BBA 66532

THE PREPARATION AND PROPERTIES OF ^{14}C-CARBOXAMIDOMETHYLATED SUBUNITS FROM A_2/1957 INFLUENZA NEURAMINIDASE

ALAN P. KENDAL AND EDWARD A. ECKERT
with amino acid analysis by PHILIP COHEN*
*Department of Epidemiology, School of Public Health, University of Michigan, 109 Observatory Street, Ann Arbor, Mich. 48104 (U.S.A.) and *Department of Biochemistry, University of Washington, Seattle, Wash. 98105 (U.S.A.)*

(Received August 30th, 1971)

SUMMARY

A_2/1957 influenza neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18) was purified 15-fold from a recombinant virus, with about 25% overall yield of enzymic activity. Neuraminidase contained glucosamine, and a high proportion of serine and threonine. The partial specific volume was 0.713 cm^3/g. Reduced neuraminidase was isotopically labeled *in vitro* by reaction with iodo[^{14}C]-acetamide. When carboxamidomethylated in the absence of urea, enzymically inactive labeled material was obtained with a maximum size similar to native neuraminidase. When carboxamidomethylated in the presence of 6 M urea, labeled, dissociated subunits were obtained that did not associate or regain enzymic activity on removal of urea. The molecular weight of dissociated subunits was determined by sedimentation–diffusion methods as 50 000–54 000, and by sodium dodecyl sulfate–acrylamide gel electrophoresis as about 50 000. Thus native neuraminidase (mol. wt. about 200 000) is probably a tetramer. Neuraminidase contained about 21 cysteine residues per subunit. These appear to be present as disulfide bonds in the native enzyme.

INTRODUCTION

Proteolytic degradation of influenza virus has shown that neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18) is located in an outermost layer of functional viral proteins, which also contains the viral hemagglutinin[1–7]. Removal of this protein layer leaves biologically inactive degraded virus particles, containing about 50% of the total viral protein in the structural layer of the virus envelope[3–5]. Although neuraminidase thus represents only about 5–10% of the total viral protein[1,4], it is antigenic[8,9], and neuraminidase antibodies appear to partially protect animals against influenza infection[10,11]. However, considerable variation in antigenic and enzymic properties has been observed between different influenza neuraminidases[8,12,13], and the chemical basis for those differences needs to be

established. We report here on the amino acid composition and subunit structure of $A_2/1957$ neuraminidase, highly purified from the recombinant virus X-7(FI). A procedure for the *in vitro* ^{14}C labeling of neuraminidase was developed, in order that microgram quantities of enzymically inactive material could be characterized.

MATERIALS

Nagarse protease (subtilisin BPN[1], EC 3.4.4.16) was obtained from Miles-Seravac, Maidenhead, England; fetuin from Nutritional Biochemicals and Colorado Serum Company; ovalbumin from Nutritional Biochemicals; bovine serum albumin and yeast alcohol dehydrogenase from Pentex. Molecular weights (M_r) and sedimentation coefficients ($s_{20,w}$) of reference proteins were taken as: ovalbumin, $M_r = 43\,500$, $s_{20,w} = 3.66$ S; bovine serum albumin, $M_r = 67\,000$, $s_{20,w} = 4.4$ S; and yeast alcohol dehydrogenase $M_r = 150\,000$, $s_{20,w} = 7.4$ S (refs. 14,15). The Stokes' radius was calculated for each protein from the modified Svedberg equation (Eqn. 1, see RESULTS) assuming $\bar{V} = 0.733$ cm^3/g. "Ultrapure" grade urea (Schwarz/Mann) was used without further purification. All buffers contained 0.02% sodium azide. Iodo[1-^{14}C]acetamide (specific activity 52–58 mC/mmole) and reference standard *n*-[^{14}C]hexadecane were products of Amersham-Searle.

METHODS

Virus purification

Recombinant virus X-7(FI), grown in chicken eggs, was concentrated from allantoic fluid by pelleting at 70 000 × *g* for 1 h. Virus was resuspended in 0.1 M Tris–HCl, pH 7.5, briefly sonicated, and centrifuged for 45 min at 25 000 rev./min through a discontinuous sucrose density gradient in the Spinco SW 25.1 rotor. The gradients were prepared by layering in transparent tubes 2 ml of 60%, 7 ml of 48%, 36% and 24%, and 5 ml of 12% sucrose (w/v in 0.1 M Tris–HCl, pH 7.5). Virus recovered from a white opalescent band midway down the gradient was diluted with 0.1 M Tris–HCl, pH 7.5, pelleted as before, resuspended in 0.1 M Tris–HCl, pH 8.5, containing 10^{-3} M $CaCl_2$, and briefly sonicated.

