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Some Properties of Influenza Virus Nucleocapsids

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Nucleocapsids released from influenza virions by sodium deoxycholate sedimented heterogeneously in sucrose gradients. Highly infectious virus (complete) preparations yielded nucleocapsids with peak distributions at 64 and 56*S*; von Magnus type virus (incomplete) lacked 64*S* nucleocapsids. Treatment of influenza virus nucleocapsids with pancreatic ribonuclease rendered the associated viral ribonucleic acid (RNA) molecules acid-soluble, indicating that capsid proteins do not completely surround the viral RNA's. However, the capsid proteins remained associated after enzymatic hydrolysis of the RNA, as judged by persistently high sedimentation rates. Sedimentation rates of viral nucleocapsids reflected the sedimentation rates of the associated RNA's: 64*S* nucleocapsids contained 18*S* RNA, whereas 56*S* nucleocapsids contained 15*S* RNA, although in both cases RNA's sedimenting at 4 to 13*S* were also recovered. Furthermore, just as incomplete virions lacked 64*S* nucleocapsids, they also lacked 18*S* RNA. These findings support the hypothesis that the influenza virus genome is divided among several distinct pieces of RNA.

Many recent analyses of ribonucleic acid (RNA) extracted from influenza virions (3, 6, 7, 19, 20, 22, 23, 27) indicate that the viral genome is not a single large RNA molecule, but is divided among several small RNA molecules. If different viral genes reside in different RNA molecules, they might simply reassort in mixed infections, explaining the extraordinarily high genetic recombination frequencies observed in influenza virus crosses (10).

RNA is a very labile polymer, and it is possible that suitable techniques have not yet been applied to prevent a large influenza virus genome from breaking during extraction. To obtain more information on this problem, we have examined properties of influenza virus nucleocapsids, expecting that the viral RNA might be preserved in its native state if kept in association with capsid proteins, and that the true size of the viral RNA might then be revealed upon removal of the capsid proteins.

Thus, with another virus having rod-shaped nucleocapsids enclosed in a lipid and protein envelope, Newcastle disease virus (NDV), disruption of virions with sodium deoxycholate (DOC) released the large nucleocapsids apparently intact (16). NDV nucleocapsids were shown to sediment homogeneously and to contain homogeneous 50*S* RNA (16).

Now, using DOC, we have found that several sedimenting classes of nucleocapsids are released from influenza virions and that different viral RNA is associated with each class. In further contrast to NDV nucleocapsids, which were ribonuclease resistant, influenza virus capsid proteins did not prevent the hydrolysis of the viral RNA by pancreatic ribonuclease.

Similar findings have recently been made by P. Duesberg (J. Mol. Biol., *in press*).

MATERIALS AND METHODS

Virus. The WSN strain of influenza virus (9) was propagated by intra-allantoic inoculation of 10-day embryonated hens' eggs with 10^2 to 10^3 EID_{50} (50% egg infectious doses) of virus, followed by incubation at 37 C for 40 hr. Allantoic fluid containing virus was centrifuged at 1,500 × *g* for 20 min to remove debris, and virus was concentrated by centrifugation at 40,000 × *g* for 30 min. Virus pellets were suspended in phosphate-buffered saline (PBS) containing 1% gelatin and stored at −70 C. The final preparation had 10^9 EID_{50} and 1.8×10^3 hemagglutinating units (HAU) per ml.

Radioisotopically labeled virus. To produce labeled virus with a low ratio of EID_{50} to HAU [incomplete or von Magnus (28) virus], primary chick embryo fibroblast monolayer cultures in plastic dishes (150 mm in diameter) were infected with 3×10^8 EID_{50} of WSN in 3 ml of PBS, an input multiplicity of approximately 3 EID_{50}/cell. After 30 min at 30 C, the inoculum was removed and replaced by 10 ml of one of several labeling media described below. Incubation at 37 C in an atmosphere of 5% CO_2 in air was continued for 24 hr when virus was collected from the fluid.

Ratios of EID_{50} to HAU for such preparations were approximately 10^3.

