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Electrophoretic Distribution of the Proteins and Glycoproteins of Influenza Virus and Sendai Virus

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The proteins of influenza (WSN) and Sendai virus have been separated by polyacrylamide gel electrophoresis into five components. In both cases, three of these components were shown to be glycoproteins containing fucose, galactose, and glucosamine. Two protein components of each virus were probably free from these sugar residues, including the structural unit of the viral ribonucleoprotein (molecular weight of about 60,000 daltons).

Myxoviruses contain between 5 and 8% carbohydrates (1, 16, 22, 24, 30) in addition to the ribose of their ribonucleic acid (RNA). The carbohydrates of influenza virus have been shown to include galactose, mannose, fucose, and glucosamine (1, 16, 26).

It is generally believed (14) that the carbohydrates of myxoviruses are associated with the viral envelope for the following reasons: Zillig et al. found that the hemagglutinin of ether-disrupted fowl plaque virus contained about 10% carbohydrates (33), whereas Frisch-Niggemeyer and Hoyle (15) found that the hemagglutinin of ether-disrupted influenza virus contained 4.2% carbohydrate, and the soluble antigen appeared to contain only RNA-derived sugar. The host antigen of influenza virus, which was first discovered on the surface of purified virus (23), was shown to be carbohydrate material by Harboe (17). Electrophoretic analysis of the viral proteins showed that the host antigen was covalently bound to the viral hemagglutinin and another unidentified structural protein of the virus, and that it contained 28% galactose and 20% glucosamine (26). No host antigen was associated with the internal antigen(s) of the virus. Antibody against host antigen inhibited the viral hemagglutinin (26).

The myxoviruses have been divided on the basis of many structural and biological differences into two subgroups (32). The proteins of influenza virus, a subgroup I myxovirus (2, 19, 21, 25, 31), and of Newcastle disease virus (NDV) and simian virus 5 (SV5), which are subgroup II myxoviruses (2, 6, 13, 18), were recently resolved into three or more major electrophoretic components. Identification of the functional properties of the distinct electrophoretic components of these myxoviruses, however, remained uncertain. The studies listed above all indicated that the structural unit of the helical ribonucleoproteins of both types of myxoviruses consists of a single protein molecule with an approximate molecular weight of 60,000 daltons, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. However, in the case of influenza virus, one group (21) identified this protein as that having the lowest electrophoretic mobility of the three distinct proteins, whereas others [2; P. H. Duesberg, *in* R. Barry and B. W. J. Mahy (ed.), Biology of large RNA viruses, *in press*] concluded that it was the component with intermediate mobility. For subgroup II myxoviruses, the structural unit of the ribonucleoprotein was the intermediate of three major electrophoretic protein components of NDV (2, 13, 18), but the fastest migrating component of SV5 (6).

In the present study, we compared the glucosamine-, galactose-, and fucose-labeled macromolecules of influenza virus, a subgroup I myxovirus, and Sendai virus, a subgroup II myxovirus, with their corresponding amino acid-labeled protein components. It was found that influenza virus contains at least three glycoprotein components and that the putative structural unit of the ribonucleoprotein seems to be free or almost free from the above sugar residues. Three of the five protein components of Sendai virus were shown to be glycoproteins, and the structural

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unit of the viral nucleocapsid is probably free from these sugars.

MATERIALS AND METHODS

Viruses and cells. The WSN strain of influenza virus and the MDBK cell line of bovine kidney cells were obtained from P. W. Choppin and were used as described before (7). Confluent monolayers in plastic petri dishes were infected at a multiplicity of 0.01 to 0.1, and the virus was collected from the medium 26 to 30 hr postinfection.

For labeling of Sendai virus, confluent cultures of MDBK cells were inoculated at a high multiplicity of infection [usually with a twofold dilution of the allantoic fluid of 10-day-old embryonated eggs which had been infected as described by Blair and Robinson (3)]. After 24 hr, the virus was collected from the medium.

Virus purification. Virus was purified as described previously for influenza virus (11). Sendai virus was purified by the procedure recently described for NDV (2).

Virus labeling. ^{14}C-sugars (5 to 20 μCi) or ^{3}H-sugars (50 to 100 μCi) were added to the medium of an infected culture in a 10-cm plastic petri dish 10 to 12 hr postinfection in the case of WSN virus, and 6 hr postinfection in the case of Sendai virus. The following sugars were used: ^{14}C-D-glucosamine (52 mCi/mmole), ^{3}H-D-glucosamine (1.5 Ci/mmole), ^{3}H-D-fucose (4.3 Ci/mmole), and ^{3}H-D-galactose (5.8 Ci/mmole); these were all obtained from New England Nuclear Corp., Boston, Mass. Labeling of the viruses with amino acid was done with ^{3}H- or ^{14}C-reconstituted protein hydrolysate (New England Nuclear Corp.) as previously described (11).

Preparation of the virus-associated glycoproteins and proteins for acrylamide gel electrophoresis (12). Purified virus was disrupted by incubation for 30 min at 37 C in 0.01 M tris (hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 8.1, 2 mM ethylenediaminetetraacetate (EDTA), 5 mM dithiothreitol, and 1% SDS. After precipitation with five volumes of ethyl alcohol, in the presence of bovine serum albumin (BSA) if less than 20 to 50 μg of material was to be precipitated, the macromolecular components were pelleted by centrifugation for 10 min at 30,000 $\times$ *g*. The pellet was redissolved in 0.01 M Tris-hydrochloride (*p*H 8.1), 2 mM EDTA, 5 mM dithiothreitol, 0.05 M mercaptoethanol, 10% glycerol, and 1% SDS by boiling for 1 min. Alternatively, the pellets were twice washed with 75% ethyl alcohol and redissolved in 10 M urea, containing 0.1 M mercaptoethanol, 2.5 mM mercaptoethylamine HCl, and 1 mM EDTA.

