JOURNAL OF VIROLOGY, Nov. 1982, p. 450–457
0022-538X/82/110450-08$02.00/0

Vol. 44, No. 2

Disulfide Bonding in Influenza Virus Proteins as Revealed by Polyacrylamide Gel Electrophoresis

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Received 12 April 1982/Accepted 9 August 1982

Disulfide bonding in the major proteins of influenza virus A, WSN strain, was studied by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels under reducing and nonreducing conditions. The electrophoretic behavior of the proteins correlated with their localization in the virions and their chemical composition. The internal proteins of the viral particles, i.e. matrix and nucleoproteins, were shown to contain a relatively small number of cysteine residues. Electrophoresis under nonreducing conditions yielded multiple forms of the proteins which could be discriminated by small but readily observable, reproducible differences in their migration rates in the gel. The multiplicity of the protein forms was caused by the formation of intramolecular disulfide bonds in matrix and nucleoproteins that arose during or after solubilization in sodium dodecyl sulfate. On the other hand, we failed to detect native inter- and intramolecular linkages in matrix and nucleoproteins. External glycoproteins of the virions (HA and NA) had, in contrast to the internal ones, a higher number of cysteine residues and native disulfide bonds. At least three disulfide linkages were revealed in HA and NA in our experiments. In uncleaved HA all of the linkages were intramolecular. In NA at least one disulfide bond linked two identical polypeptides into a dimer. It was established that the reduction of the different disulfide linkages in HA and NA required different concentrations of the reducing agent.

The virions of influenza virus A contain seven species of structural proteins. The major part of two viral glycoproteins, HA and NA, and all of their carbohydrate chains are external to the lipid bilayer of the viral envelope, forming the spikes which project from the surface of the viral particle (5, 20, 25, 29). HA trimers form hemagglutinin spikes on the virions (3, 35). For the spikes to perform the entire range of their biological funct ons, HA must be cleaved into two unequal subunits, HA_1 and HA_2 (17, 22, 24). The subunits remain bound to each other by the preexisting disulfide bond (20, 34, 36). The tetramers of NA glycopolypeptides form neuraminidase spikes on the virions (5, 6, 38). There is evidence that each tetramer consists of two noncovalently bound dimers, but the monomers inside the dimer are bridged by disulfide bonds (6, 25).

The lipid bilayer of the influenza virions covers nonglycosylated proteins involved in the formation of two types of structures. Matrix protein M, the most abundant, forms the internal layer of the lipoprotein membrane of the virions (8). Nucleoprotein NP and three minor proteins of group P form the ribonucleoprotein of the virion (9, 35).

From recent advances in discovery of the primary structure of influenza virus A hemagglutinin, it has become clear that whatever the differences in the total amino acid sequence of proteins from different strains, the number and localization of the cysteine residues are similar or even invariant (11, 27, 28, 32, 33, 37). It is therefore highly probable that the cysteine residues in HA are involved in disulfide bonding and that the alignment of these linkages is identical in the hemagglutinins of different strains. It was indeed confirmed by direct analysis that all of the 12 cysteine residues in the external domain of HA form disulfide linkages, one of which holds together the HA_1 and HA_2 subunits, four of which lie in HA_1, and one of which lies in HA_2 (34). These data are interesting for several reasons. First, they show the important role of the HA disulfide bonds in the organization and maintenance of the physiologically adequate structure of the protein. Second, they show that, in spite of the broad immunological variability of the hemagglutinins from different strains and the marked divergence of the primary structure of the genes and amino acid sequences in the proteins, the three-dimensional backbone of all the influenza virus A hemagglutinins is the same. Third, the homology between the positions of the cysteines in HA from different

strains of influenza virus, as in other evolutionarily close ectoproteins, suggests that all of these proteins must have originated from a common precursor (21, 31).

The study of the number and alignment of the disulfide bonds in viral proteins is not easy methodologically because large quantities of protein and laborious procedures are required. Our study used electrophoretic analysis in polyacrylamide gels under reducing and nonreducing conditions.

