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Isolation and Characterization of Cytoplasmic Inclusions from Influenza A Virus-Infected Cells

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Influenza A viruses induce the accumulation of electron-dense inclusions in the cytoplasm of infected cells during the latter stages of the replication cycle. Cell fractionation studies showed that these inclusions could be recovered in subcellular fractions containing ribosomes and polysomes. Isolation of these inclusions was accomplished by procedures involving RNase treatment of these fractions followed by repurification, or by fluorocarbon extraction and gradient centrifugation. Electron microscopy indicated that the isolated inclusions exhibited a major periodicity of ~8 nm with minor periodicities of ~4 nm. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the influenza virus coded nonstructural protein was the only protein component present in isolated inclusions.

One of the prominent virus-induced morphological alterations in cells infected with type A influenza viruses is the appearance and accumulation of paracrystalline inclusions in the cytoplasm during the latter stages of infection. These inclusions have been observed in vivo during the infection of mouse bronchial epithelium and brain (2, 12) as well as in infected cell cultures (6, 9, 14, 20). Despite their occurrence in a variety of cell types infected with influenza viruses, no information has been obtained on their composition or their possible significance in the viral replication cycle.

In the present study, we have undertaken to purify and characterize these inclusions. The inclusions have been isolated from two cell lines after infection with the WSN strain of influenza A virus, and their protein composition has been determined.

MATERIALS AND METHODS

Virus and cells. Stock A_0/WSN (H_0N_1) virus was grown in MDBK cells (5). ^{14}C-amino acid-labeled virus for use as internal markers in polyacrylamide gel electrophoresis (PAGE) was grown and purified as described elsewhere (11). MDBK (bovine kidney) and BHK-21-F (baby hamster kidney) cells were grown according to previously described procedures (5, 13).

Infection and labeling of cells. Monolayer cultures of BHK-21-F cells grown in 100-mm Corning plastic tissue culture dishes were inoculated at a multiplicity of 10 to 30 PFU/cell in 1.5 ml of Eagle minimal essential medium supplemented with 1% bovine serum albumin per culture. After a 2- to 3-h adsorption period at 37°C, 5 ml of reinforced Eagle medium (1), supplemented with 2% calf serum and 5 μCi of [^{3}H]-leucine per ml, was added to each culture. Whole infected cells for use as internal markers in PAGE were treated similarly, except for the use of 2 μCi of ^{14}C-amino acid mixture per ml.

Monolayer cultures of MDBK cells grown in 2-liter glass roller bottles were inoculated at a multiplicity of 10 to 20 PFU/cell in 15 ml of Eagle medium with 1% bovine serum albumin per culture. After a 4-h adsorption period at 37°C, 50 ml of reinforced Eagle medium with 2% calf serum and 2 μCi of [^{3}H]leucine per ml was added to each culture.

After 37°C incubation, for approximately 20 h for BHK-21-F cells or 24 h for MDBK cells, samples consisting of 30 BHK-21-F culture dishes or 10 MDBK roller bottles were harvested by scraping with a silicone blade.

Cell fractionation. Cells were homogenized with a Dounce homogenizer, and the procedure of Caliguiri and Tamm (4) was used to fractionate cytoplasmic extracts in a discontinuous sucrose gradient as described previously (7).

PAGE. Samples were heated for 1 min at 100°C in 1% sodium dodecyl sulfate and 1% β-mercaptoethanol in 5 mM sodium phosphate buffer, pH 7.2 (16). Then, 7.5% acrylamide, 0.20% *N,N'*-methylenebisacrylamide gels, or 10% acrylamide, 0.25% *N,N'*-methylenebisacrylamide gels containing sodium dodecyl sulfate, were prepared and run as described by Caliguiri et al. (3). For the detection of protein bands, the gels were extruded and immersed in 20 ml of 0.25% Coomassie blue stain in water-methanol-acetic acid (5:5:1) and stained for 6 h at 37°C. The gels were then rinsed in water until the wash was clear and destained for 18 h at room temperature in 20 ml of water-methanol-acetic acid (5:5:1), followed by 20 ml of water-methanol-acetic acid (7:1:2) until destained sufficiently. The stained gels were stored in demineralized water until they were scanned at 590 nm using a Schoeffel spectrodensitometer.

