×10^3
4	PF	512	2.3×10^3
5	PF	256	
6	PF	256	1.7×10^5
7	LF	1024	4.1×10^6
8	LF	1024	5.6×10^6
9	S	2048	1.3×10^7
10	S	2048	1.6×10^7
11	S	2048	1.6×10^6

* PF, prominently filamentous. LF, less prominently filamentous. S, spherical

The Proportions of "+" and "−" Virus Particles in Passages of a Strain during the Transition from Filamentous to Spherical Morphology.—The marked differences in the biological properties of the two kinds of particles, designated "+" and "−," have been described in the preceding communications (20, 21). Because of the presence of two distinct morphological forms of influenza virus, and the finding that some strains are mixtures of "+" and "−" particles, the relationship between the changing morphological characteristics of a strain and the ratio of "+" to "−" particles was investigated.

The RI/5 strain was passed serially in the chick embryo as a line separate from that recorded in Table I. The allantoic fluids were examined in the electron microscope and photographed. The number of "+" and "−" particles in the passages was determined by infectivity titrations in the presence or absence of normal horse serum, as described previously (20). The hemagglutination titers of the allantoic fluids from the consecutive passages were also determined.

As can be seen in Table II, the change from prominently filamentous to almost entirely spherical morphology occurred in the course of 9 passages. The hemagglutination titers indicate an increasing yield of virus with continued passage. In general, the later passages contained a greater proportion of "+" particles, in addition to showing a change to spherical morphology. However, it should be emphasized that passages which were still prominently filamentous already had an extremely high ratio of "+" to "−" particles; *e.g.*, the ratio for the 7th passage was 1.7×10^5. Therefore, these results do not suggest a correlation between spherical morphology and "+" character of virus particles.

Morphological Characteristics of "+" and "−" Particles.—To investigate further the morphology of "+" and "−" particles, early and later passages of pure substrains of each kind of particle were examined in the electron microscope.

The "+" and "−" substrains isolated from the RI/4 and RI/5 strains (20) were serially passed in the chick embryo using an inoculum of 0.1 ml. of a 10^{-3} dilution of infected allantoic fluid. After isolation of the pure substrains they were examined and photographed in the electron microscope. After 7 to 10 passages they were reexamined.

In the early passages, both "+" and "−" substrains were prominently filamentous, and in the later passages both "+" and "−" substrains were almost entirely spherical. Thus, the changes in morphological characteristics exhibited by "+" and "−" substrains on passage in the allantoic cavity were analogous to those observed with parent strains. No correlation was found between the filamentous or spherical morphology of virus particles, and the other properties (20, 21) which differentiate "+" and "−" virus particles. In addition, it is apparent that because the virus particles differ in morphology independently from the properties that distinguish "+" from "−", four different virus populations have been obtained from a strain which originated from a single throat washing. Thus, the RI/5 strain, yielded prominently filamentous "+," prominently filamentous "−," spherical "+" and spherical "−" virus populations.

These findings are illustrated by electron photomicrographs shown in Figs. 5 and 6. As can be seen in Fig. 5, the 5th passages of $RI/5^+$ and $RI/5^-$ substrains were both prominently filamentous. It can be seen in Fig. 6 that the 14th passages of $RI/5^+$ and $RI/5^-$ substrains were almost completely spherical.

DISCUSSION

The findings presented in this communication that strains of influenza A2 virus are prominently filamentous in early passages in the chick embryo and become almost entirely spherical on continued serial passages are in agreement with results obtained with influenza A1 viruses isolated prior to 1957 (14), and results of Portocala *et al.* (24) with one strain of influenza A2 virus isolated in Rumania. The findings are compatible with the view that virus morphology is a genetic character, as was reported by Burnet and Lind (14) and Kilbourne and Murphy (18).

As stated in the preceding communication, the markers which differentiate the "+" and "−" influenza virus particles, particularly sensitivity to horse serum inhibitors, make them well suited for genetic studies. The finding that the variation in filamentous or spherical morphological characteristics is independent of the "+" and "−" characters adds another distinct marker to those that could be employed in genetic studies with these viruses. The ability to obtain both spherical and filamentous "+" or "−" virus particles, all antigenically similar, from a single human infection should be of particular advantage. However, it should be emphasized that since the change from a higher filamentous population to a spherical one can occur in relatively few passages under certain conditions, recombination studies using filamentous morphology as a marker must be carefully controlled.

