JOURNAL OF VIROLOGY, Jan. 1980, p. 152-166
0022-538X/80/01-0152/15$02.00/0

Vol. 33, No. 1

Protein-Protein Interactions Within Paramyxoviruses Identified by Native Disulfide Bonding or Reversible Chemical Cross-Linking

MARY ANN K. MARKWELL* AND C. FRED FOX

Department of Microbiology and the Parvin Cancer Research Laboratories, Molecular Biology Institute, University of California, Los Angeles, California 90024

Analysis of native disulfide-bonded protein oligomers in paramyxoviruses showed that some viral proteins are consistently present as covalent complexes. In isolated Sendai virus the hemagglutinating protein HN is present in homodimeric and homotetrameric forms, and the minor nucleocapsid protein P exists partly as a monomer and partly as a disulfide-linked homotrimer. Similar disulfide-linked complexes were observed in Newcastle disease virus (strain HP-16), in which HN exists as a homodimer and some of the major nucleocapsid protein NP exists as a homotrimer. Noncovalent intermolecular interactions between proteins were studied with the reversible chemical cross-linkers dimethyl-3,3'-dithiobispropionimidate and methyl 3-[(*p*-azidophenyl)dithio]propionimidate, which contain disulfide bridges and a 1.1-nm separation between their functional groups. The same results were achieved with both reagents. The conditions of preparation, isolation, and storage of the viruses affected the protein-protein interactions observed upon cross-linking. Homooligomers of the glycoprotein F, the matrix protein M, and the major nucleocapsid protein NP were produced in both Sendai and Newcastle disease viruses after mild cross-linking of all viral preparations examined, but NP-M heterodimer formation in both viruses was most prevalent in early harvest preparations that were cross-linked soon after isolation. The ability of NP and M to form a heterodimer upon cross-linking indicates that the matrix protein layer lies in close proximity (within 1.1 nm) to the nucleocapsid in the newly formed virion. Some noncovalent intermolecular protein interactions in Sendai and Newcastle disease viruses, i.e., those leading to the formation of F, NP, and M homooligomers upon cross-linking, are more stable to virus storage than others, i.e., those leading to the formation of an NP-M heterodimer upon cross-linking. The storage-induced loss of the ability of NP and M to form a heterodimer is not accompanied by any apparent loss of infectivity. This indicates that some spacial relationships which form during virus assembly can alter after particle formation and are not essential for the ensuing stages of the infectious process.

Sendai virus and Newcastle disease virus (NDV), members of the family Paramyxoviridae, are composed of an outer lipoprotein envelope and an internal helical nucleocapsid. The envelope which consists of a lipid bilayer, external glycoprotein spikes, and an inner layer of carbohydrate-free M protein is acquired by the viruses in the process of budding from the host cell surface (29). At present, little is known about the spatial relationships of the viral proteins in the mature virus particle and the roles that these relationships may play in viral assembly and in the ensuing stages of the infectious process. Evidence available from selective extraction (34) and phenotypic mixing experiments (20) suggests that M is involved in transmembrane recognitions between the spike and nucleocapsid proteins. Direct interactions of M with the glycoproteins or nucleocapsid proteins have not been demonstrated in paramyxoviruses, but have been demonstrated in vesicular stomatitis virus with reversible cross-linking agents (7).

Our studies were designed to test the hypothesis that M exists in close proximity to the interior nucleocapsid proteins and the exterior spike proteins in paramyxoviruses. A secondary objective was to probe for information on the spatial relationships of the individual proteins within the viral membrane and nucleocapsid of the mature virus particle by analyzing the protein complexes present as a result of native disulfide bonding or formed by chemical cross-linking.

The two cross-linking agents chosen for this

study both contain disulfide bridges and a 1.1-nm separation between their functional groups. The homobifunctional reagent dimethyl-3,3'-dithiobispropionimidate (DTBP) is a water-soluble imidoester with a half-life in the minute range at slightly alkaline pH (26). The heterobifunctional reagent methyl 3-[(*p*-azidophenyl)dithio]propionimidate (PAPDIP) contains an imidoester group and a lipophilic aryl azide portion. The imidoester group of DTBP or PAPDIP reacts with the primary amine of a protein (Fig. 1, reaction 1). With DTBP (Fig. 1) the second step (Fig. 1, reaction 2) is the same type of site-specific reaction as the first.

With PAPDIP (Fig. 2), reaction of the imidoester group with protein (Fig. 2, reaction 1) is done in the dark to prevent simultaneous activation of the second group. The second group of PAPDIP, an aryl azide, is activated by photolysis to the nitrene (Fig. 2, reaction 2a). Nitrenes do not require a specific reactive group. They can insert at N—H and at C—H bonds in proteins (Fig. 2, reaction 2b) and have half-lives in the millisecond range (26). Protein cross-links formed with either DTBP or PAPDIP can be reversed by reductive cleavage of their disulfide bridges (Fig. 2, reaction 3).

(Parts of this work have been described briefly before [16; M. A. K. Markwell, Fed. Proc. **37**: 1773, 1978].)

MATERIALS AND METHODS

Virus. Sendai (also known as hemagglutinating virus of Japan, HVJ) and NDV (strain HP-16) viruses were propagated in 11-day-old embryonated chicken eggs incubated at 38°C. Allantoic fluid was harvested at 22 h (early harvest) or 48 h (late harvest) postinfection. Virus was purified under sterile conditions by a modification of the procedure of Samson and Fox (30); this modification substantially shortened the time required for purification. Centrifuge tubes and bottles were sterilized by overnight exposure to ethylene oxide (Anprolene, H. W. Andersen Products, Inc.). Linear gradients were formed overnight by diffusion of sequential layers (increments of 5%) of sterile solutions of sucrose or Renografin. All procedures were performed at 0 to 4°C unless otherwise specified. Clarified (50,000 g·min) allantoic fluid was layered on top of a two-step gradient consisting of 20% (wt/vol) and 65% sucrose in 0.01 M Tris·hydrochloride–0.1 M NaCl–0.001 M disodium EDTA at pH 7.4 (TSE). The gradient was centrifuged for 30 min at 19,000 rpm in a Sorvall SV-288 vertical rotor. Virus concentrated at the 20/65% sucrose interface was diluted with TSE, and banded sequentially in linear 40 to 65% sucrose and 6 to 25% iodine equivalent Renografin gradients by centrifugation for 1 h at 19,000 rpm in the vertical rotor. The virus was pelleted by centrifugation for 90 min at 75,000 × *g* in a fixed-angle rotor, and the pellet was suspended in TSE at a protein concentration of 5 mg/ml and used within 3 days or stored frozen at −70°C. The dye-binding assay for proteins (2) underestimated viral proteins by two- to threefold in purified viral samples. Protein was therefore routinely measured by a modification of the Lowry procedure for membrane and lipoprotein samples (18).

Cells. Monolayer cultures of baby hamster kidney (BHK-21) cells were grown in a medium consisting of 10% tryptose phosphate broth, 10% fetal calf serum,