MATERIALS AND METHODS

The A/WSN/33 strain of influenza virus (WSN virus) was used. The virus was grown in the allantoic cavity of 9-day-old chicken embryos incubated at 37°C for 48 h. Virus was purified from the allantoic fluid as follows: the fluid was clarified by centrifugation at 5,000 rpm for 15 min, the virus was pelleted in the 35-angle rotor of a Spinco centrifuge at 24,000 rpm for 90 min, and the pellet was suspended in STE (0.1 M NaCl, 0.01 M Tris-hydrochloride, pH 7.6, 0.001 M EDTA). The suspension was layered on sucrose density linear gradients from 20 to 60% (wt/wt) prepared in STE. The gradients were centrifuged in an SW27 rotor at 20,000 rpm for 17 h. Opalescent bands of virus were collected and diluted in STE, and virus was sedimented in the angle rotor at 24,000 rpm for 2 h. The pellet was suspended in STE (2 to 5 mg/ml), distributed into test tubes, and stored at −20°C. The contents of a test tube were thawed and used immediately. This preparation was designated allantoic virus.

In several experiments, ^{14}C- or ^{35}S-labeled proteins from cultured virus were used. For cultured virus, primary monolayers of chicken embryo fibroblasts were infected with virus at a multiplicity of infection of about 10 median infectious doses per cell, and the cells were incubated in minimal essential medium without serum. At 2 h postinfection, ^{14}C-labeled chlorella protein hydrolysate (Isotope, USSR; 25 μCi/ml), [^{35}S]cysteine (Amersham Corp., England; 740 mCi/mmol, 40 μCi/ml), or [^{14}C]glucosamine (Amersham; 239 mCi/mmol, 25 μCi/ml) was added to the culture, and it was incubated for another 18 h. The virus was then collected and purified as described above.

PAGE. The system described by Laemmli (18) was used for polyacrylamide gel electrophoresis (PAGE). Proteins were solubilized in 5% sodium dodecyl sulfate (SDS). According to the goal of the experiment, the concentration of 2-mercaptoethanol (ME) varied from 0 (nonreducing conditions) to 5% (reducing conditions). Before electrophoresis, the samples were boiled in a water bath for 3 min. The gels in various experiments contained from 9 to 13% acrylamide, with an acrylamide-to-bisacrylamide ratio of 100:1.

The procedure for two-dimensional PAGE was described previously (26). Electrophoresis in nonreducing conditions was carried out in slabs with 9% gels 1 mm thick. After electrophoresis, the corresponding tracks were cut out and stored at −20°C. Before electrophoresis in the second dimension, the corresponding piece of the gel was incubated in a reducing buffer (6.25 mM Tris-hydrochloride [pH 7.6], 5% SDS, 5% ME) with constant stirring at 20°C for 30 min. The gel was then layered on top of a freshly prepared 13% slab gel, 1.25 mm thick, the material was covered with 5% acrylamide-focusing solution, and electrophoresis was carried out in the second dimension. After electrophoresis, the gels were stained with amido black or autoradiographed, or both. Autoradiography was performed with dried gels on X-ray film Orwo SS-11.

RESULTS

Estimation of cysteine residue content in the major proteins of WSN virus. The cultured virus was grown either in the presence of ^{14}C-amino acids or [^{35}S]cysteine under conditions of constant labeling. The proteins of the purified preparations were analyzed by SDS-PAGE under reducing conditions. All experimental procedures (labeling, purification, electrophoresis) were carried out with ^{35}S- and ^{14}C-labeled samples under identical conditions to rule out possible differences in the ratios of electrophoretic forms of nucleoproteins NP and NP53 (1, 39). After electrophoresis the autoradiograph was prepared and scanned (Table 1). The relative radioactivity in the proteins labeled by the two isotopes differed (Table 1). The scanogram of the proteins labeled with ^{14}C-amino acids showed a typical influenza virus pattern, with most of the label confined to uncleaved HA, NP, and M and some of it to NA. Among the ^{35}S-labeled proteins, however, the most intense was

TABLE 1. Cysteine residue content in the major proteins of WSN virus

Protein[a]	Label incorporated by protein (% of total)[b]		$^{14}C/^{35}S$ ratio	Cystein content (mol/mol of protein)[c]
	^{14}C-amino acids	[^{35}S]cysteine		
HA	20	31	1.55	15.0
NP	30	23	0.71	6.8
NA	11	23	2.10	20.3
M	39	23	0.58	5.6

[a] In the ^{14}C-labeled sample, NP protein is revealed in the gel in two positions: NP and NP53, the latter being a modification product of the former (1, 39). The ratio of ^{14}C label taken into NP and NP53 in the given experiment was 2:1. In the ^{35}S-labeled sample, NP53 was not revealed as it was overlapped by NA. Therefore in calculations a value corresponding to ½ the NP area was subtracted from the NA peak and added to NP.

