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Neuraminidase Production by a *Streptococcus sanguis* Strain Associated with Subacute Bacterial Endocarditis

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The properties of an extracellular neuraminidase produced by a *Streptococcus sanguis* strain (isolated from a confirmed case of subacute bacterial endocarditis) during growth in a defined medium was examined in this investigation. This enzyme, isolated from concentrated culture supernatants of *S. sanguis* biotype II, was active against human alpha-1 acid glycoprotein, *N*-acetylneuramin lactose, bovine submaxillary mucin, and fetuin. Neuraminidase production paralleled bacterial growth in defined medium and was maximal in the early stationary phase of growth but decreased dramatically, probably owing to protease production, during the late stationary phase. The enzyme was purified to near homogeneity by a combination of salt fractionation, ion-exchanged chromatography on DEAE-Sephacel, and gel filtration on Sephadex G-200. These procedures yielded an enzyme preparation that possessed a specific activity of 174.4 μmol of sialic acid released per min per mg of protein against human alpha-1 acid glycoprotein. The K_m value for this enzyme with human alpha-1 acid glycoprotein as substrate was 2.5×10^{-3} M, and the enzyme possessed a pH optimum of 6.5. The *S. sanguis* neuraminidase had a molecular weight of approximately 85,000 as estimated by gel filtration and approximately 90,000 when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was stable at temperatures of 4 and 37°C for 3 h, but approximately 50% of the enzymatic activity was lost within 30 min at 50°C, with 100% of the enzymatic activity being destroyed within 10 min at temperatures of ≥65°C.

The production of neuraminidase has long been thought to play a role in the infectious process caused by several microorganisms. Hirst (16) first suggested that this enzyme played an important role in the disease process caused by influenza virus. Isacsen (17) later suggested the importance of neuraminidase as the causative factor in autoimmune disease in which myxoviruses were implicated. There have been many reports of neuraminidase production by bacteria (31) as well. The production of this enzyme by the streptococci was first reported by Hayano and Tanaka (13, 14), Hayano et al. (15), and Pinter et al. (33). These authors demonstrated its production by streptococcus groups A through O, *Streptococcus salivarius, Streptococcus viridans, Streptococcus thermophilis,* and *Streptococcus sanguis* employing bovine submaxillary mucin (BSM) and bovine *N*-acetylneuramin lactose as substrate. These authors found, however, that these organisms produced only relatively small amounts of neuraminidase in vitro.

One of these organisms and its neuraminidase production has received extensive attention, primarily owing to the work of Müller, who demonstrated extremely high neuraminidase production by an *S. sanguis* strain isolated from a patient with septicemia (29). In the same investigation, Müller demonstrated the presence of large amounts of neuraminidase in blood cultures from patients with subacute bacterial endocarditis (SBE) caused by *S. sanguis*. In a later report (30), Müller showed that *S. viridans* retained its ability to produce neuraminidase after repeated passages on laboratory media, whereas *S. sanguis* lost this ability after several weeks in culture. Müller (30) also presented immunoelectrophoretic studies of the blood cultures of patients with SBE and septicemia which demonstrated the in vivo action of the enzyme on serum glycoproteins, resulting in the loss of sialic acid. Because of the possible role of neuraminidase in the pathogenesis of SBE caused by *S. sanguis*, we attempted to characterize this enzyme and its production.

(A preliminary portion of this work was presented previously [D. C. Straus and C. Portnoy-Duran, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B34, p. 29].)

MATERIALS AND METHODS

Bacterium. *S. sanguis* biotype II (strain 381-81) was isolated from a confirmed case of SBE and kindly

supplied by R. R. Facklam, Centers for Disease Control, Atlanta, Ga. Cultures were stored at −70°C in Todd-Hewitt Broth (Difco Laboratories, Detroit, Mich.). For routine use, frozen cultures were thawed and incubated for 24 h at 37°C on agar plus 5% sheep erythrocytes (Austin Biologicals, Austin, Tex.). The stock cultures used to inoculate the blood agar plates were only one transfer away from the original frozen culture. Colonies from the blood agar were used to initiate cultures in the chemically defined medium described below.