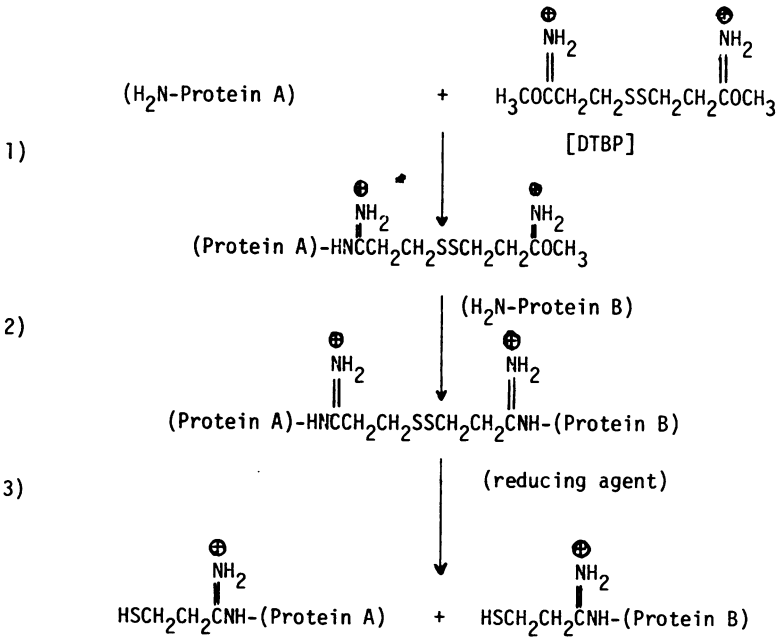

FIG. 1. *Cross-linking of proteins with DTBP.*

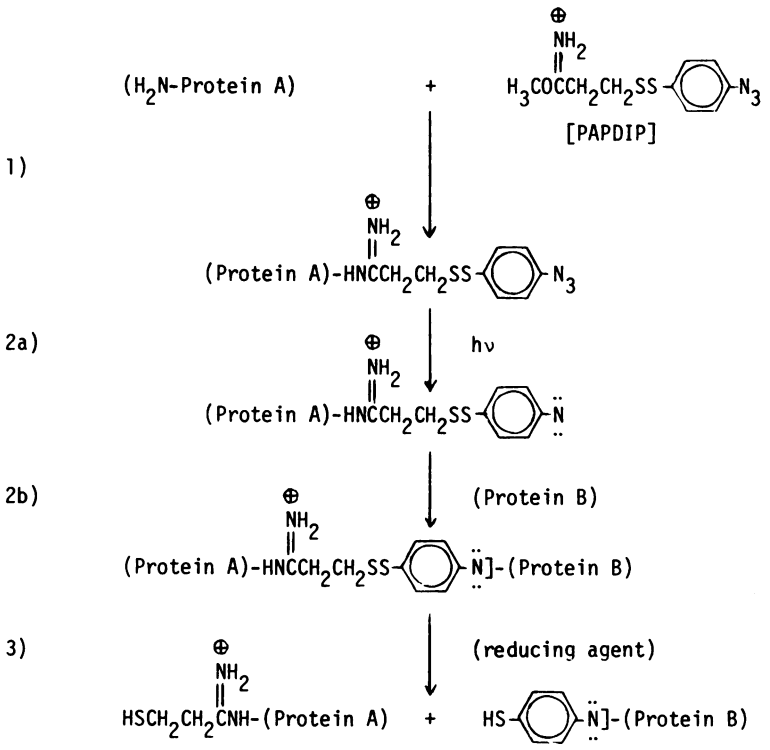

FIG. 2. *Cross-linking of proteins with PAPDIP.*

and 80% minimum essential medium from (GIBCO Laboratories, Grand Island, N.Y.). The cells were used for virus adsorption studies within 24 h of attaining confluency.

Iodination and cross-linking conditions. The externally disposed proteins of the virus envelope were iodinated with 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (chloroglycoluril, available as Iodo-gen from Pierce Chemical Co.) under conditions optimized for surface-specific labeling (17). Portions of intact virus (100 μg of protein) were reacted for 10 min at 0°C with 0.5 mCi of $Na^{125}I$ (carrier free, Amersham Corp.) in the presence of 10 μg of chloroglycoluril. The iodinated virus preparation was transferred from the reaction vessel to a test tube containing 25 μmol of carrier NaI in TSE and used immediately for cross-linking studies.

Interior as well as externally displayed proteins of the virus were iodinated by a nonvectorial chloroglycoluril method which includes detergent to disrupt the virus (17). Control and cross-linked samples were solubilized in 2% sodium dodecyl sulfate (SDS) by immersion in boiling water for 2 min and then reacted with $Na^{125}I$ in the presence of chloroglycoluril as described above. The samples were used for gel electrophoresis after the addition of 25 μmol of carrier NaI.

Paramyxoviruses were cross-linked with DTBP (Pierce Chemical Co.) by the procedure described previously for NDV (21) except that the native disulfide bonds of the virus were not reduced before cross-linking and the cross-linking reaction occurred at 0°C. The viral suspension (100 μg of protein in 25 μl of 0.2 M triethanolamine hydrochloride at pH 8.5) was made 3 mM in DTBP and incubated for 30 min at 0°C. The reaction was terminated by the addition of ammonium acetate and freshly prepared *N*-ethylmaleimide, both at a final concentration of 50 mM, and incubated for 30 min at room temperature. Samples were then made 2% in SDS and immersed for 2 min in boiling water in preparation for gel electrophoresis or nonvectorial iodination.

The same initial incubation condition was used with the heterobifunctional cross-linking reagent PAPDIP (5), but in the dark to prevent photolysis of the aryl azide group. After the 30-min incubation in ammonium acetate and *N*-ethylmaleimide, the virus was sedimented for 90 min at 75,000 × *g* through 20% sucrose onto a 65% sucrose shelf, and the PAPDIP-derivatized virus that concentrated at the interface was divided into two samples. In the first sample, the second reactive group of PAPDIP was activated by exposure to a UV lamp (Blak-ray UVL-21, peak of 366 nm, Ultra-violet Products, Inc.) for 3 min at a distance of 5 cm from the sample. The rest of the PAPDIP-derivatized virus was diluted with minimum essential medium to a protein concentration of 0.5 mg/ml and allowed to adsorb to BHK confluent monolayers for 30 min at 4°C. The monolayers were washed three times with ice-cold minimum essential medium to remove unattached virus and then exposed to UV light for 3 min.

Fractionation of BHK cells and cross-linked Sendai virus. The cellular preparation was enriched for virus cross-linked to plasma membrane by a modification of the zinc ion method used to isolate the

surface membranes of L cells (38). BHK cells were scraped with a rubber policeman from three 10-cm dishes into 15 ml of 0.025 M Tris·hydrochloride–0.14 M NaCl at pH 7.4 and centrifuged at 500 × *g* for 15 min. The pellet was gently suspended in 3 ml of 50 mM Tris·hydrochloride at pH 7.4 containing 1 mg of soybean trypsin inhibitor per ml. The cells were allowed to swell for 15 min at room temperature. An equal volume of 1 mM $ZnCl_2$ was added, and the suspension was incubated for an additional 10 min. The suspension was cooled to 4°C and homogenized with 50 to 60 strokes of a type B, tight Dounce pestle. The homogenate was layered on top of a two-step (20 and 65% sucrose in TSE) gradient and centrifuged for 30 min at 19,000 rpm in a Sorvall SV-288 vertical rotor. The membrane fraction which concentrated at the 20/65% sucrose interface was collected, diluted 10-fold with TSE, and centrifuged at 6,000 × *g* for 15 min. The resulting pellet was solubilized in 0.5 M Tris·hydrochloride–2% SDS at pH 6.8 for gel electrophoresis.

Paramyxovirus preparations cross-linked with either DTBP or PAPDIP were subdivided into envelope and nucleocapsid fractions by the selective extraction procedures of Scheid and Choppin (31) and Raghow and Kingsbury (28) but substituting Nonidet P-40 (NP-40) (Shell Oil Co.) for Triton X-100. Suspensions of the cross-linked virus (2.5 mg of protein in 2.5 ml) were dialyzed against 1 M NaCl in 0.01 M sodium phosphate buffer at pH 7.2. NP-40 was added to a final concentration of 2% (vol/vol). The virus preparations were incubated for 2 h at room temperature (21 to 22°C), then iodinated for 10 additional min at room temperature with 2.5 mCi of $Na^{125}I$ and 25 μg of chloroglycoluril. Carrier NaI (1.25 mmol) was added to the iodinated virus. The viral suspension was centrifuged for 30 min at 10,000 × *g* to separate the solubilized membrane proteins HN, F, and M from the insoluble nucleocapsid fraction. The pellet was suspended in 2 ml of 1 M NaCl in 0.01 M sodium phosphate buffer at pH 7.2 and extracted for 30 min at room temperature to selectively remove the minor nucleocapsid proteins (28). The suspension was centrifuged for 90 min at 75,000 × *g*. The resulting pellet, containing NP complexed with viral RNA, was solubilized in 0.5 M Tris·hydrochloride–2% SDS at pH 6.8 for gel electrophoresis.

Gel electrophoresis in SDS. Reagents for gel electrophoresis were purchased from Eastman Kodak Co. SDS (lauryl, sequanal grade) was obtained from Pierce Chemical Co. SDS, glycerol, and bromophenol blue were added to samples for electrophoresis to achieve final concentrations of 2, 10, and 0.008%, respectively, in a sample buffer of 0.0625 M Tris·hydrochloride, pH 6.8. Reduced samples contained 5% 2-mercaptoethanol. The molecular weight markers mollusc hemocyanin, erythrocyte spectrin bands 1 and 2, *Escherichia coli* β-galactosidase, phosphorylase a, bovine serum albumin, ovalbumin, soybean trypsin inhibitor, myoglobin, and lysozyme were used in constructing calibration curves for the 5 to 12.5% gradient gels. Hemocyanin was the gift of P. S. Linsley, University of California, Los Angeles. Spectrin bands 1 and 2 were isolated as referenced (24). β-Galactosidase, phosphorylase a, lysozyme, and trypsin inhibitor were purchased from Worthington Biochemical Corp., bovine serum albumin, and myoglobin were from Sigma Chemical Co., and ovalbumin was from Schwarz/Mann.

The procedure for two-dimensional gel electrophoresis is a modification of the one-dimensional Laemmli system (13) and has been previously described for use with erythrocyte ghosts (17). Samples were electrophoresed in the first dimension without mercaptan on a 1-mm-thick 5 to 12.5% (wt/wt) acrylamide linear gradient resolving gel. The first-dimensional slab gel was then sliced into its respective lanes, incubated for 15 min in reducing solution (0.0625 M Tris·hydrochloride at pH 6.8, 3% SDS, and 3% 2-mercaptoethanol or 5 mM dithioerythritol), and affixed with 1.5% agarose in reducing solution onto another 5 to 12.5% slab gel (1.25 mm thick) for the second dimension. To improve the resolution between the F_1 and NP proteins of NDV, a 7.5 to 10% gradient gel was used in the second dimension.