Buffers. Standard buffer contained 0.1 M NaCl, 0.01 M Tris-hydrochloride (*p*H 7.4), and 1 mM EDTA. Low-salt buffer contained 0.02 M NaCl, 0.02 M Tris-hydrochloride (*p*H 7.4), and 2 mM EDTA.

RESULTS

Pherographic comparisons of the amino acid- and sugar-labeled macromolecules of influenza virus. The amino acid-labeled macromolecules of influenza virus were resolved into three major electrophoretic components, 1, 2, and 3, on polyacrylamide gels containing 0.1% SDS at *p*H 8.1 (Fig. 1) or 8 M urea at *p*H 3.8 (Fig. 3a and b). These results confirm previous findings (2, 9, 19, 25, 31). It should be noted that the relative concentrations of the protein components 1, 2, and 3 of WSN virus grown in MDBK cells were consistently different from those of this virus grown in chick embryo fibroblasts as described in a previous study (2). The difference may be due to differential modes by which the two kinds of host cells produce and assemble subviral components. This is compatible with the observation that chick embryo fibroblasts were found to release about 10 to 100 times less virus than the MDBK cells after infection with WSN virus, at a multiplicity higher than 1 (*unpublished data*). Further work will be necessary to test this speculation.

The ethyl alcohol-insoluble macromolecules of fucose-, galactose-, or glucosamine-labeled influenza virus were also resolved into three major components, I, II, and III (Fig. 1a–c). One of these, III, coincided exactly with protein component 3, suggesting that protein 3 is a glycoprotein. The fastest migrating carbohydrate-containing component, I, had a lower electrophoretic mobility than protein 1, suggesting that it is a distinct macromolecule. The glucosamine-, fucose-, or galactose-labeled carbohydrate component II with intermediate electrophoretic mobility in SDS gels did almost coincide with protein 2 (Fig. 1a and b). However, the ^{14}C-amino acid to ^{3}H-glucosamine ratio varied across the peak, suggesting the presence of two or more components. This is directly demonstrated in Fig. 1c, which illustrates co-electrophoresis of ^{3}H-glucosamine- and ^{14}C-amino acid-labeled influenza macromolecules under the conditions described in Fig. 1a and b, but for a longer time. Although in this experiment (Fig. 1c) the carbohydrate-labeled II and amino acid-labeled 2 components had different maxima, it cannot be said whether the two peaks consist of two glycoproteins or a pure protein and a glycoprotein molecule, or any other combination thereof, until better resolution is obtained.

The following experiment, however, demonstrated that none of the carbohydrate-containing components of the virus were pure carbohydrate molecules. A mixture of ^{3}H-glucosamine- and ^{14}C-amino acid-labeled influenza macromolecules was treated with Pronase, a proteolytic enzyme, for 60 hr at 37 C (see Fig. 1d) and then analyzed by electrophoresis. As shown in Fig. 1d, the electrophoretic distribution of both the carbohydrate- and amino acid-derived radioactivities