[b] ^{14}C- and ^{35}S-labeled preparations of cultured WSN virus were electrophoresed, autoradiographed, and scanned in a Joyce-Loebl densitometer, and the values of the peaks were obtained by weighing. Total values of protein peaks in each virus sample were taken as 100%. P proteins were not taken into account.

[c] Absolute cysteine content in the WSN strain HA protein was taken as 15 amino acid residues per molecule (14), and cysteine content for the other major proteins was estimated relative to this value.

the band corresponding to glycoprotein HA, whereas the NA, NP, and M bands contained equal amounts of label. These results indicate a higher content of cysteine residues in the external glycoproteins than in the internal proteins of the viral particles.

We then turned to the recent data of Hiti et al. (14), who used DNA sequencing to show that there are 15 cysteine residues per mature protein molecule in the HA of the WSN virion. This value was used as a reference for estimating the cysteine content in other proteins. Our figures (Table 1) are much the same as those found for the NA and M proteins of the PR8 virus (2, 10), which is evolutionarily closely related to the WSN virus. Therefore, it is likely that the estimate for NP is also realistic.

Resolution of structural proteins by two-dimensional PAGE. A mixture of cultured ^{14}C-amino acid-labeled virus (~5 μg per gel) and allantoic virus (~100 μg per gel) was used for electrophoresis. Therefore, only the proteins of the cultured virus remained after autoradiography and only the allantoic virus proteins remained after the slab was stained.

Both major internal proteins of the virions, NP and M, were found in spots along the diagonal of the two-dimensional slab (Fig. 1). Hence, we concluded that the electrophoretic mobility of these proteins does not alter significantly after reduction of the disulfide bonds. This aspect will be dealt with in detail below.

Both glycoproteins of the virion were localized off the diagonal, NA lying beneath it, which means that their electrophoretic mobility increased after reduction of the native disulfide bonds. This increase corresponded to a decrease in the molecular weight of the protein from ~93,000 to ~50,000. This result supports the finding of Lazdins et al. (25) that NA of influenza virus B consists of dimers bridged by disulfide bonds.

The polypeptides of the viral hemagglutinins are the only ones that display a host-dependent electrophoretic pattern. In the cultured virus all of the protein was present as uncleaved HA polypeptides. This protein was localized slightly above the diagonal, which testified to its having a lower electrophoretic mobility after treatment with ME. In the allantoic virus, on the contrary, all of the protein was represented by HA_1 and HA_2 subunits. That both subunits were localized on one vertical line indicates that under nonreducing conditions they make one band. This confirms that HA_1 and HA_2 are linked by an interchain disulfide bond (20, 34). The dependence of HA cleavage on growth conditions also confirms earlier evidence (23, 24).

Microheterogeneity of NP and M in nonreducing conditions. Close scrutiny of the results

