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Functional Significance of Sialidase During Influenza Virus Multiplication: an Electron Microscope Study

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Morphological evidence has been obtained by electron microscopy in support of previous findings that one of the most important functions of sialidase is associated with the release of virus from infected host cells. Highly specific antiserum against fowl plague virus enzyme and specific antiserum against X7 recombinant influenza virus enzyme were shown to influence the morphology of cells infected with their homologous virus. In the presence of enzyme antiserum, an accumulation and aggregation of virus particles were evident on the cell surface and in the extracellular space of infected host cells. The aggregation of virus particles was interpreted to result from the inhibition of the release of virus.

Recent evidence suggests that the function of sialidase (neuraminidase; *N*-acetylneuraminate glycohydrolase, EC 3.2.1.18) is associated with the release of virus from host cells (5, 15, 18). With the use of highly specific enzyme antiserum devoid of demonstrable hemagglutinating-inhibiting and virus-neutralizing capacity, it has been shown that when the antiserum is added to chick embryo tissue culture monolayers infected with fowl plague virus (influenza A), no hemagglutinin, no infective virus, nor any sialidase is released into the tissue culture media. There was a fourfold reduction in hemagglutinin titer, 33% of the normal infective units, and a normal amount of complement-fixing antigen was synthesized in infected cells (15). Because of the reduced synthesis of some of the viral components, it is conceivable that sialidase may have another function during virus multiplication.

Electron microscopic studies were undertaken to examine by morphological means the late phase of virus multiplication in the presence of enzyme antiserum. Such studies should provide evidence of whether (i) the reduced synthesis of cell-associated viral components may have been insufficient to facilitate the release of measurable amounts into the culture media and of whether (ii) sialidase is indeed associated with the release phase (15).

This paper presents morphological evidence on the effects of specific enzyme antiserum on influenza virus multiplication in support of the concept that sialidase is involved in the release of virus from host cells.

MATERIALS AND METHODS

Virus. A2/Singapore/57, A2/Japan 305/57,X7 recombinant (6), and fowl plague virus (FPV) strain "Rostock" were used. Viruses were propagated, partially purified, and assayed for hemagglutinating activity (HA) titer, egg infectivity (EID_{50}), and plaque assay as described before (9, 13, 16). Cell-associated viral components of infected-tissue supernatant fluids from repeatedly frozen and thawed cells after centrifugation were measured (11).

Enzyme assay. Normal enzyme assays with bovine sialyllactose and enzyme inhibition assays with fetuin substrate were carried out as described previously (4, 14, 15). Sialic acid analyses were carried out by the method of Aminoff (1).

Enzyme antiserum. Specific antiserum against A2 Singapore enzyme (AS27) and FPV enzyme (AS42) preparations were the same as reported previously (4, 15). X7 virus enzyme antiserum (AS53) and nonspecific A2 Japan virus antiserum (AS54) were prepared, and their enzyme antibody titers were measured as before (4, 12, 15).

The serum dilutions of AS53 and AS54 inhibiting 50% of their homologous enzyme activity were 1:938 and 1:200, respectively (15). Hemagglutination inhibition titer of AS54 against A2 virus was 1:256. Antisera were treated with periodate (9), dialyzed overnight against 0.15 M sodium chloride at 4 C, and heated for 30 min at 56 C.

Tissue and cell cultures. Primary chick embryo tissue cultures and clone 1-5C-4 Chang conjunctival cell cultures and media used were as reported before (8, 16). FPV plaque assays for plaque-forming units (PFU) were carried out by the procedure described by Schäfer et al. (10). For the experiments on kinetics of virus multiplication and for the electron microscopic studies, virus replication was measured for viral components as before (15, 16). Adsorption period for

kinetics experiments of X7 virus was 1 to 2 hr. Input multiplicity of X7 virus was 10.

