Isolation and Purification of the Envelope Proteins of Newcastle Disease Virus

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A procedure has been developed for the isolation of Newcastle disease virus (NDV) envelope proteins. The two surface glycoproteins and the non-glycosylated membrane protein were solubilized with 2% Triton X-100 and 1 M KCl. Removal of the KCl by dialysis yielded by precipitation a pure preparation of the non-glycosylated membrane protein, which is insoluble in solutions of low ionic strength. The soluble fraction consisting of the two glycoproteins possessed full neuraminidase and hemagglutinating activities. The two glycoproteins could be separated by rate zonal sedimentation in a sucrose gradient containing 1% Triton X-100 and 1 M KCl. Under these conditions, the sedimentation coefficient of the larger glycoprotein, virus protein 1, was 9.3s, and that of the smaller, virus protein 2, was 6.1s. Both hemagglutinating and neuraminidase activities were associated with virus protein 1; virus protein 2 had neither activity. The results suggest that both activities reside on a single NDV glycoprotein. Similar results were obtained previously with another paramyxovirus, simian virus 5. These findings suggest that the association of hemagglutinating and neuraminidase activities with one glycoprotein is a general property of the paramyxovirus group.

Newcastle disease virus (NDV) contains at least six polypeptides (9, 16, 17). Two of the polypeptides, designated 1 and 2, are glycoproteins (17) with molecular weights of 74,000 and 56,000, respectively. Two other paramyxoviruses, simian virus 5 (SV5) and Sendai virus, have also been shown to contain two glycoproteins (12, 17). Evidence obtained with SV5 showed that the glycoproteins constitute the spikes on the parainfluenza virion and that these glycoprotein spikes possess hemagglutinating and neuraminidase activities (5).

Although evidence from many laboratories has indicated that with influenza virus the hemagglutinating and neuraminidase activities reside on different proteins, the situation with paramyxoviruses has been less clear. In the case of NDV, hemagglutinating activity has been ascribed to one protein (9, 10, 11), and it has been reported that neuraminidase is associated with another protein (10, 11). However, in the latter case the fractions with neuraminidase activity were not free of the protein with hemagglutinating activity; thus the results are difficult to interpret. Further, the protein to which neuraminidase activity was attributed, i.e., the smallest virion protein, has been found in this laboratory not to contain carbohydrate

(17), which makes it highly unlikely to be the neuraminidase, since the neuraminidase of paramyxoviruses would be expected to be a glycoprotein (5).

In contrast to the above reports, suggesting that with NDV the hemagglutinating and neuraminidase activities are associated with separate proteins, we have recently obtained evidence that with SV5 both of these activities are associated with the larger of the two glycoproteins in the virion (18). This glycoprotein has a molecular weight of ~67,000 (17). In order to do the studies with SV5, it was first necessary to develop a method for isolating the glycoproteins in pure form with biological activities preserved. This was accomplished by using the nonionic detergent Triton X-100 to disrupt the virus and sucrose gradient centrifugation in the presence of Triton and 1 M KCl to separate the glycoproteins (18).

To resolve the apparent discrepancy between our results with SV5 and the previous work on NDV, and to determine if there was a commonality among paramyxoviruses regarding the proteins with hemagglutinating and neuraminidase activities, the methods developed for SV5 were applied to NDV. The results which are reported here indicate that, with NDV also,

the larger viral glycoprotein has both hemagglutinating and neuraminidase activities.

MATERIALS AND METHODS

Cells. Monolayer cultures of a variant of the MDBK line of bovine kidney cells were grown in 10-cm plastic petri dishes (Falcon) in reinforced Eagle medium (REM) with 10% fetal calf serum as described previously (7).

Virus. Seed stocks of the Hickman strain of NDV were grown in the allantoic sac of 10- to 11-day-old embryonated chicken eggs inoculated with $\sim 10^6$ 50% egg infective doses (EID₅₀). Allantoic fluid was harvested at 48 hr.