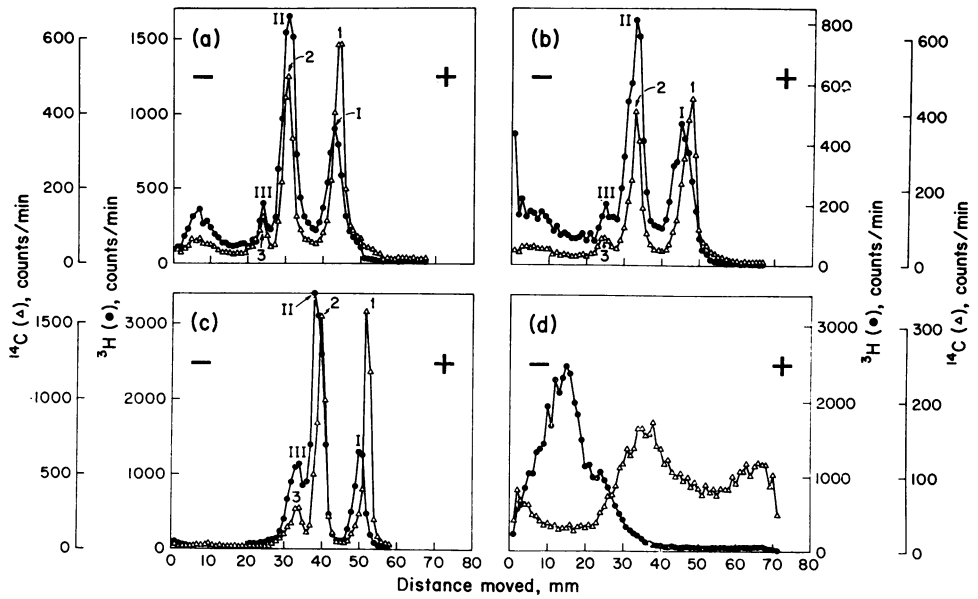

FIG. 1. *Polyacrylamide gel electrophoresis of (a) ^{3}H-fucose-labeled, (b) ^{3}H-galactose-labeled, and (c) ^{3}H-glucosamine-labeled macromolecular components of WSN virus and (d) effect of Pronase digestion on the ^{3}H-glucosamine pattern. (a and b) A mixture of ^{3}H-fucose (a) or ^{3}H-galactose (b) and ^{14}C-amino acid-labeled WSN virus was incubated with SDS and dithiotreitol at pH 8.1 in presence of 50 μg BSA as described in Materials and Methods. The ethyl alcohol-precipitable material was redissolved by heating at 100 C for 1 min in 60 μliters of buffer containing 0.01 M Tris-hydrochloride (pH 8.1), 2 mM EDTA, 0.05 M mercaptoethanol, 5 mM dithiothreitol, 1% (w/v) SDS, 10% (v/v) glycerol, and enough phenol red to serve as a pH indicator and tracking dye. Electrophoresis was for 4 hr at 8 v/cm in a 5%, diacrylate-crosslinked, polyacrylamide gel column (7 by 0.7 cm) containing 0.1 M Tris-hydrochloride (pH 8.1), 0.5 mM EDTA, and 0.1% (w/v) SDS, until the phenol red had migrated about 6 cm. After electrophoresis, the gel was frozen and divided into 1-mm slices with stacked razor blades (Diversified Scientific Instruments, San Leandro, Calif.), and each slice was dissolved in 1 ml of a solution containing one volume of 2 M piperidine and nine volumes of NCS (Nuclear-Chicago Corp.) by shaking for 4 to 8 hr at 37 C. Radioactivity was determined after addition of 5 ml of toluene-based scintillation fluid in a Tri-Carb liquid scintillation counter. (c) A mixture of ^{3}H-glucosamine- and ^{14}C-amino acid-labeled WSN virus was treated with SDS and subjected to electrophoresis as described above (a and b), but for 5 hr at 10 v/cm. (d) The ethyl alcohol-insoluble material of a mixture of ^{3}H-glucosamine- and ^{14}C-amino acid-labeled WSN virus was redissolved (as described above, a and b) in 60 μliters of a solution containing 0.01 M Tris-hydrochloride (pH 8.1), 2 mM EDTA, 0.05 M mercaptoethanol, 5 mM dithiothreitol, and 1% (w/v) SDS, and was incubated with 50 μg of Pronase for 24 hr at 37 C. Thereafter, an additional 20 μg of Pronase was added, and the incubation was continued for 36 hr at 37 C. The sample was then subjected to electrophoresis on a 5% polyacrylamide gel as described above (a and b).*

was completely changed. All of the glucosamine-derived radioactivity had a significantly lower electrophoretic mobility after Pronase digestion than the amino acid-derived radioactivity. This indicates that the glucosamine-labeled electrophoretic components of the virus are glycoproteins. The decreased electrophoretic mobility of the glucosamine-labeled material obtained after Pronase digestion may result from a reduced negative charge due to failure of SDS to bind to the carbohydrate residues of the glycopeptides. Many proteins have been shown to be uniformly charged by SDS (27), whereas similar information is not available for carbohydrates.

It was shown previously that fucose and glucosamine are not significantly metabolized in animal cells and that the sugar-derived radioactivity of viral macromolecules is still present in the respective sugar molecules after HCl hydrolysis of the viral macromolecules (12, 29). Galactose, however, was found to be metabolized slightly under these conditions (12, 29). This was confirmed for ^{14}C-glucosamine-labeled WSN grown in MDBK cells in which more than 95% of the alcohol-precipitable radioactivity could be identified as ^{14}C-glucosamine by paper chromatoggraphy as previously described (12, 29).

When ^{14}C-amino acid- and ^{3}H-glucosamine-

labeled viral proteins were subjected to co-electrophoresis at *p*H 3.8 in 8 M urea, again three protein components could be resolved: a fast-migrating protein (fractions 32–35, Fig. 2) and two incompletely resolved components of lower electrophoretic mobility (fractions 21 to 28, Fig. 2). This pattern is the same as that described previously for PR 8 influenza virus proteins resolved under similar conditions (9). Only one glucosamine-labeled component migrated as a cation in 8 M urea at *p*H 3.8. This component coincided with the intermediate protein component in the urea system, suggesting that it is a glycoprotein. The fastest and the slowest electrophoretic protein components resolved in 8 M urea at *p*H 3.8 did not undergo co-electrophoresis with significant amounts of glucosamine-derived radioactivity. A considerable fraction of the viral carbohydrate and also of the protein material did not penetrate the gel under these conditions.

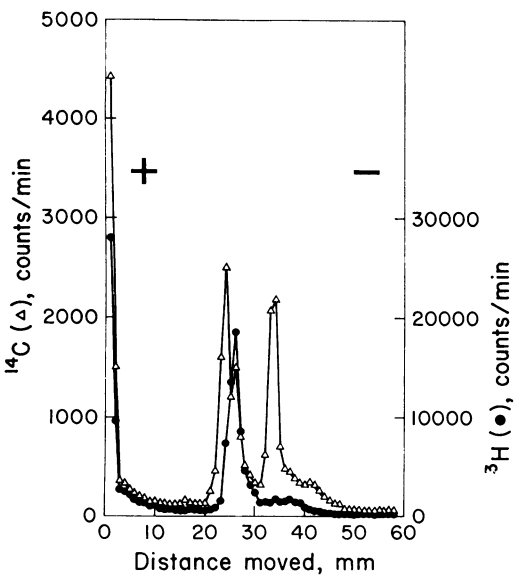

FIG. 2. *Polyacrylamide gel electrophoresis of the ethyl alcohol-insoluble material of ^{3}H-glucosamine- and ^{14}C-amino acid-labeled influenza virus in 8 M urea at pH 3.8. The ethyl alcohol-insoluble material of ^{3}H-glucosamine and ^{14}C-amino acid-labeled WSN was prepared as described in Materials and Methods. The pellet was washed two times with 75% ethyl alcohol and redissolved in 100 μliters of 10 M urea containing 0.1 M mercaptoethanol, 2.5 mM mercaptoethylamine HCl and 1 mM EDTA, and was incubated at 36 C for 5 min. Electrophoresis was on 4% diacrylate cross-linked polyacrylamide gel at pH 3.8 in a discontinuous buffer system as described previously (10). Methylene blue was used as a tracking dye. Slicing of the gel and determination of radioactivity was as described for Fig. 1.*

The selective incorporation of radioactive sugar into only some of the protein components of the virion further supports the notion that the radioactivity of the sugar precursors is not randomly incorporated in structural proteins of the virus.