Slicing and processing of gels for the determination

of radioactivity by liquid scintillation was as previously described (8).

Protein assay. Isolated inclusion samples were dialyzed against water. To 0.25 ml of sample, 0.25 g of guanidinium chloride was added, and the solution was incubated at 60°C for 60 min. A 0.1-ml volume of this solution was mixed with 5.0 ml of Bio-Rad protein assay dye reagent. After 5 min, the optical density was read at 595 nm using 0.1 ml of 50% (wt/wt) guanidinium chloride + 5.0 ml of dye reagent as a reference. Bovine serum albumin was used as a standard.

Electron microscopy. Whole cells or the nuclear fractions from homogenized cells were pelleted at 2,000 rpm for 10 min in an International model PR2 centrifuge. Gradient fractions were pelleted at 23,000 rpm for 5 h in a Spinco SW27 rotor. Sample pellets were fixed with glutaraldehyde, postfixed with osmium tetroxide, dehydrated, and embedded in epoxy resin as described previously (10). Thin sections were stained with uranyl acetate and lead citrate.

For negative staining of isolated inclusions, a drop of sample was placed on a Formvar-carbon-coated grid for 30 s and stained with phosphotungstic acid (pH 6.2) or uranyl acetate for approximately 45 s.

Specimens were examined in a Philips EM301 microscope.

Amino acid analysis. Salt-free and detergent-free samples were hydrolyzed in vacuo in 6 N HCl at 110°C for 20 h. A Durrum D-500 high-pressure amino acid analyzer controlled by a PDP-8/L computer was employed for quantitation.

Chemicals and isotopes. [4,5-^{3}H]leucine (61 Ci/mM), ^{14}C-labeled reconstituted protein hydrolysate, and density-gradient grade sucrose were obtained from Schwarz/Mann, Orangeburg, N.Y. Electronic grade Genesolv D (trichlorotrifluoroethane) was obtained from Allied Chemical Corp., Atlanta, Ga. Density-gradient grade metrizamide was obtained from Gallard-Schlesinger, Carle Place, N.Y. Triton X-100 was obtained from Sigma Chemical Co., St. Louis, Mo. Guanidinium chloride (absolute grade) was obtained from Research Plus Laboratories, Denville, N.J.

RESULTS

Location of inclusions in subcellular fractions. Cytoplasmic inclusions accumulate in large amounts at late times after influenza virus infection. An example of an MDBK cell containing numerous cytoplasmic inclusions at 24 h postinfection is shown in Fig. 1A; similar observations have been made in other cell types. To purify these inclusions, cell fractionation was initially carried out by the procedure of Caliguiri and Tamm (4). As described previously (4, 7), the discontinuous sucrose gradient employed resolves seven fractions from the cytoplasmic extracts of homogenized cells. Electron microscopic examination of these gradient fractions from BHK-21-F cells revealed small numbers of the inclusions in fractions 6 and 7 (both of which consist primarily of polysomes and free ribosomes). Few, if any, inclusions were seen in any of the other fractions. However, thin sections of the nuclear pellet remaining after preparation of cytoplasmic extracts showed that the majority of the inclusions were retained in the initial pellet with the nuclei and associated membranes (Fig. 1B).