Sucrose density gradient centrifugation

All sucrose solutions were prepared w/v in 0.1 M Tris–HCl, pH 7.5.

(a) 15–30% gradients used for purifying neuraminidase were prepared by placing 1.2 ml layers of 30, 24, 18 and 15% sucrose in 0.5 inch × 2 inch centrifuge tubes.

(b) 6–30% gradients used for determining sedimentation coefficients were prepared by placing 0.95 ml layers of 30, 24, 18, 12 and 6% sucrose in 0.5 inch × 2 inch centrifuge tubes. After centrifugation, gradients containing radioactive samples were reproducibly fractionated using a tube piercing apparatus, and equal numbers of drops collected directly into vials of scintillation fluid. Reference proteins (5 mg) were centrifuged in separate gradients, and equivalent fractions collected in tubes, diluted to 1 ml with water, and their ultraviolet absorbance measured.

Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

This was performed by the procedure of SUMMERS *et al.*[16] using 7.5% acrylamide

gels. Samples for electrophoresis were dissolved by boiling for 2–3 min in the buffer containing sodium dodecyl sulfate and β-mercaptoethanol. Gels were stained with Coomassie blue[17].

Determinations

(a) Radioactivity was counted by adding aqueous chromatography or density gradient fractions to 10 ml of scintillation fluid containing 0.9% 2,5-bis(5′-*tert.*-butylbenzoxalyl-2′)thiophene in toluene–Triton X-100 solvent (9:1, v/v)[4]. The aqueous content was made up to 4 or 5 ml with water, and ^{14}C radioactivity in the resulting emulsion was counted in an Ansitron spectrometer. Using *n*-[^{14}C]hexadecane as an internal standard, the counting efficiency was 60%.

(b) Protein was determined by the Lowry method, as described by LEGGETT-BAILEY[18], using bovine serum albumin as a standard.

(c) Viral hemagglutinin was titrated by microtitre method, using 0.5% chicken erythrocyte suspension. Hemagglutinin antibodies were titrated by standard hemagglutination-inhibition procedure[19]. Hemagglutinin-blocking antigen activity was titrated as described for A_0/PR8 virus by ECKERT[20]. In the present case, samples were tested for competition with X-7(FI) virus antigen for antibodies in rabbit anti-X-7(FI) serum.

(d) Neuraminidase enzymic activity was detected by incubation with fetuin substrate at 37° in 0.1 M phosphate buffer, pH 6.0. As the sialic acid content of different fetuin batches varied from about 4–6% (determined after 1 h hydrolysis at 80° with 25 mM H_2SO_4), fetuin concentrations were used so that a standard sialic acid concentration, 70 μg/ml, was present in enzyme reaction mixtures. A unit of neuraminidase is defined as the amount of enzyme liberating 1 mg of sialic acid from 0.5 ml of fetuin solution in 15 min under the conditions described. Free sialic acid was determined by the method of AMINOFF[21] using synthetic *N*-acetylneuraminic acid (Sigma) as a standard. Measurement of neuraminidase activity for determining yield and specific activity were made in the linear range of the enzyme assay (0–8 μg of sialic acid released). Neuraminidase antibodies were titrated as described previously, using Cohn serum Fraction IV-4 as enzyme substrate[22].

(e) Amino acids and amino sugars were determined using a Spinco-Model 120 automatic recording analyzer, equipped with electronic integrator. Neuraminidase samples were hydrolyzed for 24 and 48 h in 5.8 M HCl *in vacuo* at 110 ± 1°, after repeated flushing with nitrogen[23]. After hydrolysis the HCl was removed *in vacuo*. Total cysteine content was determined on duplicate samples after oxidation with performic acid[24]. One neuraminidase sample was hydrolyzed with 3 M *p*-toluene sulfonic acid, including 1 mg of 3-(2-aminoethyl)indole to prevent tryptophan destruction[25].

RESULTS

Stability of X-7(FI) neuraminidase

Recombinant virus X-7(FI) and A_2/1957 viruses have neuraminidases of similar antigenic specificity and enzyme kinetics[26–28]. However, recombination of influenza viruses can result in altered stability of neuraminidase without concomitant change in antigenic or enzymic properties[29]. Table I shows the similar heat stability of X-7(FI)

TABLE I

HEAT INACTIVATION OF INFLUENZA VIRUS NEURAMINIDASE AT 56°

Virus	*Neuraminidase inactivation (%)**
A_0/PR8/34	>95
A_1/FMI/47	>95
A_2/R15$^+$/57	35
Recombinant X-7 (FI)	44
A_2/Aichi/1/68 ("Hong Kong" influenza)	>95

* 1:5 dilutions of each virus allantoic fluid were incubated at pH 6.0 for 1 h at 56°, and their neuraminidase activities measured as described in METHODS.

and A_2/1957 neuraminidases, compared to other influenza neuraminidases. This further justifies the use of X-7(FI) recombinant virus as a source of A_2/1957 influenza neuraminidase.