Electron microscopy. Tissue and cell cultures were infected and treated as mentioned above. For the electron microscope experiments, cells were "fixed" in the plates with 1% glutaraldehyde in 0.1 M cacodylate buffer for 10 min and transferred into tubes. After centrifugation, the fixative was decanted, and the cells were suspended and stored in 7% sucrose also in the same cacodylate buffer overnight at 4 C. Except for experiments with FVP-infected tissue cultures which were postfixed in 2% $KMnO_4$ in phosphate buffer for 5 min, cells were postfixed in osmium tetroxide (0.5% in Veronal buffer, *p*H 7.3) for 5 min, and the reaction was stopped by dilution with Hanks basic salt solution. Cells were embedded in 2% Noble agar (Difco) containing 0.15 M sodium chloride; agar was minced into small "cubes." Tissue blocks were dehydrated in graded concentrations of acetone, infiltrated with graded concentrations of epoxy resins (Epon), and embedded in Epon. After polymerization of the Epon, sectioning was done with glass knives on an MT-1 Porter-Blum ultramicrotome. Sections were placed on Formvar-carbon-coated grids, stained with lead citrate, and examined in an RCA-EMU-3G electron microscope at 50 kv. The initial micrographs were taken at 7,500 × magnification.

RESULTS

Replication of FPV in chick embryo tissue cultures. Experiments on kinetics of FPV multiplication in chick embryo tissue cultures indicate that sialidase antibodies prevent the release of virus (15) into the culture media (Table 1). In addition to these quantitative assays, in the same experiment the effect of FPV enzyme antiserum on the morphology of the late phase of FPV multiplication was examined by electron microscopy.

FPV-infected tissue cultures, which were incubated in the presence and absence of specific enzyme antiserum, were prepared for ultrastructure analysis at various intervals during the multiplication cycle. Cell samples that were infected for 0, 1, 2, 3, 4, and 5 hr postinfection (PI) were examined in the electron microscope. The time of the appearance of typical morphological changes (budding) on the cell surface attributed to FPV maturation in control cells was readily seen beginning with the 3-hr sample. Four-hr PI budding was observed in the experimental cells, that is, cells which were infected and incubated in the presence of specific FPV enzyme antiserum. Representative electron micrographs of FPV-infected control cells and experimental cells are illustrated in Fig. 1 to 3. At 5-hr PI numerous virus particles formed extensively on the cell surface of the control cells (Fig. 1) and of the experimental cells (Fig. 2). However, some of the electron micrographs of 5-hr PI experimental cells showed virus particles in clusters or aggregates (Fig. 3). This was not observed in experimental cells infected for less than 5 hr or in any of the control cells. The number of particles or extent of budding appears to be similar in control and experimental cells, and approximately 50% of the cells observed revealed budding.

In view of the depressed synthesis of cell-associated viral components in experimental cells

TABLE 1. *Kinetics of FPV replication in chick embryo tissue cultures in the absence and presence of enzyme antiserum AS42*

AS42	Hours PI	HA titer[a]		PFU[b]		Sialidase activity[c]	
		Cell associated	Medium	Cell associated	Medium	Cell	Medium
Absent	0	0	0	5.6	4.8	0.1	0.1
	1	0	0	5.6	5.5	0.5	0.2
	2	4	0	6.3	5.8	2.4	0.6
	3	9	3	7.1	6.5	17.0	1.2
	4	9	5	7.2	6.3	23.1	4.3
	5	8	6	7.4	6.3	15.8	4.3
Present (1:750 dilution)	1	0	0	4.9	5.1	0.1	0.0
	2	3	0	5.4	5.2	2.4	0.1
	3	7	0	6.2	5.2	8.3	0.1
	4	7	0	6.7	5.6	12.7	0.1
	5	6	0	6.4	5.7	6.2	0.3

[a] Expressed as the logarithm, base 2, per milliliter.
[b] Expressed as the logarithm, base 10, per milliliter.
[c] Enzyme units × 10^{-3} per milliliter.