Isolation of inclusions. To solubilize the inclusions, the crude nuclear pellet from BHK-21-F cells was suspended in 12 ml of 1% Triton X-100 in RSB (reticulocyte standard buffer:10 mM NaCl-5 mM $MgCl_2$-10 mM Tris [pH 7.4]), held for 18 h at 4°C, and homogenized (10 to 20 strokes) in a Dounce homogenizer. After centrifugation at 800 × *g* for 10 min, the supernatant was layered onto a discontinuous sucrose-metrizamide gradient prepared in RSB (top to bottom: 3.5 ml of sample; 2.1 ml of 25% [wt/vol] sucrose; 2.1 ml of 40% [wt/vol] sucrose, 2.1 ml of 60% [wt/vol] sucrose; 2.1 ml of 56% [wt/vol] metrizamide) and centrifuged at 30,000 rpm for 90 min in a Spinco SW41 rotor. Visible bands at the density interfaces of the gradients were collected, diluted, and pelleted (25,000 rpm for 60 min in an SW41 rotor), and embedded for thin sectioning. Electron microscopic examination revealed that numerous inclusions had sedimented to equilibrium at the sucrose-metrizamide interface (Fig. 1C). PAGE analysis of [^{3}H]leucine-labeled polypeptides in this fraction (not shown) indicated that the major virus-coded polypeptide constituents were the ribonucleoprotein (NP) and nonstructural protein (NS). Prior incubation of the Triton-treated supernatant with 10 μg of RNase A per ml for 30 min at 37°C significantly reduced the number of polysomes at the sucrose-metrizamide interface without affecting the inclusions. PAGE analysis of this fraction showed NS to be the major virus-coded polypeptide present (Fig. 2).

An alternative isolation procedure was developed utilizing fluorocarbon extraction as described by Nagayama and Dales (18). These investigators reported that this procedure enriched cytoplasmic extracts for microtubular paracrystals without affecting their fine structure. To obtain cytoplasmic extracts enriched in inclusions, the nuclear pellet remaining after the initial Dounce homogenization was resuspended in RSB and homogenized for an additional 10 strokes. The supernatants from the two homogenizations were pooled and extracted first with twice the sample volume of Genesolv D and then three times with equal volumes of sample and fluorocarbon. At each extraction step, the mixture was shaken vigorously for 60 to 90 s before the fluorocarbon phase and any

