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A_2 (N2) Neuraminidase of the X-7 Influenza Virus Recombinant: Determination of Molecular Size and Subunit Composition of the Active Unit

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Neuraminidase activity of influenza virus was directly seen on sodium dodecyl sulfate polyacrylamide gels with the aid of the synthetic substrate, methoxyphenol neuraminic acid. Neuraminidase (NA) appeared as a high-molecular-weight fraction with a size in the range of 220,000 to 250,000 daltons. Isolation of this fraction from the X-7 strain of influenza virus, dissociation with sodium dodecyl sulfate, and reduction showed the presence of two polypeptides of 66,000 (NA_1) and 58,000 (NA_2) molecular weights in equimolar concentration. We postulate that the minimum active unit for the viral A_2 neuraminidase is a tetramer composed of two NA_1 and two NA_2 subunits.

The relative concentrations and molecular weights of the polypeptides of influenza virus have become well defined in the recent past with the aid of electrophoresis of the disrupted virus on polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate (SDS) (5, 2, 15). Isolation of aggregates possessing the ability to attach to red cells and determination of the subunit size has shown that the hemagglutinin (HA) consists of two polypeptides of 60,000 and 21,000 molecular weights (10). Neuraminidase (NA) of the X-7 influenza virus recombinant strain, isolated from cellulose acetate strips following electrophoresis in SDS, was found to possess one or two subunits with molecular weights of 58,000 (18).

With more precise separation of polypeptides possible on polyacrylamide gels, we have attempted to localize NA activity and determine the molecular size of the active unit by direct observation by using the substrate of Tuppy and Palese (16), 2-(3′-methoxyphenyl)-*N*-acetyl-α-neuraminic acid (MPN), a chromogenic substrate which, when incubated with NA in the presence of diazonium salt, produces a red precipitate at the site of activity (14, 16). Laver and Kilbourne (11) had shown that the A_2 NA of the X-7 strain is active after disruption of the virus with 1% SDS. By conventional means of detection with a modification of the Warren-Aminoff assay (1), we had been able to elute active NA from polyacrylamide gels of SDS-disrupted viral protein in the high-molecular-weight region of the gel (ca. 200,000), but had found no activity associated with any smaller polypeptides.

Active NA from the X-7 strain, after disruption with SDS under reducing conditions, was also found to be eluted from chromatographic columns of Bio-Gel A-5. These studies have also yielded additional information on the subunit structure of the NA.

MATERIALS AND METHODS

Preparation of virus. For our investigation we prepared influenza virus of the well-studied X-7 strain (8, 11) which grows to high titer in the allantoic sac of the chick embryo. Strain X-7 is the progeny of the A_0 (HON1) (19) strain NWS, from which it derives its HA (7), and the $A_2(H_2N_2)$ strain R15$^+$, from which it derives its NA (7). Therefore, according to the new World Health Organization system of viral nomenclature it is HA_{A_0} NA_{A_2} or HON2 (19).

Virus was prepared from the allantoic fluid of 12-day-old fertile eggs infected 45 hr previously with 10^3 EID_{50} of X-7 influenza virus. The virus was centrifuged to a 60% sucrose cushion and further purification attained by a second centrifugation through 30% sucrose to a 30/60% sucrose interface (9). All solutions were in 0.01 M sodium phosphate buffer, *p*H 7.0, containing 0.14 M sodium chloride and 0.001 M ethylenediaminetetraacetic acid.

Polyacrylamide gel electrophoresis (PAGE). Disc electrophoresis was performed by using the buffer systems of Davis (3) with 10% acrylamide gels with a low concentration (0.44%) of methylene bisacrylamide, the cross-linking agent. All solutions contained 0.1% SDS. Reducing gels were prerun for 1