Isolation of neuraminidase

0.5-ml aliquots of X-7(FI) virus concentrates, purified as described in METHODS, were incubated at 37° with 2 mg of nagarse for 6 h and then for 16 h with a further 5 mg of nagarse, thereby destroying 90–99% of viral hemagglutinating activity. The virus digests were sedimented through linear gradients of 12–60% sucrose solutions (w/v in 0.1 M Tris–HCl, pH 7.5), so that degraded virus particles[4,5] separated from the bulk of neuraminidase that had been released from the virions (Fig. 1). Neuraminidase in the upper 2 or 3 fractions was recovered, and 0.5-ml aliquots centrifuged for 16 h through 15–30% sucrose gradients. The neuraminidase separated well from the bulk of low molecular weight protein near the top of the gradient (Fig. 2). Peak fractions of neuraminidase activity were pooled, dialysed exhaustively against glass distilled water, and finally centrifuged in the SW 39 rotor for 1 h at 38 000 rev./min to remove

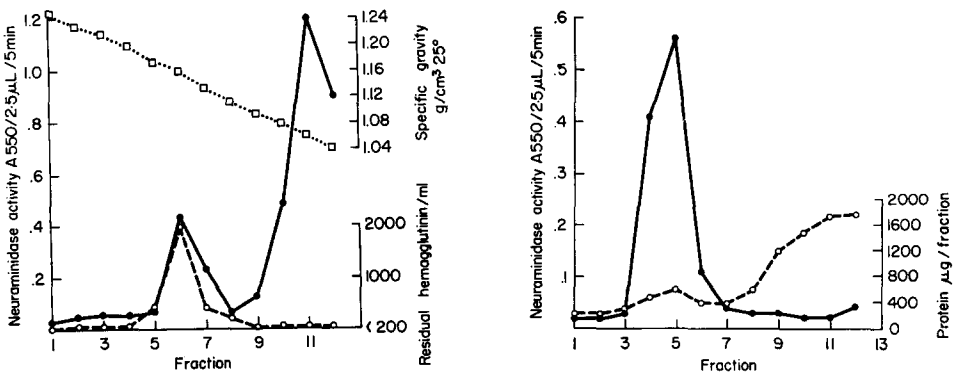

Fig. 1. Density gradient centrifugation of nagarse-treated influenza virus X-7 (FI). Sedimentation (right to left) was for 1.5 h at 38 000 rev./min in the SW39 rotor through a 12–60% (w/v) sucrose gradient. ●—●, neuraminidase activity; ○---○, residual hemagglutinating activity; □---□ specific gravity.

Fig. 2. Purification of X-7(FI) neuraminidase. Neuraminidase was centrifuged for 16 h at 38 000 rev./min in the SW39 rotor at 10° through a 15–30% (w/v) sucrose gradient. ●—●, neuraminidase activity; ○---○, protein. Sedimentation was from right to left.

any residual large impurities. Supernatant fluid was carefully withdrawn, and an aliquot assayed for enzyme activity and protein concentration, in parallel with samples of the initial untreated virus. Mean values from 12 experiments were: overall yield of neuraminidase activity, 24% (range 10 to 36); overall purification factor, 15 (range 5 to 30); and neuraminidase specific activity, 4.6 enzyme units/μg protein (range 2.4 to 7.8). Purified neuraminidase preparations were freeze-dried. This procedure for purifying neuraminidase was slightly modified from that previously described[4].

Purity of isolated neuraminidase

Purified X-7(FI) neuraminidase had no hemagglutinating activity, and did not contain serologically active hemagglutinin fragments when tested for blocking antigen activity. Immunization of rabbits failed to elicit hemagglutinin (HI) antibodies, but high-titer neuraminidase (NI) antibodies were obtained (Table II). Sodium dodecyl

TABLE II

PRODUCTION OF ANTI-NEURAMINIDASE SERUM

Time	*Action*	*Serum antibody titres*	
		*Haemagglutinin**	*Neuraminidase***
Day 0	Normal serum bleed. Foot-pad inoculation with X-7 (FI) neuraminidase, as adjuvant emulsion	<8	<20
Week 7	Sample bleed	<8	5800
Month 9	Sample bleed	<8	300
	Intravenous booster inoculation of X-7 (FI) neuraminidase		
Week 1 post boost	Sample bleed	<8	8000
Week 2 post boost	Sample bleed	<8	8000

* Periodate-treated sera *vs.* X-7(FI) antigen.

