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RADIOIODINATION OF THE ENVELOPE PROTEINS OF NEWCASTLE DISEASE VIRUS

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Lactoperoxidase-catalyzed iodination selectively labels the two glycoproteins (VP1 and VP2) of Newcastle disease virus. The low-molecular-weight, nonglycosylated major viral protein, VP6, was not iodinated in the intact virus but was iodinated in disrupted virions, suggesting a localization on the inner, rather than the outer, envelope surface. Studies on the distribution of virion proteins labeled with ^{125}I and ^{3}H-isoleucine between detergent-soluble and detergent-insoluble fractions show that the virion proteins VP4, VP5, and VP6 are solubilized to a much lesser extent than are VP1 and VP2.

INTRODUCTION

Newcastle disease virus (NDV), Simian virus 5 (SV5), and Sendai virus, members of the paramyxovirus group, have similar polypeptide patterns, as revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (1–10). Each of these viruses appears to have two glycoproteins (4, 5, 8, 9). Scheid and Choppin (11) have recently used Triton X-100 solubilization in the presence of 1.0M KCl and centrifugal techniques to isolate the glycoproteins of the Hickman strain of NDV. They reported that both the hemagglutinin and neuraminidase activities of NDV, as well as those of SV5, reside in the higher-molecular-weight glycoprotein (11, 12). Seto et al. (13) also isolated these glycoproteins from NDV-Beaudette (NDV-B) and NDV-Italien (NDV-I), using nonionic detergents in conjunction with DEAE-cellulose column chromatography. They describe observations similar to those reported by Scheid and Choppin (11). However, with the avirulent strain NDV-I, neuraminidase and antibody blocking activities resided in two discrete glycoproteins.

The present paper describes the intravirion localization of the viral proteins of NDV-L-Kansas (NDV-LK) using the lactoperoxidase-catalyzed iodination technique. This technique (14) has been used to label the surface proteins of red blood cells (15, 16), lymphocytes (17), influenza virus (18), avian tumor viruses (19), Sindbis virus (20), vesicular stomatitis virus (21), and reovirus and bluetonque virus (22). It leads to the iodination of exposed tyrosine and some histidine residues of the surface polypeptide chains (16). Due to its apparent inability to penetrate biological membranes, lactoperoxidase (MW 80,000) brings about specific labeling of proteins on the external membrane surface.

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MATERIALS AND METHODS

Growth and Purification of Labeled Virus

Allantoic sacs of 10-day-old chick embryos were inoculated with 10^3 plaque-forming units (PFU) of NDV-LK. One hour after the inoculation with the virus, radioactive label (^{3}H-isoleucine, ^{3}H-fucose, or ^{3}H-tyrosine – 50 μCi/egg; ^{14}C-amino acid mixture or ^{3}H-amino acid mixture – 10 μCi/egg) was injected into the embryonated eggs. L-Fucose [1, 5, 6–^{3}H], 5 Ci/mmole, was obtained from New England Nuclear; ^{3}H-isoleucine (7 Ci/mmole), ^{3}H-L-tryosine (7 Ci/mmole), and the ^{3}H- and ^{14}C-amino acid mixtures (no specific activity specified) were purchased from Schwartz/Mann. Chorioallantoic fluid was harvested after 48 hr of incubation at 37°C, cellular debris and chick erythrocytes were removed by centrifugation at 10,000 X g for 10 min, and virus was then pelleted at 70,000 X g for 2 hr at 4°C in a Spinco 50.1 fixed-angle rotor. The virus pellet was suspended and centrifuged to equilibrium (16 hr) in a 16–65% preformed linear sucrose-D_2O gradient in TSE buffer (0.01 M Tris-HCl, 0.01 M NaCl, 0.05 M disodium EDTA, pH 7.4) at 4°C in a Spinco SW 41 rotor at 40,000 rpm. The virus band was collected, pelleted by centrifugation, suspended, and again centrifuged to equilibrium (16 hr) in a 6–25% iodine equivalent preformed linear Renograffin gradient in TSE buffer at 4° C in a Spinco SW 41 rotor at 40,000 rpm. The purified virus band was again pelleted and suspended in TSE buffer. Virus was estimated by plaque formation on chick primary monolayers (23).