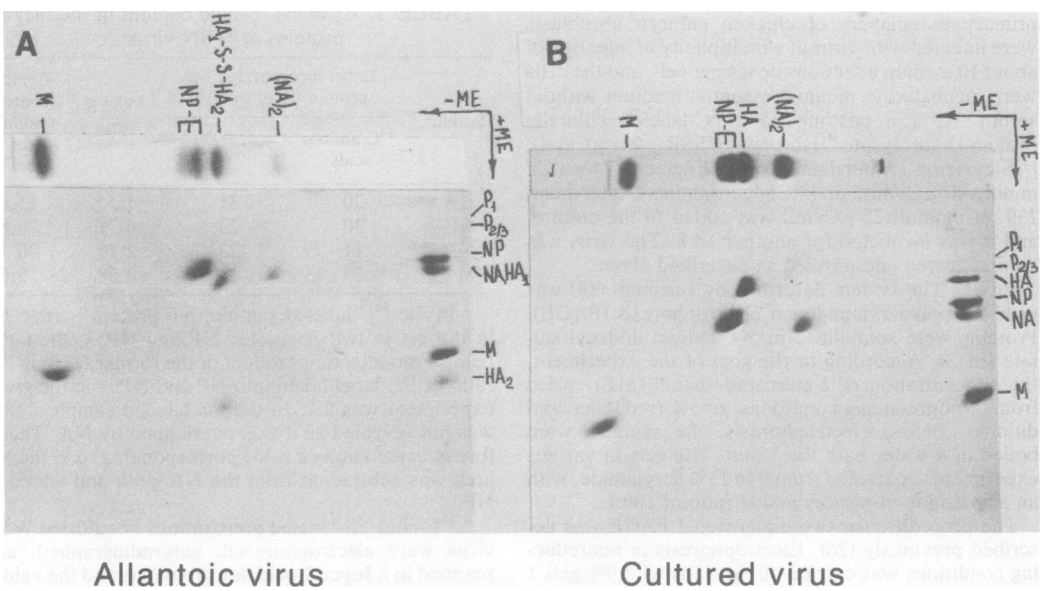

FIG. 1. Two-dimensional PAGE analysis of proteins and their disulfide complexes in WSN virus. A mixture of unlabeled allantoic and ^{14}C-labeled cultured virus was fractionated in the first dimension in the nonreducing PAGE (9% acrylamide) and in the second dimension in the reducing PAGE (13% acrylamide). The gel was then stained with amido black, and autoradiography was performed. Distribution of proteins in one-dimensional PAGE under nonreducing (−ME, top) and reducing conditions (+ME, right side) are shown. (A) Stained gel; (B) autoradiographed gel.

shown in Fig. 1 shows that M and NP are detected after PAGE under nonreducing conditions as close-lying components which differ slightly in mobility. This microheterogeneity could be studied efficiently by one-dimensional PAGE under nonreducing conditions. We studied the behavior of the viral proteins from different fractions of infected cells in this way.

At least three somewhat different components could be discerned in the position of NP under nonreducing conditions (Fig. 2). We concluded that all of them consisted of NP polypeptides after considering—and ruling out—the possibility of participation by other proteins in the microheterogeneous fractions: (i) there can be no glycoproteins, because all of the components in roughly the same ratios were detected in the nuclear fraction of infected cells (in which there were hardly any HA or NA proteins); (ii) there cannot be any nonstructural virus-specific or cellular proteins, as all of the components were detected in the purified virus preparations; and (iii) the M protein may be excluded after two-dimensional PAGE of the virion proteins (Fig. 1).

The number of detectable microheterogeneous components of NP varied from two to four in different analyses. We do not know the reason for this variability, but we are sure that it was caused by some condition of the PAGE which is beyond our control rather than by the quality of the protein preparation. In each electrophoresis experiment, the quantity of microheterogeneous components of NP was the same in all of the samples of a given slab, including the lysate of the infected cells prepared immediately after short pulsing of the proteins with radioactive precursors.

After electrophoresis in nonreducing conditions the M protein is represented by two components (Fig. 2, lane 1). The minor component was more mobile than the major one.

Other strains of influenza virus (19 strains of A and 1 strain of B; data not shown) displayed the same microheterogeneity of the NP and M proteins under nonreducing conditions. It was therefore of interest to answer the following two questions: (i) whether the difference in disulfide bonding of the components is the reason for the microheterogeneity; and (ii) if so, whether the disulfide bonds are native or arise after disruption of the proteins, as is the case for murine histocompatibility antigen (13).

To answer these questions, we treated the samples with 0.01 M iodoacetamide both before and at the same time as we disrupted them with SDS. This reagent blocks the formation of secondary disulfide bonds by binding with free sulfhydryl groups. This treatment resulted in the disappearance of the microheterogeneous components of NP and M (Fig. 3). The preliminary blocking of the sulfhydryl groups gave a better result than did simultaneous addition of SDS and iodoacetamide. Thus, heterogeneity is the result of autooxidation of NP and M that occurs during or after SDS disruption and induces the formation of the secondary disulfide linkages in vitro.