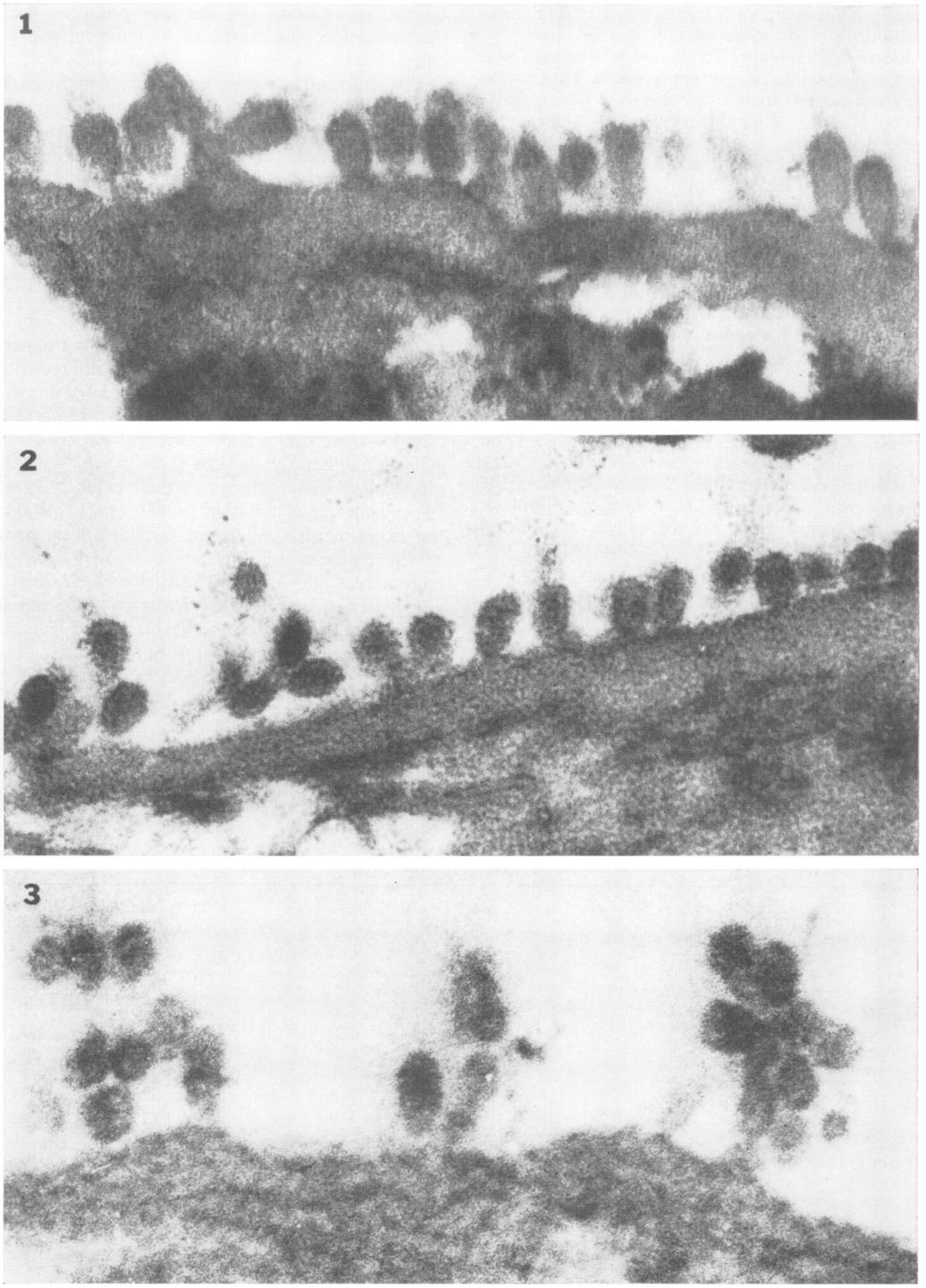

FIG. 1–3. *FPV replication in tissue cultures in the absence (Fig. 1) and in the presence (Fig. 2 and 3) of enzyme antiserum, 5 hr PI. × 73,000, × 66,000, and × 81,000, respectively.*

(Table 1), the possibility remained that perhaps viral components were not released into the tissue culture media in the presence of enzyme antiserum because of insufficient synthesis of viral components. The electron micrographs (Fig. 1 and 2) do not support this interpretation, and the morphological evidence does not suggest that the cell-associated virus in the experimental group may be partially "incomplete." The ratios of PFU and HA titer, which is an index used to characterize "incomplete" virus, are essentially similar in the controls (range of 4.2 to 5.6) and experimental cells (range of 4.0 to 5.2). To confirm the morphological evidence (Fig. 3) in support of the virus-release role of sialidase demonstrated in the FPV system (Table 1) and because of quarantine restrictions, further experiments were carried out with influenza A virus (X7 recombinant) in monolayers of conjunctival-cell cultures and chick tissue cultures.