hr with the separating gel buffer containing 0.01 M sodium thioglycolate; electrophoresis of the viral polypeptides was performed with 0.01 M sodium thioglycolate added to the 10× disc buffer of Davis (3). The virus sample was applied directly over the separating gel in 8% sucrose, containing methylene blue as tracking dye, with no sample or stacking gel used. Generally, a quantity of 50 to 100 micrograms of viral protein as determined by the Lowry assay (12) was applied to 5-mm diameter gels. For preparative purposes, the same procedure was employed, but 6-mm diameter gels were used with a protein application of 250 micrograms. Viral proteins were dissociated by incubation at 37 C for three hours in 1% SDS ± 0.01 M dithiothreitol (DTT). Electrophoresis was conducted at 2.5 ma/gel for 90 min. The length of the gel and the distance of migration of the tracking dye were measured. Proteins were fixed overnight in 7% acetic acid in 20% ethanol to remove residual SDS. Protein was stained with 0.5% Coomassie Brilliant Blue (Colab) in 7% acetic acid for several days. Destaining was performed electrophoretically in a Canalco horizontal destaining unit in 7% acetic acid. Areas and positions of the polypeptide bands were determined by scanning the gels at 580 nm for both Coomassie stain and MPN activity in a Gilford spectrophotometer equipped with linear transport.

Localization of enzymatic activity. To determine enzymatic activity after electrophoresis of viral proteins, gels were incubated in 0.1 M sodium phosphate buffer, *p*H 6.5, for 30 min. A 1.0-mg mixture of MPN and 1.0 mg of diazonium salt of 4-amino-2,5-dimethoxy-4′-nitroazobenzene (Koch and Light, Colnbrooke, England) (16) in 0.5 ml of 0.2 M sodium phosphate buffer containing 0.1 M $CaCl_2$, *p*H 6.5, was added to the gel and incubated at 37 C for 10 min. The gels were placed in 7% acetic acid for storage.

When the enzyme was isolated from 6-mm gels, the gels were stained for NA activity with the aid of MPN as described above. The active bands were sliced from the gels, placed in a solution of 0.1% SDS and 0.05% sodium azide 50 times their volume, and stirred at room temperature for 5 days to elute the NA. The supernatant fluid was concentrated to less than 1 ml for a pool of 12 gels by pervaporation and was followed by dialysis versus 0.01 M sodium phosphate buffer, *p*H 7.0, 0.14 M sodium chloride, and 0.05% sodium azide.

Determination of molecular weight. Band positions and intensities were determined by scanning the gels in a Gilford spectrophotometer equipped with linear transport (model 2410). Molecular weights were determined by coelectrophoresis of viral proteins with ribonuclease (bovine pancreas), molecular weight of 13,700; α-chymotrypsinogen A (bovine pancreas), molecular weight of 25,700; ovalbumin, molecular weight of 43,000; serum albumin (bovine), molecular weight of 68,000; and myosin (main polypeptide chain), molecular weight of 200,000 (17). The myosin was a gift from Pearl Appel. The remaining proteins were purchased from Schwarz/Mann.

Column chromatography. Bio-Gel A-5 (Bio-Rad) was equilibrated with the elution buffer, 0.02 M tris(hydroxymethyl)aminomethane (Tris)-Cl, *p*H 7.4, containing 0.1% SDS, 0.01 M sodium thioglycolate, and 0.05% sodium azide at room temperature. The chromatographic column consisted of two Pharmacia columns (2.5 × 90 cm) connected in tandem with flow adapters in an upward-flow mode maintained at a flow rate of 15 ml per hr and collected in 5-ml fractions. Virus of the X-7 strain (20 mg of protein) was dissociated by heating at 37 C with 2% SDS and 0.1 M sodium thioglycolate for 90 min and applied in a 2-ml volume. The column was calibrated with bovine serum albumin, ovalbumin, and α-chymotrypsinogen. Under these conditions of dissociation with SDS and reduction, the molecular weights of the polypeptides can be readily determined (4). The void volume was determined with Blue Dextran (Pharmacia). Fractions were collected with the aid of an LKB fraction collector and monitored at 280 nm with an LKB Uvicord with recorder.

NA was assayed directly in the fractions by a modification of the Warren-Aminoff (1) procedure with 0.050-ml samples at 0 and 30 min time intervals after incubation with fetuin at 37 C. Fractions were concentrated by pervaporation, followed by dialysis versus 0.02 M Tris-HCl buffer, *p*H 7.4, containing 0.05% sodium azide.