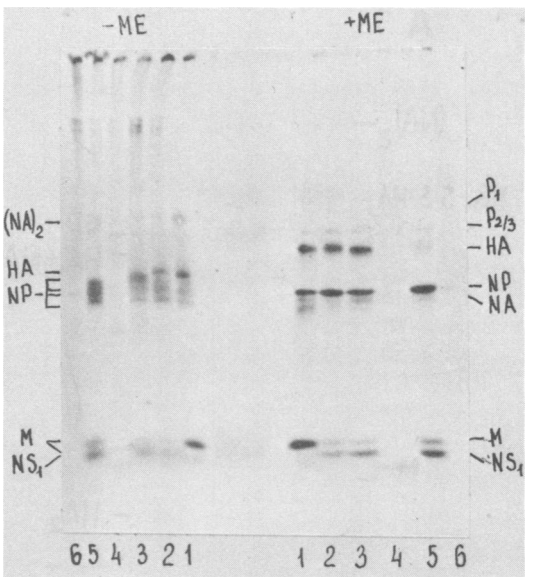

FIG. 2. Electrophoretic pattern of ^{14}C-labeled proteins of the WSN virus and virus-infected cells after electrophoresis under nonreducing (−ME) and reducing (+ME) conditions. At 4 h postinfection, chicken embryo fibroblasts were incubated with ^{14}C-amino acids for 20 min and under chase conditions for 40 min. Subcellular fractions were prepared as previously described (1), and proteins were analyzed by electrophoresis followed by autoradiography. Lanes: 1, ^{14}C-labeled proteins of virions; 2, proteins from the cytoplasm of infected cells labeled by pulse-chase, and 3, without chase; 4, pulse-labeled proteins of noninfected cells; 5, pulse-labeled proteins from the nuclear fraction of infected cells, and 6, of noninfected cells.

Step-wise reduction of disulfide bonds in HA and NA. To this point the action of ME on electrophoretic behavior was studied in terms of an all or nothing approach; the concentration of ME used was, accordingly, 5 or 0%. It was assumed that all of the disulfide bonds were reduced or retained respectively, at these concentrations. It is known that the external domain of HA contains six disulfide bonds (34, 35). Therefore, we believed it was feasible to use concentrations of ME between 0 and 5%. We expected to answer the question of whether there is cooperation in the reduction of different disulfide bonds in HA; also, it was interesting to compare HA and NA, as the latter had not been studied for disulfide bonds.

Therefore, samples of the cultured virus in

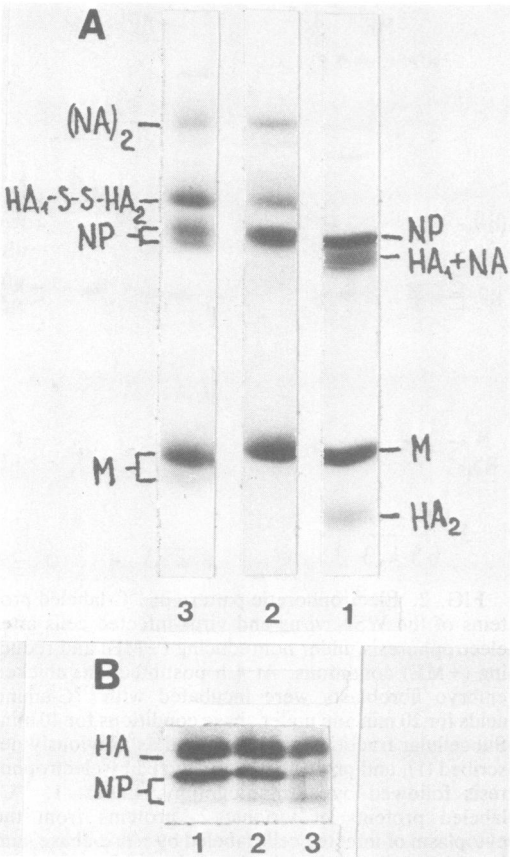

FIG. 3. Effect of iodoacetamide treatment on the electrophoretic patterns of virus proteins. (A) Samples of allantoic virus were treated with 5% ME (lane 1), 0.01 M iodoacetamide (lane 2) or not treated (lane 3). The samples were solubilized and analyzed by PAGE. (B) ^{14}C-amino acid-labeled samples of cultured virus were treated for 10 min with 0.01 M iodoacetamide before being solubilized with SDS (lane 1), treated simultaneously with 0.01 M iodoacetamide and SDS (lane 2), or solubilized with SDS without iodoacetamide treatment (lane 3). The gels were autoradiographed; only the region of the gel containing NP is shown.

which the polypeptides had been labeled with ^{14}C-amino acids or [^{14}C]glucosamine were treated with 5% SDS and with ME in consecutively decreasing concentrations; i.e., 5% (640 mM), 5% $\times$ 2^{-1}, 5% $\times$ 2^{-2}, etc; they were then heated and subjected to PAGE in a single-gel slab (Fig. 4). The use of two different isotopes and the relative stability of the electrophoretic behavior of the nonglycosylated NP and M proteins allowed us to identify HA and NA in the gel. By varying the ME concentration we were able to detect each of the two proteins as four electrophoretic components (a, b, c, and d); their apparent molecular weights are given in Table 2. It is noteworthy that the same components were revealed even when dithiothreitol or Na_2SO_3 was used instead of ME for reduction. All of this gives us grounds for believing that the components of proteins HA and NA that we detected differ from one another in the number of disulfide linkages.