Polyacrylamide Gel Electrophoresis

The method of polyacrylamide gel electrophoresis is that described by Laemmli (24) as modified by Samson and Fox (23). All gels consisted of a 3% acrylamide stacking gel (0.25 ml) layered over a 10% separating gel (2.5 ml) in siliconized glass tubes (6 mm internal diameter). Virus samples were dissolved in 0.06 M Tris-HCl (pH 7.4) containing 2% SDS and 5% 2-mercaptoethanol and heated for 60 sec at 100°C. Glycerol and bromphenol blue were added to final concentrations of 15% and 0.0005%, respectively. A total of 200 to 400 *µl* (80–175 µgm of viral protein) per gel was used in electrophoresis. Gels were initially subjected to 1 mA of current until the tracking dye entered the separating gel and then to 3 mA per gel until the tracking dye was approximately 1 cm from the bottom of the gel. Gels were then chilled at 4°C for 10–15 min before being removed from the glass tubes. The gel staining and destaining procedure of Fairbanks et al. (25) was followed. The gels were then frozen at –70°C and fractionated into 1.3 mm slices with stacked razor blades separated by spacers. Slices were shaken for 60 min at 60°C in 1.0 ml of freshly prepared 2% periodic acid solution (26). Fifteen ml of scintillation fluid was added per vial for determination of radioactivity by scintillation counting (23).

Iodination Procedures

For iodination of intact virus, purified virus grown in chick embryos (1–2 mg) was suspended in 0.5 ml of PBS solution (containing 0.01 M NaCl and 0.01 M sodium phosphate buffer of pH 7.4) plus 5 µgm of lactoperoxidase (Sigma) and 100 µCi of carrier-free ^{125}I-NaI (Schwarz/Mann). Iodination was initiated by the addition of 5 µmoles of

hydrogen peroxide. An additional 5 μmoles of H_2O_2 was added to the reaction mixture at 60 sec intervals. The addition of H_2O_2 in small amounts, minimized the undesirable oxidation of virus by free peroxides. Iodination was performed at 30°C for 5 min with constant gentle swirling. The reaction was terminated by chilling to 4°C with the addition of excess mercaptoethanol. The reaction mixture was then layered over a discontinuous sucrose gradient containing 1.0 ml of 65% and 3.0 ml of 25% sucrose in TSE buffer and centrifuged at 40,000 rpm at 4°C for 60 min in a Spinco SW 50.1 rotor. The ^{125}I-labeled virus recovered at the 65% sucrose interface was diluted with 0.01 M sodium phosphate buffer (pH 7.4) and sedimented. The virus pellet was suspended in 0.1 M sodium phosphate buffer and dialyzed against this same buffer at 4°C. For iodination of disrupted virus, approximately 1.0 mg of intact virus was first solubilized in 0.1 ml of 1.0% SDS in 0.1 M sodium phosphate buffer (pH 7.4). Deionized water containing 5 μgm of lactoperoxidase, 100 μCi of carrier-free ^{125}I-NaI, and 50 μmoles of H_2O_2 was added, bringing the final volume to 1.0 ml. Iodination proceeded at 30°C for 5 min. Additional 5 μmole portions of H_2O_2 were added at 60 sec intervals. The reaction was terminated by chilling to 4°C, and the reaction mixture was dialyzed extensively against several changes of 0.01 M sodium phosphate buffer (pH 7.4) at 4°C.