Replication of X7 virus in clone 1-5C-4 conjunctival cells and chick embryo tissue cultures. In the next series of experiments, the replication of X7 recombinant (6) virus (possessing surface antigens of A2 virus enzyme and of A0 virus HA) in conjunctival cell monolayers was followed in the presence and absence of X7 virus-specific enzyme antiserum. An abbreviated kinetics experiment representative of X7 virus in conjunctival cells is presented in Table 2. The growth cycle was purposely allowed to proceed for 12 hr PI in an attempt to enhance the effect of sialidase antiserum on the morphology of virus-infected cells. The results of HA titers give an indication of active biosynthesis of viral components, but the EID_{50} results suggest a low level of infectious X7 virus replication. Morphological analysis (Fig. 4–9) of X7 virus-infected cell cultures revealed very little budding on the cell surface characteristic of influenza virus formation (Fig. 6). On the other hand, in X7 virus-infected cell cultures in the presence of specific enzyme antiserum, filamentous particles were readily evident in aggregates or clusters (Fig. 7 and 8). Approximately 5 to 10% of X7 virus-infected cells showed evidence of virus maturation.

At zero-time infection, no virus particles could be seen attached to host cells (Fig. 5). For comparative purpose, an uninfected control cell is shown in Fig. 4. In an experiment in which cells were incubated for 4, 8, 10, and 12 hr PI, filamentous virus particles were observed beginning at 8 hr. The effect of antibody concentration was tested, and, at serum dilutions of 1:240, and 1:2400, the extent of aggregation of filamentous forms was observed to be a function of antibody titer. Furthermore, X7 virus enzyme antiserum added only during the virus adsorption period (Table 2) induced an effect as shown in the electron micrograph of X7 virus in Fig. 9. When enzyme antiserum was added during the adsorption and during the growth periods, an array of filamentous virus particles was observed similar to that shown in Fig. 9.

Additional antisera containing A2 virus enzyme antibodies were examined to confirm that the effect of enzyme antiserum was specific for A2 enzyme. A2 Singapore highly specific enzyme antiserum (AS27) and nonspecific A2 Japan virus antiserum (AS54) likewise influenced the formation of filamentous particles similar to that illustrated in Fig. 7 and 8.

Experiments were also carried out with X7 virus in chick embryo tissue cultures. The results of a representative experiment are presented in

TABLE 2. *Replication of X7 virus in conjunctival cell cultures in the absence and presence of enzyme antiserum AS53*

AS53	Hours PI	HA titer[a]		EID_{50}[b]		Sialidase activity[c]	
		Cell associated	Medium	Cell associated	Medium	Cell	Medium
Absent	0	0	0	5.50 ± 0.40	5.85 ± 0.45	0.2	0.2
	6	5	2	4.38 ± 0.30	6.30 ± 0.40	1.2	2.7
	12	8	2	5.17 ± 0.04	4.83 ± 0.39	7.0	2.4
Present (1:750 dilution)	6	2	0	3.48 ± 0.34	4.83 ± 0.39	0.6	0.1
	12	6	0	3.00 ± 0.43	4.38 ± 0.32	2.6	0.1
	12[d]	8	4	5.57 ± 0.40	5.37 ± 0.40	6.0	2.4

[a] Expressed as in Table 1.
[b] Expressed as the logarithm, base 10, ± standard error per milliliter.
[c] Enzyme units × 10^{-3} per milliliter.
[d] Enzyme antiserum (1:80 dilution) present during adsorption period only.