Gels were fixed, stained for protein, destained, and soaked in the aqueous solutions as described in the outline which follows. The gels were gently agitated on a shaker at room temperature for the stated times in tightly sealed containers. At least 500 ml of solution per gel was used for each step. (i) Fixing solution was composed of 25% isopropanol–10% acetic acid (60 min). (ii) Staining solution consisted of 0.1% Coomassie brilliant blue R-250, 25% isopropanol, and 10% acetic acid (30 to 60 min). (iii) Destaining solution was composed 5% methanol–7% acetic acid to which were added several white plastic foam plugs (diSPo plugs, Scientific Products) to adsorb the stain eluted during overnight destaining. (Gels can be soaked for at least 1 week without losing dye from the protein bands.) (iv) Soaking solution A was 10% methanol–2% glycerol (3 h). (v) Soaking solution B was 20% methanol–2% glycerol (1 h). The gels were then transferred to Whatman no. 1 chromatography paper and dried under house vacuum overnight at room temperature. Kodak single-coated SB-5 X-ray film was used for autoradiography. To determine the percent distribution of radiolabel incorporated by individual viral protein species, Coomassie brilliant blue-stained spots were cut from the dried gel and counted in a Packard gamma scintillation spectrometer.

RESULTS

Resolution of Sendai viral proteins. The major polypeptide species present in Sendai virus and their location within the virion are presented in Table 1. Identification of each species is based on approximate molecular weight, accessibility to surface-specific labeling, and fractionation by selective extraction procedures. F_2 has been shown to exist in disulfide linkage to F_1 to form the active F protein (33). The glycoproteins HN and F are the only viral components readily accessible to surface-specific iodination (15, 17). During sequential selective extraction procedures the membrane proteins HN, F, and M (fraction 1) and minor nucleocapsid proteins L and P (fraction 2) are stripped from the virus,

TABLE 1. *Proteins of Sendai virus*

Polypeptide[a]	Mol wt ($\times 10^{-3}$)[b]	Position in virion	Accessibility to surface-specific labeling[c]	Selective extraction fraction[d]
L	215	Nucleocapsid	−	2
P	78	Nucleocapsid	−	2
HN	70	Membrane exterior	+	1
NP	60	Nucleocapsid	−	3
F_1	47	Membrane exterior	+	1
M	34	Membrane matrix	−	1
F_2	15	Membrane exterior	+	1

[a] Nomenclature for paramyxovirus proteins described by Scheid and Choppin (32).

[b] Apparent molecular weights of Sendai viral proteins were calculated from molecular weight standards (see Fig. 3) coelectrophoresed with the viral proteins on 5 to 12.5% gradient slab gels.

[c] A portion of intact virus (100 μg of viral protein) was reacted for 10 min at 0°C with 0.5 mCi of $Na^{125}I$ in the presence of 10 μg of chloroglycoluril as described in the text.

[d] Sendai viral preparations were subjected to sequential selective extraction steps to fractionate the virus into its components as described in the text. Fraction I, supernatant fraction of the 2% NP-40–1 M NaCl in 0.01 M sodium phosphate extraction step; fraction 2, supernatant fraction of the 1 M NaCl in 0.01 M sodium phosphate extraction step; fraction 3, pellet of the 1 M NaCl in 0.01 M sodium phosphate extraction step.

leaving NP complexed to viral RNA (fraction 3).

Because of the greatly differing sizes of the polypeptides, a gel system was developed which could be used to approximate molecular weights of polypeptides and their complexes over a wide range of values. The Laemmli SDS gel system (13) was modified to include a 5 to 12.5% linear gradient resolving gel (17). Molecular weight standards from 14,000 to 290,000 were run with each slab gel to construct molecular weight calibration curves (Fig. 3).

By using the 5 to 12.5% gradient gel system, all the proteins of the reduced, SDS-solubilized virus were resolved (Fig. 4A). The high degree of integrity of the viral preparation is demonstrated by the fact that only the external membrane polypeptides HN, F_1, and F_2 are labeled by surface-specific iodination of the intact virus (Fig. 4B). This surface-specific technique proved useful in distinguishing between NP and F which have similar electrophoretic mobilities after SDS solubilization from the nonreduced virus (Fig. 4C and D).

Native disulfide bonding in Sendai virus. Reduction of Sendai virus destroys all its biological activities: infectivity, cell fusion, hemagglutination, neuraminidase, and hemolysis (23, 25, 33). Therefore, the polypeptides which participate in native disulfide bonding were examined. Ozawa et al. (25) had previously reported the presence of disulfide-linked high-molecular-weight oligomers of HN and P in egg-grown Sendai virus (Z strain), but were unable to accurately estimate their size. Under nonreducing conditions, three high-molecular-weight species in addition to L were observed on the 5 to 12.5% gels (Fig. 4C). Two of these (273,000 and 140,000 molecular weight) were accessible to surface-specific labeling; the third (238,000 molecular weight) was not (Fig. 4D).

Two-dimensional slab gel electrophoresis systems facilitated identification of these species. Polypeptide chains existing in native disulfide or cross-linked complexes will exhibit a greater molecular weight in a nonreducing first dimension than in a reducing second dimension. They therefore appear as spots below the diagonal formed by polypeptides which have equal molecular weights in both dimensions. The species migrating at 273,000 and 140,000 in the first (nonreducing) dimension migrated as a single polypeptide of 70,000 in the second (reducing) dimension (Fig. 5). The migration characteristics and the accessibility to surface-specific labeling (Fig. 4D) indicated that these species were tetrameric and dimeric forms of the membrane gly-

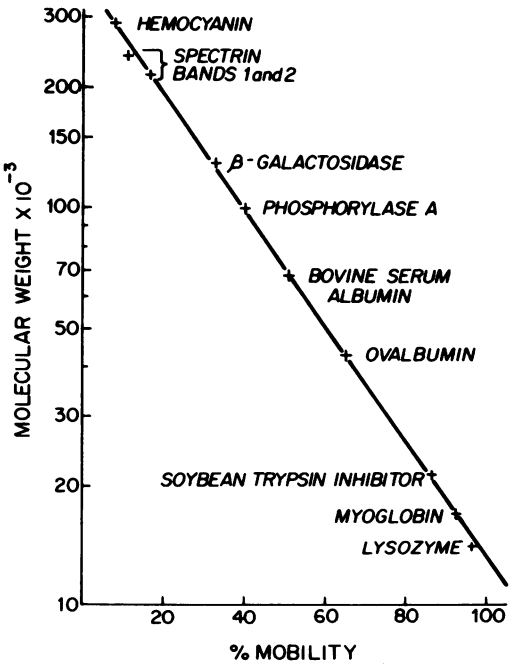

FIG. 3. *SDS-polyacrylamide gel electrophoresis of molecular weight standards. A standard consisting of mollusc hemocyanin (290,000), erythrocyte spectrin bands 1 and 2 (240,000 and 215,000), E. coli β-galactosidase (130,000), phosphorylase a (100,000), bovine serum albumin (68,000), ovalbumin (43,000), soybean trypsin inhibitor (21,500), myoglobin (17,200), and lysozyme (14,300) was electrophoresed on a 5 to 12.5% acrylamide linear gradient gel.*

coprotein HN. The homotrimeric form of HN was not observed. The incomplete reduction of the HN tetramer of Sendai virus to the dimeric form suggests that not all the disulfide linkages have equivalent sensitivity to reduction and that the tetramer may be formed from the linkage of dimers during viral assembly. When the first-dimensional gel was incubated in reducing solution (0.0625 M Tris·hydrochloride at pH 6.8, 3% SDS, and 3% 2-mercaptoethanol or 5 mM dithioerythritol) for 30 min instead of the usual 10 min, total reduction was achieved in the second dimension.

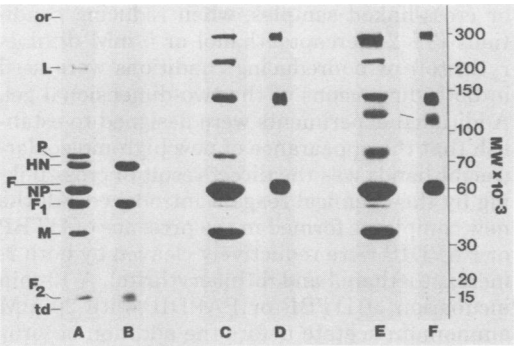

FIG. 4. *Comparison of reduced, nonreduced, and cross-linked proteins of Sendai virus by one-dimensional gel electrophoresis. A 100-μg sample of reduced egg-grown Sendai virus was electrophoresed on a 5 to 12.5% acrylamide linear gradient gel and stained with Coomassie brilliant blue (A). Samples of the same virus preparation without reducing agent (C) or after cross-linking with 3 mM DTBP (E) are shown. The autoradiographs of samples A, C, and E which had been surface-specifically labeled by the chloroglycoluril method for ^{125}I are respectively shown in B, D, and F. Letters designate the positions of the major Sendai viral proteins and their complexes, the origin (or) of the gel and the tracking dye (td) front.*

In the isolated nonreduced virion most of HN existed in disulfide-linked dimeric and tetrameric forms (54 and 38%, respectively, of the total radioactivity incorporated by HN); only 8% was detected in monomeric form. In the nine different batches of egg-grown Sendai virus examined, the percentage of HN existing as a dimer or tetramer varied by less than 5%. Formation of the disulfide bond by autooxidation during solubilization as reported for H-2 (9) can probably be excluded, since treatment of virus with freshly prepared 50 mM *N*-ethylmaleimide before solubilization did not alter the electrophoretic profile of the viral proteins.