Isolation of Nucleocapsid and Envelope Fractions

The isolation of nucleocapsid was similar to that of Mountcastle et al. (27), except highly purified doubly-labeled virions (^{3}H-isoleucine and ^{125}I) were suspended in 0.8 ml of TSE buffer (containing 0.0005 Tris-HCl buffer, 0.05 NaCl, and 0.001 M EDTA), pH 7.2; 0.2 ml of 5% Triton X-100 was added in TSE buffer; the mixture was incubated at 37°C for 25 min with frequent agitation; and finally 0.04 ml of 10% sodium deoxycholate was added in TSE buffer. This mixture (1.0 ml) was layered onto a discontinuous sucrose-CsCl gradient containing 0.5 ml of 40% CsCl (w/w) in TSE buffer, 1.0 ml of 30% CsCl in TSE buffer, 1.0 ml of 25% CsCl in TSE buffer, and 1.5 ml of 5% sucrose in TSE buffer. Gradients were centrifuged at 34,000 rpm for 90 min in a Spinco SW 50.1 rotor at 4°C. The visible band in the 30% CsCl fraction was collected and is termed nucleocapsid fraction. All remaining material in the sample above the 5% sucrose layer was also collected and is termed envelope fraction. Both fractions were dialyzed against 3 changes of cold double-distilled water for 24 hr at 4°C before solubilization for SDS-PAGE.

RESULTS

Since the peroxide used in the lactoperoxidase-catalyzed iodination of virion surface proteins might damage the virus, we first examined the effect of the iodination procedure on viral infectivity. Virus was titered by plaque formation on chick embryo fibroblasts both prior to and following the standard iodination treatment (Materials and Methods), and no significant differences were observed (data not shown). The iodination procedure also had no effect upon the hydrodynamic properties of the virions with respect to behavior in equilibrium centrifugation in a preformed density gradient (Fig. 1). A preparation of iodinated virus that had been dialyzed extensively against PBS (Materials and Methods) was combined with a preparation of ^{3}H-isoleucine-labeled NDV and the

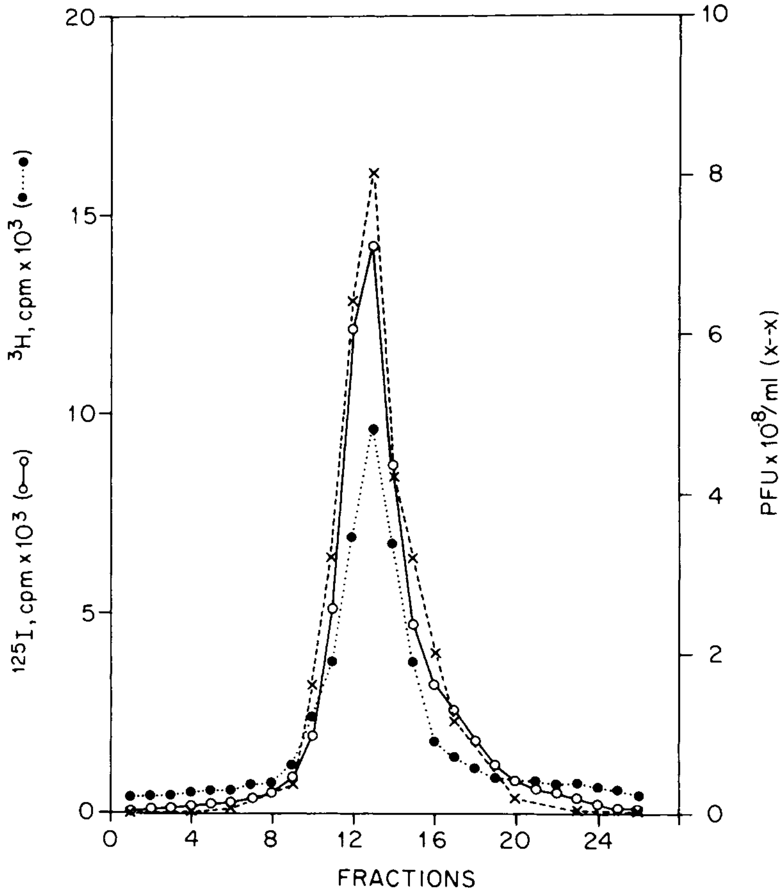

Fig. 1. Equilibrium density gradient centrifugation of a mixture of ^{125}I and ^{3}H-isoleucine labeled NDV-LK in a 16–65% preformed linear sucrose gradient. Centrifugation was in sucrose solutions containing 0.01 M potassium phosphate buffer of pH 7.5 in a Spinco SW 50.1 rotor at 37,000 rpm for 16 hr at 4°C. Twenty-six fractions of 0.2 ml each were collected for the determination of infectivity and radioactivity.

