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Structural Components of Influenza C Virions

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The genome RNA species of influenza type C virions were analyzed by polyacrylamide gel electrophoresis. The pattern obtained was found to resemble those of other influenza viruses. Six RNA species were resolved, with estimated sizes ranging from 0.37×10^6 to 1.25×10^6 daltons. The internal ribonucleoproteins of influenza C virions were found to sediment heterogeneously in glycerol velocity gradients as demonstrated previously with influenza A/WSN virus. The ribonucleoproteins possessed diameters of 12 to 15 nm, with lengths ranging from 30 to 100 nm. Of the three major virion polypeptides (molecular weights, 88,000, 66,000, and 26,000), only the largest is glycosylated. Similar polypeptide species were present in influenza C virions of five different strains. All three major proteins of influenza C virions possess electrophoretic mobilities distinguishable from those of the major polypeptides of influenza A/WSN. The 66,000-dalton protein is associated with the ribonucleoprotein components. Two additional glycosylated polypeptides, with estimated molecular weights of 65,000 and 30,000, were detected in virions grown in embryonated eggs, but not in virus particles obtained from chicken embryo fibroblasts.

Of the three immunological types of influenza viruses (A, B, and C), type A viruses have been the subject of the most detailed investigation. In contrast, very little is known about the structural components or the process of multiplication of type C influenza virus. In part this is due to the greater importance of the type A and B viruses as human pathogens and also to the availability of better systems for propagation of these viruses in cell culture.

A difference between the receptor for type C and other influenza viruses was recognized in early studies (12). Kendal (13) has recently confirmed the lack of effect of neuraminidase on cellular receptors for influenza C and has obtained evidence that the virus possesses an enzyme which destroys the influenza C receptor but does not liberate sialic acid from any substrate tested. Because of these differences, as well as the lack of information on the structure and replication process of influenza C virus, its classification as an orthomyxovirus remains uncertain.

We report here a study of the structural components of influenza C virus. In addition to determining that the viral genome consists of multiple RNA species, we have analyzed the polypeptide composition and nucleoprotein structure of the virion.

MATERIALS AND METHODS

Influenza C virus. The Johannesburg 1/66, Johannesburg 1/67, and Johannesburg 1/654 strains of influenza C virus were obtained from A. S. Beare, Medical Research Council Common Cold Research Unit, Salisbury, England. The Taylor /1233/47 strain was obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, and the Ann Arbor 1/50 strain was obtained from A. P. Kendal, Center for Disease Control, Atlanta. Virus was grown by amniotic inoculation of 0.1 to 0.2 ml of seed virus into 9- to 10-day-old embryonated chicken eggs. For radiolabeling of virions, radioisotopes were included in the inoculum. Virus was harvested from allantoic and amniotic fluids after 2 to 3 days of incubation at 33°C, and titers of 256 to 1,024 hemagglutination units/ml were obtained. For growth in chicken embryo fibroblasts (CEF), cells were seeded in 100-mm plastic petri dishes at a density of 15×10^7 cells/plate in Eagle medium with 10% calf serum. On the next day monolayers were inoculated with 1.5 ml of undiluted egg-grown virus per plate, and after 2 h the inoculum was removed and replaced with reinforced Eagle medium. In some experiments 2% calf serum was also present in the medium. Virus was harvested after incubation at 33°C for 3 days.

Influenza A virus. The WSN strain of influenza A virus was grown in MDBK cells (5) and purified as described previously (8, 15). For growth of ^{32}P-labeled influenza A/WSN, 100 μCi of [^{32}P]phosphate per ml was incorporated into the culture medium.

Purification of influenza C virions. Allantoic fluids or cell culture media were clarified at 2,000 rpm for 10 min, and virus was subsequently pelleted at 23,000 rpm for 1 h in a Beckman SW27 rotor. Pellets were resuspended in 1 ml of Eagle medium, layered on a 5 to 40% potassium tartrate gradient, and centrifuged for 2 h at 23,000 rpm in a Beckman

SW27 rotor. Visible bands of virus were collected and dialyzed against 0.01 M sodium phosphate buffer, pH 7.2.