Media and growth conditions. A chemically defined medium (FMC), previously described by Terleckyj et al. (36), was used to grow *S. sanguis*. Briefly, this medium contains amino acids, vitamins, purine and pyrimidine bases, minerals, 2% glucose, and 0.019 M sodium carbonate at an initial pH of 7.0. All experiments were performed with static aerobic cultures grown at 37°C in a circulating water bath. Starter culture inocula were grown to the mid-exponential phase of growth (0.23 mg/ml, dry weight) in the chemically defined medium in volumes that were approximately 10% of the final culture volume. Growth was measured turbidimetrically at 675 nm in a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.). The value obtained was multiplied by 1,000 and converted to adjusted optical density units so that the values would be in accordance with Beer's law and be proportional to bacterial mass (37). One adjusted optical density unit was equivalent to 0.39 μg of cells (dry weight) per ml (35). When cultures were ready for harvest, they were immediately chilled in an ice bath, a final adjusted optical density reading was made, and the culture volume was determined. Cells were removed by centrifugation at 11,300 × *g* in a J2-21 refrigerated centrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at 4°C for 30 min. Filtrates were then concentrated by a 75% ammonium sulfate saturation at 4°C, followed by suspension of the precipitate in a small volume of 0.01 M sodium citrate buffer (pH 6.5) and dialysis against 100 to 200 volumes of the same buffer at 4°C for 24 h.

Substrate preparation. A solution of human alpha-1 acid glycoprotein (0.5 to 10 mg/ml;) (Calbiochem, San Diego, Calif.) dissolved in 0.01 M sodium acetate (pH 6.5) and containing 6.2% bound sialic acid was utilized as substrate for most of the experiments performed in this study. Other substrates examined for their susceptibility to *S. sanguis* neuraminidase included BSM (8.0% bound sialic acid; Sigma Chemical Co., St. Louis, Mo.), fetuin (8.7% bound sialic acid; type III, fetal calf serum; Sigma), and *N*-acetylneuramin lactose (48.9% bound sialic acid from bovine colostrum; Sigma).

Enzyme assays. The amount of neuraminidase present in concentrated filtrates of *S. sanguis* or purified enzyme preparations was determined by measuring the amount of sialic acid released from the above-mentioned substrates. The various preparations were incubated at 37°C for time periods ranging from 1 to 60 min, depending on the enzyme preparation. Each assay mixture contained the following components in a volume of 0.2 ml: 0.05 to 1.0 mg of substrate, 11.5 mM $CaCl_2$, 100 mM sodium acetate (pH 6.5), and *S. sanguis* enzyme preparations (usually 0.01 to 0.075 ml). Each set of assays included a substrate blank, and enzyme reactions were performed in duplicate and initiated by addition of the enzyme to the remaining components. The enzyme-substrate reaction was terminated by adding 0.025 M periodic acid (in 0.125 N H_2SO_4) and placing the reaction mixture on ice. The reaction was found to be linear for 165 min. The amount of released sialic acid was quantitated by the colorimetric thiobarbituric acid assay of Aminoff (1) with *N*-acetylneuraminic acid (Sigma) as a standard. Protein concentrations were determined in duplicate by the Folin phenol assay of Lowry et al. (21) with bovine serum albumin as the standard, and specific activities were expressed as micromoles of sialic acid released per minute per milligram of protein. Neuraminic acid aldolase activity was determined by incubating enzyme samples with the sialic acid reaction product of the *S. sanguis* II neuraminidase or a commercial preparation of sialic acid and assaying for loss of thiobarbituric acid-reactive material. To prepare the *S. sanguis* neuraminidase acid substrate, neuraminidase from culture filtrates of *S. sanguis* II was incubated with 5.0 mg of human alpha-1 acid glycoprotein and the reaction was allowed to proceed as described above. The reaction mixture was then dialyzed against 10 ml of deionized water, and the resultant dialysate was concentrated by lyophilization. The liberated sialic acid (0.02 μmol) was then incubated with an enzyme sample under the previously described conditions of the neuraminidase assay. The amount of thiobarbituric acid-reactive material remaining in the reaction mixture was determined and compared with that of a negative control. For pH optimum determinations of the purified neuraminidase, a pH range of 5.0 to 8.0 was examined, with a 100 mM acetate buffer used to obtain all pH values. The neuraminidase assay employed was essentially that described above, except the pH was changed appropriately.

Purification of the extracellular neuraminidase of *S. sanguis* II 381-81. (i) Stage I: concentrated culture supernatant fluid. The extracellular neuraminidase was obtained from an early stationary-phase culture (adjusted optical density, 1,400) of *S. sanguis* II grown in 10 liters of FMC. Cells were harvested by centrifugation at 11,300 × *g* for 30 min at 4°C, and the supernatant fluid was brought to 75% ammonium sulfate saturation and stirred overnight at 4°C (stage I).

(ii) Stage II: ammonium sulfate fractionation. Stage I enzyme was brought to 20% ammonium sulfate saturation and, after stirring overnight, the suspension was centrifuged at 48,000 × *g* for 30 min and the precipitate was suspended in as small a volume as possible of 10 mM sodium citrate (pH 6.5). The resuspended precipitate was dialyzed and saved, whereas the supernatant fluid was brought to 60% ammonium sulfate saturation and treated as described above. The majority of neuraminidase activity was in the region of 20 to 60% saturation (stage II), and this fraction was utilized for further purification.