** Untreated-sera *vs.* homologous $A_2/R15^+/57$ enzyme of X-15 virus (ref. 37).

Two other rabbits immunized with different neuraminidase preparations produced similar antibody response after 6–7 weeks.

sulfate–acrylamide gel electrophoresis of reduced neuraminidase revealed a single sharp band of molecular weight about 50 000, when compared to ovalbumin and bovine serum albumin markers (Fig. 3). These results showed that neuraminidase had been completely separated from viral hemagglutinin, and that only trace amounts of contaminating proteins could be present in purified neuraminidase, which was therefore considered suitable for further study.

Amino acid composition of neuraminidase

Reproducible analyses were made on 5 samples of 100–150 μg from 3 different neuraminidase preparations, as described in METHODS. From the amino acid composition summarized in Table III, the partial specific volume ($\bar{V}$) of neuraminidase was calculated as 0.713 cm^3/g.

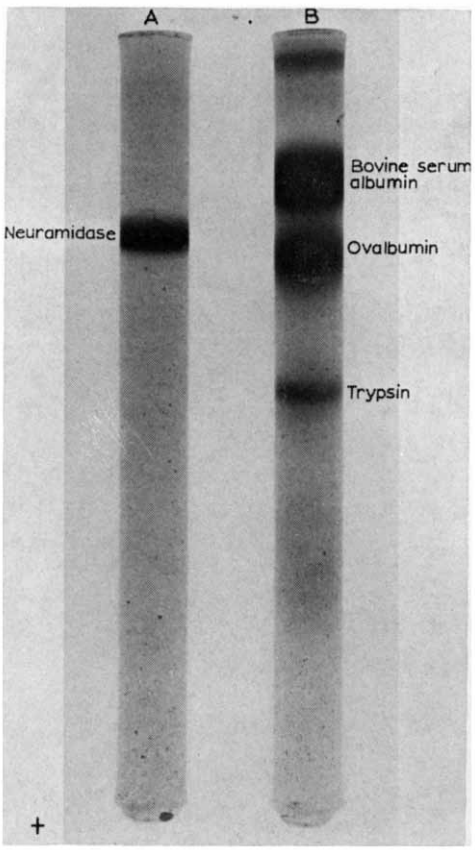

Fig. 3. Sodium dodecyl sulfate–acrylamide gel electrophoresis of reduced X-7(FI) neuraminidase. (A) 20 μg of neuraminidase. (B) Marker proteins bovine serum albumin, ovalbumin and trypsin.

Although tryptophan was never detected, this is not conclusive evidence for its absence. Glucosamine was present, confirming by direct analysis that influenza neuraminidase is a glycoprotein[6,7]. The high proportion of serine and threonine in neuraminidase may be relevant to its glycoprotein nature. Total cysteine was also present in high proportion.

^{14}C-Carboxamidomethylation of isolated neuraminidase

Stock Tris–HCl buffer was maintained in a degassed state under partial vacuum before use in the following procedure. Aliquots (20–60 μg) of purified neuraminidase in 75-μl volumes of 6 M urea, 0.066 M Tris–HCl, pH 7.5, were heated to 100° for 15 min in the presence of 0.4 μmole of dithiothreitol. To lower the dithiothreitol concentration, the denatured, reduced neuraminidase was then chromatographed through a 0.38 cm^2 × 5 cm column of Sephadex G-50, equilibrated with 6 M urea in the Tris–HCl buffer and 0.04 mM dithiothreitol. Material of molecular weight greater than about 10 000 eluting from the column was collected in a 0.6-ml volume, and immediately 0.1 μmole of iodo[1-^{14}C]acetamide (specific activity 52–58 mC/mmole) was added, and

TABLE III

AMINO ACID COMPOSITION OF A_2/1957 INFLUENZA VIRUS NEURAMINIDASE

Amino acid	*Molar ratio relative to aspartate*		*Residues (M_r per 54 000)*
	Experimental range	*Mean value*	
Lysine	0.263–0.278	0.273	20.3
Histidine	0.122–0.136	0.130	9.7
Arginine	0.383–0.409	0.400	29.8
Aspartate	1.000	1.000	74.3
Threonine	0.388–0.432*	0.413*	30.7
Serine	0.755–0.849*	0.792*	59.0
Glutamate	0.525–0.549	0.537	40.0
Proline	0.295–0.329	0.313	23.3
Glycine	0.673–0.703	0.681	50.6
Alanine	0.200–0.211	0.204	15.3
Valine	0.471–0.512	0.486	36.1
Methionine	0.120–0.129	0.125	9.3
Isoleucine	0.451–0.463	0.459	34.1
Leucine	0.238–0.284	0.258	19.4
Tyrosine	0.163–0.176	0.169	12.6
Phenylalanine	0.196–0.205	0.199	14.8
Total cysteine	0.287–0.289	0.288	21.4
Tryptophan	Not detected		
Amino sugars			
Glucosamine	0.075–0.077	0.076	5.7
Galactosamine	Not detected		

* Assumes 5% loss of threonine and 10% loss of serine during analysis.

the mixture incubated in the dark at 37° for 4 h. No neuraminidase activity was recovered after this treatment.