Preparation of antisera. Six-week-old rabbits were immunized by intravenous injection of 10^4 hemagglutinating units of freshly prepared virus. The animals were bled before and 21 days after the injection of the antigen. Sera were stored at −12°C without preservative.

Hemagglutination inhibition tests. Hemagglutination inhibition tests were performed as described by Fazekas de St. Groth and Webster (11). Before use, serum was heated at 56°C for 30 min. Preimmunization serum was used in control tests. Starting from a dilution of 1:20, serial twofold dilutions were tested. Table 1 shows the lack of relationship between influenza C and other influenza virus serotypes.

Isolation of ribonucleoproteins. Virus was disrupted with 0.5% Triton X-100 and 0.7% sodium deoxycholate at 37°C and extracted twice with ether, and the ribonucleoprotein components were resolved on a 15 to 30% glycerol gradient as described previously (7, 9).

Polyacrylamide gel electrophoresis of viral RNA and polypeptides. For protein gel electrophoresis, virus samples in 0.005 M sodium phosphate, 1% sodium dodecyl sulfate and 1% β-mercaptoethanol were boiled for 1 min at 100°C. Ten percent acrylamide–0.2% *N,N'*-methylenebisacrylamide gels containing sodium dodecyl sulfate were prepared and used as described by Caliguiri et al. (4). Gels were sliced into 1-mm sections, and the distribution of radioactivity was determined as described previously (6). Slab gels containing a 10 to 20% gradient of acrylamide were prepared in a discontinuous Tris-glycine buffer as described by Laemmli (14). RNA was extracted from purified virus and resolved by 2% polyacrylamide gel electrophoresis as described elsewhere (2).

Electron microscopy. Virions in 0.01 M phosphate buffer, pH 7.2, were applied to grids with carbon-coated Formvar films and stained with 2% sodium phosphotungstate, pH 6.8. Specimens were examined in a Philips EM301 microscope.

RESULTS

Morphology of virions. Electron micrographs of influenza C virions after purification from allantoic fluid and CEF culture medium are shown in Fig. 1. Virions from allantoic fluid are usually roughly spherical and possess diameters ranging from 75 to 100 nm, including a layer of surface projections ~10 nm in length. In contrast, virions from CEF cultures consist predominantly of long filaments with a similar diameter as well as pleiomorphic particles. The surface projections on filamentous particles are usually found in a regular hexagonal arrangement (Fig. 1C). Very long filaments, such as the particle shown in Fig. 1B, which measures over 7 μm in length, are frequently observed.

Viral RNA. The genome RNA species of the JHB 1/66 strain of influenza C virions were analyzed by coelectrophoresis with RNA from influenza A/WSN virions (Fig. 2). The results indicate that the genome of influenza C consists of multiple segments of RNA, with at least six RNA species resolved. The overall pattern, as well as most of the individual peaks of the influenza C viral RNA, can be clearly distinguished from those of influenza A/WSN. Noteworthy is the observation that influenza C virions contain RNA species of lower electrophoretic mobility than any of the species present in the A/WSN strain; also, the smaller-molecular-weight RNA species of A/WSN (molecular weight, 3.5×10^5) migrate more rapidly than the corresponding RNAs of influenza C.

The molecular weights of the RNA genome segments of influenza C virions were estimated, using values obtained for A/WSN viral RNA as standards (Table 2). The values range from 1.25 to 0.37×10^6, and the sum molecular weight for the six RNA species is 5.15×10^6.

The relative molar amounts of each RNA species are also listed in Table 2. These values are presented recognizing that the virus was labeled with [^{3}H]uridine, and no account can be made for differences in base ratios of the different species. The two high-molecular-weight peaks are present in approximately half the relative molar amounts of segments 3 to 5, whereas peak 6 is present in twice the molar

TABLE 1. *Hemagglutination inhibition of influenza C virus with antisera to various influenza virus serotypes*

Determination	Hemagglutination inhibition[a] at 1/serum dilution:							
	20	40	80	160	320	640	1,280	2,560
Antiserum[b]								
H_0N_1	Trace	+	++	++	++	++	++	++
H_1N_1	Trace	+	++	++	++	++	++	++
H_2N_2	Trace	+	++	++	++	++	++	++
B	Trace	+	++	++	++	++	++	++
C	−	−	−	−	−	−	−	−
Virus[c] C/1/66	++	±	−	−				

[a] ++ denotes complete agglutination; + denotes partial agglutination.
[b] Antisera to A/WSN/H_0N_1, A/CAM/H_1N_1, A/Jap/305/H_2N_2, B/Lee, and C/1/66 strains were used.
[c] Similar results were obtained using other strains of influenza C as the test virus.