Density gradient centrifugation. Density gradient centrifugation was performed according to the method of Martin and Ames (13), modified by the addition of 0.1% SDS with 0.01 M sodium thioglycolate in 5 to 20% linear sucrose gradients in 0.02 M Tris-HCl buffer, *p*H 7.4. Bovine serum albumin (with 0.1% SDS and 0.01 M sodium thioglycolate) was used as the calibration protein, having an assumed sedimentation rate of 4.4*S*. Sedimentation was performed at 40,000 rev/min in a Beckman Spinco L2-65B preparative ultracentrifuge with an SW41 rotor. NA activity was assayed in 0.025-ml samples directly from the sucrose gradient (1).

RESULTS

Localization of enzyme activity. Early assays, using MPN as the substrate, showed a high-molecular-weight band of enzymatic activity of about 200,000 daltons on the polyacrylamide gels after disruption of the virus with 1.0% SDS However, a large quantity of active enzyme remained at the origin, and a significant amount of activity was distributed from the 200,000 band to the top of the gel. As measured by a modification of the Warren-Aminoff assay (1), 50% of the enzymatic activity remained at the top of the gel, even when a 2.5% polyacrylamide gel was used. With the addition of reductant, 0.01 M DTT, all of the enzyme migrated as a single band with no residual enzymatic activity remaining at the top of the gel. Similar results were obtained with a direct-activity stain with MPN on PAGE, although resolution of the protein bands was poor on this set of gels. The gels are shown in Fig. 1.

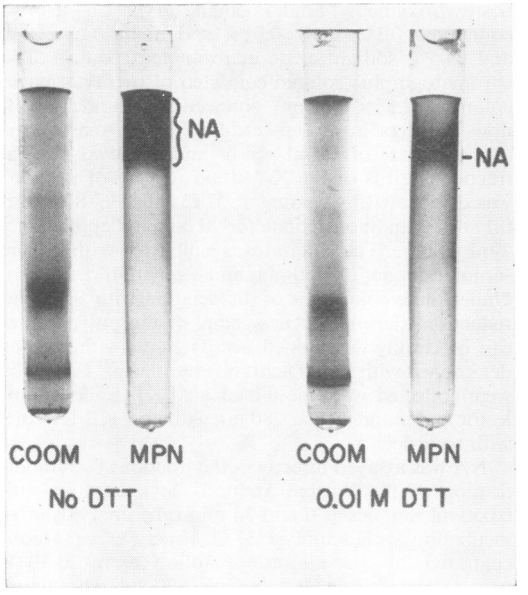

FIG. 1. *Neuraminidase activity shown for X-7 strain after dissociation with 1.0% SDS ± 0.01 M DTT. Electrophoresis was performed in the presence of 0.1% SDS, no sodium thioglycolate. The direction of electrophoresis is downward. Gels were stained for protein with Coomassie Brilliant Blue (COOM) or for neuraminidase activity (MPN).*

Strain comparison. Strain X-7 and its parental strains, RI5$^+$ and NWS, were disrupted with 1% SDS and 0.01 M DTT. After PAGE, the gels were stained for protein and activity with MPN determined. Results are presented in Fig. 2. Strains X-7 and RI5$^+$ both possessed strong MPN activity bands as anticipated, because the X-7 NA is derived from the RI5$^+$ parent. NWS subjected to electrophoresis under these conditions had no demonstrable activity reminiscent of the failure to resolve a polypeptide fraction with NA activity in earlier studies (11). Furthermore, there were no high-molecular-weight polypeptides in any appreciable concentration on the NWS gel, unlike the gels for X-7 and RI5$^+$. Differences in polypeptide composition have been consistently noted in these strains. The dark areas in the center of the NWS and X-7 MPN gels were brown rather than red, indicating incomplete removal of the diazonium salt, not activity of the enzyme. Activity with MPN was also detected in the same area for other strains containing the A_2 NA, including RI5$^-$ and the recombinant strains X-7 (F1) and X-31, the latter being antigenically indistinguishable from HK/Aichi/68 (H3N2) (9).