Labeled virus with a high ratio of EID_{50} to HAU was obtained similarly, but an infecting input multiplicity of approximately 3×10^{-3} EID_{50}/cell was used, and labeling media were added after preliminary incubation for 24 hr at 37 C in bicarbonate-buffered Earle's saline containing 0.5% lactalbumin hydrolysate. Scant virus was produced during the first 24 hr after infection at this low multiplicity, but high titers accrued in the next 24 hr. Preparations with EID_{50} to HAU ratios of 7×10^4 to 2×10^5 were obtained. These EID_{50} to HAU ratios were significantly higher than the ratio of virus produced after infection at high multiplicity, but were lower than the ratio of our egg-grown virus. However, we believe our results are representative of infectious virus, since nucleocapsids from these virions sedimented like those described by Paucker et al. (21), and P. Duesberg (J. Mol. Biol., *in press*), and the RNA sedimented as described by others (3, 7, 19, 20, 22).

Labeling media. The medium for labeling viral proteins contained Eagle's salts (8), dextrose, vitamins, and glutamine, with 10% of the recommended concentrations of amino acids other than glutamine. Either 5 μc/ml of a mixture of 15 ^{14}C-amino acids (New England Nuclear Corp., Boston, Mass.; average specific activity approximately 100 mc/mmole) or 25 μc/ml of a mixture of 16 ^{3}H-amino acids (Schwarz BioResearch, Orangeburg, N.Y.; average specific activity approximately 1,000 mc/mmole) were present. When virus with ^{14}C-labeled proteins and ^{3}H-labeled RNA was desired, 100μc/ml of ^{3}H-5-uridine (Schwarz BioResearch, specific activity 20 c/mmole) was included with the ^{14}C-amino acids.

To label virus with ^{32}P, 100 μc/ml of carrier-free ^{32}P-orthophosphate was included in phosphate-free, bicarbonate-buffered Earle's saline containing 0.5% lactalbumin hydrolysate.

Isolation of radioisotopically-labeled virus. This was performed as described for NDV (15), employing centrifugation onto 30% (w/w) potassium tartrate and centrifugation through 16% (w/w) potassium tartrate. The virus pellets were suspended in TE buffer [10^{-3} M sodium ethylenediaminetetraacetate, 5×10^{-3} M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.4], and were kept at 4 C for up to 3 weeks with no change in properties studied in the present work.

Sucrose gradient rate zonal centrifugation. For sedimentation analysis of viral nucleocapsids, 0.1 ml of 10% DOC (*p*H 7.4) was added to 2 ml of virus suspension in TE, and the mixture was immediately layered on a 29-ml linear 5 to 20% sucrose gradient in TE. After centrifugation at 18,000 rev/min 16 hr (or equivalent rev/min^2 $\times$ time) in a Spinco model SW 25.1 swinging bucket rotor at 4 C, 1-ml fractions were collected with an ISCO density gradient fractionator (Instrumentation Specialties, Inc., Lincoln, Neb.). TE buffer was used routinely in place of the phosphate-buffered saline used in the earlier study of NDV nucleocapsids (16), since aggregation of DOC occasionally occurred in the saline-containing buffer (24). However, the sedimentation properties and ribonuclease susceptibility of influenza virus nucleocapsids were identical in both buffers.

To prepare RNA from virions, virus was suspended in phosphate-buffered saline containing 0.25% Pronase [Calbiochem, Los Angeles, Calif. (14)], incubated at 37 C for 3 hr, made 1% with respect to sodium dodecyl sulfate (SDS), and extracted with phenol at room temperature. The aqueous phase from the phenol extraction was directly layered on a 29-ml linear 5 to 20% sucrose gradient in TE and centrifuged at 4 C for 42 hr at 22,500 rev/min. Since ultraviolet absorbance was not monitored, the presence of phenol was of no consequence

Determination of radioactivity. Acid-insoluble radioactivity was measured by precipitation of up to 1 ml of sample with 3 ml of 5% trichloroacetic acid in the presence of 500 μg of bovine serum albumin as carrier. When DOC was present in samples, it was precipitated by the acid, but this did not affect the counting efficiencies of any of the isotopes employed. Precipitates were collected by centrifugation, washed once with 3 ml of 5% trichloroacetic acid, dissolved in 1 ml of NCS (Amersham-Searle, Chicago, Ill.), mixed with 10 ml of a toluene-based scintillant, and counted in a liquid scintillation spectrometer. Gain controls were adjusted as indicated by Bush (4) for optimal resolution of ^{3}H-^{14}C or ^{3}H-^{32}P combinations.