Growth, isotopic-labeling, and purification of virus. Confluent monolayers of MDBK cells were inoculated at a multiplicity of approximately 20 EID₅₀ per cell, and 6 ml of REM with 2% calf serum containing either ³H-leucine (3 μ Ci/ml) or ¹⁴C-amino acid mixture (1.2 μ Ci/ml) was added. After three days, the medium was harvested and clarified at $3000 \times g$. Polyethylene glycol and sodium chloride were added to concentrations of 7% and 2.3% (w/v), respectively, and the suspension was stirred for 2 hr at 4 C (15). The precipitated virus was collected by centrifugation at $700 \times g$ for 30 min and suspended in REM. The virus was purified by two successive centrifugations to equilibrium ($100,000 \times g$ for 2 hr) in preformed 10 to 45% (w/w) potassium tartrate gradients. Virus from the gradients was concentrated by sedimentation onto a cushion of 45% potassium tartrate. Except where otherwise stated, virus was dialyzed against 0.01 M sodium phosphate buffer, pH 7.2. The volume of the virus suspension was adjusted to contain 0.8 to 1.5 mg of protein per ml.

Chemicals and isotopes. Bovine plasma albumin was obtained from Armour Pharmaceutical Co., Chicago, Ill., *N*-acetyl-neuraminic acid, from Sigma, St. Louis, Mo., Triton X-100 from Beckman Instruments, Fullerton, Calif., and polyethylene glycol 6000 from Amend Drug and Chemical Co., New York, N.Y. ¹⁴C-reconstituted protein hydrolysate and [4,5-³H]-leucine (51 Ci/mmole) were purchased from Schwarz BioResearch, Orangesburg, N.Y. *N*-acetyl-neuraminic acid-D-lactose (neuraminlactose), prepared from bovine colostrum, was kindly supplied by Marcel Pons. ¹²⁵I-labeled rabbit gamma G-globulin was a gift of Victor Bokisch.

Triton fractionation of virus. In most experiments, suspensions of purified NDV in potassium tartrate were dialyzed against 1 M KCl in 0.01 M phosphate buffer, pH 7.2. One-tenth volume of a solution of 20% (v/v) Triton X-100 in the same phosphate buffer was added. After mixing, the solution was allowed to stand for 10 to 20 min at room temperature. The further fractionation steps are described under Results.

In some experiments, Triton treatment was carried out in the absence of KCl. In these experiments, Triton X-100 was added in a final concentration of 10% (v/v) to a virus suspension in 0.01 M phosphate buffer, pH 7.2. After stirring for 10 to 20 min at room temperature, the Triton had dissolved completely,

and the still turbid solution was centrifuged at $100,000 \times g$ for 30 min. The clear supernatant fluid was collected, and the pellet was suspended in the original volume of 0.01 M sodium phosphate buffer, pH 7.2, by sonic oscillation.

Isolation of proteins from Triton-containing solutions. A 10-fold volume of chilled *n*-butanol was added, and the two phases were mixed by tilting or, with small samples and when losses of protein due to adhering to the upper portions of the tube were to be avoided, by vibration (Vibration type stirrer, Buchler Instruments, Fort Lee, N.J.). After holding for 30 min on ice, the precipitate formed was sedimented at $3,000 \times g$ for 20 min at 0 C. The supernatant fluid was removed, and the remaining butanol in the pellet was dissolved in peroxide-free ether. After centrifugation at $3,000 \times g$, the ether was discarded, 0.01 M phosphate buffer was added, and remaining traces of ether were removed by exposure to a gentle stream of nitrogen at room temperature. The precipitate was finally dispersed by gentle sonic treatment (Branson Instruments, Model LS 75, setting 1) twice for 10 sec at 0 C.

Hemagglutination titrations. Hemagglutination titrations were done at 4 C as described previously (6). Since Triton interferes with the hemagglutination reaction, the detergent was first removed from the samples by the butanol-ether precipitation described above. The final precipitate was dispersed in phosphate-buffered saline.

Neuraminidase assay. Neuraminlactose was used as a substrate. Free neuraminic acid was measured by the thiobarbituric acid procedure (1) with *N*-acetyl-neuraminic acid as a standard. Triplicate samples of virus or viral proteins were diluted with water to 0.1 ml, and 0.2 ml of 0.2 M sodium phosphate buffer (pH 6.0) and 0.2 ml of neuraminlactose in water (400 μ g/ml) were added. The incubation temperature was 37 C. One neuraminidase unit was defined as the amount of enzyme that releases 1 nmole of *N*-acetyl-neuraminic acid in 1 min at 37 C.