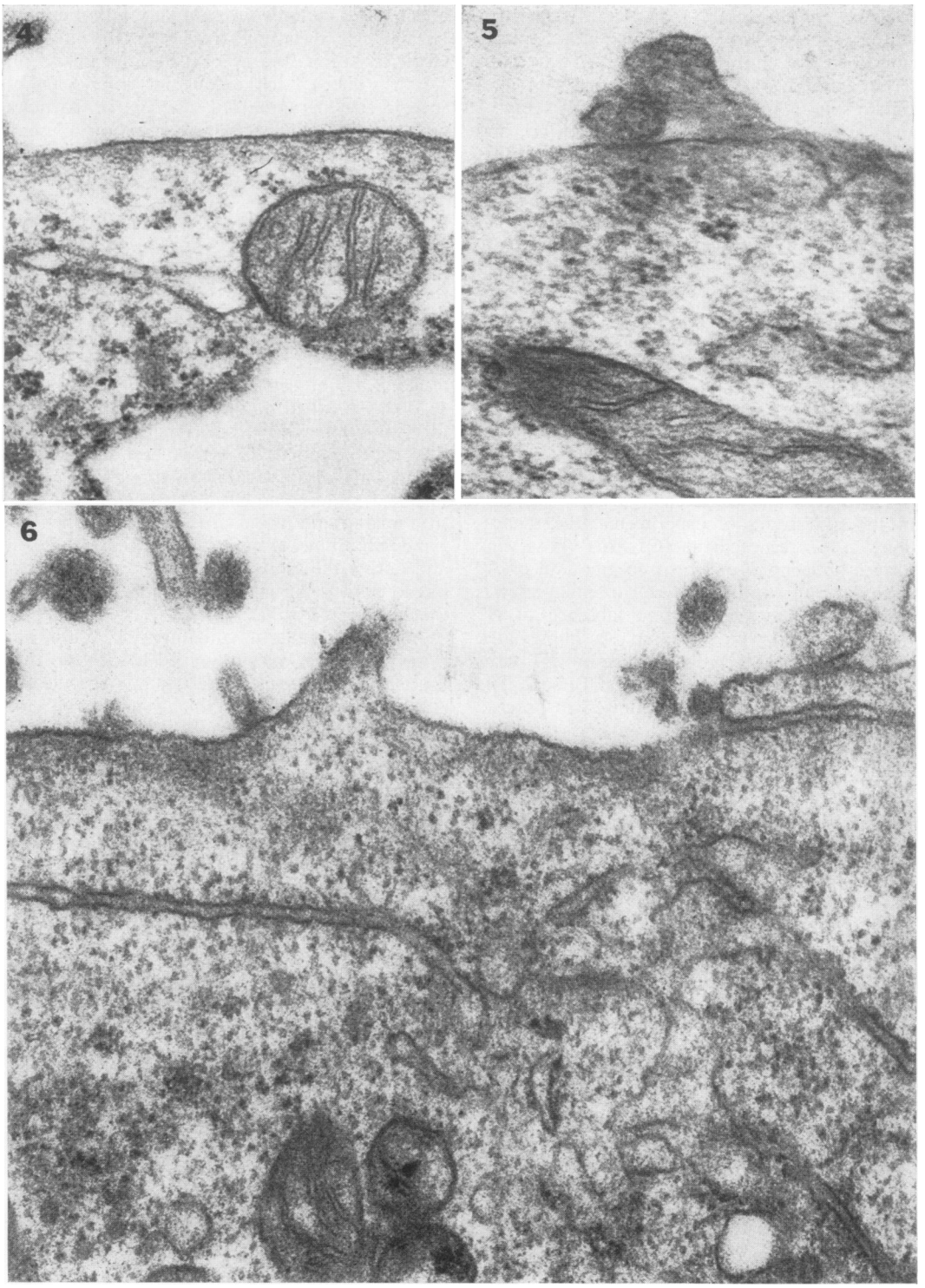

FIG. 4. *Uninfected conjunctival cell culture.* × *54,000.*
FIG. 5–6. *X7 virus infected cell cultures at 0 hr PI (Fig. 5) and 12 hr PI (Fig. 6).* × *69,000 and* × *62,000.*