Molecular weight determination. The molecular weight of the enzyme-active band with MPN was determined to be 220,000 to 250,000, based on the molecular weights of myosin, the serum albumin dimer, and serum albumin. With increased time of electrophoresis and migration through the gel, the enzyme band broadened beyond that expected from diffusion and decreased in total MPN activity. This behavior suggested to us that the enzyme was dissociating on the gel.

Subunit composition. When the enzyme was isolated from preparative gels and resubjected to electrophoresis after being subjected to mild reduction with 0.01 M DTT and dissociation with 1.0% SDS, the enzyme appeared as a single high-molecular-weight band with faint representation of two smaller polypeptides as shown in Fig. 3. With more stringent conditions of reduction, dissociation with 0.1 M DTT, and electrophoresis in the presence of 0.01 M sodium thioglycolate, two polypeptides were found migrating slightly ahead of serum albumin and the high-molecular-weight band had disappeared (Fig. 4). The molecular weights were determined to be 66,000 (NA_1) and 58,000 (NA_2). The Na_1 band was relatively sharp, whereas the NA_2 band was diffuse.

The Coomassie-stained gels were scanned in the spectrophotometer, and the ratio of the areas of NA_2-NA_1 measured by triangulation was found to be 0.87 ± 0.05, based on an average of three preparations as shown in Fig. 4. If the chains are represented equally in the functioning unit of enzyme, the ratio predicted would be 0.88, based on the molecular weights of 58,000 and 66,000. From the value of 220,000 to 250,000 minimum molecular weight for a functioning unit of enzyme, we would suggest that the enzyme is a tetramer composed of two NA_1 chains and two NA_2 chains in its active enzymatic form with a total molecular weight of 248,000.

No enzymatic activity could be detected in the area in which the polypeptides of 50,000 to 70,000 daltons would be expected to elute from preparative gels under reducing or nonreducing conditions. No activity was seen in any region other than the area of gel to which the 220,000 to 250,000 polypeptides would be expected to migrate.

Percent of composition. An estimate of the percent of the total virus protein represented by the NA was made by measuring enzymatic activity by the Warren-Aminoff assay and comparing that with the percent of protein represented by the high-molecular-weight band. Assuming that the enzyme maintains the same specific activity after isolation, we estimate that because the

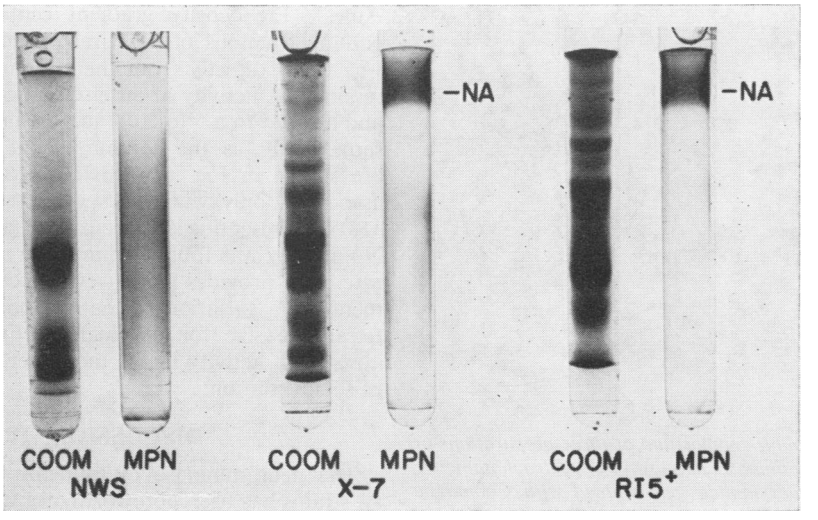

FIG. 2. *Localization of activity for three strains of influenza virus after dissociation with 1.0% SDS and 0.01 M DTT. Electrophoresis was performed in the presence of 0.1% SDS, no sodium thioglycolate. The direction of electrophoresis was downward. Gels were stained for protein with Coomassie Brilliant Blue (COOM) and for neuraminidase activity (MPN) for each strain. These gels afforded superior resolution of the polypeptides as compared with those in Fig. 1.*