Properties of ^{14}C-carboxamidomethylated neuraminidase

(a) Sephadex gel filtration. Neuraminidase–iodoacetamide reaction mixtures described above were examined after 4 h incubation by chromatography through a column of Sephadex G-100 (2 cm^2 × 60 cm) eluted in series with a column of Sephadex G-200 (2 cm^2 × 30 cm). Protein that had incorporated ^{14}C label eluted as a single sharp peak, well separated from the bulk of excess iodoacetamide at the total volume of the column series (Fig. 4A). Calibration of the columns with reference proteins showed that the ^{14}C-labeled material obtained from neuraminidase reproducibly eluted from the columns in about the same position as native bovine serum albumin (Fig. 4B), and had a Stokes' radius of 37 Å determined by graphical interpolation using the relationship of LAURENT AND KILLANDER (see ref. 15). Untreated neuraminidase eluted slightly behind the void volume (V_0) of the column series, ahead of yeast alcohol dehydrogenase (Fig. 4B) in agreement with the previously observed behavior of active neuraminidase on Sephadex G-200 columns[4]. From these results it was clear that by means of the carboxamidomethylation procedure described above, isotope labeled, dissociated subunits were prepared from neuraminidase. The subunits did not reassociate on removal of urea during gel chromatography.

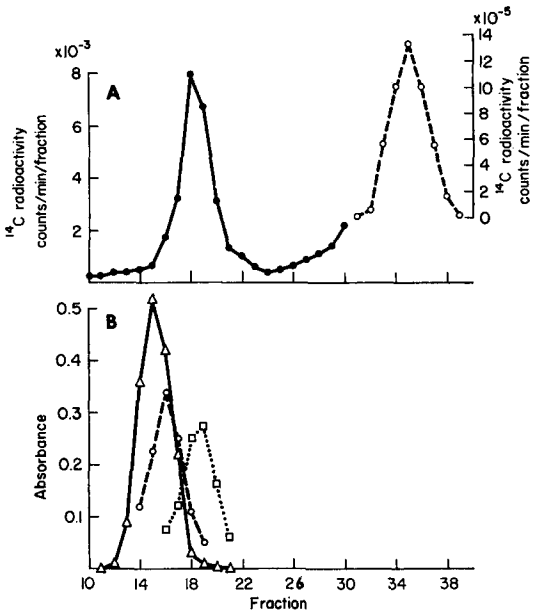

Fig. 4. Chromatography of ^{14}C-labeled dissociated neuraminidase subunits on a series of Sephadex G-100 and G-200 columns. (A) Elution of: ●—●, labeled subunits (× 10^3 scale); and ○---○, unincorporated iodo[^{14}C]acetamide, (× 10^5 scale). (B) Elution of: △—△, native neuraminidase, enzymic activity $A_{550\ nm}$; ○---○, yeast alcohol dehydrogenase $A_{280\ nm}$; and □...□, bovine serum albumin, $A_{280\ nm}$. V_0 = Fraction 12, determined with Dextran blue 2000. Columns were eluted at 10 ml/h with 0.15 M NaCl, and 5-ml fractions collected.

(b) Rate zonal centrifugation. After incubation of denatured, reduced neuraminidase with iodo[^{14}C]acetamide for 4 h as described above, 0.3-ml aliquots were taken, and a small amount of Dextran blue 2000 solution added as a visible marker. Urea and unincorporated iodo[^{14}C]acetamide were removed from these samples by "desalting" chromatography on disposable columns of Sephadex G-50, 0.38 cm^2 × 3 cm, equilibrated in 0.1 M Tris–HCl, pH 7.5. Eluant containing the Dextran blue marker, and thus all material excluded from the gel, was recovered in about 0.4 ml, and 0.1-ml aliquots of these preparations were used as samples for rate zonal density gradient centrifugation. A single peak of sedimenting radioactivity was always found (Fig. 5) and by comparison with the sedimentation of native ovalbumin and bovine serum albumin markers the sedimentation coefficient ($s_{20,w}$) of ^{14}C-labeled neuraminidase polypeptides was reproducibly determined in this way as 3.7 S. These results confirmed that ^{14}C-labeled dissociated subunits had been obtained from neuraminidase, which has a sedimentation coefficient of about 8 S as the active enzyme[4].