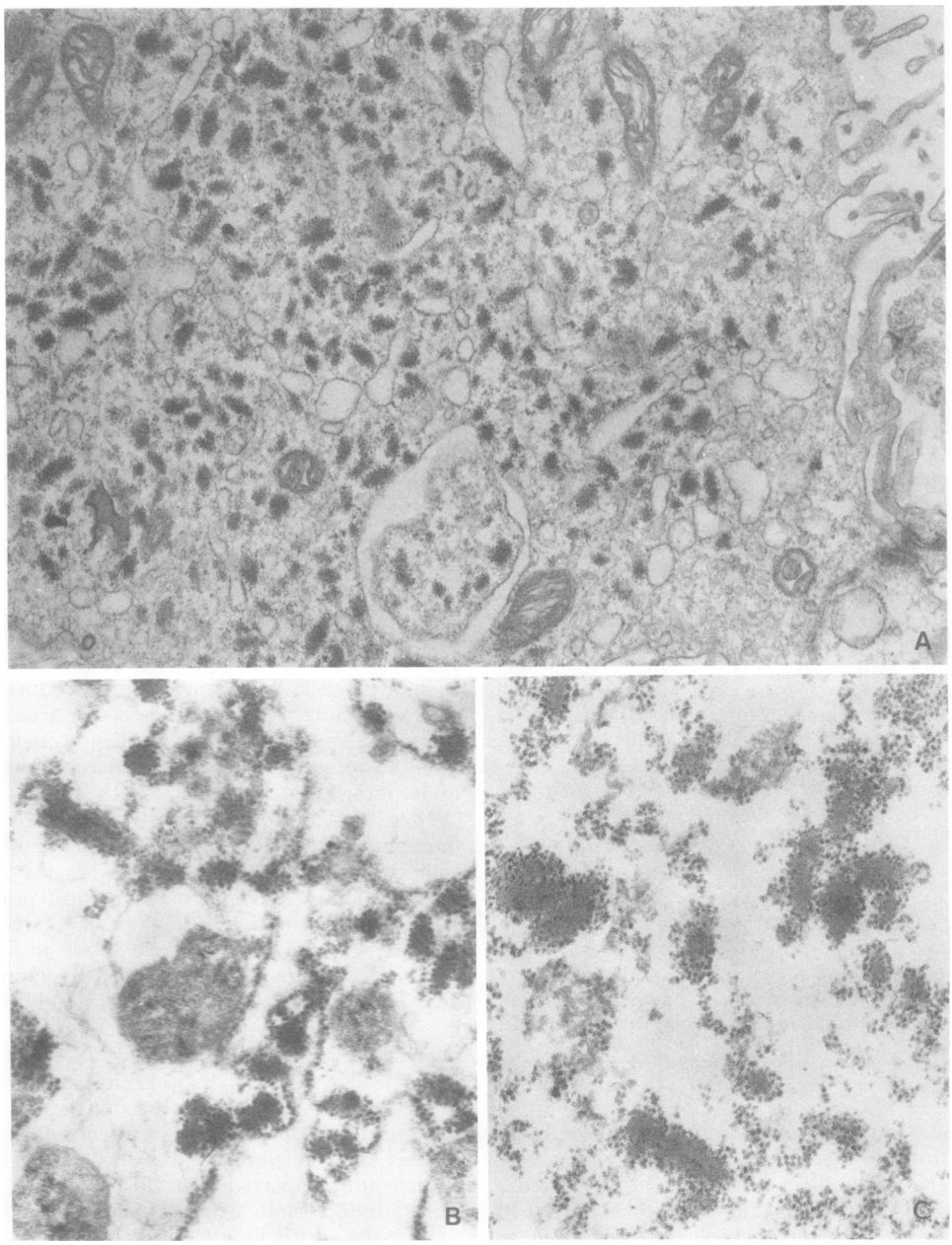

FIG. 1. *Electron micrographs of thin sections of influenza virus-infected cells and cell fractions. (A) Infected MDBK cell, 20 h postinfection, showing a large number of electron-dense inclusions in the cytoplasm (×18,000). (B) Nuclear pellet obtained after the Dounce homogenization, showing the association of the inclusions with the perinuclear membranes and polysomes (×18,000). (C) Sucrose-metrizamide interface fraction from a discontinuous gradient after Triton treatment and further homogenization of the nuclear pellet. Polysomes are the major visible contaminant (×38,000).*

membranous material forming at the interface were discarded. Low-speed centrifugation was sometimes necessary to facilitate the phase separation. The resultant aqueous fraction was pelleted at 25,000 rpm for 60 min in a Spinco SW27 rotor.

The pellet was suspended in 0.01 M Tris-hydrochloride–0.001 M EDTA (pH 8.5) and ad-

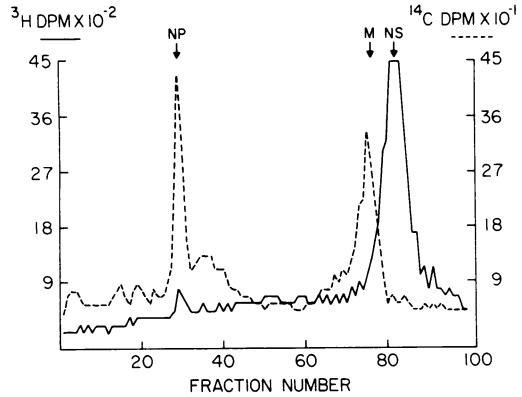

FIG. 2. *Polyacrylamide gel electropherogram of [^{3}H]leucine-labeled polypeptides in an inclusion fraction similar to that shown in Fig. 1C, treated with RNase A before gradient fractionation. ^{14}C-amino acid-labeled WSN viral polypeptides were included as internal markers.*