(iii) Stage III: chromatography on DEAE-Sephacel. A column (2.5 by 20 cm) of DEAE-Sephacel (Pharmacia Fine Chemicals, Inc., Pistcataway, N.J.) was poured and equilibrated overnight with 10 mM sodium phosphate (pH 7.5). Stage II enzyme (after concentration by lyophilization and equilibration overnight with the starting buffer) was then placed on the DEAE-Sephacel column. The column was washed twice with the starting buffer, and the adsorbing material was eluted with a linear NaCl (0 to 0.5 M) gradient in the starting buffer. The eluting peaks were monitored at 280 nm

with a flow-through 2138 Uvicord S monitor (LKB Instruments, Rockville, Md.) and recorded by a 6520-5 chopper-Bar six-channel recorder (LKB Instruments). Fractions of 100 drops (ca. 4.2 ml) were collected. Every fifth tube was dialyzed against 10 mM sodium citrate (pH 6.5) and tested for neuraminidase activity by the procedure of Aminoff (1). All tubes containing neuraminidase activity were pooled, and this pool was designated stage III.

(iv) Stage IV: chromatography on Sephadex G-200. Stage III enzyme was then applied to an ascending flow column (2.6 by 90 cm) of Sephadex G-200 (Pharmacia) equilibrated with 10 mM sodium citrate (pH 6.5) at 4°C. Fractions of 100 drops (ca. 4.6 ml) were collected and protein peaks were monitored and recorded at 280 nm. Every third tube was assayed for neuraminidase activity as described above. The resultant pool was designated stage IV.

(v) Stage V: ion exchange chromatography on hydroxylapatite. Stage IV enzyme was then concentrated to dryness by lyophilization and equilibrated against 5 mM sodium phosphate buffer (pH 7.0). A column (2.5 by 20 cm) of hydroxylapatite (Bio-Rad Laboratories, Richmond, Calif.) was poured and equilibrated with 5 mM sodium phosphate buffer (pH 7.0) that had been prepared from boiled deionized water. Stage IV enzyme was then placed on the hydroxylapatite column, which was washed twice with the starting buffer, and the adsorbing material was eluted with a linear phosphate gradient (0.005 to 0.505 M). The eluting peaks were monitored at 280 nm and fractions of 100 drops (ca. 4.2 ml) were collected. Every third tube was then dialyzed against 10 mM sodium citrate buffer (pH 6.5). The neuraminidase activity of each of the tubes was assayed as described above.

PAGE. All neuraminidase preparations were subjected to polyacrylamide gel electrophoresis (PAGE) on 7% acrylamide gels by the procedure of Davis (6). Samples containing 20 to 30 μg of protein in a maximum volume of 100 μl were mixed with 20 μl of 0.2% bromophenol blue and 100 μl of unpolymerized stacking gel, layered onto a stacking gel, and electrophoresed at 2 to 3 mA per gel at 0 to 4°C for 2 h. All gels were immediately fixed and stained for 2 h at 25°C in 0.2% Coomassie brilliant blue R in methanol-acetic acid-water (5:1:5) and destained by diffusion in a solution containing 5% (vol/vol) methanol and 7.5% (vol/vol) acetic acid. For localization of neuraminidase activity, a gel was immediately sliced into 0.5-cm sections after electrophoresis, and each fraction was mashed and eluted at 4°C for 72 h with 0.5 ml of 0.01 M sodium citrate buffer (pH 6.5). The gel residue was then removed, and the eluate was dialyzed against 200 volumes of the same buffer for 24 h and examined for neuraminidase activity by the procedure of Aminoff (1).

Molecular weight estimations. (i) SDS-PAGE. After elution from 7% polyacrylamide analytical gels, the purified neuraminidase preparations and reference proteins were subjected to sodium dodecyl sulfate (SDS)-PAGE by the discontinuous slab system described by Laemmli (19). Samples were dissociated and reduced in 0.01 M Tris-hydrochloride buffer (pH 8.0) containing 1% 2-mercaptoethanol and 1% SDS for 2 min at 100°C and directly loaded onto a 12% acrylamide separation gel with a stacking gel composed of 5% acrylamide. Standard proteins with molecular weights ranging from 60,000 to 220,000 (Pharmacia) were run simultaneously with the purified neuraminidase preparations. Plots of percent migration of standards and unknown protein samples versus $\log_{10}$ molecular weights were prepared, and unknown molecular weights were estimated by interpolation (38).

