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Proteins of Newcastle Disease Virus and of the Viral Nucleocapsid

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Newcastle disease virus was found to contain three major proteins. The structure unit of the viral nucleocapsid appears to be monomeric and to consist of a single large protein of an approximate molecular weight of 62,000.

Newcastle disease virus (NDV), a subgroup II myxovirus, consists of a helically arranged ribonucleic acid (RNA) containing nucleocapsid which is enclosed by a lipoprotein envelope (18, 24). The viral envelope contains at least two distinct active proteins, a hemagglutinin and a viral neuraminidase, but the total number of the different proteins of the viral envelope is unknown.

The structures of the nucleocapsids of several parainfluenza viruses including NDV are similar to that of tobacco mosaic virus (TMV) with regard to several physical and chemical properties. The nucleoproteins of parainfluenza viruses and of TMV have a diameter of about 20 nm (11, 24) and a density of about 1.3 g/ml (in sucrose or CsCl) (1, 21), and contain about 4 to 5% RNA and 95 to 96% protein (11, 13). The lengths of the two kinds of nucleoproteins were found to be proportional to the size of the RNA molecules which they include. Tobacco mosaic virus has a length of 0.3 μm and contains an RNA of a molecule weight of 2×10^6 (11) and the nucleoprotein of parainfluenza viruses contains an RNA of about 6.5×10^5 (3, 18) and has a length of about 1 μm (1, 12).

Although it is known that the structure units of TMV consist of monomeric proteins, it is not known whether the capsomers of the nucleocapsids of parainfluenza viruses also consist of single proteins or whether they consist of several proteins.

The subject of the present investigation was the isolation of the different proteins of the virus. Three distinct major proteins of NDV were isolated by polyacrylamide gel electrophoresis. A single protein was found to be associated with the viral nucleocapsid. Similar independent findings were recently reported by Evans and Kingsbury (6).

METHODS AND MATERIALS

Virus growth. The NDV strain, NDV-Beaudette, obtained from D. W. Kingsbury, St. Jude Hospital, Memphis, Tenn., was used in all experiments. The virus was grown on lung cultures of 15- to 17-day-old chick embryos. The lungs were dispersed by stirring in tris(hydroxymethyl)aminomethane (Tris) saline containing Pronase (2.5 mg/ml) for 30 to 60 min at room temperature. Cultures were seeded at 2×10^7 to 4×10^7 cells per 10-cm plastic dish and cultured for 1 day prior to infection (19). After the medium was removed, the cells were incubated with about 3 ml of a twofold dilution of stock virus in Tris-saline. Stock virus consisted of allantoic fluid of infected chick embryos containing about 10^9 plaque-forming units (PFU)/ml (4). After incubation for 30 min at 37 C, the inoculum was replaced by 6 ml of amino acid-free (except for glutamine) medium 199 supplemented with 0.1% (w/v) lactalbumin hydrolysate, 0.1% (w/v) glucose, and 0.2 μg of Fungazone per ml. Incubation was continued for 24 hr in the presence of 10 to 50 μc/plate of ^{3}H-amino acids (specific activity, 5 c/mmole), or ^{14}C-amino acids (specific activity, 0.3 c/mmole) or 50 to 100 μc/plate of ^{3}H-uridine. At the end of this period, the medium was removed for virus purification. The cells had become rounded and partly detached from the plate. The hemagglutinin (HA) titer of such medium was usually about 160 HA units per ml.

Virus purification. This process was a modification of the procedure described previously (4). Virus was purified in essentially two steps. First, the virus was concentrated from the medium of virus-producing cells by precipitation with an equal volume of saturated ammonium sulfate. The pellet was then redissolved in standard buffer [0.1 M NaCl, 0.01 M Tris (*p*H 7.4), 1 mM ethylenediaminetetraacetate (EDTA)] and concentrated by sedimentation on a sucrose cushion of a greater density than that of the virus. The concentrated virus was then transferred from the density interface and after appropriate dilution layered on a preformed sucrose density gradient in the same buffer. After centrifugation, viral infectivity coincided with radioactivity and optical density in a density range from 1.20 to 1.25 g/ml. From 50 to 100% of the

starting infectivity can thus be recovered as purified virus.

Disintegration of the virus. One A_{260} or about 10^4 HA units of purified NDV in 100 μliters of low salt buffer [0.01 M NaCl, 0.01 M Tris (*p*H 7.4), and 1 mM EDTA] were mixed with an equal volume of 2 to 4% (w/v) Na deoxycholate (DOC) in the same buffer and incubated for 30 to 60 min at room temperature (2, 12, C. Blair, Ph.D. thesis, University of California, 1968). If higher virus to detergent ratios were used, incomplete degradation or aggregation of split products was observed, suggesting that a certain stoichiometry of detergent and virus substrate is necessary for complete disruption.