Although no correlation has been found between filamentous or spherical morphology and the properties which distinguish "+" and "−" virus particles, investigation of the fine structure of the virus with high resolution electron microscopic techniques might reveal differences between the "+" and "−" particles.

The highly filamentous appearance of influenza virus in early passage in the chick embryo raises the question of the morphology of the virus in the human host. At the present time there is no direct evidence in this regard, and the results with early chick embryo passages do not necessarily indicate that the virus is filamentous in human infection.

SUMMARY

Seven strains of influenza A2 virus were serially passed in the chick embryo, and morphological characteristics of the passages were examined in the electron microscope. With serial passage there was a change from a prominently filamentous appearance in early passages to an almost entirely spherical appearance in later passages. The number of passages required for the conversion to spherical morphology varied with different strains. The filament-sphere variation was found to be independent of the properties which differentiate "+" and "−" influenza A2 virus particles, and both highly filamentous and spherical populations of "+" and of "−" virus particles were obtained. The usefulness of these pairs of independent markers for genetic studies is discussed.

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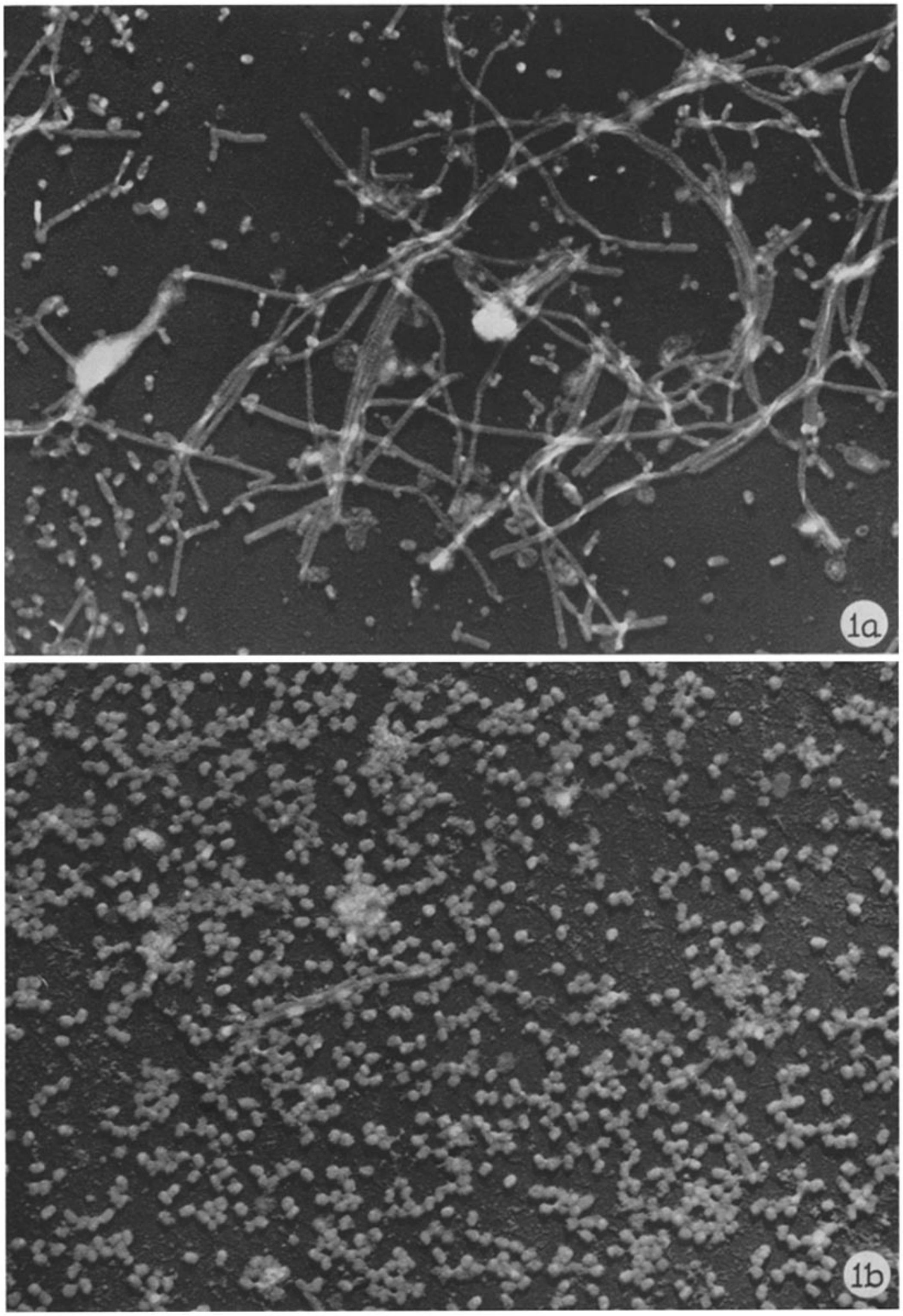
EXPLANATION OF PLATES