(ii) Gel filtration on Sephadex G-200. The Sephadex G-200 gel filtration column used to purify extracellular neuraminidase was calibrated with proteins of known molecular weights (Pharmacia). Elution volumes were determined for blue dextran (molecular weight, 2×10^6), aldolase (145,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and RNAase (13,700) by recording the volume at which these proteins or dextran displayed maximal absorbance at 280 nm. The molecular weight of the purified neuraminidase was then estimated by interpolation from a graph of partition coefficients between the liquid phase and the gel phase (K_{av}) values versus the $\log_{10}$ molecular weights, utilizing the K_{av} values of the standard proteins and the peaks containing neuraminidase activity (10).

RESULTS

Relationship between *S. sanguis* II growth curve and neuraminidase production. Experiments were performed to determine the optimal phase of the growth curve for maximum yield of extracellular neuraminidase produced by *S. sanguis* II. Cells were grown in complete FMC as described previously, and at various times, 200-ml samples were withdrawn and the cell-free supernatant material was concentrated by 75% ammonium sulfate fractionation. Figure 1 shows the neuraminidase activities of enzyme present in supernatants at various stages of the growth curve. The pattern observed for production of neuraminidase in FMC at 37°C closely paralleled the growth curve of the organism. Enzyme production increased dramatically as the organisms entered the logarithmic phase of growth and increased through the exponential and early stationary phases. A decline in enzyme activity was seen when the cells were allowed to incubate at 37°C past the early stationary phase of growth. This decrease in enzyme activity in the late stationary phase could have been due to the production of an extracellular neuraminic acid aldolase (4). However, no neuraminic acid aldolase activity could be demonstrated when either a commercial preparation of sialic acid or the actual sialic acid product liberated by the *S. sanguis* neuraminidase was used (data not shown).

Substrate specificity and kinetics of enzyme activity. To examine the specificity of the *S. sanguis* II neuraminidase, we compared the ability of this enzyme to cleave sialic acid from a variety of glycoprotein substrates (Table 1). In each case, 0.05 mg of glycoprotein preparation was treated with a 75% ammonium sulfate fractionated supernatant fluid from an early-station-

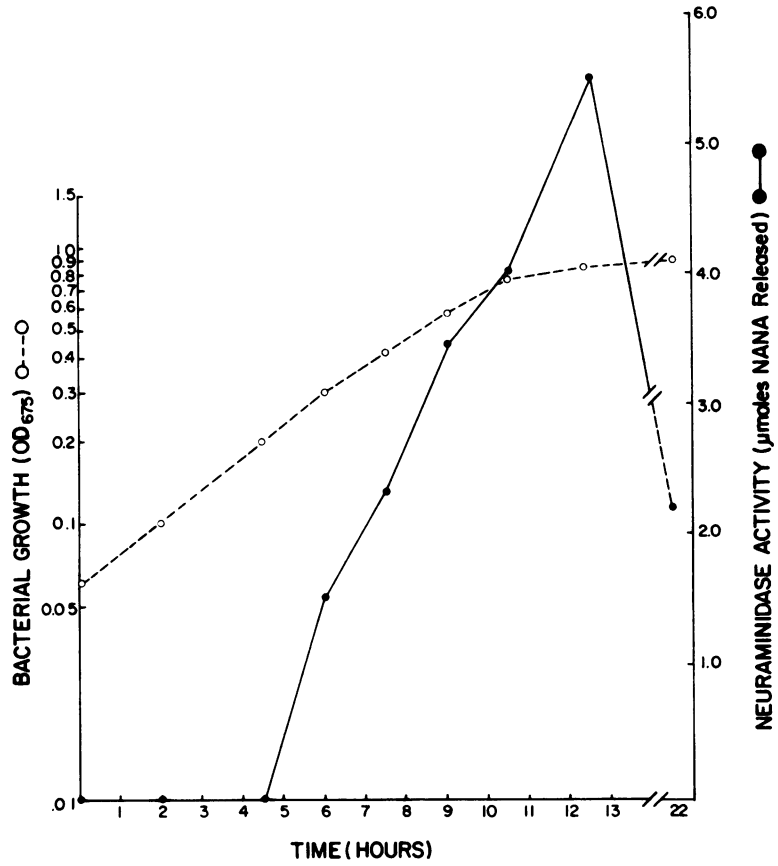

FIG. 1. Correlation of extracellular production of neuraminidase with bacterial growth for *S. sanguis* II cultured at 37°C in FMC.

ary-phase culture of *S. sanguis* II, and specific activity was calculated for each preparation. The *S. sanguis* II neuraminidase was able to hydrolyze sialic acid from all four substrates examined. The enzyme was active against human alpha-1 acid glycoprotein, *N*-acetylneuramin lactose, and BSM, whereas it was somewhat less active against fetuin.