Isolation of viral RNA. Isolation of the viral RNA was as described previously (4).

Isolation of proteins of virus or nucleocapsid. Isolation of the proteins of virus or nucleocapsids and polyacrylamide gel electrophoresis were as described recently (5) with the following modifications. After electrophoresis, the gels were sliced in stainless-steel gel slicers (Diversified Scientific Instruments, Mountain View, Calif.). The slices were dissolved by the addition of 50 μliters of 1 M piperidine and 0.5 ml of NCS (Nuclear-Chicago Corp., Des Plaines, Ill.) and by shaking for 4 hr at 37 C or incubating at room temperature overnight. Thereafter, 5 ml of toluene-based scintillation fluid was added and each sample was counted in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.)

RESULTS

Isolation and characterization of the viral nucleocapsid. The nucleocapsid of NDV was released from the virion by incubation with 1 to 2% DOC as described above. The nucleocapsid was then isolated from the split products of the viral envelope by sucrose density gradient centrifugation (*see* legend of Fig. 1). Under the conditions of this experiment, equilibrium density was attained by the fast sedimenting nucleocapsid (*see* below), whereas the slowly sedimenting split products of the viral envelope remained on top of the gradient. As shown in Fig. 1A, the density of the nucleocapsid of NDV was 1.27 g/ml which is higher than that of the intact virion (1.23 g/ml) under the same conditions (4).

The density of the nucleocapsid of NDV is the same as that of the nucleocapsid of Sendai virus in sucrose (C. Blair, Ph.D. thesis, University of California, 1968) and very similar to the density of 1.30 g/ml of the nucleocapsids of other parainfluenza viruses, including NDV in CsCl (1, 9). Alternatively, the nucleocapsid of

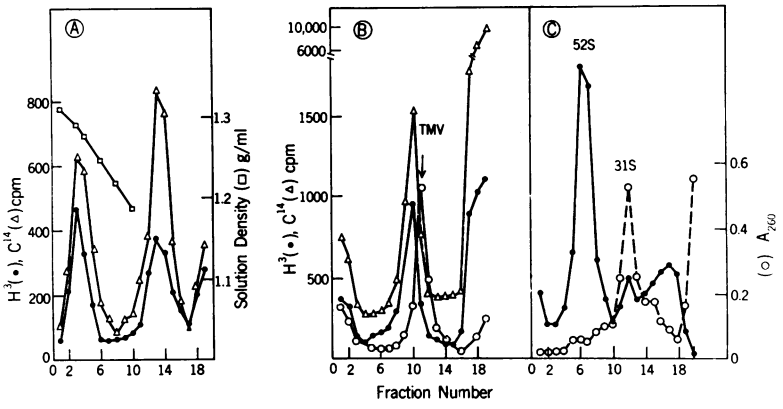

FIG. 1. *Characterization of the nucleocapsid of NDV. (A) Equilibrium sucrose-density gradient sedimentation of DOC-disrupted NDV. ^{14}C-amino acid- and ^{3}H-uridine-labeled NDV in low salt buffer (0.5 ml) containing 1% DOC were layered over a sucrose-D_2O density gradient (4) 20 to 65% (w/v) in standard buffer containing 0.1 M NaCl, 0.01 M Tris, pH 7.4, and 1 mM EDTA. After centrifugation for 3 hr at 300,000 × g in a SW-65 Spinco rotor at 4 C, 6 drop fractions were collected. Solution density was determined by weighing 100 μliter samples of fractions. Radioactivity was determined by counting appropriate samples after 1 to 1 dilution with H_2O in 5 volumes of NCS and 5 ml of toluene-based scintillation fluid. Symbols: ▲, ^{14}C-amino acid-labeled NDV; ●, ^{3}H-uridine-labeled NDV; □, solution density. (B) Velocity sedimentation of DOC-disrupted NDV. A sample of the DOC-treated virus preparation used in (A) was mixed with 600 μg of TMV and 20 μg of pancreatic-ribonuclease and centrifuged through a 10 to 25% (w/v) sucrose density gradient in standard buffer. After centrifugation in a Spinco SW-65 rotor for 16 min at 65,000 rev/min at 7 C, fractions were collected. Absorbancy at 260 nm (○) was measured and radioactivity was determined from samples as described for A. (C) Sedimentation of the RNA of the nucleocapsid of NDV. The remainder of fractions 8–12 of the experiment described in B were pooled and the RNA was extracted by the phenol-SDS method (4). The RNA was redissolved in 200 μliters of standard buffer and analyzed by sucrose gradient sedimentation as described for B. Sedimentation was for 2 hr at 65,000 rev/min at 7 C.*

disrupted virus was isolated from the viral split products by velocity sedimentation.