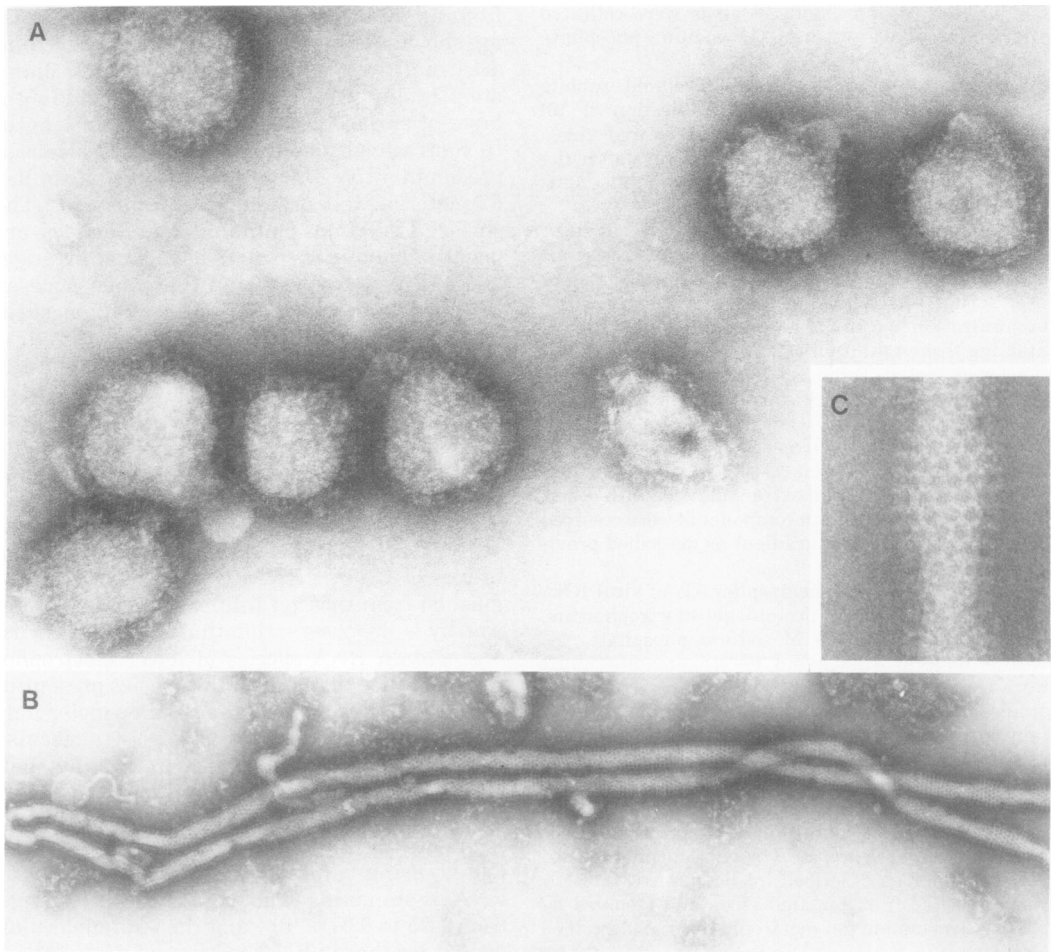


FIG. 1. *(A) Influenza C virions (JHB/1/66) purified from allantoic fluid. ×220,000. (B) Influenza C filament obtained from CEF cell culture fluids. The particle measures over 7 μm in length. ×40,000. (C) Higher magnification of the same particle, showing regular hexagonal arrangement of the surface glycoproteins. ×180,000. Negative staining with sodium phosphotungstate, pH 6.8.*