PLATE 75

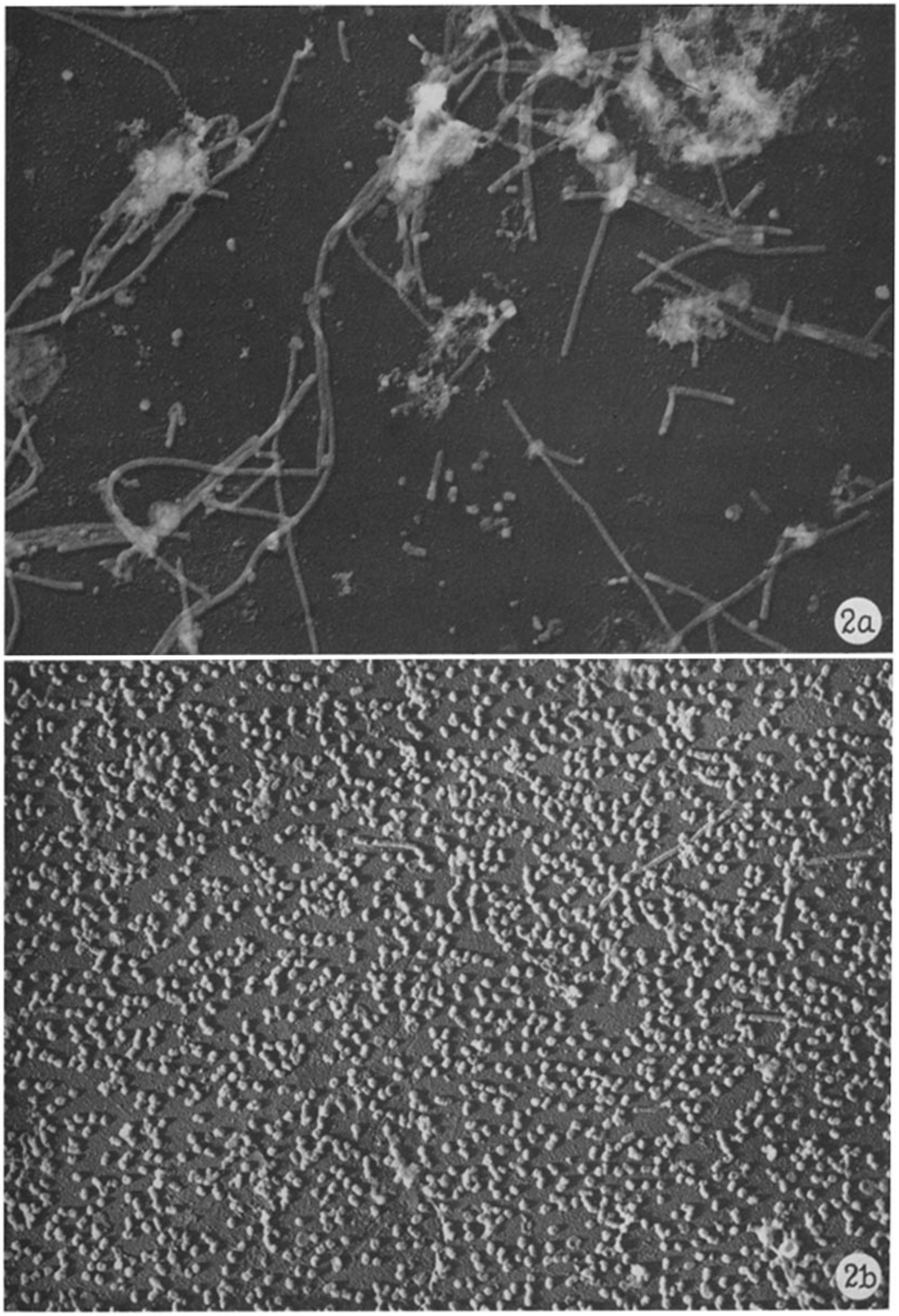
FIG. 1. (*a*) RI/1 strain of influenza A2 (2nd passage) showing filamentous morphology. × 8,500. (*b*) RI/1 strain of influenza A2 (12th passage) showing spherical morphology. × 12,500.