The sedimentation coefficient (S_w) of the NDV nucleocapsid could be estimated by the method of Martin and Ames (17) to be about 225*S* (Fig. 1B) using TMV as a 200*S* (21) sedimentation marker. This S_w is compatible with the values of about 200*S* determined by Kingsbury and Darlington (12) and about 250*S* determined by Hosaka (9) and C. Blair (Ph.D. thesis, 1968).

By definition, a nucleocapsid contains viral RNA (15). The 225*S* component released from NDV by DOC can therefore be identified as the viral nucleocapsid by isolation of its RNA. As shown in Fig. 1C, The RNA of the 225*S* component had a S_w of about 52*S* as determined by the method of Martin and Ames (17) using TMV-RNA as a 31*S* sedimentation marker (11). A S_w of 57*S* has previously been determined for the intact RNA of NDV by sedimentation in the analytical ultracentrifuge (4).

Electrophoresis of the proteins of the virion and of the nucleocapsid. The proteins isolated by the phenol-SDS method (5) of ^{3}H-amino acid-labeled NDV were coelectrophoresed with ^{14}C-TMV protein and bovine serum albumin (BSA) on polyacrylamide gel at *p*H 8.1 containing 0.1% SDS (Fig. 2). Three major viral proteins (NDV_1, NDV_2, NDV_3) were obtained (Fig. 2). An estimate of the molecular weight of the NDV proteins can be made on the basis of the relationship between electrophoretic mobility and molecular weight as described by Shapiro et al. (22). Using BSA as a 67,000 molecular weight marker and TMV protein as a 16,500 molecular weight marker, the approximate molecular weight of NDV_1 was estimated to be 45,000, NDV_2 to be 62,000, and NDV_3 to be about 100,000.

To determine which and how many of the three major viral proteins are components of the nucleocapsid, the proteins of ^{3}H-amino acid-labeled NDV and density gradient purified ^{14}C-amino acid-labeled nucleocapsid were isolated together by the phenol method (5) and analyzed by polyacrylamide gel electrophoresis. The result indicates that the nucleocapsid predominantly contains protein NDV_2 and very small amounts of proteins NDV_1 or NDV_3 (Fig. 3). Proteins NDV_1 and NDV_3 are presumably proteins of the viral envelope such as the hemagglutinin and the neuraminidase, although this has not been demonstrated.

The protein of the nucleocapsid obtained from virus treated simultaneously with DOC and Pronase (100 μg/ml, 30 min, 20 C) had a higher electrophoretic mobility than the protein of

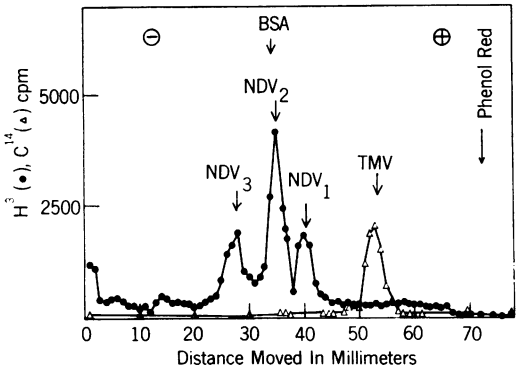

FIG. 2. *Electropherogram of the proteins of ^{3}H-amino acid-labeled NDV (●), ^{14}C-labeled TMV (△), and BSA. The radioactive proteins were isolated by phenol extraction (5). The proteins were dissolved in about 100 μliters of buffer containing 0.01 M Tris (pH 8.1), 1 mM EDTA, 2 mM dithiothreitol, 0.2% (w/v) SDS, 10% (v/v) glycerol and phenol red. Electrophoresis was for 4 hr at 10 v/cm in a 5% polyacrylamide gel as described previously (5) until the phenol red marker had migrated about 6 cm. Subsequent to electrophoresis the gel was incubated for 30 min in 10% trichloroacetic acid until the BSA band could be located. The gel was then divided into 1 mm slices and counted.*