mixture was centrifuged to equilibrium. Fractions were collected and ^{125}I and ^{3}H radioactivity and infectivity were determined. The ratios ^{125}I: PFU and ^{3}H:PFU were constant within the limits of error for all fractions tested.

Solubilization of a mixture of virus preparations labeled with an amino acid mixture or with ^{125}I followed by SDS-PAGE revealed that only two of the proteins are likely to have a surface localization (Fig. 2, bottom). One of these proteins (VP1) is known to have surface function since it elicits both hemagglutinin and neuraminidase activities (11). The second protein labeled with ^{125}I migrated in SDS-PAGE in the region occupied by VP2 and VP3. The peak fraction of ^{125}I radioactivity, however, consistently migrated one gel fraction less than the peak fraction of radioactivity derived from the amino-acid-labeled viral protein. The experiment in Fig. 2 (top) shows that the protein of lesser mobility in the VP2-VP3 region is glycosylated. Taken together, these data indicate that

both virion proteins labeled by lactoperoxidase-catalyzed labeling with radioactive iodine are glycoproteins. The major virion protein VP 6 was not labeled with ^{125}I under conditions where VP1 and VP2 were, indicating that VP6 either is not localized on the virion surface or has few groups reactive to labeling with iodine. Little iodine label appeared to be associated with the region of the gel where the minor virion proteins VP4 and VP5 are known to migrate – that is, the region of the gel between the VP3 and VP6 peaks. Radioiodine was also detected in some experiments in a region of 130,000–160,000 daltons. This "aggregate" band has been observed by others (1) and probably arises from the type of aggregation that occurs between certain polypeptide species when dissociated erythrocytes are heated in the presence of SDS (25). T. Steck (personal communication) has suggested to us that sulfenyl iodides (R-SI) formed during iodination might react with remaining sulfyhydryl groups to form disulfides (R-SI + R′-SH → RSSR′). When cross links of this sort form between hydrophobic proteins, the resultant product might be resistant to reduction, even in the presence of dodecyl sulfate.

The experiments in Fig. 3 show that VP6 became labeled when NDV proteins were labeled with ^{3}H-tyrosine during virus propagation and that VP6 was readily labeled with radioactive iodine when the virions were solubilized with SDS prior to lactoperoxidase-catalyzed iodination. Thus the failure to observe lactoperoxidase-catalyzed iodination of VP6 in the intact virion is caused by inaccessibility of reactive groups rather than by their absence. While this is not definitive proof of internalized localization of VP6, it is nevertheless indicative of this possibility. Chen et al. (4) have reached similar conclusions on the external localization of the glycoproteins and the putative internal localization of VP6. They demonstrated that the former but not the latter are digested when intact virions are incubated with a proteolytic enzyme.

Another experiment consistent with localization of VP6 on the inner, rather than the outer, surface of the viral envelope is described in Fig. 4. Here, a ^{3}H-labeled virus preparation was labeled with ^{125}I by lactoperoxidase-catalyzed iodination, and the viral envelope and nucleocapsid fractions were separated prior to solubilization of the proteins with SDS and resolution by SDS-PAGE. The most striking observation in the experiment is the presence of large quantities of VP4, VP5, and VP 6 in the nucleocapsid fraction under conditions where all or virtually all of VP1 and VP2 are solubilized. The data in Fig. 4 were tabulated, and the percentage of radioactivity of the virion proteins in these two fractions is presented in Table I. It is obvious from the figure itself that little radioactivity derived from VP1 or VP2 is present in the nucleocapsid fraction. Thus the high proportions of VP4, VP5, and VP6 in the nucleocapsid fraction suggest that these three proteins may share a localization in both the envelope and nucelocapsid.