Identification of the protein of the influenza ribonucleoproteins. Protein 2 in the SDS system (Fig. 1) has previously been tentatively identified as the structural unit of the viral ribonucleoprotein (2; Duesberg, *in press*). This was confirmed by the following experiments. ^{14}C- and ^{3}H-amino acid-labeled virus was disrupted and fractionated by a modification of a procedure described previously (see Fig. 5).

Samples of the radioactive protein of the ribonucleoproteins were then mixed with unfractionated amino acid-labeled WSN, again incubated with SDS and dithiothreitol as described in Materials and Methods, and ethyl alcohol-precipitated. The precipitates were then redissolved and subjected to electrophoresis in either 8 M urea at *p*H 3.8 or 0.1% SDS at *p*H 8.1, as shown in Fig. 3a and b. It can be seen in Fig. 3a that the protein of the viral ribonucleoproteins consists of one major electrophoretic component under the two different electrophoretic conditions used. It coincides with protein 2 in the SDS system (Fig. 3a) but with the protein of the lowest electrophoretic mobility in the urea system (Fig. 3b). From this it is concluded that the ribonucleoproteins of influenza virus (11) contain most likely a single protein as structural unit. Its molecular weight was estimated by the method of Shapiro et al. (27) to be 60,000 daltons based on its electrophoretic mobility in SDS-polyacrylamide gels (Fig. 1 and 3) with BSA (67,000 daltons) and tobacco mosaic virus protein (16,500 daltons) as molecular weight markers.

Is the structure unit of the influenza ribonucleoprotein a pure protein or a glycoprotein? It is illustrated in Fig. 1 that the protein part of the viral ribonucleoproteins (protein 2, Fig. 1) roughly undergoes co-electrophoresis with a glycoprotein (II, Fig. 1). It could not be determined, however, from these experiments whether the protein of the viral ribonucleoproteins contains carbohydrate or not. The following experiments were designed with this question in mind.

The ethyl alcohol-insoluble material of ^{14}C-amino acid-labeled ribonucleoprotein (prepared as described above) and unfractionated ^{3}H-glucosamine-labeled WSN was mixed and subjected to electrophoresis in 8 M urea at *p*H 3.8. As shown in Fig. 4a, the ^{14}C-amino acid-labeled protein of the viral ribonucleoproteins does not coincide but overlaps with the only ^{3}H-glucosamine-labeled component of the virus which migrates under these conditions.

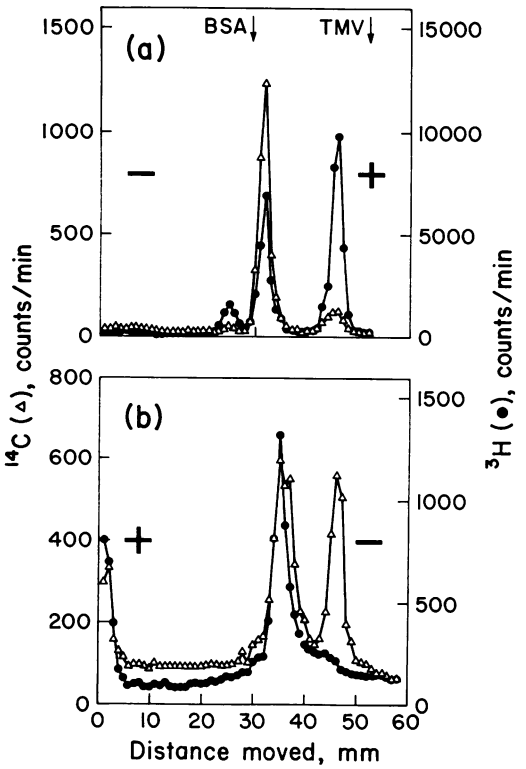

FIG. 3. *Co-electrophoresis of the ethyl alcohol-insoluble proteins of unfractionated amino acid-labeled WSN with the amino acid-labeled material of the viral ribonucleoproteins. The ribonucleoproteins of WSN were prepared as described for Fig. 5, but sedimentation of the disrupted virus was for only 60 min. (a) Electrophoresis of a mixture of ^{14}C-amino acid-labeled ribonucleoprotein with ^{3}H-amino acid labeled WSN protein in SDS-polyacrylamide as described for Fig. 1. Prior to slicing, the gel was incubated in 10% trichloroacetic acid until the BSA band could be located. (b) A mixture of ^{3}H-amino acid-labeled ribonucleoprotein and ^{14}C-amino acid-labeled WSN was analyzed by electrophoresis in 8 M urea as described for Fig. 2.*