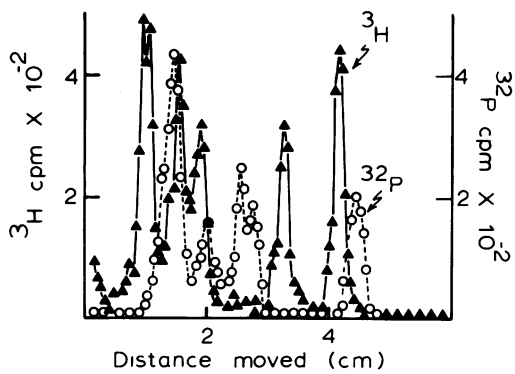


FIG. 2. *Coelectrophoresis of RNA species of influenza C/JHB/1/66 virions labeled with [^3H]uridine and influenza A/WSN virions labeled with $^{32}PO_4$. Symbols: ▲, ^3H dpm; ○, ^{32}P dpm.*

quantity. The slight separation of the two high-molecular-weight peaks and their occurrence in a relatively lower amount could indicate that a single RNA species exhibiting conformational differences is present in this region. By using gels of a lower concentration, two bands have been partially separated, although until these bands are fingerprinted we will not know whether they represent different nucleotide sequences. Similarly, using gels of a higher concentration, the smallest influenza C RNA peak has been resolved into two partially separated bands (data not shown).

Ribonucleoprotein components. The internal ribonucleoprotein components of influenza A viruses sediment heterogeneously in velocity gradients, and three size classes with sedimentation coefficients ranging from 38–70S have

TABLE 2. *Genome RNA species of influenza C virions*[a]

RNA species	Mol wt[b] (×10⁶)	Relative amt[c] (M)
1	1.25	0.5
2	1.20	0.5
3	0.98	1.0
4	0.85	0.8
5	0.50	1.1
6	0.37	2.0

[a] JHB/1/66 strain.

[b] Molecular weight estimates are based on a comparison of electrophoretic mobility with influenza A/WSN genome RNAs, using molecular weights determined by Bishop et al. (2) for the calculations.

[c] Molar amounts of each RNA species were calculated relative to species no. 3 = 1.0.

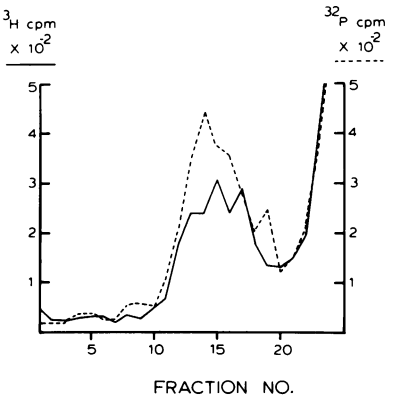


FIG. 3. *Cosedimentation of ribonucleoproteins of influenza C/JHB/1/66 virions labeled with [³H]-uridine and influenza A/WSN virions labeled with $^{32}PO_4$. Virions were mixed and disrupted with Triton X-100 and sodium deoxycholate, followed by two ether extractions, and analyzed on a 15 to 30% glycerol gradient containing 0.05% sodium deoxycholate as described previously (7). Sedimentation was for 2 h at 40,000 rpm in a Beckman SW50.1 rotor.*

been reported (3, 7, 9, 10, 18). Cosedimentation of [³H]uridine-labeled ribonucleoproteins of influenza C virions with those of [³²P]phosphate-labeled influenza A/WSN indicated that influenza C ribonucleoproteins also sediment heterogeneously (Fig. 3). The fast-sedimenting region of the influenza C ribonucleoproteins contained a lower proportion of the total label than that observed with WSN virus.

The morphology of influenza C ribonucleoproteins is shown in Fig. 4 and 5. The strand-like structures measure about 12 to 15 nm in width in negatively stained preparations and appear similar to the ribonucleoproteins obtained from influenza A viruses (7, 18). The lengths of the ribonucleoprotein strands were variable, ranging from 30 to 100 nm. A preponderance of longer ribonucleoproteins was found in the fast-sedimenting region of the gradient, but complete separation into distinct size classes was not achieved.