The third high-molecular-weight disulfide-bonded species migrated at a molecular weight of 238,000 in the first dimension and comigrated with P monomer (78,000 molecular weight) in the second dimension. This and its lack of accessibility to surface-specific labeling indicated that it was a trimeric form of minor nucleocapsid protein P. Approximately equal amounts of P

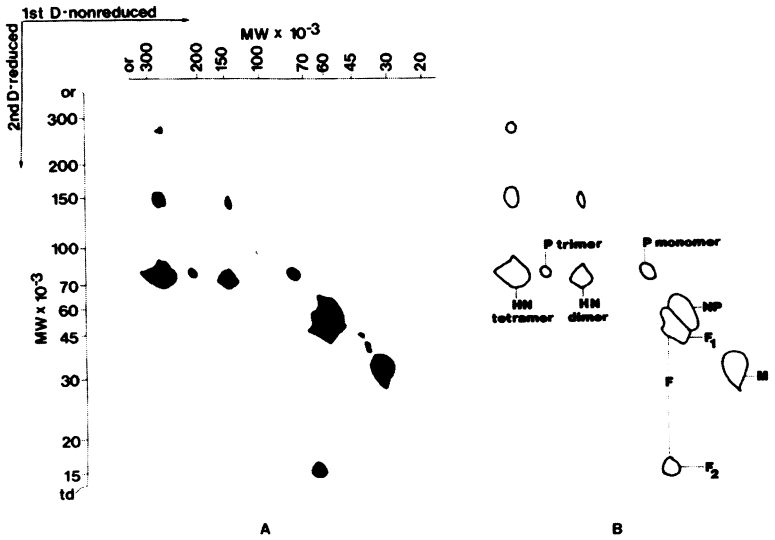

FIG. 5. *Two-dimensional electrophoretic analysis of proteins and their native disulfide complexes present in egg-grown Sendai virus. Approximately 50 μg of viral protein which had been iodinated in the presence of SDS were fractionated in the first dimension without reducing agent on a 5 to 12.5% polyacrylamide slab gel. The first-dimensional gel was sliced into its respective lanes, reduced with 2-mercaptoethanol, and affixed onto another 5 to 12.5% slab gel for the second dimension. The autoradiograph of the gel is shown on the left, and its corresponding schematic diagram is shown on the right. Refer to Table 2 for identification of proteins and their native disulfide complexes.*

existed in the monomeric and trimeric forms (49 and 51%, respectively), and this distribution varied by less than 5% among the different batches of Sendai virus examined. Disulfide-linked homodimers of P were not observed.

Reduction of the disulfide-linked F protein (60,000 molecular weight) to its component polypeptides F_1 (47,000 molecular weight) and F_2 (15,000 molecular weight) is clearly defined on the two-dimensional 5 to 12.5% gradient slab gels. Several minor proteins were sometimes seen on or below the diagonal. These differed in intensity from batch to batch of egg-grown virus and could be cellular contaminants or proteolysis products of the viral proteins. A summary of the proteins and their native disulfide complexes present in egg-grown Sendai virus is presented in Table 2.

Cross-linking of Sendai virus. Noncovalent protein-protein interactions were elucidated with reversible chemical cross-linkers. Intact, nonreduced preparations of Sendai virus were cross-linked as described in Materials and Methods with either DTBP or PAPDIP. Both reagents contain disulfide bridges and a 1.1-nm separation between functional groups. The individual components of cross-linked complexes formed with these reagents appeared as new spots below the diagonal on two-dimensional gels.

TABLE 2. *Proteins and their native disulfide complexes present in egg-grown Sendai virus*

Mol wt ($\times 10^{-3}$)[a]	Present in:		Accessible to surface-specific labeling[b]	Identity[c]
	Nonreduced gels	Reduced gels		
273	+	−	+	HN homotetramer
238	+	−	−	P homotrimer
215	+	+	−	L monomer
140	+	−	+	HN homodimer
78	+	+	−	P monomer
70	+	+	+	HN monomer
60	+	−	+	F monomer (F_1 + F_2 oligomer)
60	+	+	−	NP monomer
47	−	+	+	F_1 monomer
34	+	+	−	M monomer
15	−	+	+	F_2 monomer

[a] Molecular weights of Sendai viral proteins were calculated from molecular weight markers (see Fig. 3) coelectrophoresed on 5 to 12.5% slab gels in the first (nonreduced) and second (reduced) dimension.

[b] A 100-μg portion of intact virus was reacted for 10 min at 0°C with 0.5 mCi of $Na^{125}I$ in the presence of 10 μg of chloroglycoluril as described in the text.

[c] The individual components of protein complexes were identified by their molecular weight, accessibility to surface-specific iodination, and selective extraction procedures.

Experiments were performed to ensure that the off-diagonal spots arose from the reductive cleavage of complexes stabilized by native disulfide bonds or by disulfide-containing cross-links. No off-diagonal spots were observed with control or cross-linked samples, when reducing conditions (3% 2-mercaptoethanol or 5 mM dithioerythritol) or nonreducing conditions were used in both dimensions of the two-dimensional gel. Additional experiments were designed to establish that the appearance of new high-molecular-weight bands was the direct result of cross-linking by the chemical reagent introduced. All the new complexes formed in the presence of DTBP or PAPDIP were reductively cleaved by both 2-mercaptoethanol and dithioerythritol. A 10-min incubation of DTBP or PAPDIP with 20 mM ammonium acetate before the addition of virus prevented the appearance of new high-molecular-weight complexes. These complexes were also not formed when viral samples were irradiated for 3 min with UV light in the absence of PAPDIP. When PAPDIP-derivatized virus was solubilized in 2% SDS before photolysis, no cross-linking was observed. The requirement for photolysis eliminated the possibility of cross-linking by monofunctional imidoesters (3, 26, 36) or by DTBP produced from PAPDIP through an exchange reaction: 2PAPDIP $\rightleftharpoons$ DTBP + 4,4′-dithiobisphenylazide.

Disulfide reagents (R-SS-R) such as DTBP and PAPDIP are susceptible to SH-SS interchange (26). During incubation of such reagents with virus at pH 8.5 an exchange reaction (protein-SH + R-SS-R $\rightleftharpoons$ protein-SS-R + R-SH) can occur between the reagent disulfide and free sulfhydryl groups of viral proteins. The requirement for photolysis to obtain cross-linking with PAPDIP suggests that significant amounts of

$$\text{protein-SS-CH}_2\text{CH}_2\text{C}(\overset{\oplus}{\text{NH}_2})\text{CH}_3$$

had not been formed by mercaptan-disulfide interchange between protein and reagent. Also, incubation of viral proteins with 50 mM *N*-ethylmaleimide before cross-linking did not alter the cross-linking results. These controls indicate that none of the cross-linked products was formed by SH-SS interchange.

Chemical cross-linked homodimers of F and NP. DTBP treatment of Sendai virus preparations stored frozen for 3 to 12 months at −70°C before cross-linking produced the one-dimensional pattern seen in Fig. 4E. Under the mild cross-linking conditions used, little or no protein remained at the origin of the first-dimensional gel. The same results were obtained when cross-linking was performed at 0, 11, 22, or

37°C over a range of DTBP concentrations (0.5 to 10 mM) or when PAPDIP (0.1 to 0.5 mM) was used as the cross-linking reagent. A new band (120,000 molecular weight) appeared in the one-dimensional profile as the result of cross-linking. This complex was selectively labeled by surface-specific iodination, indicating the presence of HN or F (Fig. 4F).