Precipitation of viral nucleocapsids with antiserum. The procedure was patterned according to the method of Scharff et al. (26). After sucrose gradient centrifugation of DOC-treated ^{3}H-amino acid-labeled virus, gradient fractions were dialyzed against TE. To 0.25 ml of each fraction was added 0.05 ml of 1/100 dilution of guinea pig serum containing antibodies to influenza A (PR 8) soluble antigen. This serum, a gift from H. G. Pereira, had been prepared according to the method of Lief and Henle (18), and did not inhibit WSN hemagglutination when diluted more than 1:10. The mixture was incubated at 37 C for 2 hr. To form sedimentable precipitates, 0.05 ml of rabbit antiserum to guinea pig gamma globulin was added to each sample at optimal concentration, as determined by equivalence tests, and incubation was continued for 2 hr at 37 C and 18 hr at 4 C. The precipitates were collected by centrifugation at $1,000 \times g$ for 20 min, washed twice with 3 ml of 0.14 M NaCl, precipitated with trichloroacetic acid, and were processed for counting as described above. To correct for volume changes during dialysis, a portion of each dialyzed sample was precipitated directly with acid and the counts were compared with acid-precipitable counts in each sample before dialysis, as described by Scharff et al. (26).

RESULTS

Sedimentation properties of influenza virus nucleocapsids. In view of evidence that incomplete (28) influenza virions contain less RNA and nucleocapsid protein ("soluble antigen") than complete virions (1, 17), both types of virions were prepared in labeled form to examine the sedimentation behavior of subviral structures containing RNA. After disruption of virions with

DOC, some of the labeled RNA sedimented more rapidly than viral RNA isolated with SDS and phenol (see below). The most rapidly sedimenting radioactive component of DOC-treated ^{3}H-uridine-labeled complete virus was approximately 64*S* (Fig. 1, fraction 20), with another component discernible at 56*S* (Fig. 1, fraction 17), relative to 74*S* ribosomes and 60*S* and 45*S* ribosomal subunits from chick embryo fibroblasts centrifuged in 0.01 M Tris-hydrochloride, 0.01 M KCl, 0.0015 M $MgCl_2$, and 0.5% DOC (*p*H 7.4). In contrast, ^{32}P-labeled incomplete virus yielded little 64*S* material relative to material sedimenting at 56*S* (Fig. 1, fraction 17). Occasionally, minor components were seen at about 45*S* (Fig. 1, fraction 14), and at about 35*S* (Fig. 3A, fraction 11) in incomplete virus preparations. Radioactivity remaining in the upper 10 ml of such gradients varied in amount from preparation to preparation (see also Fig. 2, 3, and 6); although slow sedimenting, virus-specific components may be present there, it is likely that obscuring contaminants were present as well.

Some labeled proteins sedimented with the viral RNA's (Fig. 2A, 3A, and 4, fractions 14 to 30) as expected if the RNA's were associated with viral capsid proteins in nucleocapsids.

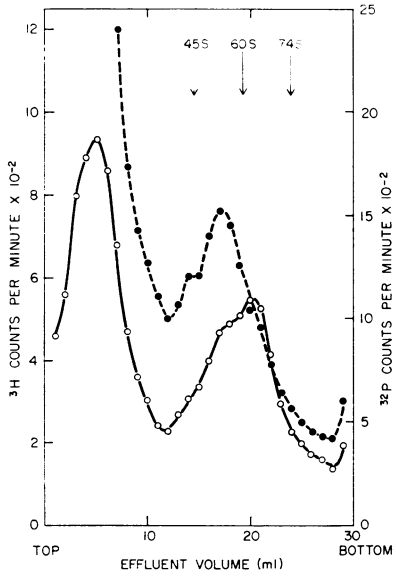

FIG. 1. *Sucrose gradient rate zonal centrifugation of influenza virus nucleocapsids. A mixture of complete virions, labeled with ^{3}H-uridine and incomplete virions, labeled with ^{32}P, was treated with deoxycholate, centrifuged, and analyzed for acid-precipitable radioactivity, as described in Materials and Methods. Symbols: ○, ^{3}H counts/min (complete virus); ●, ^{32}P counts/min (incomplete virus).*