DISCUSSION

Of the six discernible virion proteins of NDV-L-Kansas, two (VP1 and VP2) have a distinct localization on the outer surface of the viral envelope, three others (VP4, VP5, and VP6) are apparentlv localized in or on the inner membrane surface, perhaps at the envelope-nucleocapsid junction, and a sixth (VP3), "nucleocapsid" protein, may have a distinct localization in the capsid. The finding that VP1 and VP2 are exposed on the external surface of the envelope is consistent with the nature of the biological activities of these proteins. VP1 is known to possess both neuraminidase and HA activities (11), and VP2,

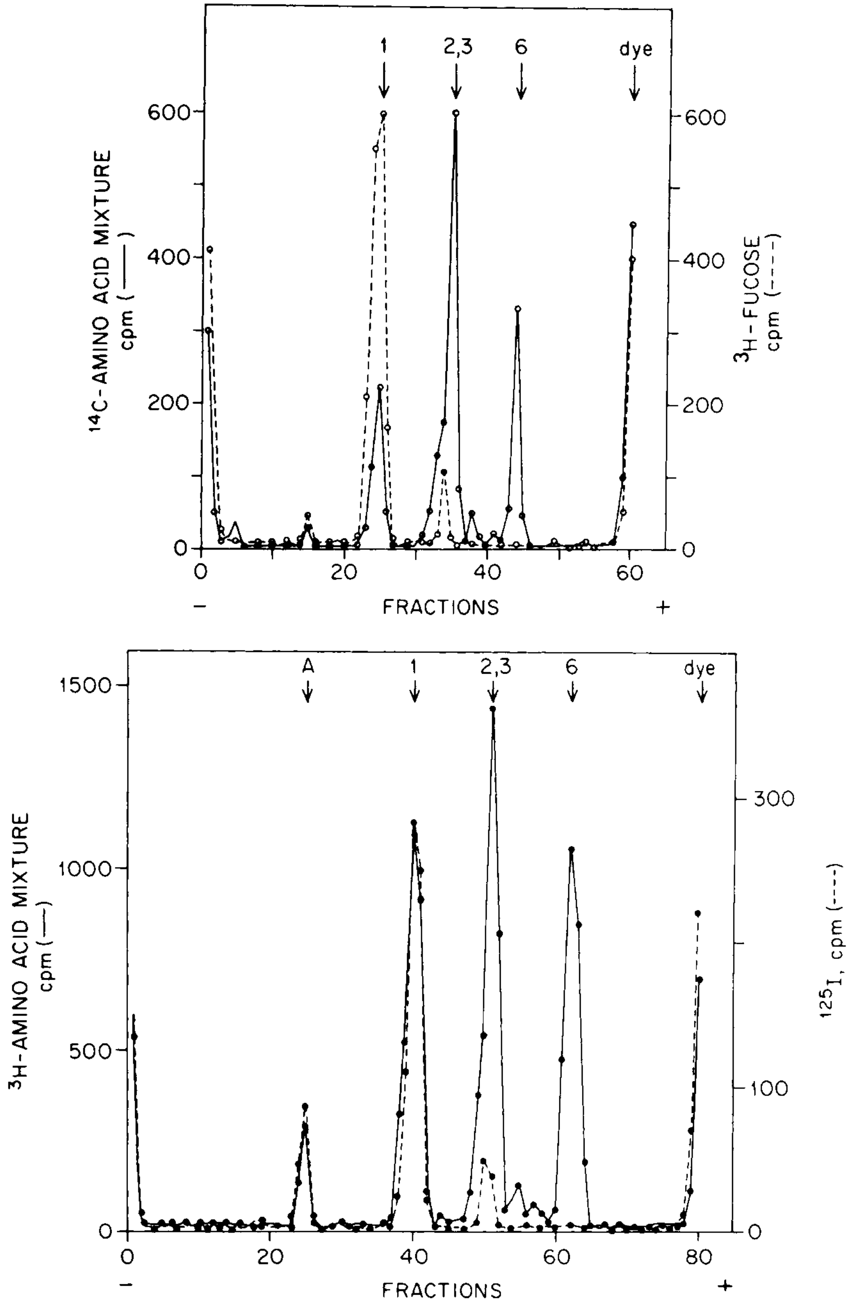

Fig. 2. Polyacrylamide gel electrophoresis of the polypeptides of NDV. (Top) NDV preparation labeled with ^{3}H-fucose or a ^{14}C-amino-acid mixture were disrupted in SDS-mercaptoethanol solution (Materials and Methods) for SDS-PAGE. (Bottom) a virion preparation was labeled during growth with a ^{3}H-amino-acid mixture and subsequently with ^{125}I in a lactoperoxidase-catalyzed reaction and purified and solubilized for SDS-PAGE as described in Materials and Methods. In this and subsequent gel patterns the anode and cathode are indicated (+) and (−), respectively.