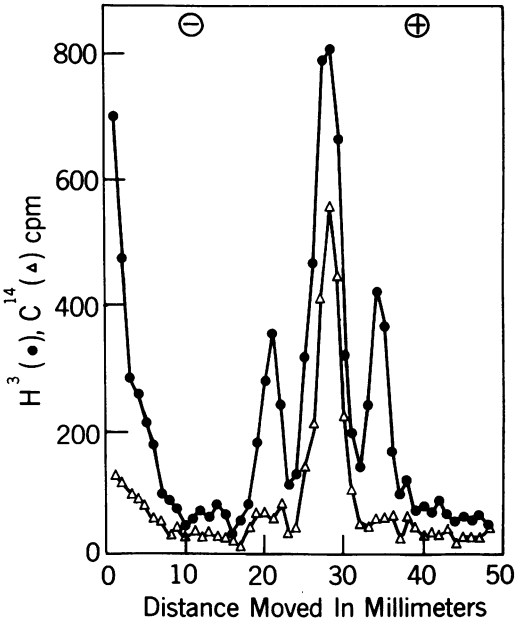

FIG. 3. *Coelectrophoresis of the total protein of ^{3}H amino acid-labeled NDV (●) and ^{14}C amino acid-labeled NDV nucleocapsid (△). The viral nucleocapsid was released from the virus by incubation with 2% (w/v) DOC and purified by sucrose gradient sedimentation (Fig. 1B). Electrophoresis was as described in Fig. 2.*

nucleocapsid untreated with this enzyme (Fig. 4), although the S_w of the nucleocapsid was little affected by such pronase treatment. This indicates that the protein of the nucleocapsid is susceptible to degradation with Pronase, whereas the RNA of the nucleocapsid is resistant to ribonuclease [Fig. 1B and C, (12)].

If the total protein of ^{3}H-amino acid-labeled virus was coelectrophoresed with ^{14}C-labeled protein of the viral nucleocapsid at *p*H 3.8 in 8 M urea, a different pattern was obtained. Only two ^{3}H-protein components were resolved, which migrated as distinct components (Fig. 5). The rest of the ^{3}H-NDV protein failed to penetrate the gel or formed a rather high background between the top of the gel and the two ^{3}H-protein peaks. The ^{14}C-protein of the viral nucleocapsid, however, migrated as a single component. It coincided with a single peak of the total ^{3}H-NDV protein. The peak is presumably the protein component which was defined as NDV_2 when the proteins of the virion were electrophoresed in SDS (Fig. 2).

Preliminary experiments indicate that the protein subunits of the three distinct helical nucleoproteins of influenza virus (3*a*) also consist of only one single kind of protein (Duesberg,

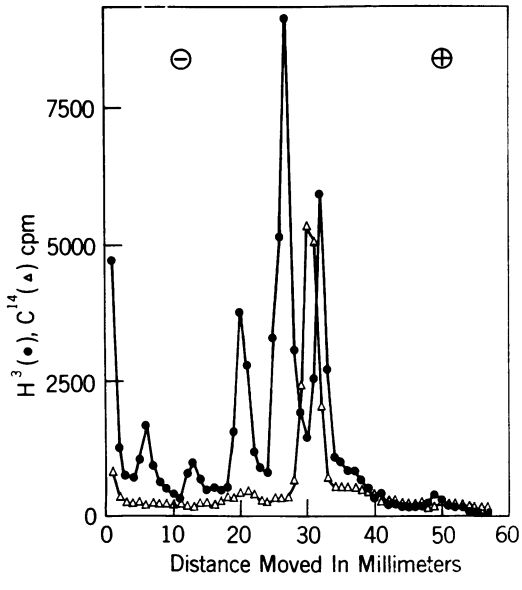

FIG. 4. *Coelectrophoresis of the total protein of ^{3}H amino acid-labeled NDV (●) and ^{14}C amino acid-labeled NDV nucleocapsid (△). The nucleocapsid was incubated with Pronase (100 μg/ml) for 30 min at room temperature prior to sucrose gradient purification (Fig. 1B). Electrophoresis was as described for Fig. 2.*