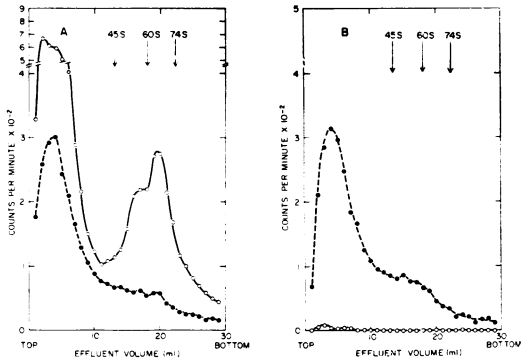

FIG. 2. *Sucrose gradient rate zonal centrifugation of influenza virus nucleocapsids. Complete virions, labeled with ^{14}C-amino acids and ^{3}H-uridine, were treated with deoxycholate, centrifuged, and analyzed, as described in Materials and Methods. (A) Treated with ribonuclease. (B) Treated with 10 μg of pancreatic ribonuclease A per ml for 10 min at 23 C before centrifugation. Symbols: ○, ^{3}H counts/min (RNA); ●, ^{14}C counts/min (protein).*

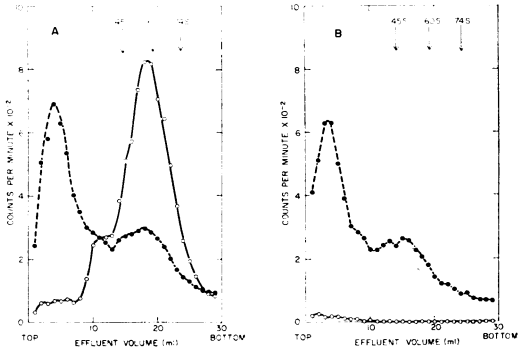

FIG. 3. *Sucrose gradient rate zonal centrifugation of nucleocapsids from incomplete influenza virions. Virus labeled with ^{14}C-amino acids and ^{3}H-uridine was analyzed as in Fig. 2. Fig. 3(A) Not treated with ribonuclease. Symbols: ○, ^{3}H counts/min (RNA); ●, ^{14}C counts/min (protein). Fig. 3(B) Treated with 10 μg of pancreatic ribonuclease A per ml for 10 min at 23 C before centrifugation. Symbols: ○, ^{3}H counts/min (RNA); ●, ^{14}C counts/min (protein).*

Thus, methods which yielded homogeneous 200*S* nucleocapsids from NDV (16) gave structures from influenza virus which sedimented significantly more slowly and heterogeneously.

Identification of the protein cosedimenting with influenza virus RNA. The capsid proteins of type A influenza viruses cross-react immunologically (18). Therefore, serum containing antibodies to type A (PR 8) influenza virus capsid proteins was mixed with fractions from a sucrose gradient sep-

aration of DOC-treated ^{3}H-amino acid-labeled complete WSN to precipitate viral capsid proteins. Despite unavoidable and variable losses of portions of the precipitates during the saline washes, more than 60% of the labeled proteins sedimenting more rapidly than 40*S* (Fig. 4, fractions 14 to 30) was recovered as immune precipitates. Less than 20% of the labeled proteins at the top of the gradient (Fig. 4 fractions 1 to 7) was precipitated; much of the virus-specific proteins in this region must be envelope elements, such as viral hemagglutinin and neuraminidase, but it is possible that some nucleocapsids or capsid polypeptides occur here as well, since only 6% of labeled virus proteins was precipitated by nonimmune guinea pig serum in control experiments.

It appears that most of the viral proteins sedimenting with influenza virus RNA's at 40*S* to 64*S* are capsid proteins, and that DOC releases nucleocapsids from influenza virions. Attempts to precipitate labeled viral RNA with anticapsid serum were unsuccessful; after incubation with antiserum the labeled RNA became acid-soluble, presumably due to hydrolysis by nucleases in serum (see below).

Hydrolysis of viral RNA by ribonuclease treatment of nucleocapsids. It appeared reasonable to expect that the capsid proteins of influenza virus might protect the associated viral RNA from digestion by bovine pancreatic ribonuclease, since the RNA in NDV nucleocapsids was not made acid-soluble when the enzyme was added to DOC-treated virus (16). However, using the conditions previously employed with NDV, we found that the RNA's in influenza virus nucleocapsids were hydrolyzed by ribonuclease (Fig. 2B, 3B). This indicates that influenza virus RNA's are not completely surrounded by capsid proteins.