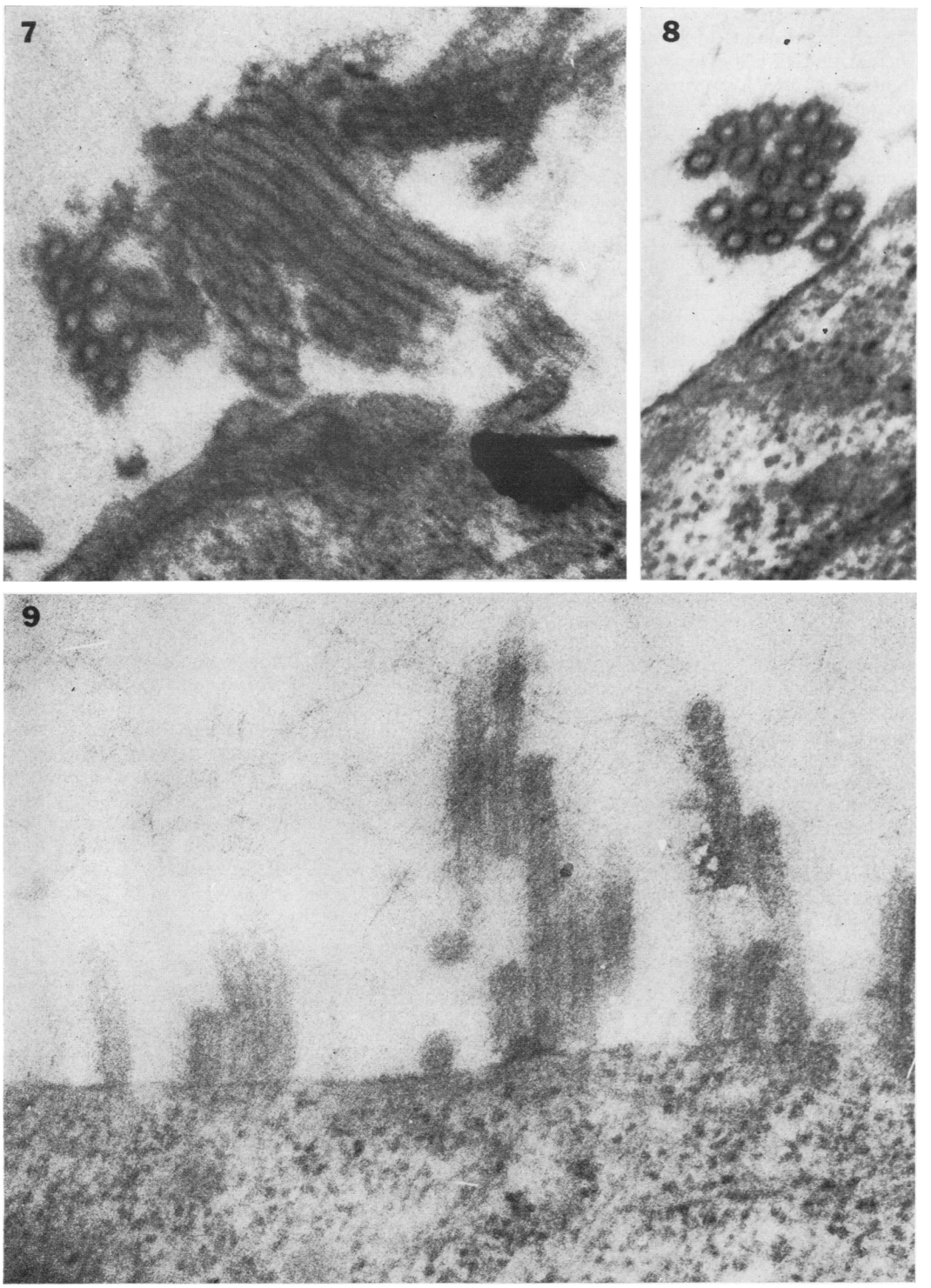

FIG. 7–9. *X7 virus infected cell cultures in presence (Fig. 7 and 8) of enzyme antiserum during replication, antiserum present during adsorption period (Fig. 9), 12 hr PI. × 68,000.*

Table 3. Virus assays of the media (EID_{50}) suggest an infectious cycle of virus replication, whereas that of cell-associated virus indicates a decrease in infectious virus synthesis. Perhaps this may be attributed to technical problems (17), since, in other experiments, the EID_{50} and PFU assayed with conjunctival cells were similar in 0- and 18-hr PI preparations of cell-associated virus. Electron micrographs of 18 hr PI cells do indeed illustrate maturation of X7 virus (Fig. 10). Predominately spherical virions were produced in the control cells (Fig. 10); however, in the experimental cells an accumulation of virus particles was observed in the extracellular space (Fig. 11). This was attributed to the inhibition of the release of virion by enzyme antiserum into the tissue culture media (15). In this same experiment (Table 3), enzyme antiserum present exclusively during the adsorption period induced the formation of filamentous virus particles (Fig. 12) and is essentially akin to that of X7 virus illustrated in Fig. 9 in conjunctival cell cultures.