justed to 1% Triton X-100 in a final volume of 2.5 ml per BHK-21-F cell sample or 7.5 ml per MDBK cell sample, and then shaken vigorously and left for 18 to 20 h at 5°C. These samples were layered onto discontinuous sucrose gradients prepared in Tris-EDTA (top to bottom: 2.5 ml of sample; 2.5 ml of 45% [wt/wt] sucrose; 2.5 ml of 50% [wt/wt] sucrose; 2.5 ml of 55% [wt/wt] sucrose; 2.5 ml of 60% [wt/wt] sucrose) and centrifuged at 35,000 rpm in an SW41 rotor for 90 min. Several fractions were obtained with well-defined peaks of radioactivity occurring at the 50–55% and 55–60% sucrose interfaces (Fig. 3). These fractions were diluted, washed by pelleting (three times for 15 min at 25,000 rpm in an SW41 rotor) in Tris-EDTA buffer, and resuspended in that buffer. Electron microscopic examination of the fractions from BHK-21-F cells showed the inclusions to be present in large amounts in both the 50–55% and 55–60% sucrose interface fractions and also in the 55% sucrose zone separating them (Fig. 4). Similar results were obtained using MDBK cells, where the 50–55% sucrose interface fraction was found to be the purer of the two; however, both MDBK fractions appeared to contain considerably more contaminating material than similar fractions from BHK-21-F cells. A total of 0.75 to 1.0 mg of protein of purified inclusions was obtained from 30 BHK-21-F cell monolayers.

Fine structure of isolated inclusions. The morphology of the paracrystalline inclusions isolated as described above was examined by negative staining. As seen in the high-magnification inset of Fig. 4, major striations were observed running longitudinally, and at least two other orientations of striations were observed. Measurements of such negatively stained images yielded approximate values of 8 nm for the periodicity of the major striations and 4 nm for the minor striations. The minor striations run at angles of approximately +55° and −55° to the major striations. Some inclusions also appeared fragmented (particularly in MDBK isolates) but still possessed the characteristic striations.

Composition of isolated inclusions. Sodium dodecyl sulfate-PAGE analyses of the purified inclusion are shown in Fig. 5 and 6. Radioactivity profiles of the electrophoresis of polypeptides in the 50–55% sucrose interface fraction from MDBK cells (Fig. 5A) show that NS is the only viral protein detectable. By 23 h postinfection, the only viral proteins observed in significant amounts in WSN-infected BHK-21-F cells were NP and NS, which are seen in the profile of ^{14}C marker proteins used in Fig. 5B. The NS protein in infected BHK-21-F cells comigrated with the protein found in isolated inclusions. PAGE analysis of the 55–60% interface fraction from MDBK cells, or of either fraction from BHK-21-F cells, all showed virtually identical results when radioactivity was analyzed (data not shown). However, only the fractions from BHK-21-F cells contained no detectable contaminating unlabeled proteins when examined in stained gels (Fig. 6). MDBK inclusion isolates contained minor stained bands not detected in radioactivity profiles (data not shown).

Amino acid analysis. The amino acid composition of pure NS protein isolated from BHK-21-F cells is shown in Table 1. For comparison,

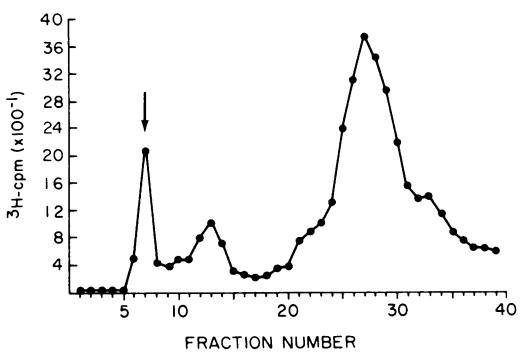

FIG. 3. *Discontinuous sucrose gradient centrifugation of [^{3}H]leucine-labeled inclusions. Cytoplasmic extracts of infected MDBK cells were extracted with fluorocarbon and treated with Triton before centrifugation. Fractions of 25 drops each were collected by puncturing the tube. The bottom of the gradient is on the left, and the arrow indicates the peak of radioactivity at the 55–60% sucrose interface.*