Protein. Protein determinations were done by the method of Lowry and co-workers (13) with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis. The procedures employed in the preparation of gels and electrophoresis have been described (4); 7.5% acrylamide gels, approximately 10 cm long, with a 0.5-cm stacking gel of 2.5% acrylamide, were used. Since the presence of Triton in the sample was found to accelerate the migration of proteins, the detergent was removed from the sample by a modification of the precipitation procedure described above, in which 1% (v/v) mercaptoethanol was added to the *n*-butanol, and the ether wash was omitted. For determination of radioactivity 1-mm slices of gels were processed as described previously (18).

To determine the radioactivity in fractions from sucrose gradients, samples were diluted in 0.5 ml of water, and 4 ml of a scintillation fluid consisting of 1,200 ml of Triton X-100, 2,000 ml of toluene, and 140 ml of Liquifluor (New England Nuclear Corp., Boston, Mass.) was added.

RESULTS

Fractionation of NDV virions with Triton X-100. The electrophoretic pattern of the NDV polypeptides obtained in this laboratory on sodium dodecyl sulfate-mercaptoethanol-polyacrylamide gels has been described in detail previously (16, 17). Figure 1 illustrates the radioactivity profile of these proteins labeled with ^3H -leucine. This pattern is shown here because this preparation of ^3H -leucine-labeled virus was used throughout the experiments to be illustrated below. The marker virus preparations were labeled with ^{14}C -amino acids. In previous studies (16, 17), the following points concerning NDV proteins were established. Protein 1 is a glycoprotein, with a molecular weight of approximately 76,000, and there is a second glycoprotein, protein 2, with a molecular weight of $\sim 62,000$ which migrates with the nucleocapsid subunit, protein 3, and which can be demonstrated as a protein distinct from the nucleocapsid protein by the use of a carbohydrate label such as glucosamine. Protein 4 is a non-glycosylated protein which is usually seen as a shoulder on the front of the 2 + 3

FIG. 1. Polyacrylamide gel electrophoresis of the polypeptides of NDV. Virions labeled with ^3H -leucine were disrupted with sodium dodecyl sulfate (SDS) and mercaptoethanol and subjected to electrophoresis for 14 hr as described in Materials and Methods. In this and subsequent gel patterns, the origin is on the left and the anode on the right. Arrows indicate the positions of the six polypeptide species of the virion which are described in the text.

peak. Protein 5 is a minor component which is not resolved in the ^3H -leucine-labeled preparation in Fig. 1, but can be detected in the virus marker labeled with ^{14}C -amino acid mixture (Fig. 2). Protein 6 is a non-glycosylated protein which the available evidence suggests is the major nonspike protein associated with the paramyxovirus membrane (8, 17).

To disrupt the virus and separate the proteins, purified ^3H -leucine-labeled virus in 1 M KCl and 0.01 M phosphate buffer, pH 7.2, was treated with Triton X-100 at a final concentration of 2% as described in Materials and Methods. On the addition of Triton, the homogeneous cloudiness of the suspension cleared, and a flocculent precipitate appeared. The suspension was centrifuged at $10,000 \times g$ for 20 min, and a clear supernatant fluid and a pellet were obtained. Polyacrylamide gel analysis of the proteins in the pellet (Fig. 2) revealed one major

FIG. 2. Polyacrylamide gel electrophoresis of the NDV polypeptides in the $10,000 \times g$ pellet fraction after treatment of virions with Triton X-100 in the presence of 1 M KCl. Virus proteins 3 and 4 were present in this pellet. A suspension of 1.3 mg of purified ^3H -leucine-labeled NDV in 0.01 M phosphate buffer and 1 M KCl was treated with Triton X-100 at a final concentration of 2% as described in Materials and Methods, and centrifuged at $10,000 \times g$ for 20 min. The pellet was suspended in 0.5 ml of 0.01 M phosphate buffer by sonic treatment, and a 20- μ liter sample was prepared for electrophoresis by precipitation of the proteins with *n*-butanol and 1% mercaptoethanol. Virions labeled with ^{14}C -amino acids were added as a co-electrophoresis marker, and the mixture was treated with SDS and mercaptoethanol and subjected to electrophoresis as described in text.

peak comigrating with proteins 2 and 3 of ^{14}C -marker virus. This pellet fraction contained 40% of the radioactivity in the unfractionated virus, and consisted mainly of protein 3, the nucleocapsid protein, and protein 4. The nature of the association of these two proteins which are found in the pellet remains to be determined. That the pellet fraction did not contain a significant proportion of the smaller glycoprotein, protein 2, was shown by the lack of staining of the gel with Schiff's stain, and by the recovery of protein 2 in the supernatant fraction as will be described below.