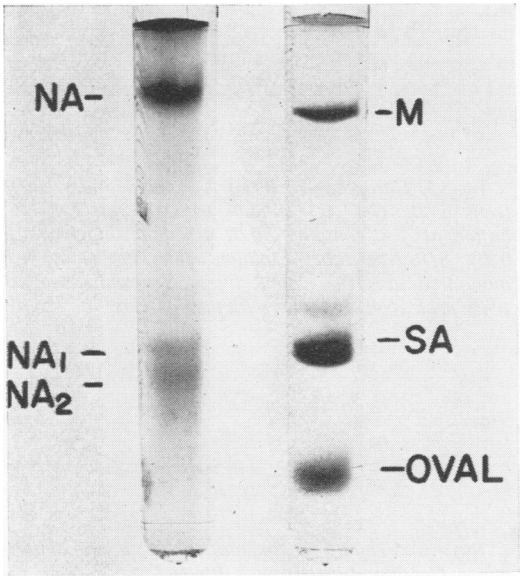

FIG. 3. *PAGE of enzyme preparation following treatment with 1.0% SDS and 0.01 M DTT showing a single band for the enzyme (NA) and the minor presence of the subunits NA_1 and NA_2. Electrophoresis was performed in the presence of 0.1% SDS, no sodium thioglycolate. The direction of electrophoresis was downward. The gel on the right was subjected to electrophoresis in parallel and shows the positions of myosin (M), serum albumin* (SA), *and ovalbumin (OVAL).*

enzyme accounts for 25% of the original activity (activity before electrophoresis) in the high-molecular-weight band with the SDS disruption and represents 1.5 to 2.5% of the total protein as measured by scanning of Coomassie-stained gels, 6 to 10% of the original associated protein was NA. Gel analysis of X-7 virus, dissociated with 1% SDS plus 0.01 M DTT in which 35% of original activity is associated with 3 to 4% of the total protein, suggests that 9 to 12% of the total viral protein is NA.

Column chromatography. Addition of a reductant appeared to be necessary to maintain activity of the NA on elution from the column. With SDS alone added to the Tris buffer, no activity was found in the eluant in any fraction. If the virus preparation was reduced before application to the column but no reductant was present in the buffer, the activity was diminished and variable. With reduction of the virus preparation and elution with a buffer containing 0.01 M sodium thioglycolate, enzymatic activity was recovered in an essentially 100% yield.

The NA activity was centered at fraction 110 and represented a molecular weight, at elution, of about 53,000. The other main optical density peaks were: fraction I, >250,000 (the void volume); fraction II, 61,000; fraction III, 42,000; and fraction IV, 24,000. The elution pattern is shown in Fig. 5.

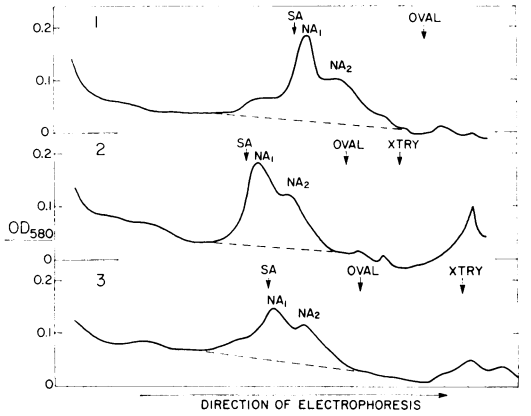

FIG. 4. *Subunit composition of three preparations of isolated neuraminidase, as shown by spectrophotometric scans of polyacrylamide gels stained with Coomassie Brilliant Blue. The enzyme was dissociated in the presence of 1.0% SDS and 0.1 M DTT. Electrophoresis was performed in buffer containing 0.1% SDS and 0.01 M sodium thioglycolate. Positions are shown for marker proteins subjected to electrophoresis on parallel gels, including serum albumin (SA), ovalbumin (OVAL), and α-chymotrypsinogen (XTRY). The broken line shows baseline used for triangulation of peaks for determination of areas of NA_1 and NA_2. The ratios of areas found for NA_2/NA_1 were 1 = 0.87, 2 = 0.84, and 3 = 0.93.*