It was noteworthy that with both incomplete and complete virions the ribonuclease treatment did not markedly alter the sedimentation rates of the capsid proteins (Fig. 2B, 3B, fractions 12 to 30) suggesting that substantial nucleocapsid structure persists after hydrolysis of the RNA. The forces holding the capsid polypeptides together in these rapidly-sedimenting RNA-free structures were partially overcome when DOC-treated virions were centrifuged in sucrose gradients at *p*H 12. At high *p*H, most of the labeled proteins (and all of the RNA) were recovered in the top 15 ml of the gradients.

Properties of RNA's recovered from viral nucleocapsids. There were striking similarities between the sedimentation patterns of influenza virus RNA's isolated free from protein and the sedimentation patterns of viral nucleocapsids. Just as the most rapidly sedimenting nucleocapsid species of complete virions was 64*S*, with less 56*S* material (Fig. 1), so the most rapidly sedimenting RNA was 18*S* (Fig. 5, fraction 23), with a 15*S* RNA present in smaller quantity (Fig. 5, fraction 20). With incomplete virus, there was little 18*S* RNA, but a large peak at 15*S* (Fig. 5, fraction 20), correlating with reduced amounts of 64*S* nucleocapsids relative to 56*S* nucleocapsids (Figs. 1). Heterogeneously sedimenting RNA in the region of 10*S* to 13*S* was also found in incomplete virus (Fig. 5, fractions 12 to 17). In both complete and incomplete virus preparations, there was some RNA sedimenting at 4*S* to 6*S* (Fig. 5, fraction 7).

To examine more closely the relationship between viral nucleocapsids and RNA's 56*S* and 64*S* nucleocapsids labeled with ^{3}H-uridine were isolated separately from a sucrose gradient. After treatment with SDS, the RNA's were analyzed in sucrose gradients. RNA from the top of the nucleocapsid gradient sedimented heterogeneously at up to 12*S* after SDS treatment (Fig. 6, A and B). Nucleocapsids sedimenting at 56*S* yielded 15*S* and 8*S* RNA's, whereas 64*S* nucleocapsids gave RNA's sedimenting sharply at 18*S* and heterogeneously at up to 12*S*. Thus, though more than one sedimenting class of RNA was recovered from either 64*S* or 56*S* nucleocapsids, the more rapidly sedimenting nucleocapsids yielded more rapidly sedimenting RNA's.

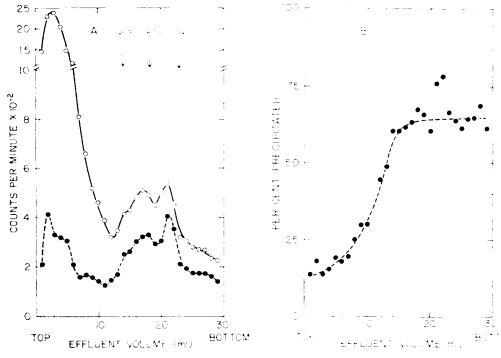

FIG. 4. *Precipitation of influenza virus capsid proteins with specific antiserum. A sample of complete virions, labeled with ^{3}H-amino acids, was treated with deoxycholate, and centrifuged on a sucrose gradient to separate viral nucleocapsids, as described in Materials and Methods. A portion of each fraction from the gradient was treated with antiserum to influenza A capsid ("soluble") antigen, as described in Materials and Methods. (A) Shows distribution of radioactivity in the gradient. Symbols: ○, ^{3}H counts/min in 10% of each gradient fraction; ●, ^{3}H counts/min in the antibody precipitate obtained from 10% of each gradient fraction. (B) Shows the radioactivity precipitated by antiserum to capsid proteins as percentage of total radioactivity in each density gradient fraction.*

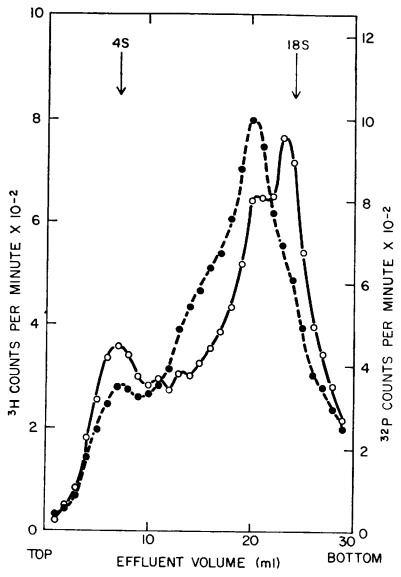

FIG. 5. *Sucrose gradient rate zonal centrifugation of influenza virus RNA's. A mixture of complete virions, labeled with ^{3}H-uridine, and incomplete virions, labeled with ^{32}P, was treated with Pronase, SDS, and phenol as described in Materials and Methods and centrifuged 42 hr at 51,500 × g, 4 C on a sucrose gradient. Symbols: ○, ^{3}H counts/min (complete virus); ●, ^{32}P counts/min (incomplete virus).*