The supernatant from the $10,000 \times g$ centrifugation was centrifuged at $200,000 \times g$ for 1 hr to remove any remaining nucleocapsid or incompletely disrupted virus. The pellet obtained contained only 16% of the total radioactivity of the initial virus preparation. Polyacrylamide gel electrophoresis of this material revealed only small amounts of each of the viral proteins (not shown). This pellet was discarded. The supernatant fluid from the $200,000 \times g$ centrifugation was dialyzed against 0.01 M phosphate buffer in order to remove the potassium chloride. A precipitate formed during dialysis, and this was collected by centrifugation at $10,000 \times g$ for 20 min. As shown in Fig. 3, this precipitate was found to consist of

FIG. 3. Polyacrylamide gel electrophoresis of NDV protein which was solubilized with Triton and 1 M KCl and then rendered insoluble by removal of the salt. A pure preparation of protein 6 was obtained. The $200,000 \times g$ supernatant fluid after treatment of virus with 2% Triton X-100 and 1 M KCl was dialyzed against 0.01 M phosphate buffer for 16 hr. During dialysis a visible precipitate formed which was collected at $10,000 \times g$ for 20 min. This pellet was suspended in 2.0 ml of 0.01 M phosphate buffer by sonic treatment, and a 100- μ l sample was processed for polyacrylamide gel electrophoresis as described in the legend to Fig. 2.

the smallest virus protein, protein 6, and was not contaminated by any of the other virus proteins. Thus, this procedure can be used to obtain a pure preparation of this envelope protein, which is insoluble in solutions of low ionic strength.

The proteins which remained in the supernatant fluid after removal of the potassium chloride by dialysis were also analyzed by polyacrylamide gel electrophoresis as shown in Fig. 4. The predominant species is protein 1, the larger glycoprotein. A smaller peak which comigrated with the protein 2 + 3 peak of the ^{14}C -labeled marker virus is also present. Since, as described above, the nucleocapsid was pelleted in the initial $10,000 \times g$ centrifugation after the Triton treatment, this second peak consists largely of the smaller virus glycoprotein, protein 2. This conclusion was also supported by the staining of this second band in a polyacrylamide gel such as that shown in Fig. 4 with Schiff's stain. Figure 4 also shows that, in addition to glycoproteins 1 and 2, there is a small amount of protein 6 in this supernatant fraction.

The biological activities of the virus components isolated by this procedure and the amount of radioactivity recovered at each step are shown in Table 1. As expected, the fraction which consisted mainly of the nucleocapsid (Table 1, line 2, and Fig. 2) had no

FIG. 4. Polyacrylamide gel electrophoresis of NDV proteins which remained soluble in 2% Triton X-100 in the absence of KCl. These proteins were predominantly virus proteins 1 and 2. A sample of the supernatant fluid after the sedimentation of insoluble protein as described in the legend to Fig. 3 was prepared for gel electrophoresis as described in the legend to Fig. 2.

hemagglutinating activity and had an insignificant amount of neuraminidase activity. The fraction of the $200,000 \times g$ supernatant fluid which precipitated after the KCl was removed by dialysis, and which contained virus protein 6 (Table 1, line 3, and Fig. 3), had neither activity. The fraction that contained the two virion glycoproteins (Table 1, line 4, and Fig. 4) contained the bulk of the hemagglutinating and neuraminidase activities. The neuraminidase activity recovered with this fraction was even higher than that of the undisrupted virus. An increase of the neuraminidase activity after disruption of virions with Triton X-100 has also been found with SV5 (18).

In other experiments, purified NDV was treated with Triton X-100 in the absence of 1 M KCl as described in Materials and Methods. A supernatant fluid was obtained which contained only the two glycoproteins; the polyacrylamide gel pattern of this fraction was identical to that shown in Fig. 4. The non-glycosylated membrane protein, protein 6, was not present in this Triton supernatant fraction, because, as described above, it is insoluble in solutions of low ionic strength, as is the non-glycosylated membrane protein of SV5 (18). This method of isolation of NDV glycoproteins without KCl was not adopted because a significant amount of the glycoproteins (30–50%) was lost in the first high-speed centrifugation. Thus, Triton treatment in the presence of 1 M KCl, as described above, was selected as the method of choice for isolating NDV glycoproteins.