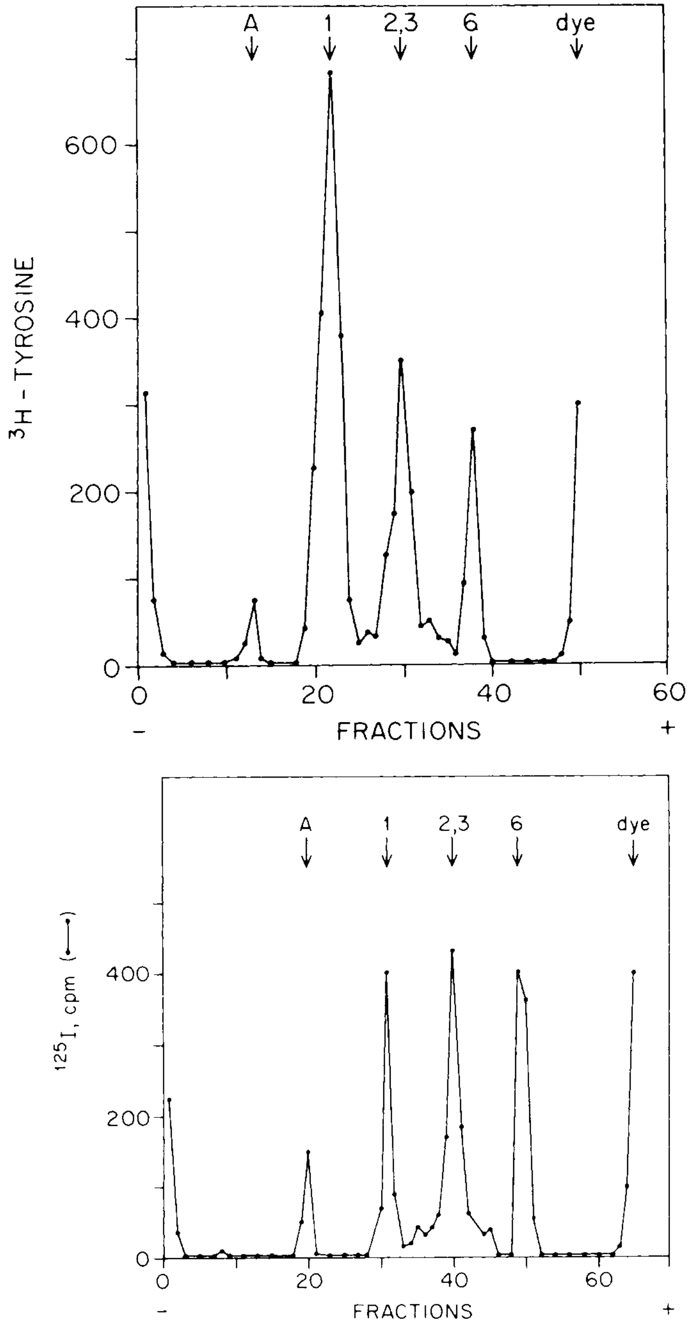

Fig. 3. Polyacrylamide gel electrophoretic patterns of NDV labeled with ^{3}H-tryosine or by lactoperoxidase-catalyzed iodination. (Top) virions labeled during growth with ^{3}H-tyrosine. (Bottom) virions were disrupted with 1.0% SDS in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C to remove free ^{125}I before solubilization with SDS-mercaptoethanol solution and analysis by SDS-PAGE.

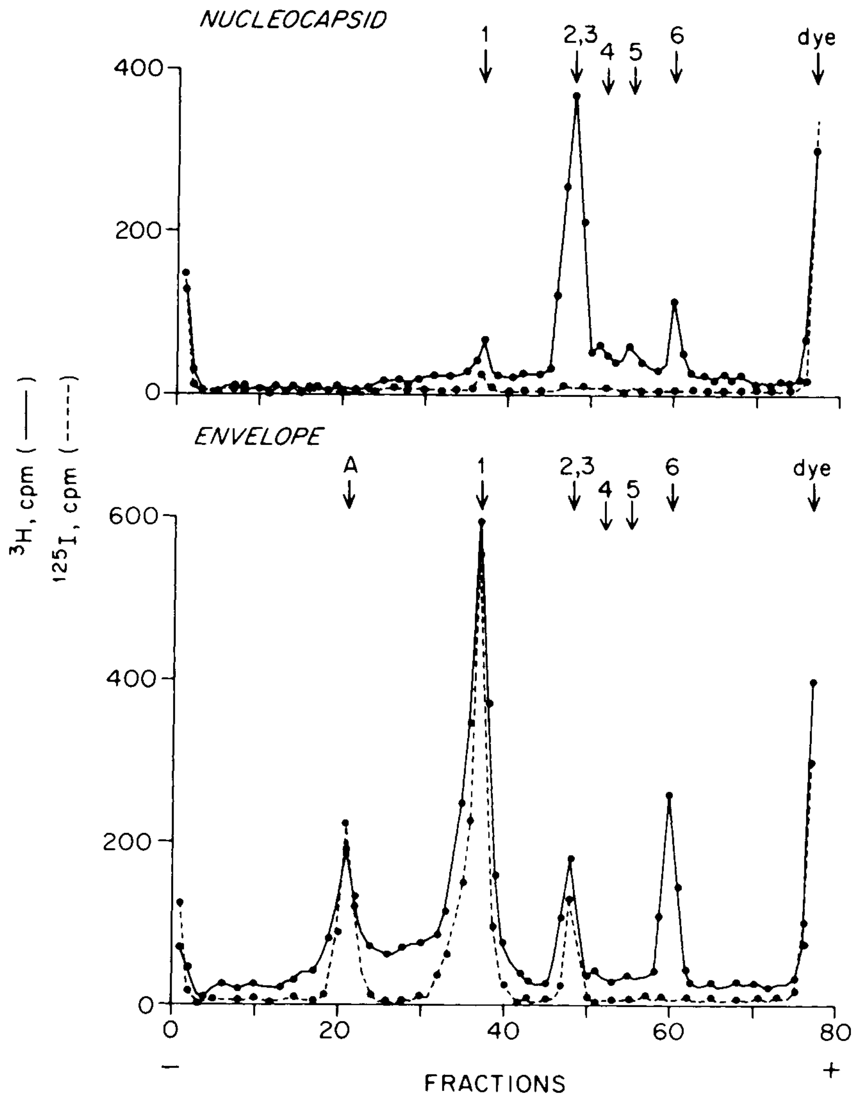

Fig. 4. Polyacrylamide gel patterns of ^{3}H-isoleucine-labeled NDV which was iodinated and purified before resolution of the nucleocapsid and envelope fractions by the methods of Mountcastle et al. (27). (Top) nucleocapsid fraction. Note the absence of VP1, VP2 (^{125}I labeled), and the aggregate (A) and the presence of VP4 and VP6. (Bottom) envelope fraction. Note the presence of both ^{3}H and ^{125}I labels in VP1, VP2, and aggregate and the lesser, relative abundance of VP4 and VP5 as compared with the nucleocapsid fraction.