DISCUSSION

Morphological evidence obtained by electron microscopy is presented in support of previous studies with FPV and its specific enzyme antiserum, with which the role of sialidase was demonstrated (15). In this study, the electron micrographs provide further evidence suggesting that the function of sialidase involves the final stages of the influenza virus cycle in vertebrate cells, a stage involving the release mechanism of the virion from the host.

Kinetics experiments with FPV and abridged experiments with X7 virus, carried out in the presence of their respective specific sialidase antisera, illustrate a morphological effect of enzyme antiserum on the late phase of virus multiplication cycle. The effect was particularly striking with X7 virus in conjunctival cell cultures (Fig. 7 and 8) and also in chick embryo tissue cultures (Fig. 11). Aggregates of filamentous particles (Fig. 7 and 8) and an accumulation of particles (Fig. 11) were observed in systems of the two cultures, respectively. Since enzyme antiserum inhibits the release of virus from infected host cells (*see* Tables 1–3), cell-associated titers of viral components of experimental cells should be greater than that of virus controls. Although the synthesis of viral components is actually reduced as discussed below, the morphological evidence illustrated in Fig. 7, 8, and 11 is suggestive of the above supposition.

One consistent effect of enzyme antiserum on the synthesis of viral components in kinetics experiments was the reduction in synthesis of cell-associated HA, enzyme, and PFU or EID_{50} (Tables 1–3; 15). Thus, it may be possible that a sufficient amount of virus was not synthesized to facilitate the release of measurable virus components into the tissue culture medium. The extent of budding observed in FPV and X7 virus experimental infected cells does not lend support to this possible interpretation (Fig. 3, 7, 8, and 11). Alternatively, it is conceivable that an aggregate of virions (Fig. 3, 7, and 11) might be assayed as 1 infectious unit (17), which would serve to explain the reduction in infectivity assays of cell-associated virus materials of experimental cells (Tables 1–3). Another possibility is that, in X7 virus infections, the EID_{50} to HA ratios which are suggestive of "incomplete" virus or an abortive infection may represent abnormal maturation of virus. An electron micrograph showing a cross section of the filamentous particles which apparently lack electron-dense cores (2) is illustrated in Fig. 8. Further work is in progress in an attempt to resolve this problem by biochemical analysis.

TABLE 3. *Replication of X7 virus in chick embryo tissue cultures in the absence and presence of enzyme antiserum AS53*

AS53	Hours PI	HA titer[a]		EID_{50}[b]		Sialidase activity[c]	
		Cell associated	Medium	Cell associated	Medium	Cell	Medium
Absent	0	0	0	2.38 ± 0.30	4.63 ± 0.32	0.2	0.3
	18	6	5	<1.0	6.00 ± 0.43	3.6	2.7
Present (1:240 dilution)	18	4	0	<1.0	4.32 ± 0.40	1.8	0.1
	18[d]	8	5	<1.0	4.83 ± 0.39	7.0	2.6

[a] Expressed as in Tables 1 and 2.
[b] Expressed as in Table 2.
[c] Enzyme units × 10^{-3} per milliliter.
[d] Enzyme antiserum (1:80 dilution) present during adsorption period only.