Separation of the two NDV glycoproteins. To investigate the biological activities of each of the two glycoproteins, it was first necessary to separate them from each other. This was achieved by rate zonal centrifugation in the presence of Triton X-100 and 1 M KCl.

The fraction containing the two soluble glycoproteins (see Fig. 4) was layered onto a continuous gradient of 10 to 25% sucrose which contained 1% Triton, 1 M KCl, and 0.01 M phosphate buffer throughout, and centrifuged for 15 hr at $297,000 \times g$, as described in the legend to Fig. 5. Two nearly symmetrical peaks and a broad area of radioactivity at the top of the gradient were obtained (Fig. 5). The *s* values for the peaks, calculated from co-sedimentation with ^{125}I -labeled gamma G-globulin as a marker by the method of McEwen (14), were 9.3 for peak I and 6.1 for peak II. By

FIG. 5. Separation of the NDV glycoproteins on a sucrose gradient. A 0.2-ml sample of the supernatant fraction containing the two glycoproteins in 2% Triton X-100 (Fig. 4) was layered onto a linear preformed gradient of 10 to 25% sucrose in 0.01 M phosphate buffer with 1% Triton X-100 (v/v) and 1 M KCl, and centrifuged at $297,000 \times g$ (55,000 rev/min in an SW56 rotor) for 15 hr at 5 C. Samples (10 μ liters) of each fraction were assayed for radioactivity.

TABLE 1. Neuraminidase and hemagglutinating activities of NDV proteins obtained by treatment with Triton X-100 in 1 M KCl followed by differential centrifugation and precipitation

Proteins present in virus fraction	Radioactivity (counts/min $\times 10^3$)	Neuraminidase (units)	Hemagglutinin (HAU $\times 10^3$) ^a
Intact virions (Fig. 1) ^b	2,455	64.2	102.4
Proteins 3, the nucleocapsid protein, and 4 (Fig. 2)	994	1.9	<1.6 ^c
Protein 6, the non-glycosylated envelope pro- tein (Fig. 3)	314	<0.1	<1.6
Proteins 1 and 2, the glycoproteins (Fig. 4)	875	95.4	51.2

^a HAU, hemagglutination units.

^b Numbers indicate figure in which polyacrylamide gel analyses of the fractions are shown. The details of the procedure for obtaining these fractions are described in detail in the text and legends to the figures.

^c Hemagglutination on this and following samples were done after removal of Triton X-100 as described in Materials and Methods.

the same method, a value of 6.9 was obtained for the 7s gamma G-globulin marker.

The proteins in peak I and peak II from the gradient shown in Fig. 5 were analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 6, peak I consisted of a pure preparation of the larger glycoprotein, protein 1, which migrated as a single very sharp peak in the gel. Peak II (Fig. 7) contained the smaller glycoprotein, protein 2, as the predominant polypeptide, and also small amounts of proteins 1 and 6. The upper portion of the gradient (region III) consisted largely of material which, when subjected to polyacrylamide gel electrophoresis, was spread over the gel appearing as a rather high background with no apparent peaks.

Table 2 shows the hemagglutinating and neuraminidase activities recovered in gradient peaks I, II, and III. The table also shows the amount of each of the two glycoproteins, expressed as radioactivity, in the original supernatant fluid and in the peaks from the gradient. Most of the neuraminidase activity applied to the gradient was recovered in peak I, which contained electrophoretically pure protein 1, the larger glycoprotein. The same fraction also

FIG. 6. Polyacrylamide gel electrophoresis of the NDV glycoprotein in peak I of a sucrose gradient containing 1% Triton X-100 and 1 M KCl. Fractions comprising peak I in the gradient shown in Fig. 5 and in an identical companion gradient were pooled and dialyzed overnight against 0.01 M phosphate buffer containing 1% Triton X-100. The volume was then reduced to 0.75 ml by dialysis for 3 hr against the same solution with 20% polyethylene glycol added. A 100- μ liter amount was processed for polyacrylamide gel analysis as described in legend to Fig. 2.