For this experiment, one-half of the material in the nucleocapsid fraction preparation and one-third of the material in the envelope preparation were subject to SDS-PAGE. It is therefore necessary to multiply the counts per min in the gel fractions by the appropriate factors to arrive at estimates of the total counts per min in the six discernible viral proteins.

like the analogous protein from Sendai virus (28–30), apparently plays roles in both viral penetration of the cell membrane and cell membrane fusion from without (31). Both VP2 and the analogous Sendai viral glycoprotein are formed from a precursor by a post-translational cleavage process (23, 29–30, 32).

It is relatively simple to quantitatively assess the presence of VP1 and VP2 in the fraction insoluble in nonionic detergent – that is, the nucleocapsid fraction (Fig. 4 and Table I). The presence of VP1 can be assessed from ^{3}H or ^{125}I radioactivity. VP2 in nucleocapsid fraction was assessed only by radioiodine content since VP 2 and VP3 have electrophoretic mobilities too close to make the assessment on the basis of ^{3}H radioactivity. Though there appeared to be greater amounts of ^{125}I in nucloecapsid fraction for VP2 than VP1, this finding may only be the sum of the effects of the low level of radioiodine that is observed in all fractions of the gel and the lower radioiodine content in VP2 than VP1. We have no way of assessing the precise distribution of VP3 from our data. Its close similarity in electrophoretic mobility to VP2 and the lack of a unique, known, biochemical property preclude this assessment. It is apparent from the data, however, that VP3 is predominantly a nucleocapsid protein since 60% of the sum of ^{3}H radioactivity in VP2 + VP3 was localized in the nucleocapsid fraction.

Mountcastle et al. (27) also observed the presence of VP6 as well as what appeared to be VP4 and VP5, in nucleocapsids isolated from mature virions by detergent treatment, but they made no quantitative assessment of the disposition of these polypeptides in envelope and nucleocapsid fractions. Scheid and Choppin (11) have shown that VP6 is relatively insoluble in nonionic detergents in the presence of low salt, and this solubility characteristic is probably the factor which gives rise to the presence of VP6 in both nucleocapsid and envelope fractions.

The results obtained with lactoperoxidase-catalyzed radioiodination and the relative insolubility of VP6 in solutions containing low salt and nonionic detergent, as compared with the solubility characteristics of VP1 and VP2, suggest a peripheral localization for this protein on the inner surface of the virion envelope. This concept is supported by recent findings in this laboratory using the protein-perturbing agent lithium diiodosalicylate (LIS) (Miyakawa, T., Li, J.K.-K., and Fox, C.F., to be published). VP6 is selectively eluted from the virion envelope by low concentrations of LIS, whereas VP1 and VP2 are not. Steck and his associates (33–35), in an elegant series of studies on the elution of proteins from the erythrocyte membrane, have shown that nonionic detergents

TABLE I. Distribution of Viral Protein Between the Nucleocapsid and Envelope Fractions

	Virion protein					
	1	2	2 + 3	4	5	6
Nucleocapsid	8 (2)	(10)	60	52	51	26
Envelope	92 (98)	(90)	40	48	49	74

Distributions are expressed as percent of total counts per min from the combined fractions in the experiment described in Fig. 4. Values in parentheses are from ^{125}I data; all other values were calculated from ^{3}H radioactivity.

selectively elute integral membrane proteins – for example, those which span the membrane – whereas protein-perturbing agents such as LIS selectively elute peripheral membrane proteins – that is, those with little surface area exposed to a hydrophobic environment.

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