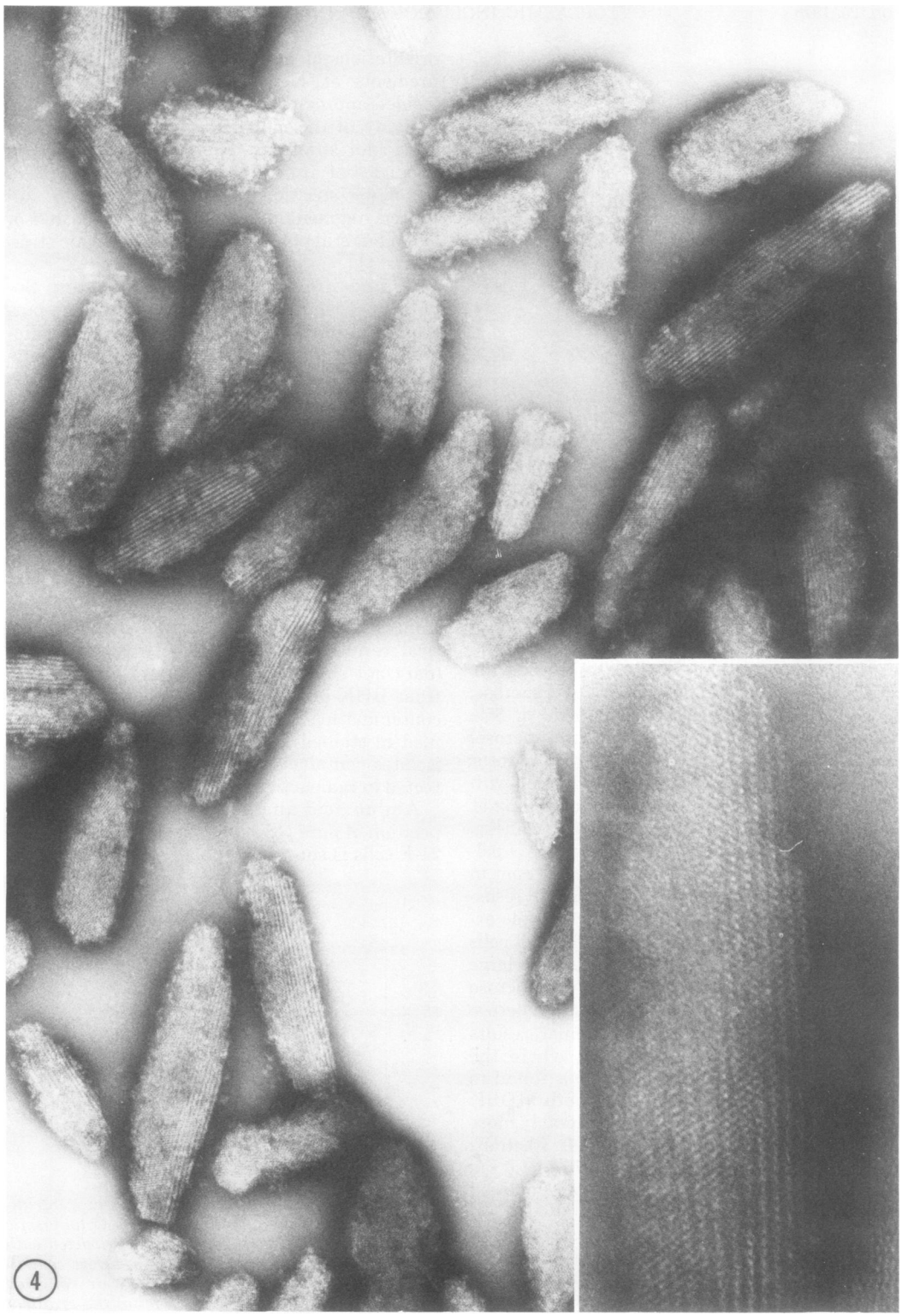

FIG. 4. *Purified inclusions isolated from infected BHK-21-F cells by fluorocarbon extraction and gradient fractionation, negatively stained with uranyl acetate. The high-magnification inset shows the fine structure of the isolated inclusions stained with sodium phosphotungstate. Magnification, ×80,000; inset, ×300,000.*