(Choppin *et al.*: Influenza A2 virus strains. III)

PLATE 76

FIG. 2. (*a*) RI/4 strain of influenza A2 (3rd passage) showing filamentous morphology. × 8,500. (*b*) RI/4 strain of influenza A2 (11th passage) showing spherical morphology. × 7,500.



(Choppin *et al.*: Influenza A2 virus strains. III)

PLATE 77

FIG. 3. (*a*) RI/5 strain of influenza A2 (3rd passage) showing filamentous morphology. × 8,500. (*b*) RI/5 strain of influenza A2 (9th passage) showing spherical morphology. × 9,000.

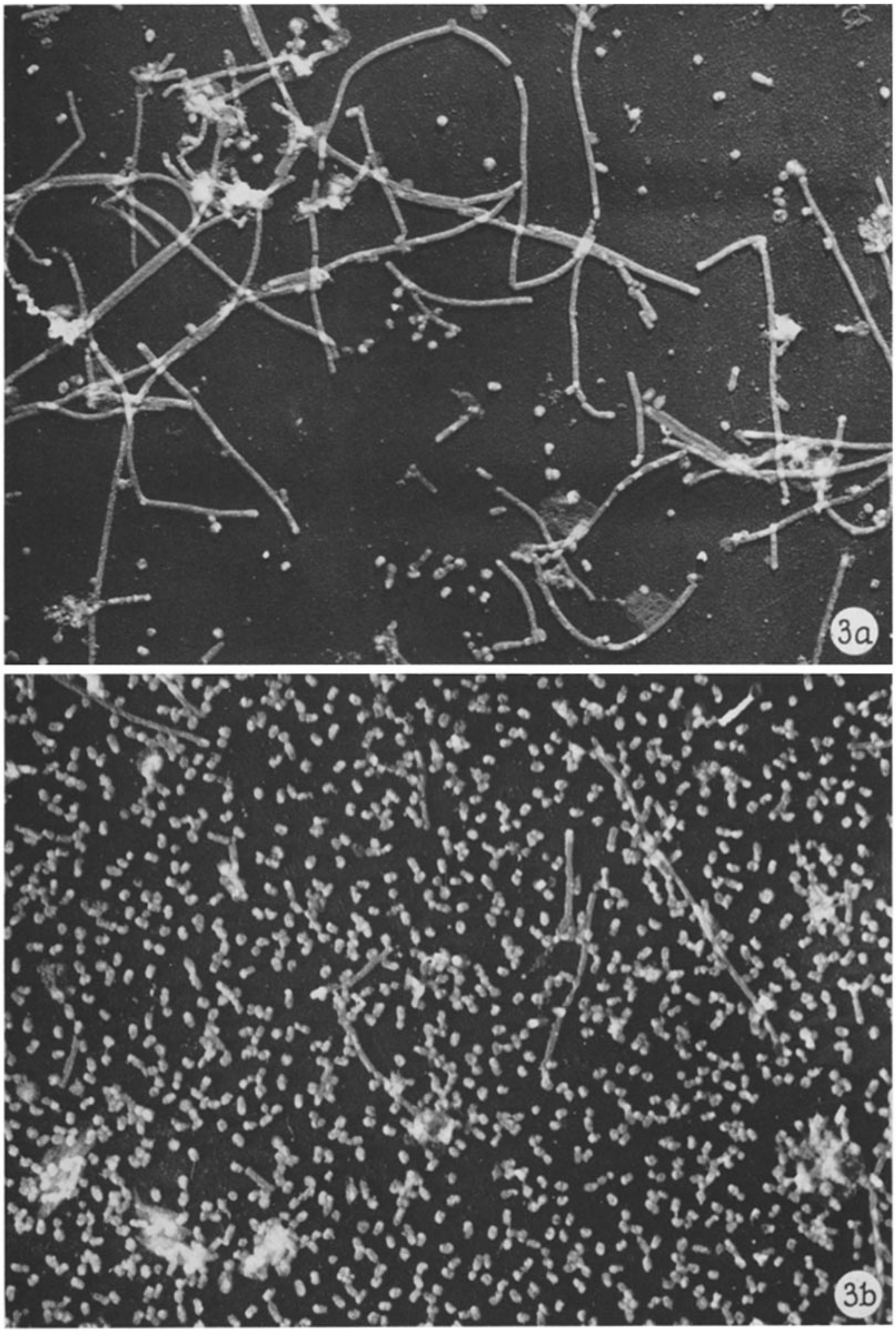


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PLATE 78

FIG. 4. Japan/305 strain of influenza A2 (10th passage) showing oblong or "bacilary" particles. × 8,500.