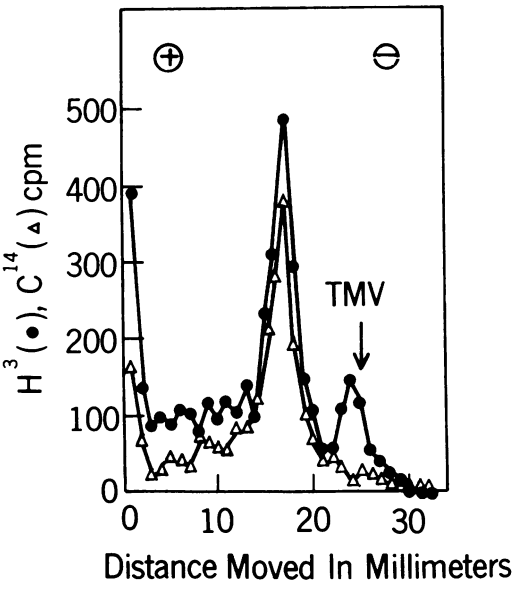

FIG. 5. *Electropherograms of the total protein of ^{3}H-amino acid-labeled NDV (●), ^{14}C-amino acid-labeled viral nucleocapsid (△) and 50 μg of TMV protein. The proteins were isolated as described for Fig. 2. After precipitation with 5 volumes of alcohol the proteins were dissolved in a solution containing 8 M urea, 0.01 M acetic acid, 2 mM dithiothreitol, 1 mM EDTA, and enough methylene blue to serve as a tracking dye. Electrophoresis was in a 6% polyacrylamide gel for 5 hr at 10 v/cm at pH 3.8 in 8 M urea (5). After electrophoresis, the gel was stained with amido black to locate the carrier TMV protein. The gel was then sliced and the radioactivity was determined.*

unpublished data; Fig. 6) after electrophoresis at *p*H 8.1 in 0.1% SDS.

DISCUSSION

That the protein of the nucleocapsid of NDV migrated as a single component as anion at *p*H 8 in 0.1% SDS, where separation is thought to be only a function of molecular weight, and as cation at *p*H 3.8 in 8 M urea, where separation is a function of both molecular weight and charge, suggests that it is a single molecule. An accidental coincidence between different proteins in one of the two employed electrophoretic systems would probably have been resolved in the other system because the electropherograms of the viral proteins were different in the two systems and because their relative electrophoretic mobilities were different in relation to the mobility of TMV protein.

It, therefore, seems likely that the helical nucleocapsid of NDV contains only a single kind of protein subunit, i.e., consists of mono-

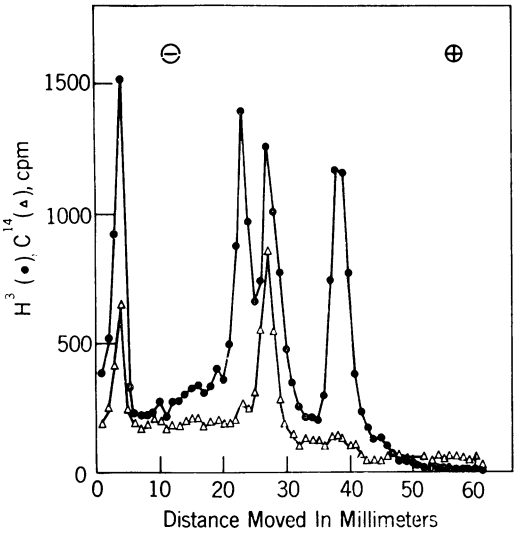

FIG. 6. *Coelectrophoresis of the total proteins of ^{3}H amino acid-labeled influenza virus protein (●) and ^{14}C-amino acid-labeled protein (△) of the three distinct components of the nucleoprotein of influenza virus (3a). The viral nucleoproteins were isolated and purified as described previously (3a) and pooled prior to isolation of the protein. Electrophoresis was for 3 hr and otherwise as described in Fig. 2.*

meric capsomers like the structurally related TMV. The molecular weight of the capsomer of the nucleocapsid of NDV is about 62,000 or about four times larger than that of TMV. The nucleocapsid of NDV differs from that of TMV in several properties, such as its dissociability by low concentrations of SDS (~0.2% w/v) and its relative flexibility evident in electron micrographs (1, 12). The helical nucleoprotein of influenza virus, which consists of three distinct subunits (3a), also contains only a single electrophoretic protein component in 0.1% SDS at *p*H 8. This result is compatible with Laver's (14) conclusion that the ribonucleoprotein-antigen of influenza A virus consists of a single protein component after electrophoresis on cellulose acetate in 0.4% SDS at *p*H 8.9.

The helical nucleoproteins of two rhabdoviruses (7), vesicular stomatitis virus (10), and rabies virus (22a), which are structurally related to myxoviruses (7) but are not members of the myxovirus group were recently shown to consist of one kind of protein molecule. Thus, all known helical nucleoproteins of RNA viruses consist of monomeric protein subunits. The nucleocapsids of several icosahedral RNA viruses, on the other hand, have been shown to contain capsommers with multiple protein subunits (16, 20).

The internal antigens of the enveloped RNA tumor viruses, which have failed to show any detectable symmetry up to date, have also been shown to consist of several distinct proteins (5, 8). On the other hand, the protein subunits of the spherical cores of arboviruses which also failed to show any detectable symmetry, were found to consist of a single type of protein molecule (23).

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