Virion polypeptides. Coelectrophoresis of polypeptides of the C/Taylor/47 strain, grown in embryonated eggs and labeled with [³H]-leucine, with polypeptides of A/WSN virions labeled with ¹⁴C-amino acids is shown in Fig. 6A. Three major peaks are observed, with a distinct shoulder on the trailing side of the fast-migrating component. In Fig. 6B the polypeptide subunit of the ribonucleoprotein is shown, which migrates just behind the NP polypeptide of the A/WSN marker and therefore corresponds to the intermediate of the three influenza C polypeptide peaks. To identify glycosylated polypeptides, influenza C virions were grown in embryonated eggs and labeled with [³H]glucosamine and ¹⁴C-amino acids (Fig. 7). Glucosamine label is associated with the largest virion polypeptide as well as the region of the nucleocapsid protein and the trailing side of the fast-migrating polypeptide. As discussed below, the nucleocapsid protein is distinct from the glycosylated component migrating in this region, so that a total of three glycoproteins and two carbohydrate-free polypeptides have been identified in the egg-grown C/Taylor virus. Two minor peaks of amino acid label at fractions 42 and 53 are not reproducibly observed (see Fig. 6A) and may be contaminants or degradation products. A variable amount of radioactivity was also observed at the top of the gel, and it is uncertain whether additional viral polypeptides are present in this region.

Because of the lack of information on specific functions of each glycoprotein, the estimated molecular weights based on coelectrophoresis with A/WSN virion polypeptides have been used to denote the glycoproteins in Fig. 7. Accordingly, the largest glycoprotein of ~88,000 daltons is designated gp 88, the glycoprotein in the region of the ribonucleoprotein subunit is estimated at 65,000 daltons and is denoted gp 65, and the smallest glycoprotein of ~30,000 daltons is denoted gp 30. The ribonucleoprotein subunit is denoted NP, and the lowest-molecular-weight polypeptide is denoted M; the latter designation was suggested by Kendal (13) based on the resistance of this polypeptide to proteolytic digestion. The molecular weights of these components were estimated to be 66,000 for NP and 26,000 for M.

The major polypeptides of influenza C virions of four other strains are shown in Fig. 8. Similar profiles were observed for all of the strains, although the NP polypeptide of the C/JHB/1/66

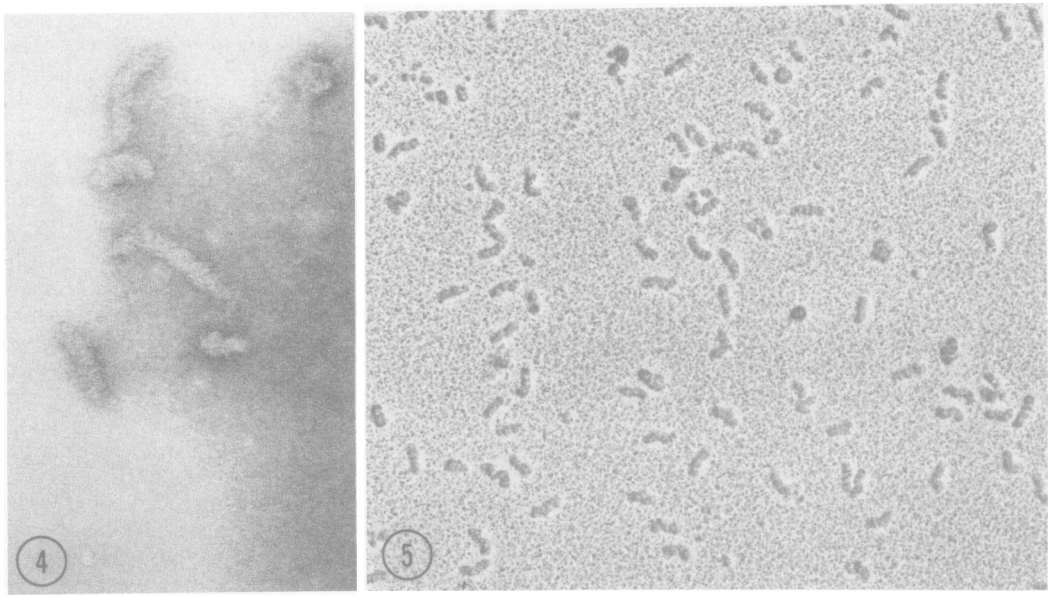


FIG. 4. *Ribonucleoproteins of influenza C virions (JHB/1/66) negatively stained with uranyl acetate. ×180,000.*