DISCUSSION

The structural proteins of influenza virus can be divided by a few important properties into two categories. The first contains the glycopeptides HA and NA, and the second includes all of the remaining proteins, i.e., M, NP, and P's. The distinguishing properties are: (i) HA and NA are exposed in the virion, whereas all of the other proteins are coated with lipid bilayer (16); (ii) only NA and HA contain covalently bound sugars (8, 16); and (iii) HA and probably NA are synthesized in the infected cells in membrane-bound polysomes, whereas nonglycosylated proteins are synthesized in free polysomes (7, 12). Characteristically, the external and internal proteins of many other enveloped viruses show the same differences. Moreover, such differences are to a certain extent typical of the secreted or ectoproteins of eucaryotes and of their internal proteins.

The data obtained in this study show that analysis of disulfide bonding allows better discrimination among the influenza virus proteins. The external glycoproteins contained essentially more cysteine residues than did the internal M and NP proteins and hence are potentially more capable of forming disulfide bonds. The external proteins do form disulfide bonds in vivo, and no such bonds were found to exist in M or NP. On the contrary, only internal proteins are able to form secondary disulfide bonds in vitro via autooxidation after disruption of the proteins by SDS. The question of why internal proteins do not form disulfide bonds in vivo and external glycoproteins do then arises. Disulfide bonding occurs very rapidly and effectively in the latter: after the infected cells were labeled by a short pulse (20 min), all of the glycoproteins were detected in the a form (Table 2 and Fig. 2), which means that they were saturated with disulfide bonds. Logic prompted us to assume that it is the microenvironment of the synthesizing or just synthesized molecules that plays a role in the disulfide bonding. The microenvironments may be different because the synthesis and processing of the external protein are membrane bound, whereas in the internal proteins these events have an extramembrane location. This is in agreement with the known data that a membrane fraction of eucaryotic cells possesses a special enzyme(s) that stimulates the thiol-