The total radioactivity of the electrophoretic ^{14}C-protein component 2 and ^{3}H-glucosamine component II (fractions 37–42, Fig. 1c) was eluted from the SDS-polyacrylamide gel (shown in Fig. 1c) and subjected to electrophoresis in 8 M urea at *p*H 3.8. The pherogram (Fig. 4b) indicated that the maxima of both radioactivities differed by one fraction, which is comparable to the results shown in Fig. 4a and also in Fig. 2. Moreover, the glycoprotein component II, which had a slightly lower electrophoretic mobility than the protein component 2 in the SDS system, had a slightly higher electrophoretic mobility than protein 2 in urea at *p*H 3.8. This result also agrees with those illustrated in Fig. 2 and 3b which indicated that the protein of the viral ribonucleoproteins is the component with the lowest electrophoretic mobility in the urea system and that the viral glycoprotein forms only a single electrophoretic component in the urea system. The experiments depicted in Fig. 4a and b further suggest that the glycoprotein component II (Fig. 1) is part of or identical with the only glycoprotein component that migrates in the urea system. [The other glycoprotein components (I and III, Fig. 1) were not analyzed in the urea system because their electrophoretic mobilities were com-

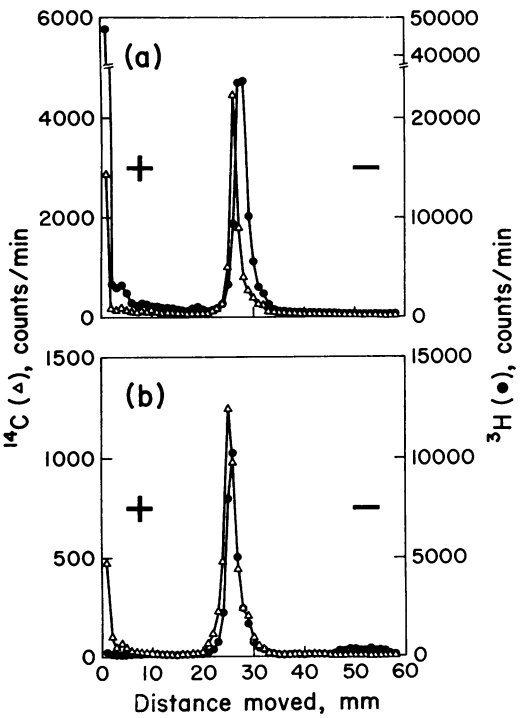

FIG. 4. *(a) Co-electrophoresis of the ethyl alcohol-insoluble material of the ribonucleoproteins of ^{14}C-amino acid-labeled WSN (prepared as described for Fig. 5) with the ethyl alcohol-insoluble material of unfractionated WSN, which was labeled with ^{3}H-glucosamine. Electrophoresis was in 8 M urea as described for Fig. 2. (b) Co-electrophoresis of protein component 2 and glycoprotein component II obtained by elution of the polyacrylamide gel described in Fig. 1c. Fractions 37–42 (Fig. 1c) were each suspended in 1 ml of a solution containing 0.1 M Tris-hydrochloride (pH 7.4), 1 mM EDTA, 0.1% mercaptoethanol, and 1% SDS. After being shaken for 40 hr at 37 C, the eluant was mixed with 50 μg of BSA carrier, and the ethyl alcohol-insoluble material was recovered by precipitation with five volumes of ethyl alcohol. The pellet was redissolved in 10 M urea and subjected to electrophoresis as described for Fig. 2.*

pletely different from that of the protein (2, Fig. 1) of the influenza ribonucleoprotein.]

When a mixture of ^{3}H-uridine- and ^{14}C-glucosamine-labeled virus was disrupted with deoxycholate, Triton X-100, and ether (see legend of Fig. 5) and analyzed by sucrose gradient sedimentation, the distribution reported in Fig. 5 was obtained. A minimum of ^{14}C-glucosamine-derived radioactivity coincided with the peak of ^{3}H-ribonucleoprotein subunits (70*S*, 60*S*, and 50*S*) of the virus.

Since none of these experiments completely separated carbohydrate-derived radioactivity from the respective radioactivities of the ribonucleoprotein, it is not possible to exclude the possibility that the protein structural unit of the ribonucleoprotein of influenza virus contains a small amount of carbohydrate. These experiments suggest, however, that the structural unit of the ribonucleoprotein contains at most a minor fraction of the total glucosamine of the virus, because under none of the observed conditions (electrophoresis in urea or SDS and sedimentation) did the ribonucleoprotein or its protein coincide with a peak of glucosamine-derived radioactivity.

Glycoproteins of Sendai virus. By electrophoresis in 0.1% SDS-containing polyacrylamide gels at *p*H 8.1, the proteins of Sendai virus could be resolved into five components: three major components, 1, 3, and 4, and two minor components, 2 and 5, which were not always completely separated (Fig. 6a–c and 8a). Some variations in the relative concentration of component 1 and 3, for example, were observed with different working stocks of virus labeled with ^{14}C- or ^{3}H-amino acid.

The ethyl alcohol-insoluble macromolecules of fucose-, galactose-, or glucosamine-labeled Sendai virus could all be resolved into two major components, II and III, and one minor component, I (Fig. 6a–c). In the case of galactose-labeled virus, a small amount of material was found which migrated in the region corresponding to the protein component 1 (Fig. 6b and 8b). This may be because galactose, in contrast to fucose and glucosamine, is partially metabolized by the cell (12, 29). As shown in Fig. 6d, the Pronase sensitivity of the electrophoretic mobilities of all carbohydrate-containing components indicated that they are glycoproteins. Component II coincided with an amino acid-labeled component, 4, in the protein pattern, suggesting that it is a single glycoprotein (Fig. 6a–c). Components I and III coincided approximately with protein components 2 and 5, respectively. They could be mixtures of a protein and a glycoprotein or two glycoproteins, as we discussed above for the influenza virus.