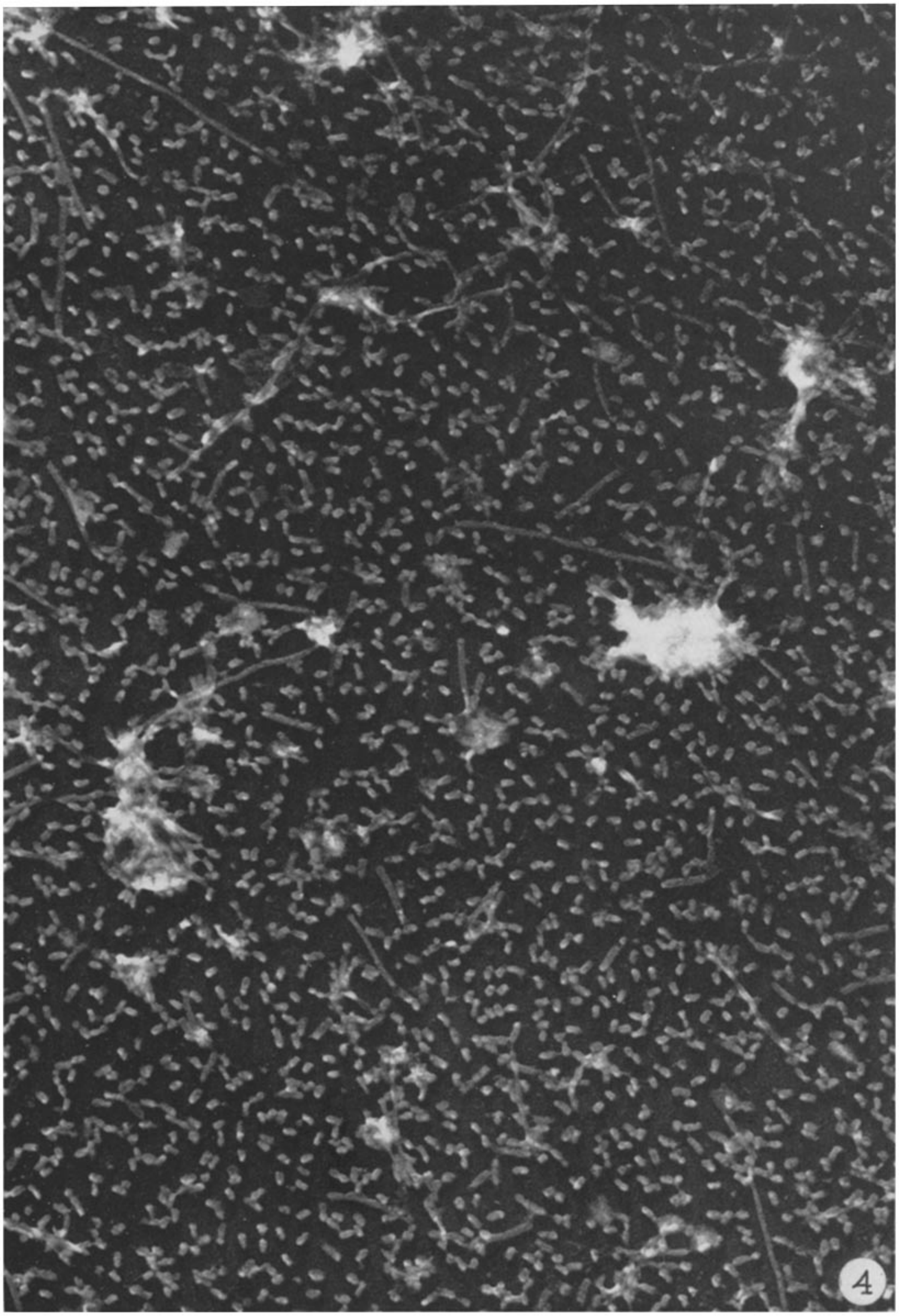

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