(c) Calculation of the molecular weight of neuraminidase polypeptides. The values obtained above for the Stokes' radius (a), sedimentation coefficient (s) and partial specific volume ($\bar{V}$) of neuraminidase subunits were substituted in the equation:

$$M_r = 6\pi\eta\, Nas/(1 - \bar{V})q \qquad (1)$$

where η = viscosity of water, N = Avagadro's number, and q = density of water. The molecular weight (M_r) of the subunits from A_2/1957 influenza neuraminidase was determined as 54 000.

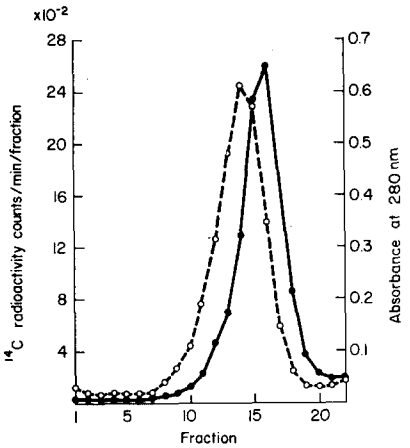

Fig. 5. Sedimentation of ^{14}C-labeled dissociated neuraminidase subunits. Centrifugation (right to left) was for 16 h at 38 000 rev./min in the SW39 rotor at 10° through a linear 6–30% sucrose gradient. ●—●, labeled neuraminidase subunits, radioactivity; ○---○, bovine serum albumin marker, $A_{280\ nm}$.

(d) Estimation of the accuracy of molecular weight determination. 50-μg samples of reference proteins were treated to produce ^{14}C-carboxamidomethylated derivatives, whose molecular parameters were then determined by Sephadex gel filtration and rate zonal centrifugation. Procedures throughout were identical to those described above for neuraminidase subunits. The molecular weights of ^{14}C-carboxamidomethylated ovalbumin and bovine serum albumin polypeptides were thus calculated as 50 000 and 74 000, respectively (Table IV), about 10% higher than their "true" molecular weights. This suggests that the molecular weight of neuraminidase subunits similarly determined probably lies in the range of about 50 000–54 000.

TABLE IV

PHYSICO CHEMICAL DATA FOR NATIVE AND ^{14}C-CARBOXAMIDOMETHYLATED REFERENCE PROTEINS

Protein	*Sedimentation coefficient (S)*	*Stokes' radius (Å)*	*Molecular weight*
Native ovalbumin	3.66	28*	43 500
^{14}C-carboxamidomethylated ovalbumin	3.4	34.5	50 000*
Native bovine serum albumin	4.4	36*	67 000
^{14}C-carboxamidomethylated bovine serum albumin	3.9	44.5	74 000*

* Calculated from the relationship $M_r = \frac{6\pi\eta Nas}{(1-\bar{V})q}$ (Eqn. 1) assuming a value of 0.733 ml/g for $\bar{V}$.

Preparation of ^{14}C-carboxamidomethylated associated neuraminidase subunits

After denaturation–reduction of neuraminidase by heating in urea–dithiothreiol as before, urea was removed from the samples by chromatography on a 0.38 cm^2 × 5 cm column of Sephadex G-50, equilibrated with 0.1 M Tris–HCl buffer, pH 7.5, and 0.04 mM dithiothreitol, but no urea. Material of molecular weight greater than about 10 000 recovered from this step was reacted with iodo[^{14}C]acetamide and un-

incorporated ^{14}C label removed by "desalting" chromatography as before. The product contained ^{14}C-labeled material with sedimentation coefficients ranging between that of dissociated subunits and native neuraminidase (Fig. 6). Sephadex gel chromatography confirmed the presence of labeled material in this size range. These findings showed that if urea was removed from reduced neuraminidase prior to blocking exposed sulfhydryl groups, the subunits could reassociate to produce aggregates up to about the size of native neuraminidase. However, some sulfhydryl groups remained free for reaction with iodoacetamide, and the final product was enzymically inactive.