On analysis by PAGE, fraction I would not enter the gel and was comprised of a mixture of ribonucleic acid and protein, II consisted mainly of a protein containing no carbohydrate and was believed to represent the nucleoprotein (2, 15), III contained carbohydrate and was considered to be the large glycopeptide of the (HA) (2, 15), and the main protein of IV contained no carbohydrate and was thought to be the small internal protein (2, 15).

On PAGE of the NA-active fraction, MPN activity was found to be associated only with a high-molecular-weight band of about 240,000. No activity was found associated with any smaller unit. Apparently the enzyme was dissociated to its monomeric units during gel filtration and reassociated upon elution from the column.

Preparative electrophoresis of the NA-active fraction from the column, isolation of the high-molecular-weight band, and re-electrophoresis of this fraction again demonstrated the existence of two subunits of 66,000 and 58,000 molecular weights present in a 1:1 ratio.

Density gradient centrifugation. Additional evidence of a reassociated NA after column chromatography was provided by Martin and Ames' (13) density gradient centrifugation of column fractions of the viral protein. A sample was taken directly from the column eluate possessing NA activity at an elution point of 53,000 and applied to a 5 to 20% sucrose gradient in the same buffer as the column eluant. The results are shown in Fig. 6. Activity was found to be associated only with the species sedimenting at 11.0S, representing a molecular weight of 270,000. No activity was found sedimenting at any slower rate. This provides further evidence of a requirement for association of the monomeric units to tetrameric size for enzymatic activity and the absence of activity in the monomeric range (4S) of sedimentation.

DISCUSSION

The neuraminidase of influenza virus of the A_2 strains has been noteworthy for its remarkable property of maintenance of activity under the usual denaturing conditions of heat and disruption with SDS. This property has enabled us to

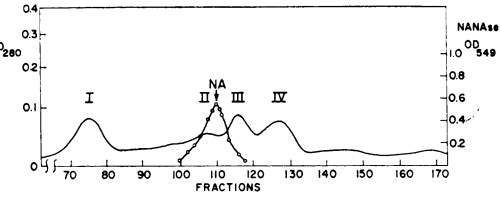

FIG. 5. *Chromatography of X-7 polypeptides eluted from a Bio-Gel A-5 column with 0.02 M Tris-HCl buffer, pH 7.4, containing 0.01 M sodium thioglycolate, 0.1% SDS, and 0.05% sodium azide. The eluate was monitored at OD_{280} (solid line). Neuraminidase activity peak (NA) is shown with open circles.*

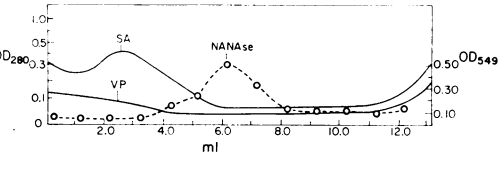

FIG. 6. *Velocity sedimentation of neuraminidase active fraction eluted from a Bio-Gel A-5 column run on a sucrose density gradient in 0.02 M Tris-HCl buffer, pH 7.4, containing 0.01 M sodium thioglycolate, 0.1% SDS, and 0.05% sodium azide. Centrifugation was for 8 hr at 40,000 rev/min with a Beckman SW41 rotor at 18 C. Elution of the gradient was monitored at OD_{280} for the viral protein fraction (VP) which contained neuraminidase activity. The absorption profile is also shown for serum albumin (SA). Based on an assumed sedimentation rate of 4.4S for serum albumin, the neuraminidase had a sedimentation rate of 11.0S.*

directly observe enzymatic activity on acrylamide gels following electrophoresis in the presence of SDS and to determine the molecular weight of the active unit of enzyme. By our techniques we have estimated this unit to be 220,000 to 250,000 daltons.