Two-dimensional analysis indicated that the 120,000-molecular-weight band contains NP, F_1, and F_2 (Fig. 6A). From molecular weight considerations alone, the band could be explained by the formation of a heterodimer of the two 60,000-molecular-weight proteins NP and F or by their homodimers. The fact that the F_1 spot at molecular weight 47,000 contained more radioactivity than the NP spot at molecular weight 60,000 suggested the homodimer explanation. Also, a species just below the diagonal is visible, migrating at molecular weight 120,000 in the first

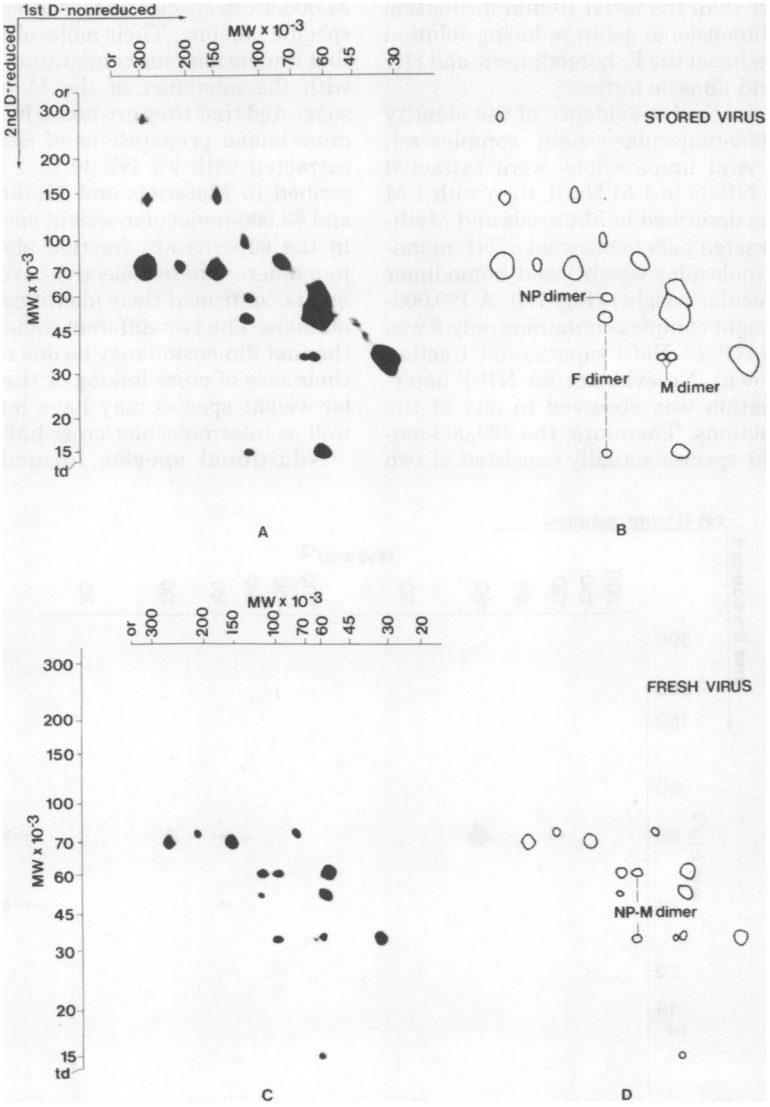

FIG. 6. *Reversible cross-linking of proteins in egg-grown Sendai virus by DTBP. Sendai virus (100 μg of protein) was treated with 3 mM DTBP for 30 min at 0 to 4°C. The same preparation of early harvest Sendai virus was used in Fig. 4A and 4C, but the stored virus of 4A was kept frozen 3 months at −70°C before cross-linking. The fresh virus of 4C was kept at 4°C and used for cross-linking experiments within 3 days of isolation. Cross-linked products were iodinated in the presence of SDS and analyzed by two-dimensional polyacrylamide electrophoresis as described in Fig. 5.*

dimension and molecular weight 97,000 in the second dimension. From its molecular weight and accessibility to surface-specific iodination (data not shown), it appeared to be a homodimer of F_1, which was not totally reduced to its monomer form. The presence of an F_1 homodimer supports the homodimer explanation of the 120,000-molecular-weight species. The presence of some HN in tetrameric and dimeric forms in the second dimension further indicates incomplete reduction of the first-dimensional gel. A 30-min rather than the usual 10-min incubation of the first-dimensional gel in reducing solution completely reduced the F_1 homodimeric and HN tetrameric and dimeric forms.

To obtain conclusive evidence of the identity of the 120,000-molecular-weight complex(es), cross-linked viral preparations were extracted first with 2% NP-40 in 1 M NaCl, then with 1 M NaCl alone as described in Materials and Methods. The extracted pellet consisted of NP monomer (60,000 molecular weight) and homodimer (120,000 molecular weight) (Fig. 7A). A 120,000-molecular-weight complex containing only F was found in the NP-40–NaCl supernatant fraction (data not shown). No evidence for NP-F heterodimer formation was observed in any of the extracted fractions. Therefore, the 120,000-molecular-weight species actually consisted of two different types of protein complexes: an NP homodimer and an F homodimer which comigrated.

Chemical cross-linked homodimers of M. Two additional products of cross-linking were detected as off-diagonal spots on two-dimensional gels. These migrated at molecular weights 67,000 and 63,000 in the first dimension and thus were obscured by the F and NP bands on one-dimensional gels. In the second reducing dimension, they both migrated at molecular weight 34,000. Both species were inaccessible to surface-specific labeling. Their molecular weights in the first dimension plus comigration upon reduction with the monomer of the M protein strongly suggested that they are homodimers of M. When cross-linked preparations of Sendai virus were extracted with 2% NP-40 in 1 M NaCl as described in Materials and Methods, the 67,000- and 63,000-molecular-weight species were found in the supernatant fraction along with the M monomer. These selective extraction experiments confirmed their identification as M homodimers. The two different molecular weights in the first dimension may be due to a difference in their axes of cross-linking or the 63,000-molecular-weight species may have intramolecular as well as intermolecular cross-links.

Additional species formed by chemical

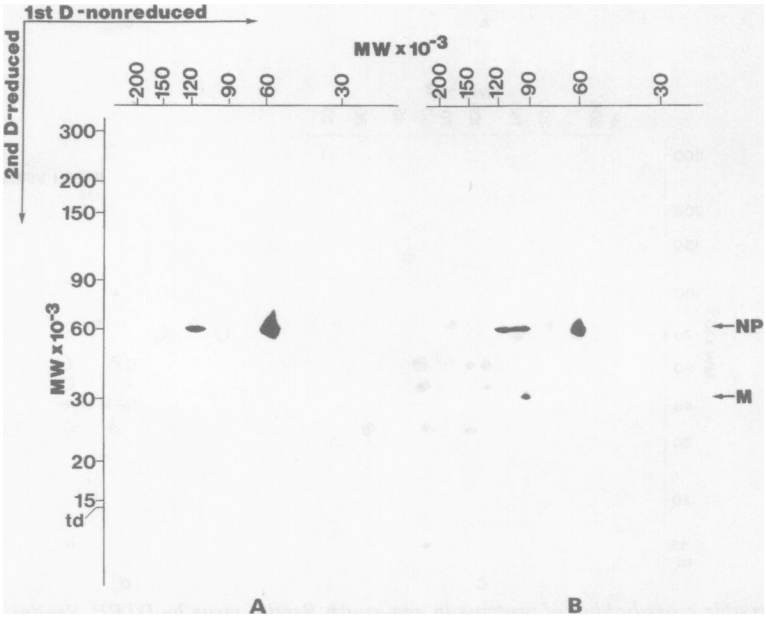

FIG. 7. *Selective extraction of cross-linked Sendai virus. The stored (A) and fresh (B) virus samples that had been cross-linked for Fig. 6A and C were extracted for 2 h at room temperature in 2% NP-40–1 M NaCl in 0.01 M sodium phosphate buffer at pH 7.2 and then iodinated in the extraction buffer. The insoluble nucleocapsid fraction was then further extracted with 1 M NaCl. The resulting pellet was analyzed by two-dimensional polyacrylamide gel electrophoresis as described in Fig. 5.*

cross-linking. Production of homodimers of NP, F, and M upon cross-linking was observed with six independent batches of late harvest Sendai virus and three independent batches of early harvest Sendai virus. No differences in cross-linking products were noted between early and late harvests after 3 to 12 months of storage at −70°C or in the cross-linked complexes produced by DTBP and PAPDIP. Evidence for the formation of small amounts of M trimeric (105,000 molecular weight), F tetrameric (245,000 molecular weight), NP trimeric (178,000 molecular weight) and tetrameric (250,000 molecular weight) forms was found when the gel which produced the autoradiograph shown in Fig. 6A was exposed for several weeks (data not shown).

Effect of storage on cross-linking: formation of an NP-M heterodimer upon cross-linking of freshly harvested preparations. When freshly harvested (not frozen, stored no more than 3 days at 4°C) Sendai virus preparations were subjected to cross-linking, an additional band was observed in the first dimension at molecular weight 97,000 (data not shown). This band was more predominant in the two early harvest preparations examined than in the two late harvest preparations grown in the same batches of eggs as the early ones but harvested 26 h later. Reduction of the 97,000-molecular-weight complex in the second dimension produced two spots which comigrated with NP (60,000 molecular weight) and M (34,000 molecular weight), indicating the possible formation of a NP-M heterodimer (Fig. 6C).