DISCUSSION

Heterogeneously sedimenting influenza virus nucleocapsids have been observed before. Using ether to disrupt influenza virions, Paucker et al. (21) obtained "soluble antigen" (nucleocapsid) preparations which sedimented at 68*S*, 56*S*, and 48*S* in the analytical ultracentrifuge. Although it might have been arguable that the heterogeneity and relatively slow sedimentation rates of the ether-extracted subunits resulted from shearing or some other artifact of a two-phase system for virus disruption, this seems less likely in the single-phase deoxycholate system we used; the detergent appears simply to dissolve the virus envelopes. The congruence of our data and the data of Paucker et al. (21) indicates that both methods extract unaltered influenza virus nucleocapsids.

There is also precedent for our finding that, unlike the situation with NDV (16), the RNA in influenza virus nucleocapsids is accessible to ribonuclease. Again, using ether, Schafer and Wecker (25) extracted ^{32}P-labeled internal (gebundenes) antigen from fowl-plague virus and showed that pancreatic ribonuclease rendered all the label acid-soluble and nonprecipitable by immune serum. It is remarkable that this unambiguous demonstration has been neglected over the years, in view of its implications. For example, the accessibility of influenza virus RNA to ribonuclease when in the nucleocapsid may mean that the capsid protein performs a function other than protection of the RNA from nucleases at some stage in virus replication. Perhaps the capsid protein is necessary for the infecting RNA's to reach their sites of replication, or it may be essential for assembly of the progeny RNA's into virions. Another implication of the nuclease susceptibility is that the viral RNA's can enjoy noncovalent interactions even though encapsidated. The poorly defined morphology of influenza virus nucleocapsids (13) compared to paramyxovirus nucleocapsids (11, 12) may reflect a relatively open structure. This could provide a mechanism, via specific base-pairing between some nucleotides of each RNA chain, for coordinating the selection of a complete cohort of pieces of a divided genome in virus assembly. The 38*S* to 40*S* influenza virus RNA "aggregates" obtained from infected cells or virions under certain circumstances (2, 7, 19, 22) may actually represent such specific intermolecular complexes.

The persistently rapid sedimentation rates of influenza virus capsid proteins after ribonuclease digestion of the RNA's indicates that the capsid proteins interact mutually with enough avidity to maintain a good deal of quarternary structure in the absence of the RNA, an idea which is consonant with known strengths of structural protein interactions in the rod-shaped RNA plant virus, tobacco mosaic virus (5).

Similarities in sedimentation displays of the viral nucleocapsids and RNA's suggest that each RNA molecule is contained in a separate nucleocapsid, instead of several RNA molecules being combined in a large nucleocapsid. Parallel loss of the most rapidly sedimenting RNA and the most rapidly sedimenting nucleocapsid in incomplete virus supports this idea. The slower sedimenting RNA's and nucleocapsids have broader distributions on the gradients (Fig. 6), indicating incomplete resolution of several species, probably corresponding, in the case of the RNA's to species resolved by polyacrylamide gel electrophoresis (6, 23). We did not find complete correspondence between sedimentation rates of nucleocapsids and RNA's derived from them, perhaps because some degradation of RNA occurred during the lengthy sedimentation to separate the nucleocapsids.