FIG. 5. *Ribonucleoproteins after rotary shadowing with Pt/Pd for 15 s at a 10° angle. ×45,000.*

strain migrated slightly more rapidly than the corresponding polypeptide of other strains. The NP and M polypeptides of A/WSN virions, included together with a sample of C/Ann Arbor 1/50 in lane E, are observed to migrate distinctly faster than the corresponding influenza C polypeptides. In a previous study with the C/Ann Arbor 1/50 strain of influenza C virus, Kendal (13) also observed three major components, as well as two minor glycoproteins, with electrophoretic mobilities similar to those we report here.

Polypeptides of influenza C virions grown in CEF cell cultures and labeled with [^{3}H]leucine or [^{3}H]glucosamine are shown in Fig. 9. These cells produced low yields (~32 hemagglutination units/ml) of virus, which did not undergo multiple cycles of CEF replication and was highly pleomorphic, as shown in Fig. 1. The largest glycoprotein (gp 88) was the major polypeptide species present in the CEF-grown virions. NP and M were less abundant components of the virions grown in cell culture; however, they were reproducibly observed, whereas the smaller glycoproteins gp 65 and gp 30 were not detected by glucosamine labeling. Therefore, virus particles obtained from CEF cells appear to be composed predominantly of a single type of glycoprotein with lower levels of internal proteins and no other detectable glycoproteins.

DISCUSSION

Because of limited knowledge of the structure and replication of influenza C virus, its classification within the myxovirus group has remained a matter of some uncertainty. The demonstrated difference between the receptor-destroying activity of influenza C and the neuraminidase of influenza types A and B (12, 13) has raised the possibility that other biochemical differences may exist between these virus types. The present results, however, indicate certain basic similarities between the structural components of type C and prototype influenza A viruses: the segmented genome, the size and number of the RNA species, the morphology and sedimentation properties of the ribonucleoproteins, and the sizes of the NP and M polypeptides. Although we do not know whether the six or seven RNA species so far observed for the influenza C virus represent all of the RNA species (eight have been observed for influenza A and B viruses [19]), the total molecular weight of the genome (5×10^6 to 6×10^6) of influenza C virus is also quite similar to values obtained for type A virus. Since only five polypeptide components have been identified and it is uncertain whether they all represent primary gene products, it is not yet possible to suggest any relationships between specific genome RNA species and polypeptide products.

Virion RNAs of the GL/1167/54 strain of influenza C were recently described by Ritchey et al. (19). Their results differ from those reported here in that only four RNA species were resolved and also in the size range observed.

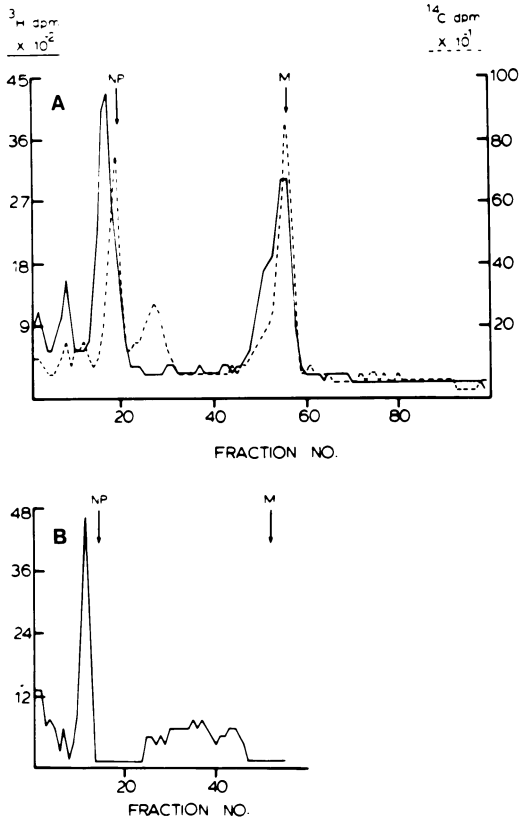


FIG. 6. *(A) Coelectrophoresis of polypeptides of influenza C (Taylor) virions, grown in embryonated eggs and labeled with [^{3}H]leucine, with polypeptides of influenza A/WSN virions labeled with ^{14}C-amino acids. The NP and M polypeptides of the WSN strain are indicated by arrows. (B) Polyacrylamide gel electrophoresis of the polypeptide subunits of ribonucleoproteins isolated from [^{3}H]leucine-labeled influenza C (Taylor) virions as described in the legend to Fig. 3. Polypeptides of influenza A/WSN virions labeled with ^{14}C-amino acids were included as markers, and the positions of the NP and M polypeptides are indicated.*