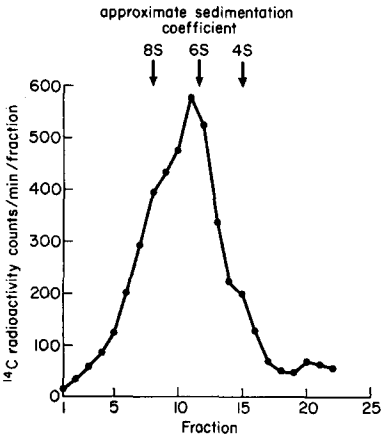

Fig. 6. Sedimentation of ^{14}C-labeled associated neuraminidase subunits. Centrifugation (right to left) was for 16 h at 38 000 rev./min in the SW39 rotor at 10° through a linear 6–30% sucrose gradient. Approximate sedimentation coefficients were by comparison with bovine serum albumin and IgG centrifuged in similar gradients.

Reactivity of neuraminidase sulfhydryl groups

Native neuraminidase incubated at pH 7.5 with iodo[^{14}C]acetamide at similar concentrations to before neither lost its enzymic activity, nor incorporated any ^{14}C label as judged by chromatography on Sephadex G-100 and G-200 columns. Neuraminidase denatured by heating in 6 M urea without dithiothreitol-reducing agent lost its enzymic activity completely, but did not incorporate any ^{14}C label on reaction with iodo[^{14}C]acetamide in the presence of 6 M urea. These results indicated that the sulfhydryl groups of native $A_2/1957$ influenza neuraminidase may all be present as disulfide bonds, and that the neuraminidase enzymic activity is also dependent on structural conformations maintained by noncovalent interactions. These conclusions are in agreement with HOYLE's[30] description of $A_2/1957$ influenza virus neuraminidase reactivity.

DISCUSSION

This report confirms that $A_2/1957$ influenza neuraminidase may be solubilized from virions by treatment with the protease nagarse, and conveniently purified by a series of two density gradient centrifugation steps[4]. Neuraminidase activity was recovered with an overall yield about twice that obtained by the alternative methodology of DRZENIEK *et al.*[31]. Overall purification factors using either technique are

about 15–18-fold, consistent with findings that neuraminidase comprises 5–10% of the total virus protein[1,4]. Stable A_2/1957 neuraminidase may also be purified after disrupting virus particles with detergent[32] but there is insufficient data available to evaluate the efficiency of that method.

Using similar techniques to those adopted here, the molecular weight of active neuraminidase was previously determined to be 220 000, assuming $\bar{V} = 0.733$ cm^3/g (ref. 4). As amino acid analysis indicates that $\bar{V} = 0.713$ cm^3/g (excluding carbohydrate and tryptophan content), the molecular weight of neuraminidase may now be corrected to about 200 000. By two independent procedures neuraminidase subunit size was shown here to be close to 50 000. Hence the active enzyme probably contains 4 subunits. However, it is not known whether the subunits are identical.

An average of about 21 total-cysteine residues are present in each subunit (Table III). This would explain the ready reassociation of subunits when efficient denaturing and reducing agents are removed from reduced neuraminidase without first having blocked sulfhydryl groups (Fig. 6 and ref. 33). Since the sulfhydryl residues appear to be present in the native enzyme as disulfide bonds, the high total-cysteine content is also probably a major factor in stabilizing A_2/1957 neuraminidase to heat, proteolytic enzymes and detergent.

WEBSTER[33] reported that neuraminidase solubilized from virus with ionic detergent could be dissociated into subunits with a size of 58 000. The larger subunit size compared to the present results may not be significant when intrinsic experimental errors are considered. Additionally, active neuraminidases isolated by proteolytic and detergent methods do not appear to differ significantly in size as judged by sedimentation studies[34,35]; hence their subunits should be similar. One difference between neuraminidases isolated by proteolytic and detergent methods is that in the latter case both the enzyme and its dissociated subunits can form very high molecular weight aggregates[33,36]. However, it has been suggested that the component responsible for the aggregation of detergent-solubilized neuraminidase is not an integral component of the enzyme molecule, and that proteolysis does not destroy any significant-sized portion of neuraminidase[35].

Procedures described here have enabled monomeric enzymically active A_2/1957 neuraminidase and its dissociated subunits to be prepared for further study. It is hoped that similar techniques will prove suitable for use with other influenza neuraminidases, so that ultimately structural changes in the enzyme subunits may be correlated with variation in the antigenic and enzymic properties between different virus neuraminidases.

ACKNOWLEDGMENT

Support for this research was provided by the Navy Department through a contract, N00014-67-A-0181-0029, with the Office of Naval Research and the Bureau of Medicine and Surgery. Seed of X-7(FI) virus was kindly supplied by Dr. E. D. Kilbourne.