Although the actual identity of the reaction product of the *S. sanguis* II neuraminidase acting on any of the substrates has not been established, it is probably a member of the sialic acid family of compounds. Data to support this hypothesis comes from absorption spectra of the complexes obtained when a stage IV enzyme preparation was allowed to react at 37°C with human alpha-1 acid glycoprotein. The absorption spectra of complexes obtained from the *S. sanguis* neuraminidase reaction product and *N*-acetyl neuraminic acid were essentially identical (Fig. 2).

The kinetics of the enzyme were studied with human alpha-1 acid glycoprotein used as substrate. As determined from the Lineweaver-Burk plot (20), the K_m value of this bacterial neuraminidase was 2.5×10^{-3} M (data not shown).

Temperature stability of the enzyme. The stability of the enzyme in complete FMC after incubation at various periods of time and various

TABLE 1. Ability of *S. sanguis* II neuraminidase to cleave sialic acid from various glycoprotein preparations

Glycoprotein prepn	*S. sanguis* II neuraminidase sp act[a]
Fetuin	1.95
BSM	2.77
Human alpha-1 acid glycoprotein	3.84
N-Acetylneuramin lactose	4.85

[a] The neuraminidase preparation was obtained from a culture of early-stationary-phase cells by 75% ammonium sulfate fractionation. Results are the averages of duplicate determinations. Specific activity is expressed as micromoles of sialic acid released per minute per milligram of protein.

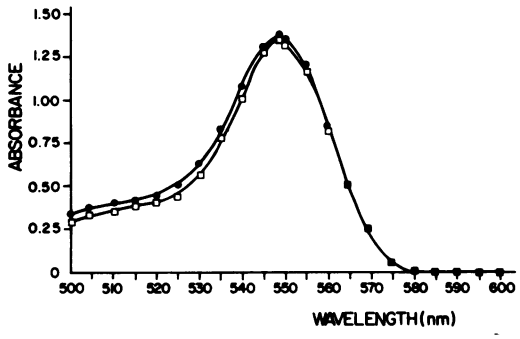

FIG. 2. Absorption spectra of complexes obtained from *S. sanguis* II neuraminidase reaction product (●) and *N*-acetylneuraminic acid (□) with the thiobarbituric acid reagent.

temperatures was examined. Preincubation of the enzyme at 4 to 37°C for up to 30 min before assay did not significantly alter the activity of the enzyme. A loss of enzyme activity of ca. 50% occurred after incubation of the enzyme in 100 mM sodium acetate (pH 6.5) for 30 min at 50°C, but total loss of enzyme activity occurred after incubation of the enzyme for ≥10 min at ≥65°C (data not shown).

Enzyme purification. Stage I material exhibited a specific activity of 9.6 μmol/min per mg of protein; the majority of this activity resided in the 20 to 60% $(NH_4)_2SO_4$-saturated fraction and possessed a specific activity of 9.8 μmol/min per mg of protein (Table 2). Figure 3 shows the elution profile of stage II *S. sanguis* neuraminidase on DEAE-Sephacel. All of the neuraminidase activity was found to elute from this column at a NaCl concentration of 0.25 to 0.30 M. This material, which had a specific activity of 82.0 μmol/min per mg of protein (Table 2), was then applied to a Sephadex G-200 column; the resultant elution profile is shown in Fig. 4. All of the neuraminidase activity eluted from the G-200 column in pool II (stage IV) and possessed a specific activity of 174.4 μmol/min per mg of protein (Table 2). This material was then applied to a hydroxylapatite column, and after concentration by lyophilization and dialysis against 0.01 M sodium citrate buffer (pH 6.5), the neuraminidase was shown to elute from the hydroxylapatite column at a phosphate concentration of approximately 0.15 M. However, >90% of the specific activity of the stage V enzyme preparation was lost after this step, attesting to the instability of the enzyme in its pure state. The substrate profile of the stage IV enzyme was identical to that of the stage I preparation. PAGE of the stage IV enzyme preparation demonstrated that there were two protein components present in this preparation (Fig. 5). Neuraminidase activity was always found to be associated with the gel slice containing the upper Coomassie blue staining component.

Molecular weight and pH optimum of *S. sanguis* II neuraminidase. The molecular weight of the *S. sanguis* neuraminidase was calculated by its elution profile on the Sephadex G-200 column. The *S. sanguis* neuraminidase had an apparent molecular weight of approximately 85,000 by chromatography on sephadex G-200 (Fig. 4). When the purified *S. sanguis* neuraminidase was subjected to SDS-PAGE, the molecular weight of the highest band was approximately 90,000 (data not shown). However, lower-molecular-weight fragments were seen on the SDS-PAGE separation and probably represented fragments of the enzyme generated during the purification procedure.