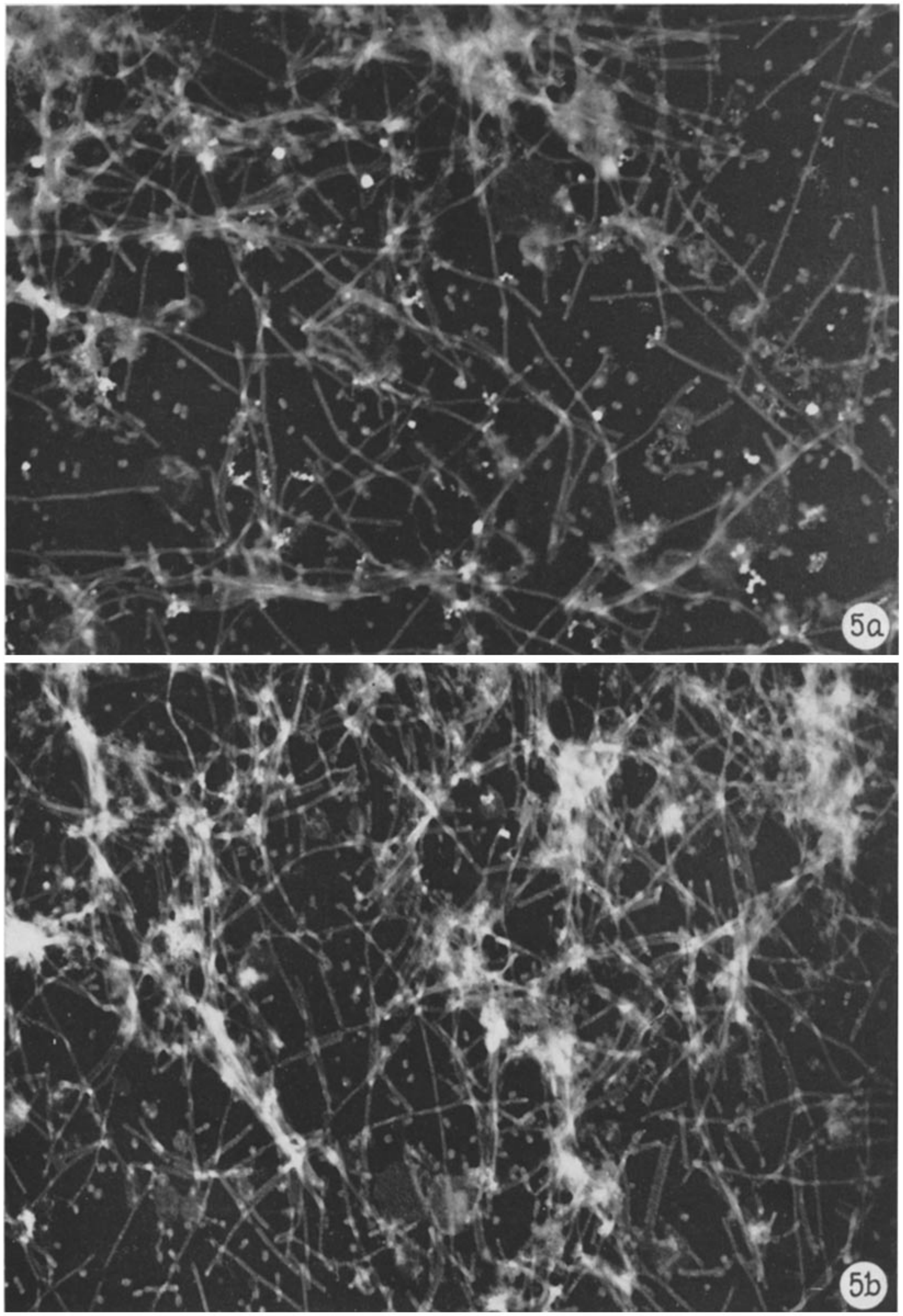
PLATE 79