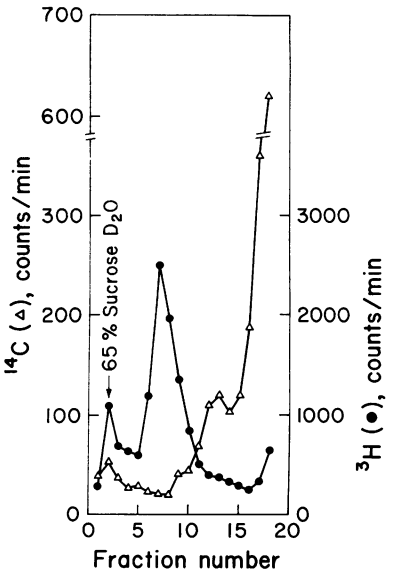

FIG. 5. *Sucrose gradient sedimentation of ether-detergent treated influenza virus. A mixture of ^{3}H-uridine- and ^{14}C-glucosamine-labeled influenza virus in 100 μliters of low-salt buffer (Materials and Methods) containing about 10 to 20% sucrose was mixed with an equal volume of this buffer containing 1% Triton X-100 and 1.4% deoxycholate. The mixture was homogenized two times with two volumes of ether. Subsequently, the ether was pipetted off and removed completely at reduced pressure, and the solution containing the disrupted virus was layered on a linear 15 to 30% sucrose gradient in low-salt buffer containing 0.05% (w/v) sodium deoxycholate. The gradient was poured on 0.5 ml of a dense (1.32 g/ml) sucrose solution containing 65% sucrose in low-salt buffer made up in D_2O. Centrifugation was for 110 min at 65,000 rev/min in a Spinco SW65 rotor at 5 C. Samples of each fraction of the gradient were counted after the addition of five volumes of NCS and 5 ml of toluene-based scintillation fluid in a Tri-Carb liquid scintillation counter. [The small peak (fraction 2) on top of the dense sucrose solution represents intact or incompletely disrupted virus.] The fractions containing the subunits of the viral ribonucleoproteins (11) were mixed with carrier protein (BSA or tobacco mosaic virus protein), solubilized in SDS in the presence of dithiothreitol, and alcohol-precipitated as described in Materials and Methods. It was found important not to sediment the ribonucleoproteins too close to the interface to avoid contamination by faster sedimenting split products of the virus. The absence or small amount of fast sedimenting material collecting at the 65% sucrose-D_2O interface or sedimenting faster than the ribonucleoproteins could be used as a measure of the completeness of the disruption of the virus.*

Nucleocapsid protein of Sendai virus. The nucleocapsid of Sendai virus was released from the virus by incubation with deoxycholate (4) and was isolated from the split products of the viral