A selective extraction method was used to demonstrate conclusively that the 97,000-molecular-weight species was a heterodimer and not homooligomers of NP and M with similar electrophoretic mobilities. Cross-linked preparations were extracted with 2% NP-40 plus 1 M NaCl and then with 1 M NaCl as previously described in Materials and Methods. The insoluble pellet contained the NP monomer and homodimer plus the NP-M heterodimer (Fig. 7B). The monomer and homooligomers of M were not present in the pellet, having been previously extracted into the 2% NP-40–1 M NaCl supernatant fraction.

The ability of NP and M to form a heterodimer upon treatment with cross-linking reagents diminished upon freezing and prolonged storage of the sample. The same preparation of early harvest Sendai virus was used in Fig. 6A and C, but the sample in 6A was frozen and stored for 3 months at −70°C before cross-linking. In Fig. 6C the amount of NP-M heterodimer formed was about the same as the amount of NP homodimer formed. In Fig. 6A only NP homodimer formation was observed.

Cross-linking of Sendai virus was also performed after its adsorption to host cells. Both freshly harvested and stored Sendai virus preparations were derivatized in the dark with PAPDIP and then adsorbed to BHK monolayers as described in Materials and Methods. Unadsorbed virus and excess cross-linking reagent were removed before photolysis. Cross-linking of stored Sendai virus preparations adsorbed to BHK cells produced the same viral protein complexes as seen in Fig. 6A, i.e., homodimers of NP, F, and M. Cross-linking of the freshly harvested virus preparation adsorbed to host cells additionally produced the NP-M heterodimer as in Fig. 4C. These results indicate that PAPDIP-derivatized virus retained its ability to adsorb to host cells and that the cross-linked products obtained with isolated virus were also obtained with virus adsorbed to host cells. No new band formed to give an indication of cross-linking of HN or F to host membrane proteins.

Protein-protein interactions in NDV. Protein-protein interactions within NDV, another member of the paramyxoviruses, were investigated with the same approach used with Sendai virus. The major polypeptide species of NDV have the same nomenclature as those of Sendai virus but differ slightly in molecular weight (see Table 3). The most noticeable differences between the Coomassie patterns of the reduced viral proteins were the absence of P and greatly reduced amount of F_1 in NDV. (Compare Fig.

TABLE 3. *Proteins and their native disulfide complexes present in egg-grown NDV (HP-16)*

Mol wt ($\times 10^{-3}$)[a]	Present in:		Accessible to surface-specific labeling[b]	Identity[c]
	Nonreduced gels	Reduced gels		
220	+	+	−	L monomer
154	+	−	+	HN dimer
147	+	−	−	NP trimer
74	+	+	+	HN monomer
61	+	−	+	F monomer ($F_1 + F_2$ oligomer)
55	+	+	−	NP monomer
53	+	+	+	F_1 monomer
34	+	+	−	M monomer
15	−	+	±	F_2 monomer

[a] Molecular weights of NDV viral proteins were calculated from molecular weight markers (see Fig. 3) coelectrophoresed on 5 to 12.5% slab gels in the first (nonreduced) and second (reduced) dimension.

[b] A 100-μg portion of intact virus was reacted for 10 min at 0°C with 0.5 mCi of $Na^{125}I$ in the presence of 10 μg of chloroglycoluril as described in the text.

[c] The individual components of protein complexes were identified by their molecular weight, accessibility to surface-specific iodination, and selective extraction procedures.

4A to Fig. 8A.) Although NP and F_1 of NDV have similar electrophoretic mobilities, they were easily distinguished by surface-specific labeling (Fig. 8B). Most of the radiolabel was incorporated into the major glycoprotein HN, and the rest was incorporated into F_1. The same results were obtained for NDV (L. Kansas) with lactoperoxidase-catalyzed iodination (15). F_2 of NDV, unlike F_2 of Sendai virus, incorporated little radioiodine.

Under nonreducing conditions the F band was observed just above NP (Fig. 8C and D). Some F_1 was also present in the nonreduced preparation. Two bands were present in the high-molecular-weight region of the nonreduced gel (Fig. 8C). The one at molecular weight 154,000 was accessible to surface-specific iodination; the one at molecular weight 147,000 was not (Fig. 8D). Two-dimensional analysis (Fig. 9A) indicated that the 154,000-molecular-weight species was a homodimer of glycoprotein HN. Most (95%) of HN was present in the dimeric form; the rest (5%) was present as a monomer. The 147,000-molecular-weight species was identified as homotrimer of NP. Most (92%) of NP existed in the monomeric form in the nonreduced virus, but a significant amount (8%) formed a disulfide-linked homotrimer in the six viral preparations examined. No evidence for a homodimer form of NP was observed. The 5 to 12.5% gradient gel employed in these studies resolved the homooligomers of HN and NP into two discrete bands, but on a 7.5% gel such as that used previously for cross-linking studies in this laboratory (21, 37), the two homooligomers comigrated at an apparent molecular weight of 140,000.

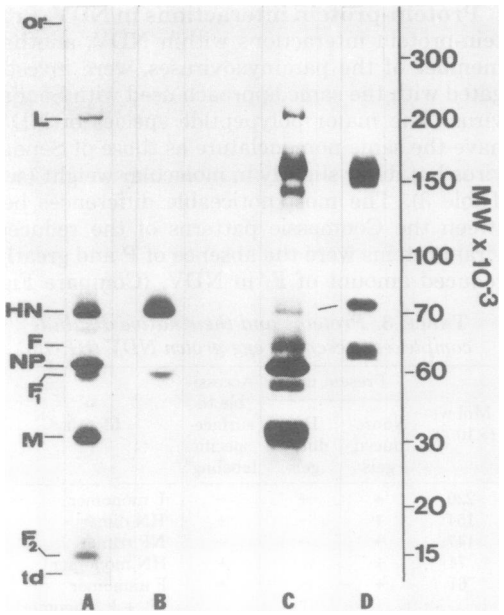

FIG. 8. *Comparison of reduced and nonreduced proteins of NDV by one-dimensional gel electrophoresis. A 100-μg sample of reduced egg-grown NDV was electrophoresed on a 5 to 12.5% linear gradient resolving gel and stained with Coomassie brilliant blue (A). A sample of the same virus preparation without reducing agent is shown in C. The autoradiographs of samples A and C which have been surface specifically labeled by the chloroglycoluril method for ^{125}I are shown in B and D, respectively. Letters designate the positions of the major viral proteins. Refer to Table 3 for identification of proteins and their native disulfide complexes.*

DTBP-induced cross-linking of freshly harvested (not frozen, stored no more than 3 days at 4°C) preparations of NDV produced the two-dimensional pattern observed in Fig. 9C. The same results were obtained when cross-linking was performed at 0, 11, 22, or 37°C over a range of DTBP concentrations (0.5 to 10 mM) or when PAPDIP (0.1 to 0.5 mM) was used as the cross-linking reagent. The homodimers of F, NP, and M produced by cross-linking were identified by their molecular weight and accessibility to surface-specific labeling (data not shown). NP-M heterodimer formation was further verified by selective extraction experiments described previously for identification of the NP-M heterodimer in Sendai virus. There was slightly greater heterodimer formation in early harvest than in late harvest preparations of NDV, but the difference between early and late harvest was not nearly so dramatic as with Sendai virus. NDV which was stored for 3 months or more at −70°C before cross-linking showed little or no evidence of NP-M heterodimer formation.

DISCUSSION

The protein-protein interactions identified in these studies expand our conceptual knowledge of the molecular architecture of the paramyxoviruses. Analysis of native disulfide bonding in isolated egg-grown viruses demonstrates that some of the proteins of Sendai virus and NDV consistently form covalent complexes. The size of the complex and the percentage of the particular protein found in complex form appear to depend on the infecting virus rather than the host cell. The HN of egg-grown Sendai virus is found mostly in tetrameric and dimeric forms, with only a small amount present as a monomer. This same distribution of HN in complexes was observed with Sendai virus produced by MDBK cells (unpublished data, Markwell and Fox). The HN of egg-grown NDV, strain HP-16, is present mostly as a disulfide-linked dimer, but the HN of a related strain of NDV, L-Kansas, is present