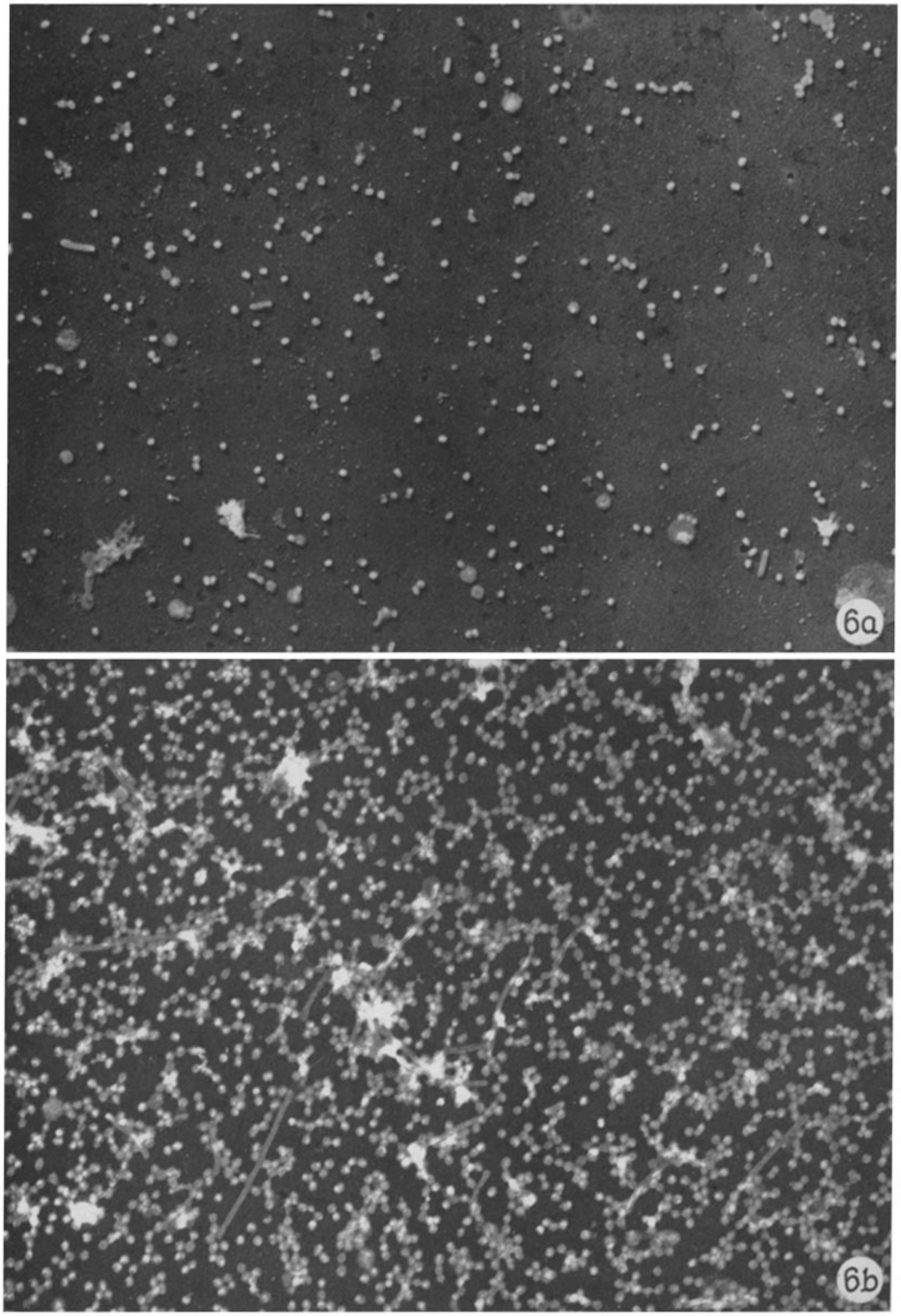
FIG. 5. (*a*) RI/5$^+$ substrain of influenza A2 (5th passage) showing filamentous morphology. $\times$ 8,500. (*b*) RI/5$^-$ substrain of influenza A2 (5th passage) showing filamentous morphology. $\times$ 8,500.



(Choppin *et al.*: Influenza A2 virus strains. III)

PLATE 80

FIG. 6. (*a*) RI/5$^+$ substrain of influenza A2 (14th passage) showing spherical morphology. $\times$ 7,500. (*b*) RI/5$^-$ substrain of influenza A2 (14th passage) showing spherical morphology. $\times$ 9,000.



(Choppin *et al.*: Influenza A2 virus strains. III)