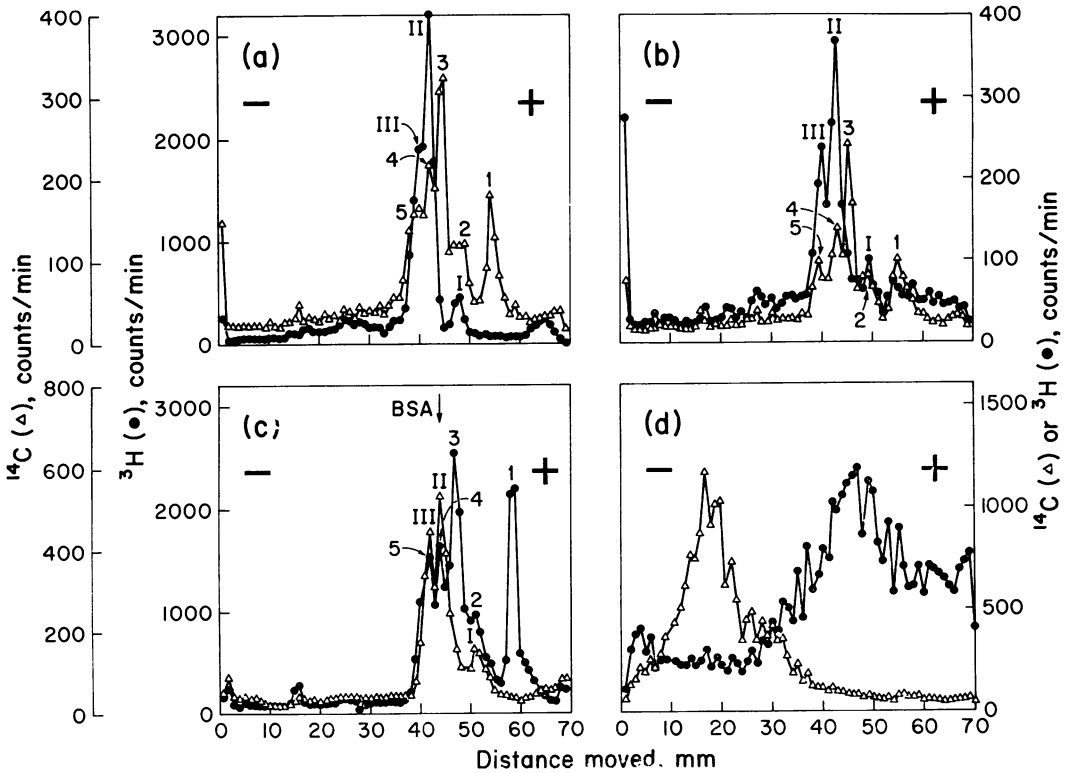

FIG. 6. *Polyacrylamide gel electrophoresis of ^{3}H-fucose, ^{3}H-galactose, and ^{14}C-glucosamine-labeled macromolecular components of Sendai virus and effect of Pronase digestion on the ^{14}C-glucosamine pattern. The conditions were as described in Fig. 1 except that electrophoresis was for 5 hr at 10 v/cm. (a) Co-electrophoresis of the ethyl alcohol-insoluble components of ^{3}H-glucose- and ^{14}C-amino acid-labeled Sendai virus. (b) Co-electrophoresis of the ethyl alcohol-insoluble components of ^{3}H-galactose and ^{14}C-amino acid-labeled Sendai virus. (c) Co-electrophoresis of the ethyl alcohol-insoluble material of ^{14}C-glucosamine- and ^{3}H-amino acid-labeled Sendai virus. The BSA band was located as described in Fig. 3a. (d) Electrophoresis of the material described above (c) after Pronase digestion (see Fig. 1d).*

envelope by sucrose or potassium tartrate density gradient centrifugation (see Fig. 7). If a mixture of disrupted ^{3}H-fucose- and ^{14}C-amino acid-labeled virus was fractionated in a sucrose density gradient, less than 5.7% of the radioactive fucose was found in the density range of the nucleocapsid (Fig. 7). Similar results were obtained with galactose-labeled (<6.7%) and glucosamine-labeled (<5.9%) virus. Electrophoretic comparison of nucleocapsid protein of Sendai virus with the total amino acid- or carbohydrate-labeled macromolecules of unfractionated virus is shown in Fig. 8. A single electrophoretic protein component, 3, was identified as the protein of the viral nucleocapsid (Fig. 8a). (The small amount of ^{3}H-labeled material moving faster than component 3 in Fig. 8a was not present in all preparations, as shown in Fig. 8b.) The molecular weight of the protein of the nucleocapsid of Sendai virus was estimated from an included BSA marker to be about 60,000 daltons (27). This molecular weight is very similar to those of the nucleocapsid protein of NDV (2, 13, 18) and of influenza virus (see above), but it differs from that reported for the nucleocapsid protein of SV5 (6). In Fig. 8b, it is shown that the nucleocapsid protein of Sendai virus did not coincide with a distinct carbohydrate-containing component of the virus. Since glycoprotein II overlapped with the protein of the nucleocapsid, it is difficult to say whether the minor amount of carbohydrate undergoing electrophoresis with the nucleocapsid protein is part of the structural unit of the viral nucleocapsid or whether it belongs to a separate structural component.

However, the failure of a distinct viral carbohydrate component to coincide with the nucleocapsid in sucrose gradients and with the nucleocapsid protein in polyacrylamide gels suggests that the nucleocapsid contains at most 5 to 7%

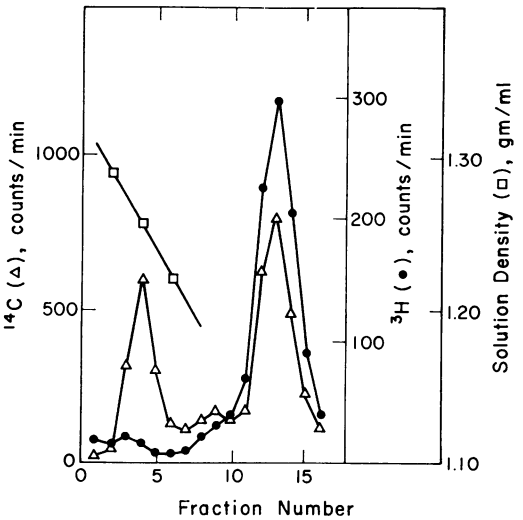

FIG. 7. *Isopycnic sucrose gradient centrifugation of the nucleocapsid of deoxycholate-treated, ^{14}C-amino acid- and ^{3}H-fucose-labeled Sendai virus. The virus was disrupted by sodium deoxycholate as described for Fig. 8. The solution was then layered on a preformed sucrose gradient containing standard buffer and 0.05% (w/v) deoxycholate [prepared by mixing 2.2 ml of a solution of 15% (w/v) sucrose with 2.2 ml of a solution of 65% sucrose in D_2O] and was centrifuged for 135 min at 64,000 rev/min in a Spinco SW65 rotor at 4 C. Six-drop fractions were collected. Solution density was determined by weighing 100 μliter samples. Trichloroacetic acid-precipitable radioactivity was determined by plating on membrane filters, as described before (9).*