The pH optimum was determined for the enzyme with human alpha-1 acid glycoprotein as substrate. A peak of maximal activity occurred at pH 6.5 in 100 mM sodium acetate containing 11.5 mM $CaCl_2$ (Fig. 6).

DISCUSSION

Although there have been several studies documenting the production of neuraminidase by *S. sanguis*, the production of the enzyme and its characteristics have not been described. Pinter et al. (33) first examined *S. sanguis* for neuraminidase production but were unable to detect the presence of this enzyme in the supernatant fluids of five different strains employing *N*-

TABLE 2. Purification of *S. sanguis* II extracellular neuraminidase

Stage	Enzyme prepn	Total amt (mg) of protein	Neuraminidase sp act (μmol/min per mg of protein (increase [fold])[a]	Total neuraminidase activity (μmol/min)
I	0 to 75% $(NH_4)_2SO_4$	42.3	9.6	406.1
II	$(NH_4)_2SO_4$ fractionation (20 to 60%)	20.8	9.8 (1.0)	203.8
III	DEAE-Sephacel chromatography	1.4	82.9 (8.5)	116.1
IV	Sephadex G-200 chromatography	0.3	174.4 (18.2)	52.3

[a] Compared with stage I.

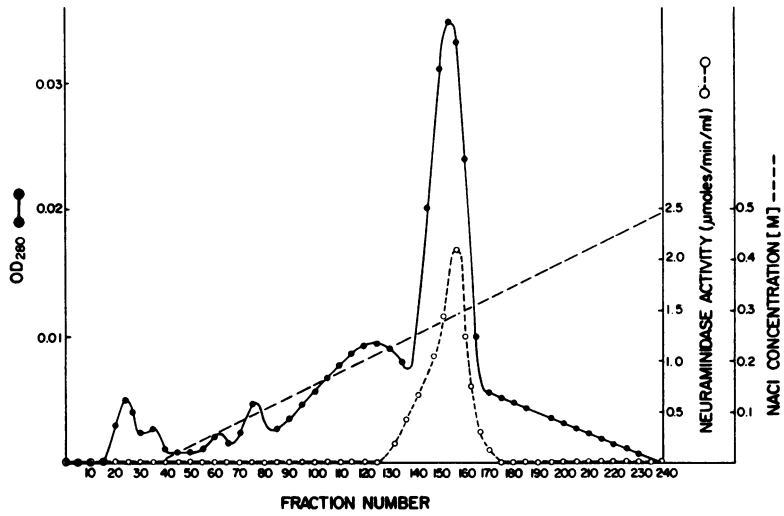

FIG. 3. Chromatography of stage II neuraminidase on DEAE-Sephacel. The peaks were monitored at 280 nm and eluted with an increasing NaCl gradient from 0 to 0.5 M in 0.01 M potassium phosphate buffer (pH 7.5) at 4°C. Every fifth tube was assayed for neuraminidase activity by the method of Aminoff (1).

acetylneuramin-lactose as substrate. This is surprising in light of the fact that we were able to demonstrate the degradation of this compound by the neuraminidase of *S. sanguis* II 381-81 (Table 1). Hayano and Tanaka (14) and Hayano et al. (15) were the first workers to demonstrate neuraminidase production by *S. sanguis* employing bovine submaxillary mucin as substrate. They reported that their streptococcal enzymes (streptococcal sialidases) were incapable of liberating *N*-acetylneuraminic acid from *N*-acetylneuramin lactose, but our data (Table 1) show clearly that the neuraminidase produced by *S. sanguis* II is active against this substrate. A neuraminidase preparation obtained from a culture of early-stationary-phase cells by a 0 to 75% ammonium sulfate fractionation possessed a specific activity against that substrate of 4.85 μmol of sialic acid released per min per mg of protein. Since we attempted to perform our studies under conditions which were as similar as possible to those of Hayano and Tanaka (14)