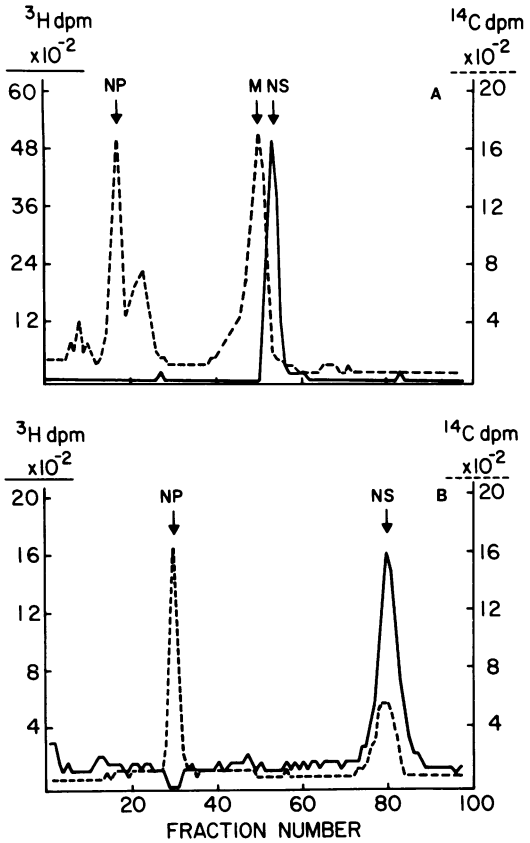

FIG. 5. *(A) Analysis of the polypeptides of [^{3}H]-leucine-labeled purified inclusions from the 50–55% sucrose interface fraction from MDBK cells. ^{14}C-amino acid-labeled polypeptides of influenza (WSN) virions are included as markers. (B) The same preparation shown in A, analyzed by coelectrophoresis with ^{14}C-amino acid-labeled marker polypeptides from infected BHK-21-F cells harvested at 23 h post-infection.*

the amino acid composition of the membrane (M) protein purified from the WSN strain is shown. The results indicate that there is only a single tyrosine residue in the NS protein. NS and M are clearly distinct in their content of serine, glycine, alanine, and tyrosine. In addition, analyses of NS have consistently shown a significant ninhydrin-positive peak that elutes just before lysine, which is not included in the usual standards. Further information is required to identify this component, and its possible contribution to the amino acid composition is not included in Table 1.

DISCUSSION

The present results indicate that NS forms cytoplasmic inclusions in influenza virus-infected cells. The isolated crystalline structures contained no other proteins in detectable amounts; however, we have observed that in preliminary experiments RNA also may be associated with the inclusions. A significant amount of [^{3}H]uridine label is associated with inclusions and is resistant to RNase A. Further

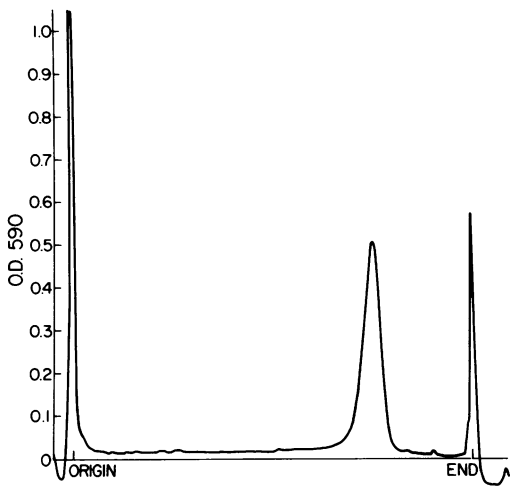

FIG. 6. *Densitometer scan of Coomassie blue-stained 10% sodium dodecyl sulfate-polyacrylamide gel electropherogram of polypeptides of purified inclusions from the 50–55% sucrose interface fraction from BHK-21-F cells.*