REFERENCES

1 H. NOLL, T. AOYAGI AND J. ORLANDO, *Virology*, 18 (1962) 154.
2 MONIQUE REGINSTER, *Acta Virol. Prague*, 9 (1965) 462.

3 F. BIDDLE, *J. Gen. Virol.*, 2 (1968) 19.
4 A. P. KENDAL, F. BIDDLE AND G. BELYAVIN, *Biochim. Biophys. Acta*, 165 (1968) 419.
5 A. P. KENDAL, K. APOSTOLOV AND G. BELYAVIN, *J. Gen. Virol.*, 5 (1969) 141.
6 R. W. COMPANS, H. D. KLENK, L. A. CALIGUIRI AND P. W. CHOPPIN, *Virology*, 42 (1970) 880.
7 IRENE T. SCHULZE, *Virology*, 42 (1970) 890.
8 M. E. RAFELSON, M. SCHNEIR AND V. W. WILSON, *Arch. Biochem. Biophys.*, 103 (1963) 424.
9 G. L. ADA, PATRICIA E. LIND AND W. G. LAVER, *J. Gen. Microbiol.*, 32 (1963) 225.
10 J. L. SCHULMAN, M. KHAKPOUR AND E. D. KILBOURNE, *J. Virol.*, 2 (1968) 778.
11 W. H. ALLAN, C. R. MADELEY AND A. P. KENDAL, *J. Gen. Virol.*, 12 (1971) 79.
12 C. K. J. PANIKER, *J. Gen. Virol.*, 2 (1968) 385.
13 A. P. KENDAL AND C. R. MADELEY, *Biochim. Biophys. Acta*, 185 (1969) 163.
14 H. A. SOBER, *Handbook of Biochemistry—Selected Data for Molecular Biology*, Chemical Rubber Co., 1968, Section C, c. 10.
15 L. M. SIEGAL AND K. J. MONTY, *Biochim. Biophys. Acta*, 112 (1966) 346.
16 D. F. SUMMERS, J. V. MAIZEL AND J. F. DARRELL, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 505.
17 K. WEBER AND M. OSBORNE, *J. Biol. Chem.*, 244 (1969) 4406.
18 J. LEGGETT-BAILEY, *Techniques in Protein Chemistry*, Elsevier, Amsterdam, 1962, p. 293.
19 F. M. DAVENPORT AND E. MINUSE, in E. H. LENNETTE AND N. J. SCHMIDT, *Diagnostic Procedure for Viral and Rickettsial Diseases*, American Public Health Association, New York, 1964, p. 462.
20 E. A. ECKERT, *J. Bacteriol.*, 92 (1966) 1430.
21 D. AMINOFF, *Biochem. J.*, 81 (1961) 384.
22 A. P. KENDAL, C. R. MADELEY AND W. H. ALLAN, *J. Gen. Virol.*, 13 (1971) 95.
23 S. MOORE AND W. H. STEIN, *Methods Enzymol.*, 6 (1963) 819.
24 S. MOORE, *J. Biol. Chem.*, 238 (1963) 235.
25 T. Y. LIU AND Y. H. CHANG, *J. Biol. Chem.*, 246 (1971) 2842.
26 R. G. WEBSTER, W. G. LAVER AND E. D. KILBOURNE, *J. Gen. Virol.*, 3 (1968) 315.
27 E. D. KILBOURNE, F. S. LIEF, J. L. SCHULMAN, R. I. JAHIEL AND W. G. LAVER, in M. POLLARD, *Perspectives in Virology*, Vol. 5, Academic Press, New York, 1967, p. 87.
28 A. P. KENDAL AND C. R. MADELEY, *Arch. Ges. Virusforsch.*, 31 (1970) 219.
29 G. C. SCHILD, D. MCCAHON AND A. P. KENDAL, in R. D. BARRY AND B. W. J. MAHY, *The Biology of Large RNA viruses*, Academic Press, London, 1970, p. 638.
30 L. HOYLE, *J. Hyg., Cambridge*, 67 (1969) 301.
31 R. DRZENIEK, J. T. SETO AND R. ROTT, *Biochim. Biophys. Acta*, 128 (1966) 547.
32 W. G. LAVER, *J. Mol. Biol.*, 9 (1964) 109.
33 R. G. WEBSTER, *Virology*, 40 (1970) 643.
34 K. APOSTOLOV, T. H. FLEWETT AND A. P. KENDAL, in R. D. BARRY AND B. W. J. MAHY, *The Biology of Large RNA Viruses*, Academic Press, London, 1970, p. 3.
35 R. ROTT, R. DRZENIEK AND H. FRANK, in R. D. BARRY AND B. W. J. MAHY, *The Biology of Large RNA Viruses*, Academic Press, London, 1970, p. 75.
36 W. G. LAVER AND R. C. VALENTINE, *Virology*, 38 (1969) 106.
37 E. D. KILBOURNE, *Science*, 160 (1968) 74.