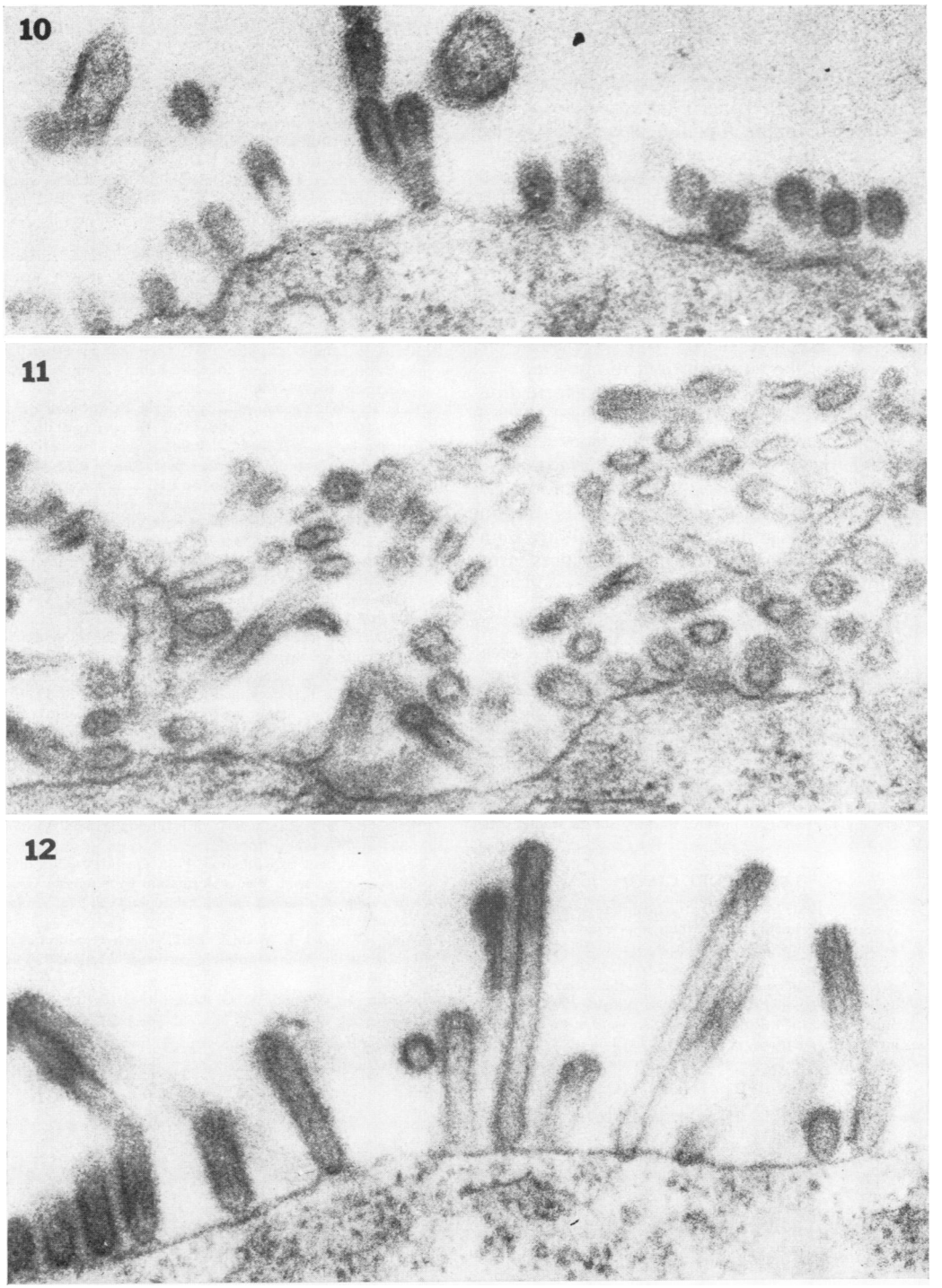

FIG. 10–12. *X7 virus replication in chick embryo tissue cultures in the absence (Fig. 10) and in the presence (Fig. 11) of enzyme antiserum and with antiserum present during adsorption period (Fig. 12), 18 hr PI.* × *68,000.*

The extensive filamentous X7 virus formation in cells which were infected and with enzyme antiserum present solely during the adsorption period (Fig. 9 and 12) is also suggestive that perhaps a factor(s) other than virus release may be attributed to the function of sialidase, since (i) filamentous structures were much more numerous than in their respective virus control cells; (ii) the particles were orientated in a radial fashion (Fig. 9 and 12) unlike that observed in the experimental cells (Fig. 7 and 11); (iii) viral components were released into the tissue culture media (Tables 2, 3). The lack of neutralization by the enzyme antiserum is additional evidence against the possible role of viral sialidase in the early phase of the virus multiplication cycle.

It has been reported previously that formation of filamentous virion is under the control of viral genome (7) and filamentous particles can be produced by cells treated with surface-active agents (3). The formation of filamentous X7 virus in the presence of enzyme antiserum in conjunctival cells and in chick tissue cultures is an example of another factor which influences virus morphology.

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