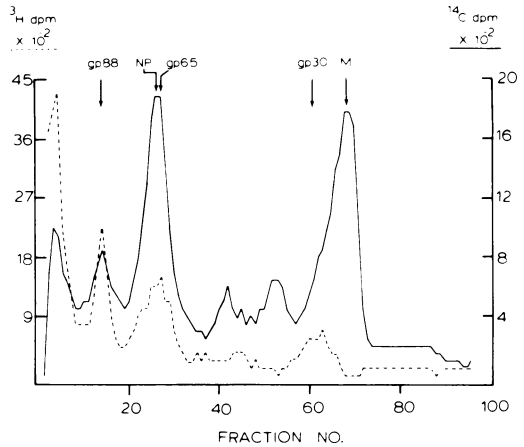


FIG. 7. *Polyacrylamide gel electrophoresis of polypeptides of influenza C (Taylor) virions grown in embryonated eggs and labeled with [^{3}H]glucosamine and ^{14}C-amino acids. The major polypeptides and glycoproteins that have been clearly identified are indicated by arrows and designated as described in the text.*

Their largest species was estimated to be 2 × 10^6 daltons, which is significantly larger than any RNA species we have observed. The reasons for these differences remain to be established.

The major polypeptides observed in the present study are in agreement with those described by Kendal (13) for the influenza C/AA 1/50 strain. Two minor glycoprotein species were also reported (molecular weights, 62,000 and 28,000), which are similar to species that we have observed using glucosamine labeling of virions in embryonated eggs, but not in virions grown in CEF cells. Since the NP polypeptide in virions grown in CEF cells was not glycosylated, as shown by [^{3}H]glucosamine labeling, we conclude that the glycosylated polypeptide gp 65 observed in virions grown in embryonated eggs is distinct from the NP polypeptide. The relationship between the molecular weights of the three glycoproteins in egg-grown virions and the fact that only the largest species is found in virions grown in chicken fibroblasts both suggest the possibility that gp 65 and gp 30 may be generated by proteolytic cleavage of gp 88. However, in preliminary experiments we have been unable to demonstrate such cleavage by treatment of CEF-grown virions with 1 to 10 μg of trypsin per ml, which causes complete cleavage of the hemagglutinin polypeptide of influenza A virions (16).

The pleomorphic and filamentous particles produced by CEF infected with influenza C virus clearly contain large amounts of the glycoprotein gp 88 and smaller amounts of the internal proteins. Since these particles are unable to undergo multiple cycles of infection, they appear analogous to incomplete or von Magnus-type virus observed on serial undiluted passage of other influenza viruses. In previous analyses of the polypeptides of incomplete virus obtained with influenza A/WSN, an increase in the relative amounts of the virion glycoprotein species was also demonstrated (17), although the differences were less striking than those found with influenza C.

In a previous study of thin sections of influenza C virions, Apostolov et al. (1) reported a difference between the nucleoprotein diameter of influenza C virions (9 nm) in comparison with that of A and B virions (6 nm). We find a