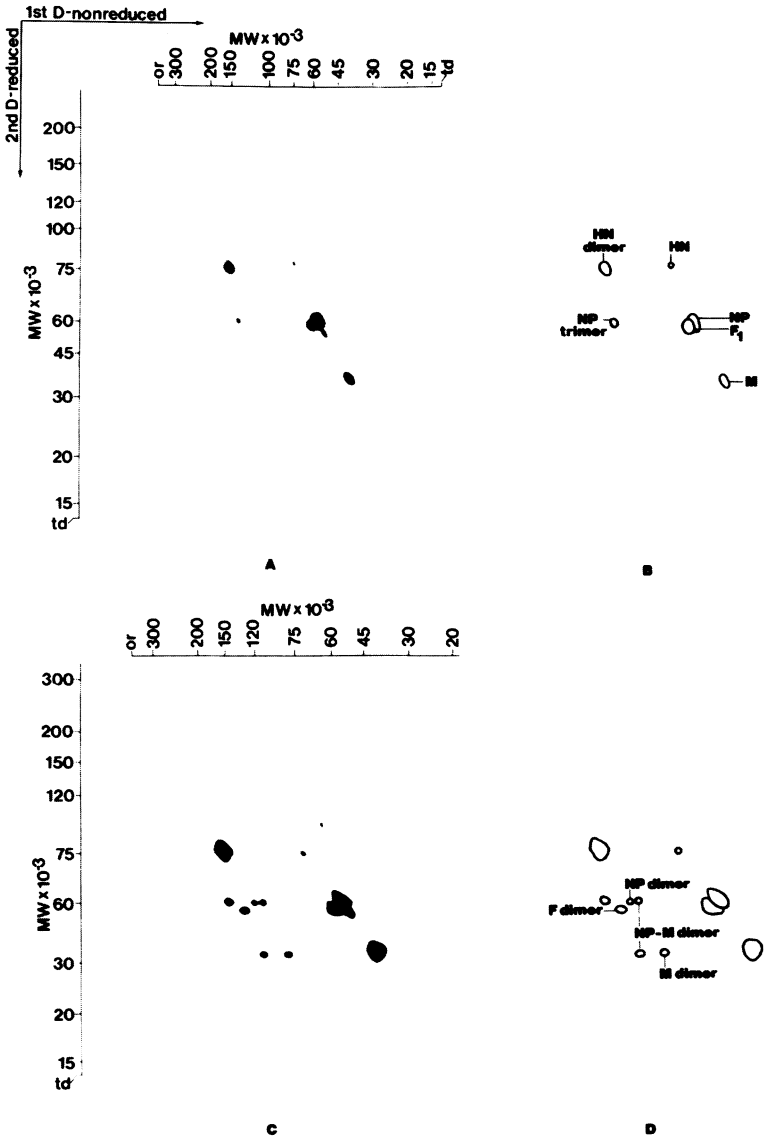

FIG. 9. *Two-dimensional analysis of native disulfide and chemically cross-linked protein complexes of egg-grown NDV. Approximately 50 μg of viral protein from untreated (A) or DTBP-cross-linked (C) virus were iodinated in the presence of SDS and then fractionated in the first dimension without reducing agent on a 5 to 12.5% polyacrylamide slab gel. The first-dimensional gel was sliced into its respective lanes, reduced with 2-mercaptoethanol, and affixed onto 7.5 to 10% slab gel for the second dimension. The autoradiograph of the gel is shown on the left, and its corresponding schematic diagram is shown on the right.*

only as a monomer when produced in embryonated eggs (unpublished data, Markwell and Fox). To what extent these native disulfide linkages influence the number of HN polypeptide chains which assemble to form the HN spike is not currently known.

At the onset of these studies, it was recognized that the validity of the cross-linking results obtained would depend to a large extent on the integrity of the virus preparations used. From hemolysis studies, Homma et al. (10) concluded that Sendai virus obtained from harvests just at the end of the one-step growth cycle (early harvests) had more intact envelopes than virus obtained from late harvests. A comparison of early and late harvest preparations was therefore included in the present studies. Use of the SV-288 vertical rotor greatly shortened the time re-

quired for virus purification and produced preparations of virus that were more intact as judged by surface-specific iodination than the previous procedure (30) with swinging bucket rotors and a number of pelleting steps. Sterile, purified virus preparations that were stored for longer than 3 days were frozen at high protein concentrations (5 mg/ml) at −70°C and thawed only once, immediately before use. The importance of native disulfide bonding in Sendai virus has been emphasized by reports of the loss of infectivity, cell fusion, hemagglutinin, neuraminidase, and hemolytic activities upon treatment of Sendai virus with reducing agents (23, 25, 33). Therefore, virus preparations used in the present cross-linking studies were not pretreated with reducing agent as described in previous reports (21, 37), and cross-linking was performed under conditions designed to preserve native disulfide bonding. Moreover, under the mild cross-linking conditions used in this study, little or no protein remained at the origin of the first-dimensional gel. This indicates that we are looking at the initial products of cross-linking and that the cross-linking process has only minimally perturbed the native structure of the virion.

The same products were formed when cross-linking was induced with DTBP or PAPDIP. Both reagents have an 1.1-nm separation between their functional groups (4, 26), and both diffuse across the viral membrane as demonstrated by their ability to form homodimers of NP. The imidoester group of both reagents is specific for primary amino groups (26); the nitrene generated by photolysis of the aryl azide group of PAPDIP does not require a specific reactive group. Recent evidence suggests that the imidoesters and nitrenes preferentially interact with extrinsic membrane components (1, 11).

Our studies indicate that the protein-protein interactions identified by reversible cross-linking are influenced by the conditions of production, isolation, and storage of these membrane-enveloped viruses. Some of the interactions, namely the ability to form homooligomers of F, NP, and M upon cross-linking, appear to be very stable and can be observed in preparations that have been stored at −70°C for as long as 2 years. This type of interaction has been previously reported for the paramyxoviruses (22, 28). Other interactions such as those between NP and M are best observed in preparations with the greatest degree of biological integrity, i.e., early harvest preparations isolated by the vertical rotor procedure and cross-linked immediately after isolation. This type of interaction has not been demonstrated previously by cross-linking in paramyxoviruses. The ability of NP and M to form a heterodimer appears to be the reflection of a stable complex rather than a transient one formed momentarily by diffusion of components for a number of reasons. Comparable complex formation was demonstrated at 37 and 0°C, where diffusion is severely limited, and also with a photoactivated reagent which yields a reactive species with a lifetime on the order of milliseconds (6).

This report of NP-M heterodimer formation in paramyxoviruses demonstrates that the M protein layer is in close proximity (1.1 nm or less) to the nucleocapsid and could serve as its recognition site during viral assembly. The progressive loss of the ability of NP and M to form a heterodimer as the virus is stored without a corresponding loss in infectivity suggests that this spatial relationship alters after particle formation and is not necessary for the ensuing stages of the infectious process. This structural change is not simply due to freezing and thawing. Viral preparations thawed after 1 week of storage at −70°C still formed some NP-M heterodimer upon treatment with cross-linking reagents. Rather, it appears that the preparations age upon storage even at −70°C. The progressive loss of the interaction between NP and M may reflect either a general breakdown of weak, noncovalent interactions between proteins or inactivation of particular binding sites between the nucleocapsid and membrane components of the virus. A recent electron microscopic study of Sendai virus (12) indicates that as the virions age in ovo (late harvest preparations) or in vitro, the nucleocapsid becomes irregularly folded and detaches from the viral envelope.

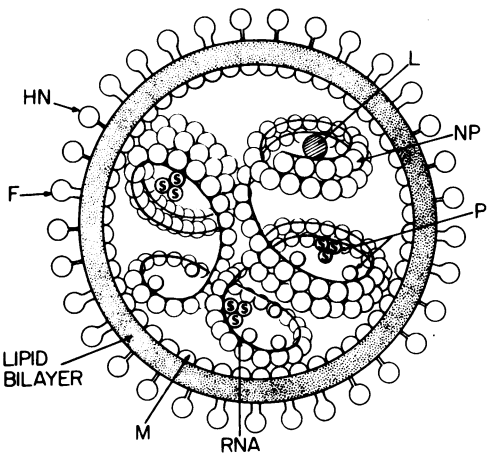

FIG. 10. *A structural model of protein-protein interactions within the Sendai virion. Refer to Table 2 and text for description of protein complexes produced by native disulfide bonding or reversible chemical cross-linking.*

Figure 10 depicts our model of the topographical organization of proteins within the paramyxoviruses, specifically Sendai virus, based on the protein-protein interactions discussed in this paper and current knowledge of paramyxovirus structure. The glycoproteins HN and F form separate spikes on the outer surface of the membrane envelope. Each spike appears to be an oligomer whose component polypeptides associate through noncovalent interactions or disulfide bonding or both. Shimizu et al. (35) have separated two types of dumbbell-shaped spikes (HN+ and HN−) from detergent-solubilized Sendai virus that differed in their biological, serological, morphological, and chemical properties.

The glycoproteins of membrane-enveloped viruses appear to associate with the lipid bilayer through a relatively small, predominantly hydrophobic peptide (14). Cross-linking studies with Semliki Forest virus (8) and vesicular stomatitis virus (7) suggest that in these lipid-containing viruses the anchor peptide tail spans the bilayer. The selective extraction experiments described in this paper suggest that HN and F of Sendai and Newcastle disease viruses are embedded in the lipid bilayer. However, no evidence for transmembrane cross-linking between the glycoproteins and M was found for the two paramyxoviruses examined in this study, and previous reports from this laboratory of transmembrane cross-linking between HN and NP in NDV (HP-16) (21, 37) were shown to be a misinterpretation of data due to the inadequate resolution of the two-dimensional gel system used. The lack of cross-linking between HN or F and M by no means eliminates the possibility that the glycoproteins span the bilayer. Cross-linking reagents with different site specificities than those used in this study might demonstrate this topographical relationship.