FIG. 7. Polyacrylamide gel electrophoresis of the NDV glycoprotein in peak II of a sucrose gradient containing 1% Triton X-100 and 1 M KCl. Fractions comprising peak II from the gradient shown in Fig. 5 were processed as described in the legend to Fig. 6. A 100- μ liter amount of a final volume of 0.95 ml was subjected to electrophoresis.

TABLE 2. Neuraminidase and hemagglutinating activities of the NDV glycoproteins separated by velocity sedimentation in a sucrose gradient containing Triton X-100 and 1 M KCl^a

Fraction	Protein content		Activity	
	Protein 1 (counts/ min $\times$ 10^3)	Protein 2 (counts/ min $\times$ 10^3)	Neura- minidase (units)	Hemag- glutinin (HAU)
Sample ap- plied to gra- dient	49.9	44.9	19.1	1024
Peak I	33.5	0.4	15.0	1920
Peak II	2.5	29.2	0.23	<38
Peak III	1.8	1.7	<0.1	<25

^a The sample applied to the gradient was a portion of the fraction analyzed in Fig. 4. The gradient peaks were those analyzed in Fig. 6 and 7. The radioactivity in each of the glycoproteins was calculated from the total present in the various fractions and from the electropherograms. Portions of the three dialyzed gradient fractions were assayed directly for neuraminidase and for hemagglutinating activity after butanol-ether precipitation as described in Materials and Methods.

contained all demonstrable hemagglutinating activity. The recovery of protein 1 closely followed the recovery of the neuraminidase. In this experiment, peak I contained 67% of total amount of protein 1 and 78% of total of

the neuraminidase applied to the gradient. In two other experiments, similar recoveries were obtained. Peak II, which contained most of the smaller glycoprotein, protein 2, contained only a very small amount of neuraminidase activity and no demonstrable hemagglutinating activity. No activity could be detected in region III, the top part of the gradient. These results indicate that both hemagglutinating and neuraminidase activities of NDV are associated with the larger glycoprotein of the virion, and neither is associated with the smaller.

DISCUSSION

A procedure has been developed which permits the separation of the glycoproteins of NDV from the non-glycosylated virion proteins and from each other with full recovery of neuraminidase and hemagglutinating activities. The non-glycosylated membrane protein can also be obtained in pure form by this procedure. The method is based on the following physical characteristics of the virion proteins which have been determined in the present studies. (i) In the presence of Triton X-100 and high ionic strength, i.e., 1 M KCl, the two glycoproteins and protein 6, a non-glycosylated membrane protein, are soluble whereas the other virion proteins are insoluble. (ii) Protein 6 is insoluble at low ionic strength, and it therefore can be selectively precipitated from a solution of this protein and the glycoproteins in Triton and 1 M KCl by dialyzing away the salt. (iii) In the presence of Triton, the sedimentation coefficients of the two glycoproteins are sufficiently different to allow separation by rate zonal centrifugation. Similar properties of virion proteins were found previously with another paramyxovirus, SV5 (18). The sedimentation coefficients of the larger and smaller NDV glycoprotein were 9.3s and 6.1s, and those of SV5 were 8.9s and 6.7s, respectively. A minor difference between the two viruses was that with NDV complete recovery of the two glycoproteins could only be obtained if Triton treatment was done in the presence of 1 M KCl, whereas with SV5 this could be accomplished with Triton X-100 alone, although high salt concentration did facilitate the subsequent separation of the two glycoproteins with SV5 also.

Once the two NDV glycoproteins were separated, it was found that both neuraminidase and hemagglutinating activities were associated with the larger glycoprotein, virus protein 1. Neither activity was found

associated with the smaller glycoprotein or with any of the non-glycosylated virus proteins. The present assignment of the hemagglutinating activity of NDV to virus protein 1 is in agreement with previous results. Using fractions of deoxycholate (DOC)-disrupted NDV, Haslam et al. (10) ascribed hemagglutinating activity to their viral protein 1, which was probably identical with the larger NDV glycoprotein described in the present and previous (17) studies from this laboratory. A protein of similar size was also found by Evans and Kingsbury (9) to be the major component of an NDV fraction which had been released by ether treatment and which adsorbed to and eluted from erythrocytes. A similar protein was found to adsorb to erythrocytes by Iinuma et al. (11) using DOC-disrupted NDV. Thus all the available evidence indicates that NDV protein 1, the larger glycoprotein, possesses hemagglutinating activity.