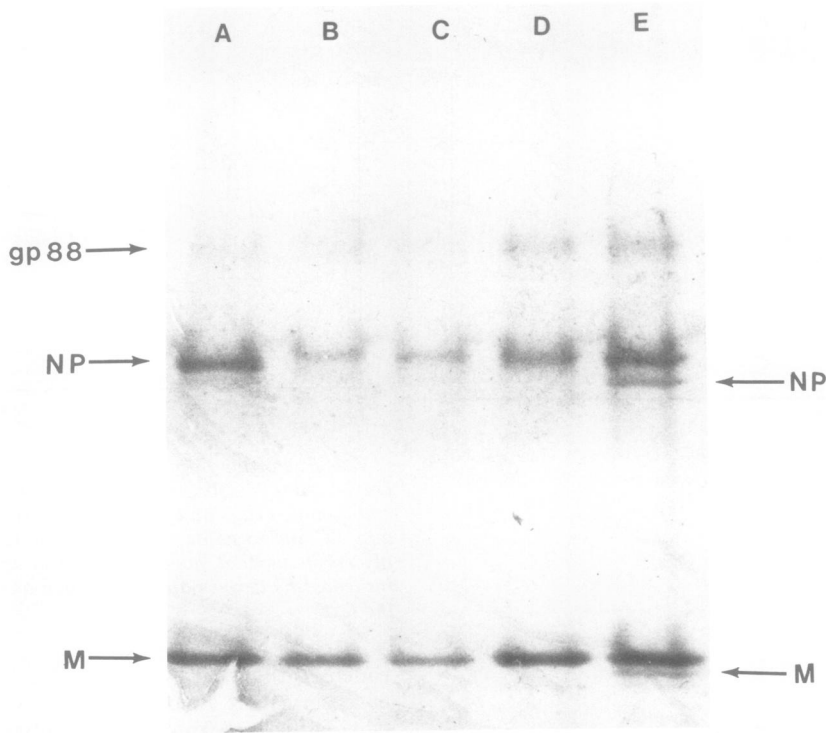
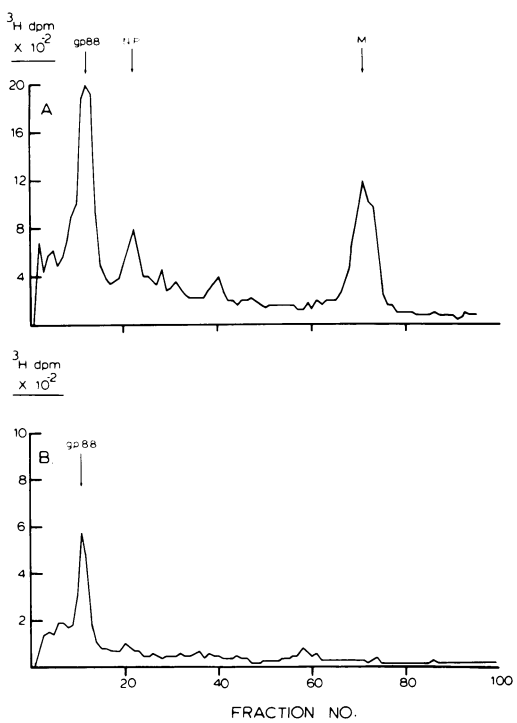


FIG. 8. *Polyacrylamide gel electrophoresis of polypeptides of influenza C virions on a 10 to 20% gradient gel. (A) JHB/1/66 strain; (B) JHB/1/67 strain; (C) JHB/1/654 strain; (D) AA/1/50 strain; (E) coelectrophoresis of AA/1/50 and A/WSN strains. Polypeptides of influenza C virions are indicated on the left margin, and the NP and M polypeptides of A/WSN are indicated on the right margin.*



diameter of 12 to 15 nm for isolated influenza C ribonucleoproteins by negative staining, which corresponds to values obtained previously with influenza A. These results are not necessarily in conflict, however, since the morphology of the nucleoprotein may be modified by the extraction procedure. Also, the diameter seen in a thin section probably reflects binding of stain to the viral RNA, whereas a negative stain reveals the diameter formed by the polypeptide subunits. Therefore, the ribonucleoproteins may differ in the radius at which the RNA is located without differing in the overall diameter of the structure.

The results presented here as well as previous studies of the biochemistry of influenza C virions (13) indicate many similarities to influenza A and B viruses. Further studies on the mode of replication of the virus will be necessary, however, before the nature of influenza C

FIG. 9. *(A) Polypeptides of influenza C (JHB/1/66) virions obtained from CEF cell cultures labeled with [^{3}H]leucine. Positions of the three major virion polypeptides gp 88, NP, and M are indicated. (B) A similar preparation labeled with [^{3}H]glucosamine, showing label associated with the major glycoprotein gp 88.*

and its relationship to other orthomyxoviruses can be clearly established.

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