Our cross-linking data indicate that the nucleocapsid lies in close proximity to the M protein layer in freshly harvested paramyxoviruses. The ease of extraction of P and L from the nucleocapsid suggests a more superficial location in or weaker association with the nucleocapsid for these two proteins than the firmly attached NP. Both the P protein of Sendai virus and the NP protein of NDV (HP-16) exist as monomers and as disulfide-linked homotrimers. A functional role(s) for these trimers is not known.

Cross-linking of intact virus revealed little more about the organization of the paramyxovirus nucleocapsid beyond the relationship of NP as nearest neighbor to itself. No cross-linking of P or L which are also part of the transcriptive apparatus of Sendai virus (19) was observed. Whether P and L interact with NP or directly with viral RNA or both is still open to question. Cross-linking of isolated Sendai virus nucleocapsids with DTBP, tetranitromethane, or dimethyl suberimidate has been previously reported to produce homooligomers of NP and to convert P and L to high-molecular-weight complexes (27, 28). In our study, cross-linking of intact virus led to formation of very small amounts of NP homotrimer and homotetramer, but not to high-molecular-weight complexes of P or L. This may be due to the presence of the viral membrane which could either limit the accessibility of cross-linking reagents to the nucleocapsid or subtly change the structure of the nucleocapsid by associating with it during the process of viral assembly.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Rochelle Weiss in preparing the egg-grown Sendai virus and of Tokichi Miyakawa for synthesizing the cross-linking reagent PAPDIP used in this study. We also wish to thank Debi P. Nayak and Joseph T. Seto for their helpful comments on the proposed structural model of Sendai virus.

This work was supported by Public Health Research grant GM-18233 from the National Institute of General Medical Sciences and by a grant from the Muscular Dystrophy Association, Inc. M.A.K.M. is the recipient of postdoctoral fellowship grant DRG 118-FT from the Damon Runyon-Walter Winchell Cancer Fund.

LITERATURE CITED

1. **Bayley, B., and J. R. Knowles.** 1978. Photogenerated reagents for membrane labeling. I. Phenylnitrene formed within the lipid bilayer. Biochemistry **17**:2414–2423.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Crain, R. C., and G. V. Marinetti.** 1978. Cross-linking of membrane phospolipid and protein using putative monofunctional imidoesters. Chem. Phys. Lip. **21**:195–204.
4. **Das, M., and C. F. Fox.** 1977. Specific radiolabeling of a cell surface receptor for epidermal growth factor. Proc. Natl. Acad. Sci. U.S.A. **74**:2790–2794.
5. **Das, M., and C. F. Fox.** 1978. Molecular mechanism of mitogen action: processing of receptor induced by epidermal growth factor. Proc. Natl. Acad. Sci. U.S.A. **75**: 2644–2648.
6. **Das, M., and C. F. Fox.** 1979. Chemical cross-linking in biology. Annu. Rev. Biophys. Bioeng. **8**:165–193.
7. **Dubovi, E. J., and R. R. Wagner.** 1977. Spatial relationships of the proteins of vesicular stomatitis virus: induction of reversible oligomers by cleavable protein cross-linkers and oxidation. J. Virol. **22**:500–509.
8. **Garoff, H., and K. Simons.** 1974. Location of the spike glycoproteins in the Semliki Forest virus membrane. Proc. Natl. Acad. Sci. U.S.A. **71**:3988–3992.
9. **Henning, R., R. J. Milner, K. Reske, B. A. Cunningham, and G. M. Edelman.** 1976. Subunit structure, cell surface orientation, and partial amino-acid sequences of murine histocompatibility antigens. Proc. Natl. Acad. Sci. U.S.A. **73**:118–122.
10. **Homma, M., K. Shimizu, Y. K. Shimizu, and N. Ishida.** 1976. On the study of Sendai virus hemolysis. I. Complete Sendai virus lacking in hemolytic activity. Virology **71**:41–47.
11. **Ji, T. H.** 1979. The application of chemical crosslinking

for studies on cell membranes and the identification of surface receptors. Biochim. Biophys. Acta **559**:39–69.
12. **Kim, J., K. Hama, Y. Miyake, and Y. Okada.** 1979. Transformation of intramembrane particles of HVJ (Sendai virus) envelopes from an invisible to visible form on aging of virions. Virology **95**:523–535.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**:680–685.
14. **Lenard, J.** 1978. Virus envelopes and plasma membranes. Annu. Rev. Biophys. Bioeng. **7**:139–165.
15. **Li, J. K.-K., and C. F. Fox.** 1975. Radioiodination of the envelope proteins of Newcastle disease virus. J. Supramol. Struct. **3**:51–60.
16. **Markwell, M. A. K., and C. F. Fox.** 1978. Protein-protein interactions within the Sendai virion. J. Supramol. Struct. (Suppl. 2), p. 261.
17. **Markwell, M. A. K., and C. F. Fox.** 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochemistry **17**:4807–4817.
18. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
19. **Marx, P. A., A. Portner, and D. Kingsbury.** 1973. Sendai virion transcriptase complex:polypeptide composition and inhibition by virion envelope proteins. J. Virol. **13**:107–112.
20. **McSharry, J. J., R. W. Compans, and P. W. Choppin.** 1971. Proteins of vesicular stomatitis virus and of phenotypically mixed vesicular stomatitis virus-simian virus 5 virions. J. Virol. **8**:722–729.
21. **Miyakawa, T., L. J. Takemoto, and C. F. Fox.** 1976. Supramolecular organization of proteins in Newcastle disease virus, p. 485–497. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), Animal virology. Academic Press Inc., New York.
22. **Nagai, Y., T. Yoshida, M. Hamaguchi, M. Iinuma, K. Maeno, and T. Matsumoto.** 1978. Cross-linking of Newcastle disease virus (NDV) proteins. Arch. Virol. **58**:15–28.
23. **Neurath, A. R., S. K. Vernon, R. W. Hartzell, and B. A. Rubin.** 1973. Virocidal cleavage of disulphide bonds within structural proteins of Sendai virus. J. Gen. Virol. **19**:9–20.
24. **Nicolson, G. L., and R. G. Painter.** 1973. Anionic sites of human erythrocyte membranes. II. Antispectrin-induced transmembrane aggregation of the binding sites for positively charged colloidal particles. J. Cell Biol. **59**:395–406.
25. **Ozawa, M., A. Asano, and Y. Okada.** 1976. Importance of interpeptide disulfide bond in a viral glycoprotein with hemagglutination and neuraminidase activities. FEBS Lett. **70**:145–149.
26. **Peters, K., and F. M. Richards.** 1977. Chemical cross-linking. Annu. Rev. Biochem. **46**:523–551.
27. **Raghow, R., D. W. Kingsbury, A. Portner, and S. George.** 1979. Topography of a flexible ribonucleoprotein helix: protein-protein contacts in Sendai virus nucleocapsids. J. Virol. **30**:701–710.
28. **Raghow, R., and D. W. Kingsbury.** 1976. The molecular organization of Sendai virus nucleocapsids, p. 471–484. *In* D. Baltimore, A. S. Huang, and C. F. Fox (ed.), Animal virology. Academic Press Inc., New York.
29. **Rott, R., and H. D. Klenk.** 1977. Structure and assembly of viral envelopes, p. 47–81. *In* G. Poste and G. L. Nicolson (ed.), Virus infection and the cell surface. North-Holland Publishing Co., New York.
30. **Samson, A. C. R., and C. F. Fox.** 1973. Precursor protein for Newcastle disease virus. J. Virol. **12**:579–587.
31. **Scheid, A., and P. W. Choppin.** 1973. Isolation and purification of the envelope proteins of Newcastle disease virus. J. Virol. **11**:263–271.
32. **Scheid, A., and P. W. Choppin.** 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology **57**:475–490.
33. **Scheid, A., and P. W. Choppin.** 1977. Two disulfide-linked polypeptide chains constitute the active F protein of paramyxoviruses. Virology **80**:54–66.
34. **Shimizu, K., and N. Ishida.** 1975. The smallest protein of Sendai virus: its candidate function of binding nucleocapsid to envelope. Virology **67**:427–437.
35. **Shimizu, K., Y. K. Shimizu, T. Kohama, and N. Ishida.** 1974. Isolation and characterization of two distinct types of HJV (Sendai virus) spikes. Virology **62**:90–101.
36. **Sweadner, K. J.** 1977. Crosslinking and modification of Na,K-ATPase by ethyl acetimidate. Biochem. Biophys. Res. Commun. **78**:962–969.
37. **Takemoto, L. J., T. Miyakawa, and C. F. Fox.** 1977. Analysis of membrane protein topography of Newcastle disease virus and cultured mammalian fibroblasts, p. 605–614. *In* J. P. Revel, U. Henning, and C. F. Fox (ed.), Cell shape and surface architecture. Alan R. Liss, Inc., New York.
38. **Warren, L., and M. C. Glick.** 1969. Isolation of the surface membranes of tissue culture cells, p. 66. *In* K. Habel and N. P. Salzman (ed.), Fundamental techniques in virology. Academic Press Inc., New York.