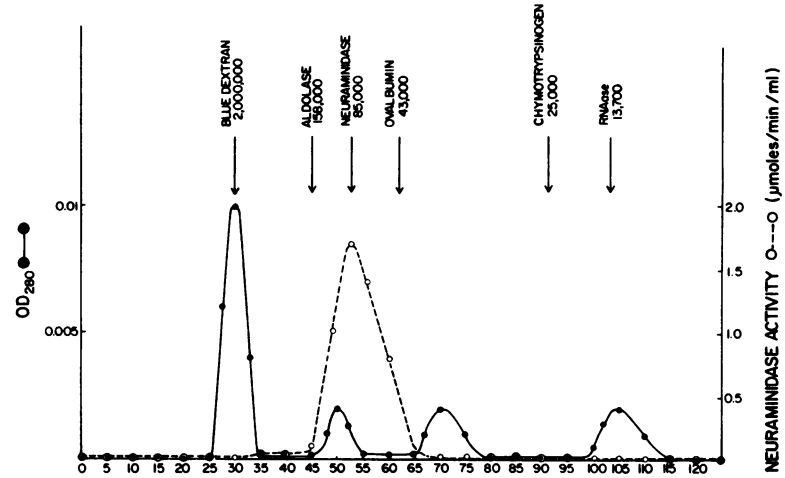

FIG. 4. Chromatography of stage III neuraminidase on Sephadex G-200 column (2.5 by 90 cm). The peaks were monitored at 280 nm and eluted with 0.01 M sodium citrate buffer (pH 6.5) at 4°C. Every third tube was assayed for neuraminidase activity by the procedure of Aminoff (1). Calibration of the column with blue dextran, proteins, and glycoproteins of known molecular weights was performed in the same buffer.

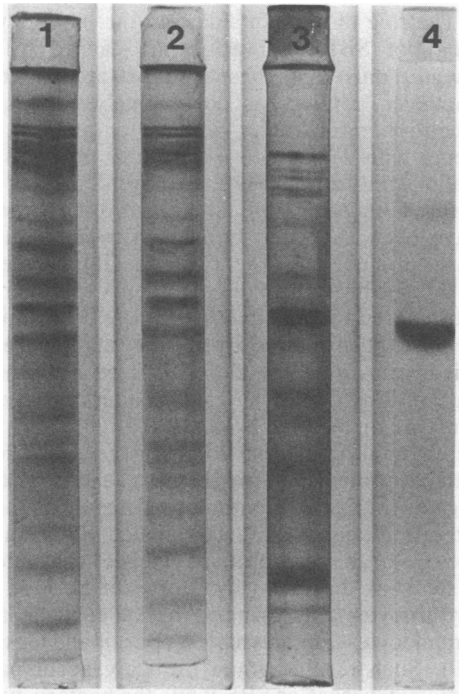

FIG. 5. Electrophoresis of various stages of enzyme purification on 7% polyacrylamide gels. Gels were loaded and electrophoresed as described in the text with 20 to 30 μg of the following materials: stage I neuraminidase preparation (gel 1), stage II neuraminidase preparation (gel 2), stage III neuraminidase preparation (gel 3), and stage IV neuraminidase preparation (gel 4).

and Hayano et al. (15), the only explanation for these discrepancies would be differences in the strains used. *S. sanguis* strains can now be divided into two biotypes on the basis of several physiological attributes (9). Since this classification scheme was not available to Hayano and Tanaka, we do not know the biotype of their *S. sanguis* strain. Great physiological differences have been shown to exist between the two biotypes of *S. sanguis* (9).

We have confirmed the findings of other investigators (14, 15, 29, 30) that *S. sanguis* produces an extracellular neuraminidase. Our data would appear to indicate that the best time to harvest *S. sanguis* cells for maximal enzyme yield is the late exponential or early stationary phase (Fig. 1). Extracellular neuraminidase production paralleled the growth of the organism, as was reported by Davis et al. (7) for group A streptococci. However, as observed in other streptococci (7, 23), neuraminidase activity declined dramatically after the organisms had entered the mid-stationary phase (ca. 12 to 13 h after inoculation). This observation could be an important consideration when attempting to quantitate or purify this enzyme. The destruction of neuraminidase activity in the stationary phase of growth is probably due to protease production by this organism. Straus et al. (35) and Labib et al. (18) recently showed that *S. sanguis* strains are capable of producing proteolytic enzymes, and more recently, Straus (34) has shown that *S. sanguis* II 381-81 produces most of its proteolytic activity in the stationary phase.

Partial purification of the *S. sanguis* neuraminidase was facilitated by the use of a completely defined medium (36) and the fact that the enzyme appeared to be stable in this medium until the organisms were past the early stationary phase of growth. The enzyme preparation eluted from Sephadex G-200 demonstrated two bands on PAGE and possessed a specific activity of 174.4 μmol of sialic acid released per min per mg of protein when assayed with alpha-1 acid glycoprotein as substrate. The specific activity of neuraminidase in this investigation is considerably greater than that of the neuraminidase produced by *Streptococcus pneumoniae* (12). Glasgow et al. purified this enzyme from *S. pneumoniae* and showed that it had a specific activity of 45 μmol/min per mg of protein when alpha-1 acid glycoprotein was used as the substrate. However, when the *S. sanguis* neuraminidase examined in this study was purified to homogeneity by elution from polyacrylamide gels or by the hydroxylapatite purification step, the specific activity dropped drastically, indicat-