TABLE 1. *Comparison of amino acid composition of NS and M proteins*

Amino acid	NS protein[a]		M protein[b]	
	Residues per 100 residues	Residues per molecule[c]	Residues per 100 residues	Residues per molecule[d]
Asp	9.97	21.12	8.42	18.96
Thr	5.89	12.47	6.34	14.27
Ser	8.79	18.61	6.06	13.64
Glu	11.67	24.72	12.02	27.07
Pro	3.56	7.55	4.52	10.19
Gly	10.89	23.06	7.27	16.36
Ala	7.33	15.52	9.73	21.92
Val	6.30	13.35	6.30	14.19
Met	3.33	7.04	4.20	9.45
Ile	4.59	9.72	4.85	10.93
Leu	8.96	18.97	10.43	23.50
Tyr	0.62	1.32	2.30	5.19
Phe	3.16	6.69	3.07	6.91
His	2.26	4.78	2.38	6.36
Lys	5.58	11.83	5.60	12.61
Arg	7.12	15.07	6.48	14.60

[a] Results represent averages of four analyses.

[b] Results calculated from B. H. Robertson, A. S. Bhown, R. W. Compans, and J. C. Bennett, *in* R. D. Barry and B. W. J. Mahy (ed.), *Negative Strand Viruses and the Host Cell*, in press.

[c] Assuming a molecular weight of 23,000.

[d] Assuming a molecular weight of 25,000.

information is needed to determine whether these molecules are an integral part of the inclusion structure.

While this report was in preparation, we learned that Morrongiello and Dales (17a) have independently isolated influenza virus-induced inclusions from infected chicken embryo fibroblasts. Their data also show that NS is the protein component that is present in the inclusion structure. They also observed large cylindrical helices in the nuclei and cytoplasm of infected chick cells and in isolated inclusion preparations. We did not observe such structures, although the WSN strain of virus was employed in both laboratories, which suggests that the formation of such helices may be a host cell-dependent phenomenon.

Previous cell fractionation studies have shown that large quantities of the NS protein are present in subcellular fractions which were not observed to contain inclusions (7, 15, 17, 19). These include polysome fractions examined at early times postinfection as well as fractions containing nucleoli. Therefore, much of the NS protein in infected cells may be present in a form other than inclusions. Since the inclusions are detectable only during the latter stages of infection (6, 9, 15, 20), it is possible that the association of NS molecules into such paracrystalline arrays is due to continued production and accumulation of that protein in the infected cell. It remains uncertain whether the inclusions are of functional significance in virus replication. The apparent absence of any cellular proteins in electrophoretically pure, microscopically intact inclusion isolates should eliminate the possibility that NS is aggregating into these structures in association with cellular proteins. The consistency of the periodicities observed in isolated inclusions regardless of the cell of origin indicates that the structural regularity seen is an inherent characteristic of NS, possibly in association with nucleic acid.

Previous results of cell fractionation studies (7, 19) indicated that the NS protein was recovered in association with ribosomes and polysomes. Subsequent results (15) indicated that soluble NS protein bound to both ribosomal subunits by salt-dissociable bonds, raising the possibility that the observed association of NS with polysomes may have been an artifact occurring after cell disruption. Since ribosomes can be seen in close association with the cytoplasmic inclusions in thin sections of infected cells, the present results suggest that extensive association between ribosomes and NS protein occurs under physiological conditions.

The availability of purified cytoplasmic inclusions provides milligram quantities of pure NS protein, which should facilitate further studies of its structure and function. In addition, the fact that the protein forms crystalline arrays under physiological conditions suggests that it may be feasible to obtain larger crystals suitable for crystallographic analysis.

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