On the other hand, in contrast to the present results, others have reported that the neuraminidase activity of NDV could be separated from hemagglutinating activity by rate zonal centrifugation of DOC-disrupted virus (10, 11) or by exclusion chromatography of Triton-disrupted virus (3). However, these studies did not provide conclusive evidence for separation. In the studies of the DOC-disrupted virus (10, 11), 60 to 80% of the neuraminidase activity was inactivated by the treatment used, and the preparations with neuraminidase activity also contained the protein to which hemagglutinating activity was attributed. Under these circumstances, a definite conclusion as to the identity of the protein with neuraminidase activity cannot be made. Indeed, the protein assumed to be the neuraminidase in these studies was the smallest virion protein, which has been shown in this laboratory to be non-glycosylated (17). The available evidence suggests that in paramyxoviruses this protein is not associated with the surface projections, but rather is a membrane protein which is covered by the lipid layer of the envelope (5, 8, 17). It was found in the present studies that neither this non-glycosylated protein nor the smaller NDV glycoprotein had neuraminidase activity; all of the activity was found associated with the larger glycoprotein.

In the studies of Brostrom et al. (3), in which separation of neuraminidase and hemagglutinin was attempted by exclusion chromatography of Triton-disrupted virus, the protein composition of the virus fractions was not analyzed, and there was significant overlap

between the peaks of the two activities obtained in the column effluent. Therefore, the possibility of aggregates of different sizes producing apparent partial separation of activities could not be excluded. Aggregation is a serious problem in the isolation of pure virion proteins, and pure preparations of individual proteins must be obtained before an evaluation of their activities can be made. When this was done in the present studies with NDV, and in previous studies with SV5 (18), both activities were clearly associated with the larger virion glycoprotein. The finding with two different paramyxoviruses that hemagglutinating and neuraminidase activities are associated with one glycoprotein suggests that this is a general property of this group of viruses.

The conclusion that both hemagglutinating and neuraminidase activities reside on a single polypeptide is based on the finding that the viral component with both activities consistently migrates as a single sharp peak on a polyacrylamide gel. This does not exclude completely the possibility that the component identified as the larger glycoprotein consists of two different polypeptides with identical electrophoretic mobilities. However, the improbability of this explanation is increased by the fact that, with SV5 also (18), both activities are associated with the larger viral glycoprotein which behaves as a single entity in all procedures yet attempted, including polyacrylamide gel electrophoresis in a discontinuous buffer system. It seems highly unlikely that, in each of two different paramyxoviruses, neuraminidase and hemagglutinating activities would reside on two different glycoproteins, which fortuitously have identical electrophoretic mobilities, particularly since the NDV and SV5 glycoproteins are not the same size and do not comigrate when they are subjected to co-electrophoresis. The final proof that the larger glycoproteins of NDV and SV5 consist of single polypeptides will come from amino acid analyses and peptide mapping, and it now should be possible to obtain sufficient quantities for these studies.

One of the interesting questions that remains is the biological function of the smaller glycoprotein of paramyxoviruses, which has neither hemagglutinating nor neuraminidase activity. It could play a purely structural role in the virus spikes, or it might be involved in the hemolysis or cell fusion induced by these viruses. The immunological specificities of the two glycoproteins also remain to be established. Such studies are now in progress.

Using a simple and gentle procedure involving the nonionic detergent Triton X-100, we have thus far succeeded in isolating the surface glycoproteins in biologically active form from NDV (as described in this report), SV5 (18), influenza virus (Scheid, Caligiuri, and Choppin, *Abstr. Amer. Soc. Microbiol.*, 1972, p. 218), and vesicular stomatitis virus (McSharry, Compans, Lackland, and Choppin, *Abstr. Amer. Soc. Microbiol.*, 1972, p. 215). In each case, the glycoproteins were obtained quantitatively by a simple, one-step solubilization procedure, although with those viruses which contain more than one glycoprotein, further steps are necessary for their separation from each other. This type of simple procedure should be of value in the isolation of glycoproteins from a wide variety of enveloped viruses, not only for studies of the chemical, physical, and biological properties of such proteins, but also for vaccine production, since with many such viruses the important antigens from the standpoint of immunity to infection are the surface glycoproteins.

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