Applying the same techniques on a preparative scale for isolation of the enzyme and subjecting this isolated enzyme to re-electrophoresis under conditions of reduction, we find the enzyme to be dissociated into two subunits, NA_1 (66,000) and NA_2 (58,000). Scanning of Coomassie-stained gels showed the subunits were present in a 1:1 ratio. Our findings suggest that the enzyme is tetrameric, with a total molecular weight of 248,000.

Column chromatography of the dissociated X-7 virus resulted in the elution of active NA at a volume indicating a molecular weight of 53,000. However, gel electrophoresis followed by determination of activity showed a reaggregation of the enzyme after elution with activity associated only with the high-molecular-weight band. Analysis of the column eluate by sucrose density gradient centrifugation also demonstrated activity associated only with the aggregated state. Because earlier attempts at renaturation of activity of the enzyme after dissociation to the subunits had proved futile, the information gained from column chromatography assured us that dissociation to the monomeric units was not irreversible. Preparative electrophoresis of the partially purified enzyme from the column and re-electrophoresis under conditions of reduction again demonstrated the presence of two subunits.

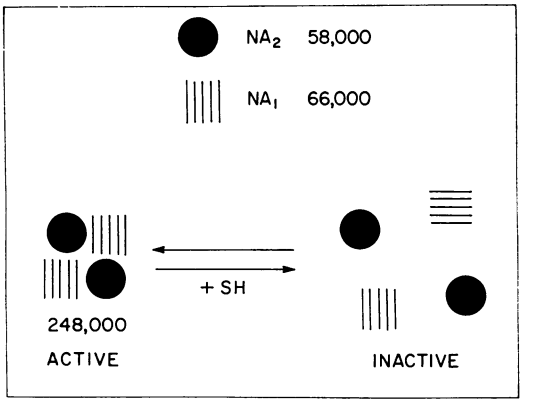

FIG. 7. *Proposed model for neuraminidase as a tetramer composed of subunits NA_1 and NA_2 requiring association to a 248,000 molecular weight unit for activity. A reversible dissociation to inactive monomers facilitated by the presence of sulfhydryl reagents is shown.*

A working model which would incorporate our experimental findings is presented in Fig. 7. Essentially, the model shows an active tetrameric enzyme in equilibrium with its monomeric subunits which are inactive. The equilibrium is apparently facilitated by the presence of a reducing agent.

The effect of reduction appears to be operative at two levels. First, there is a requirement for reduction of those disulfide bonds which restrict the release of the NA from the envelope by disruption with 1% SDS alone, as shown in our early attempts at localization of the enzyme-active band. In this case, lack of reductant resulted in about half of the enzyme remaining at the origin of the polyacrylamide gel. With the addition of 0.01 M DTT, all of the enzyme is able to enter the gel. At this level of reduction, enzymatic activity remains unaffected.

At higher levels of reduction using 0.1 M DTT, we have a second level at which disulfide bonds are broken. With reduction of these bonds, the equilibrium is shifted toward the monomeric subunits. We suggest that these are intrachain disulfide bonds since there does seem to be dissociation on the acrylamide gels in the absence of reduction. Interchain disulfides would, of course, require reduction before dissociation could proceed. In this instance, reduction seems merely to facilitate the dissociation process. At this level of reduction and dissociation of the molecule, enzymatic activity is lost.

Taking into account differences in techniques of electrophoresis and isolation of the enzyme from strain X-7, our results did not differ substantially from Webster (18), who isolated neuraminidase from cellulose acetate strips and determined the molecular weight by gel chromatography in guanidine-HCl. Webster noted the likelihood of the NA possessing two different subunits. A recent paper by Kendal and Eckert (6) suggests that X-7 influenza NA, isolated with the aid of nagarse, exists as a tetramer. However, they find only one subunit species.

To summarize, our results suggest that the minimal functioning unit of influenza virus NA is a tetramer of 248,000 molecular weight, composed of two chains of 66,000 and two chains of 58,000. Sulfhydryl reagents facilitate dissociation of the enzyme to its monomeric units.

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