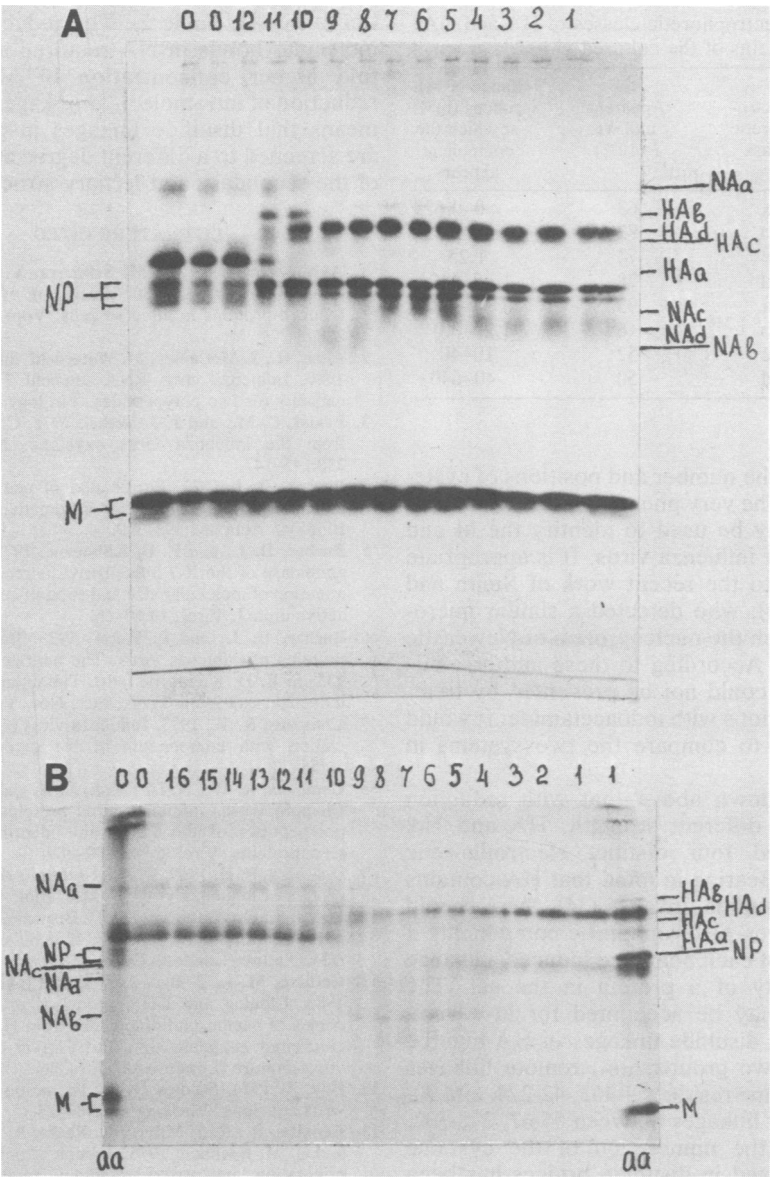

FIG. 4. Effect of the reducing action of various strengths on the electrophoretic pattern of culture virus. Samples of virus labeled with ^{14}C-amino acids (A) or [^{14}C]glucosamine (B) were treated with ME in decreasing concentrations. Lanes: 1, 5% ME (640 mM); 2, 5% × 2^{-1}; 3, 5% × 2^{-2}, etc., up to lane 16, 5% × 2^{-15} ME (~20 μM). Lane zero, no ME added. PAGE and autoradiography were then performed; lanes aa in panel B show ^{14}C-amino acid-labeled cultured virus.

disulfide exchange that is responsible for the alignment of disulfide bonds in various proteins (31).

HA and NA seem to owe their stability to the comparatively large number of native disulfide bonds. This is evidently the reason why influenza virus preparations retain hemagglutination and neuraminidase activities after solubilization by SDS and the removal of the detergent (4, 19). On the other hand, as indicated by our results, such treatment should have induced irreversible conformational changes in at least part of the internal protein molecules due to the formation of secondary disulfide bonds.

Microheterogeneity under nonreducing conditions was inherent in the M and NP proteins of all the influenza virus strains studied (20 strains of A and 1 strain of B; data not shown). This should be interpreted to mean that the M and NP proteins from different strains of influenza virus

TABLE 2. Electrophoretic classes of HA and NA proteins of the cultured virus

Protein	Electrophoretic class	Apparent mol wt ($\times 10^{-3}$)	Range of ME concns (mM) at which the component appears
HA	a	63	0–0.625
	b	82	0.625–1.25
	c	74	1.25
	d	78	1.25–640
NA	a	93	0–1.25
	b	46	1.25–5.0
	c	52	10–40
	d	50	40–640

are similar in the number and positions of cysteine residues. The very phenomenon of microheterogeneity may be used to identify the M and NP proteins of influenza virus. It is appropriate to refer here to the recent work of Smith and Hightower (30), who detected a similar microheterogeneity in the nucleoprotein of Newcastle disease virus. According to these authors, this heterogeneity could not be prevented by treatment of the virions with iodoacetamide. It would be interesting to compare the two systems in greater detail.

We have shown above that with reduction treatments of different strength, HA and NA each produced four distinct electrophoretic components. Bearing in mind that HA contains at least six disulfide linkages (34), there should have been seven electrophoretic components if the breakage of each bond had induced a change in the mobility of a protein in the gel. The discrepancy may be accounted for in several ways. The six disulfide linkages in HA may be divided into two groups, i.e., remote linkages between cysteine residues 4-463, 42-274, and 90-135 and closer linkages between 55-67, 278-302, and 470-474 (the numeration of the cysteine residues involved in disulfide bridges has been borrowed from references 34 and 36). It may be assumed that it is only the rupture of the remote disulfide linkages that brings about the transconformation of a glycoprotein sufficient for its electrophoretic mobility to be altered. An alternative explanation would be that at certain concentrations of ME more than one bond is reduced. But regardless of how the data are rationalized, they indicate the significance of the configuration of a polypeptide in the rate of its migration in SDS-PAGE.

NA is the only protein that is known to have an intermolecular disulfide bond(s). This bond(s) links identical glycopeptides into dimers. In addition to the intermolecular bond(s), we found in NA at least two intramolecular linkages (transition b→c→d, Table 2). The reduction of intramolecular bonds in NA required an almost 10-fold higher concentration of ME than did reduction of intramolecular linkages in HA. This means that disulfide linkages in glycoproteins are screened to a different degree at the expense of the secondary and tertiary structure.

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