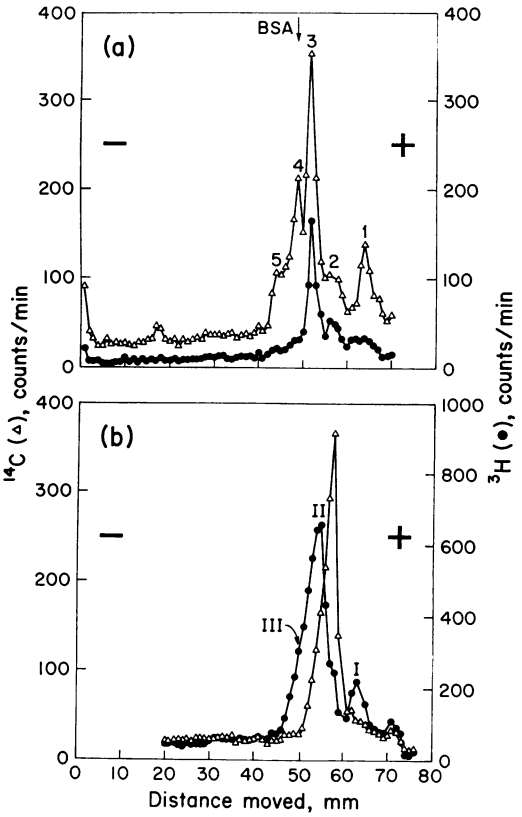

FIG. 8. *Identification of the structural unit of the viral nucleocapsid of Sendai virus. The nucleocapsid was obtained by incubating ^{3}H- or ^{14}C-amino acid-labeled virus for 30 min at 20 C in standard buffer containing 2% (w/v) sodium deoxycholate. After equilibrium centrifugation for 90 min at 64,000 rev/min in a SW65 Spinco rotor at 4 C in a preformed potassium tartrate density gradient [10 to 50% (w/v) in 0.01 M Tris-hydrochloride (pH 7.4), 2 mM EDTA], the viral nucleocapsid appeared as a sharp peak of radioactivity at a density of 1.265 g/ml (2, 4). To remove the potassium tartrate, the fractions containing the nucleocapsid were pooled, diluted with water, and centrifuged at 400,000 × g for 35 min. Under these conditions, all of the radioactivity was recovered in the pellet, which was then dissolved (as described in Materials and Methods) and mixed with amino acid- or galactose-labeled Sendai virus. After reduction by dithiothreitol, the macromolecules were ethyl alcohol-precipitated, redissolved, and subjected to electrophoresis in SDS-polyacrylamide gels as described for Fig. 1, but for 5.5 hr at 10 v/cm. (a) Co-electrophoresis of the total proteins of ^{14}C-amino acid-labeled Sendai virus with ^{3}H-amino acid-labeled protein prepared from Sendai virus nucleocapsid. (BSA was located as described in Fig. 3a.) (b) Co-electrophoresis of the total ^{3}H-galactose-labeled glycoproteins of Sendai virus with the ^{14}C-amino acid protein prepared from Sendai virus nucleocapsid.*

of any of the described carbohydrate molecules of the virus.

DISCUSSION

The proteins of influenza virus were resolved in three major electrophoretic zones consisting of probably five or more electrophoretic components. Three of the electrophoretic protein components of the virus are glycoproteins containing glucosamine, fucose, and galactose, and possibly other sugar residues. A single electrophoretic component with a molecular weight of 60,000 daltons was shown to be the structural unit of the viral ribonucleoprotein subunits.

This is in contrast to previous estimates of only three structural proteins of influenza virus (2, 10, 19, 25, 31). Our five protein components may correspond to five of the seven serological components obtained from disrupted WSN virus by Dimmock (8). Additional intracellular virus-specific antigens may be nonstructural components of the virus (8) and could also be unglucosylated or incompletely glucosylated

counterparts of the structural glycoproteins of the virus.

Because of the electrophoretic similarity of the structural unit of the ribonucleoproteins of influenza virus with glycoprotein II (Fig. 1), the question was raised whether the viral ribonucleoprotein contains some carbohydrates. Differential labeling and electrophoresis of proteins and glycoproteins in 8 M urea at *p*H 3.8 (Fig. 4) and differential sedimentation of the ribonucleoprotein and the glucosamine-labeled components of the disrupted virus (Fig. 5) suggest that the structural unit of the viral ribonucleoproteins and glycoprotein II may be different macromolecules. However, it could not be demonstrated that the structural unit of the viral ribonucleoproteins is entirely free from glucosamine-, fucose-, or galactose-derived radioactivity. Obviously, further experiments, including improved fractionation of disrupted virus and different methods of molecular analysis of structural components of the virus, such as electrofocusing, are needed to answer this question unequivocally.

Sendai virus was found to contain at least five proteins. A single protein with an approximate molecular weight of 60,000 daltons was shown to be the structural unit of the nucleocapsid. The nucleocapsid protein may contain none, or at most 5 to 7%, of virus-associated glucosamine, fucose, or galactose. In addition, the virus contains three glycoproteins, I, II, and III, which appear to be identical with the viral protein components 2, 4, and 5 (Fig. 8). By analogy with the proteins of NDV, resolved under similar conditions (13), one or two of the largest glycoproteins (I or II, or both) having a molecular weight of 70,000 to 80,000 daltons could be part of or identical with the viral hemagglutinin.

As in the case of all other enveloped viruses whose glycoproteins have been analyzed so far [vesicular stomatitis virus (5), Sindbis virus (29), herpes virus (28), RNA tumor virus (12), influenza virus (20); for general review, see 14], it appears also for the two viruses we studied as representatives of the two subgroups of myxoviruses (i) that only some of the viral structural proteins contain carbohydrate residues and (ii) that these are probably the envelope proteins of the virus. The presence of carbohydrate in only some protein components of the myxoviruses, probably only the components of the viral envelope, should be helpful in the identification of their structural and functional role in the virus particle.

It is not possible to say whether and how much of the carbohydrate part of the glycoprotein of Sendai virus or influenza virus is specified by the viral genome, the host cell, or a combination of the two. A specifically controlled addition of carbohydrate precursors to some viral macromolecules is suggested by their discrete electrophoretic patterns, which differ from those of other viral proteins. Also, it cannot be concluded from our data whether the protein parts of all viral proteins and glycoproteins are different or whether some glycoproteins and proteins share identical polypeptide chains. A role for the host cell in carbohydrate addition has been demonstrated for influenza virus (16, 17, 26). It is possible that the antigenic variability of influenza virus might be related to relatively small changes in the sugar composition of the envelope proteins.

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