It is interesting that "incompleteness" of influenza virus produced by cells infected at high multiplicity results from loss of a specific RNA's species, rather than nonspecific or random reduction in all species. Analysis of virus-specific RNA synthesized by cells infected at multiplicities high

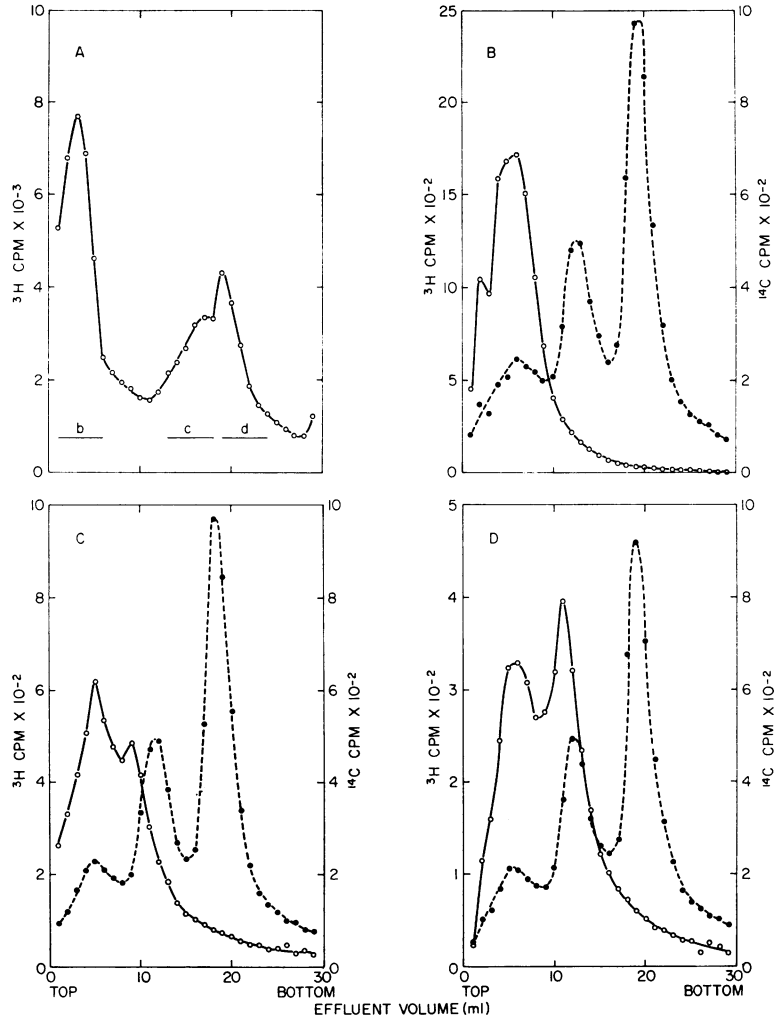

FIG. 6. *Sucrose gradient rate zonal centrifugation of influenza virus nucleocapsids and RNA's. A preparation of complete virus labeled with ^{3}H-uridine was treated with deoxycholate and centrifuged on a sucrose gradient, as described in Materials and Methods. After fractionation of the gradient and analysis of a portion of each fraction for acid precipitable radioactivity, fractions were pooled; RNA was extracted from each group of fractions with phenol and centrifuged on sucrose gradients at 51,500 × g for 24 hr, 4 C. RNA extracted from chick embryo fibroblasts labeled with ^{14}C-adenine was added to the viral RNA samples to provide sedimentation markers. (A) Deoxycholate-treated virus; (B) RNA from fractions 1 to 6 (b); (C) RNA from fractions 13 to 18 (c); (D) RNA from fractions 19 to 24 (d). Symbols: ○, ^{3}H count/min (viral RNA); ●, ^{14}C count/min (cell RNA).*

enough to produce such incomplete virus reveals reduced amounts of this very species (6, 7), suggesting that the basis of incompleteness in influenza virus is a defect in RNA biosynthesis rather than in packaging of the RNA into virions. Future work may reveal whether the largest influenza virus RNA's are simply synthesized in smaller amounts or whether their synthesis is initiated, but terminates prematurely, producing smaller RNA fragments.

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LITERATURE CITED

1. Ada, G. L., and B. T. Perry. 1956. Influenza virus nucleic acid: Relationship between biological characteristics of the virus