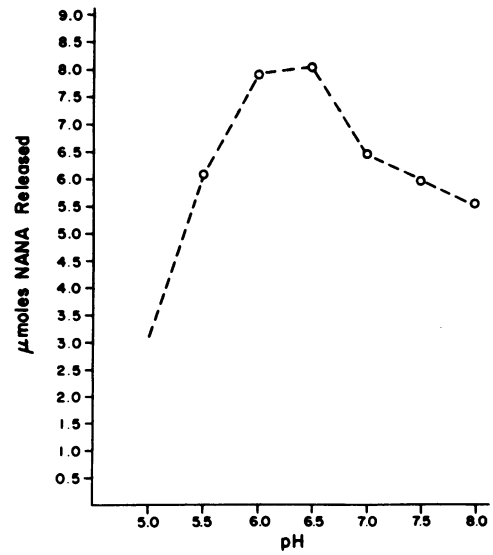

FIG. 6. pH optimum of purified *S. sanguis* II neuraminidase in 0.1 M sodium acetate buffer containing 11.5 mM $CaCl_2$.

ing that the enzyme was quite unstable in a highly purified form.

The molecular weight estimate of the *S. sanguis* neuraminidase based on gel filtration (Fig. 4) and SDS-PAGE indicated molecular weights of 85,000 and 90,000, respectively. This estimate is in agreement with the reported molecular weights of other bacterial neuraminidases. For example, the neuraminidase from *Arthrobacter sialophilus* has been reported to have a molecular weight of 87,000 by gel filtration (11), and the neuraminidase of *Streptococcus pyogenes* was estimated to have a molecular weight of 90,000 by the same technique (7). Another neuraminidase with a comparable molecular weight is that of *S. pneumoniae*, which was estimated to be approximately 88,000 (8). Some bacterial neuraminidases whose molecular weights have been estimated by gel filtration have been shown to be decidedly smaller than that of *S. sanguis*. These include neuraminidases produced by *Vibrio cholerae* (molecular weight, 68,000 [8]), *Clostridium perfringens* (molecular weight, 56,000 [3, 5]), and *Corynebacterium diphtheriae* (molecular weight, 65,000 [25]). One other bacterial neuraminidase that has a higher reported molecular weight is that of the type III group B streptococcus, which has been shown to have a molecular weight of 125,000 by gel filtration chromatography (22).

Neuraminidase and neuraminic acid aldolase production appear to vary from one *S. sanguis* strain to another. As stated previously, Hayano and Tanaka (14) and Hayano and co-workers (15) demonstrated that the strain of *S. sanguis* that they were using was able to produce a neuraminidase that could degrade BSM but not *N*-acetylneuramin lactose; however, our *S. sanguis* strain produced an enzyme capable of degrading both substrates. Müller (29, 30) demonstrated both neuraminidase and neuraminic acid aldolase production by an *S. sanguis* strain. Although we were able to demonstrate neuraminidase production by our *S. sanguis* strain, we were unable to show neuraminic acid aldolase production. In addition, Müller (29) showed that his *S. sanguis* strain was able to produce two different molecular weight forms of the enzyme, a high- and a low-molecular-weight form. Although we were able to demonstrate a high-molecular-weight neuraminidase, we were unable to show the production of a low-molecular-weight form. The extracellular neuraminidase of *S. sanguis* II 381-81 had a K_m value of 2.5×10^{-3} M against human alpha-1 acid glycoprotein. The only other K_m value that has been reported for *S. sanguis* neuraminidase is 5.2×10^{-5} M, but that was against BSM and it was from a different *S. sanguis* strain, so it is difficult to compare the two values (14).

The role of neuraminidase in the pathophysiology of SBE and septicemia caused by *S. sanguis* must remain speculative at this time. However, Müller has convincingly shown, in electrophoretic studies of blood cultures of patients with *S. sanguis* septicemia, the loss of sialic acid from several glycoproteins of the serum (30). Additional alterations of host tissue have been shown to be caused by neuraminidases produced by other bacteria. Glycoproteins in the cerebrospinal fluid of patients with meningitis caused by *S. pneumoniae* have been shown to have been altered by the neuraminidase of this organism (26). Additionally, the neuraminidase of *Clostridium perfringens* alters the glycoproteins in infected wounds (27, 28), and the neuraminidase of *Bacteroides fragilis* produces altered proteins in abscesses caused by this organism (32). One pathological result of these alterations could be a diminished life-span of these serum glycoproteins in the circulatory system, as described by Morell et al. (24), or erythrocytes, as shown by Aminoff et al. (2), and lymphocytes, as shown by Woodruff and Gesner (39). Such results would probably accompany systemic disease or SBE while the bacteria were actively multiplying in the blood or body tissues. Therefore, it would appear that the neuraminidase of *S. sanguis* is an enzyme whose role in the disease processes produced by this organism merits further attention.

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