particle and properties of the nucleic acid. J. Gen. Microbiol. 14:623–633.
2. Agrawal, H. O., and G. Bruening. 1966. Isolation of high-molecular-weight, P^{32} labeled influenza virus ribonucleic acid. Proc. Nat. Acad. Sci. U.S.A. 55:818–825.
3. Barry, R. D., and P. Davies. 1968. The sedimentation of influenza virus and its RNA in sucrose density gradients. J. Gen. Virol. 2:59–69.
4. Bush, E. T. 1964. Liquid scintillation counting of double-labeled samples. Choice of counting conditions for best precision in two-channel counting. Anal. Chem. 36:1082–1089.
5. Caspar, D. L. D. 1963. Assembly and stability of the tobacco mosaic virus particles. Advan. Protein Chem. 18:37–121.
6. Duesberg, P. H. 1968. The RNA's of influenza virus. Proc. Nat. Acad. Sci. U.S.A. 59:930–937.
7. Duesberg, P. H., and W. S. Robinson. 1967. On the structure and replication of influenza virus. J. Mol. Biol. 25:383–405.
8. Eagle, H. 1959. Amino acid metabolism in mammalian cell culture. Science 130:432–437.
9. Francis, T., Jr., and A. E. Moore. 1940. A study of the neurotropic tendency in strains of the virus of epidemic influenza. J. Exp. Med. 72:717–728.
10. Hirst, G. K. 1962. Genetic recombination with Newcastle disease virus, polioviruses, and influenza. Cold Spring Harbor Symp. Quant. Biol. 27:303–308.
11. Horne, R. W., A. P. Waterson, P. Wildy, and A. E. Farnham. 1960. The structure and composition of the myxoviruses. I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques. Virology 11: 79–98.
12. Hosaka, Y., and K. Shimizu. 1968. Lengths of the nucleocapsids of Newcastle disease and mumps viruses. J. Mol. Biol. 35:369–373.
13. Hoyle, L., R. W. Horne, and A. P. Waterson. 1961. The structure and composition of the myxoviruses. II. Components released from the influenza virus particle by ether. Virology 13:448–459.
14. Huppert, J., and M. Semmel. 1965. Suppression de l'activite ribonucleasique par la pronase. Biochim. Biophys. Acta 108:501–503.
15. Kingsbury, D. W. 1966. Newcastle disease virus RNA. I. Isolation and preliminary characterization of RNA from virus particles. J. Mol. Biol. 18:195–203.
16. Kingsbury, D. W., and R. W. Darlington. 1968. Isolation and properties of Newcastle disease virus nucleocapsid. J. Virol. 2:248–255.
17. Lief, F. S., and W. Henle. 1956. Studies on the soluble antigen of influenza virus. III. The decreased incorporation of S antigen into elementary bodies of increasing incompleteness. Virology 2:782–797.
18. Lief, F. S., and W. Henle. 1959. Methods and procedures for use of complement fixation technique in type- and strain-specific diagnosis of influenza. Bull. World Health Organ. 20:411–420.
19. Nayak, D. P., and M. A. Baluda. 1967. Isolation and partial characterization of nucleic acid of influenza virus. J. Virol. 1:1217–1223.
20. Noll, H., and E. Stutz. 1968. The use of sodium and lithium dodecyl sulfate in nucleic acid isolation, p. 129–155. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 12B. Academic Press Inc., New York.
21. Paucker, K., A. Birch-Andersen, and P. von Magnus. 1959. Studies on the structure of influenza virus. I. Components of infectious and incomplete particles. Virology 8:1–20.
22. Pons, M. W. 1967. Studies on influenza virus ribonucleic acid. Virology 31:523–531.
23. Pons, M. W., and G. K. Hirst. 1968. Polyacrylamide gel electrophoresis of influenza virus RNA. Virology 34:385–388.
24. Rich, A., and D. M. Blow. 1958. Formation of a helical steroid complex. Nature 182:423–426.
25. Schafer, W., and E. Wecker. 1958. Uber die Wirkung von Ribonuclease auf ^{32}P-markiertes, 'gebundenes Antigen', des Virus der Klassischen Geflugelpest. Arch. Exp. Veterinaermed. 12:418–422.
26. Scharff, M. D., A. J. Shatkin, and L. Levintow. 1963. Association of newly formed viral protein with specific polyribosomes. Proc. Nat. Acad. Sci. U.S.A. 50:686–694.
27. Sokol, F., and S. Schramek. 1963. Comparison of different preparations of influenza virus ribonucleic acid. Biochem. Biophys. Res. Commun. 12:21–26.
28. von Magnus, P. 1951. Propagation of the PR 8 strain of influenza A virus in chick embryos. II. The formation of "incomplete" virus following inoculation of large doses of seed virus. Acta Pathol. Microbiol. Scand. 28:278–293.