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Properties of Extracellular Neuraminidase Produced by Group A Streptococcus

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Extracellular neuraminidase production by group A streptococci was examined in 92 strains. Fourteen of these strains produced appreciable amounts of enzyme; 12 of the neuraminidase-producing strains belonged to T types 1, 4, and 12. Production of the enzyme paralleled bacterial growth in culture and was maximal in medium containing 0.2% glucose. The enzyme produced by one of these strains was partially purified by ammonium sulfate fractionation and filtration on G-200 Sephadex. Its molecular weight was estimated at 90,000. Activity was optimal at pH 5.7 and in the presence of 0.01 to 0.03 M calcium and magnesium cations. The enzyme was stable at temperatures of 4 and 37°C for at least 24 h but was inactivated within 10 min at temperatures of 50 and 65°C. The enzyme hydrolyzed 40% of the sialic acid in bovine submaxillary mucin, but was inactive on sialyllactose, porcine submaxillary mucin, oligosaccharides derived from porcine mucin, or human orosomucoid. The K_m value for this enzyme with bovine submaxillary mucin as substrate was in the order of 3.6×10^{-4} M.

Neuraminidase production has been associated with a variety of microorganisms, both bacterial and viral (3, 9). The production of "sialidase-like" enzyme by beta-hemolytic streptococci was first reported by Hayano and Tanaka (15-17). This enzyme manifested minimal activity when incubated with alkali-treated bovine submaxillary mucin, but showed enhanced activity on the acid-treated substrate (16). Among many group A beta-hemolytic serotypes tested, only strains belonging to two serotypes, Lancefield M type 4 and M type 22, produced significant amounts of this enzyme (17).

McIntosh and his co-workers (21) reported the loss of sialic acid from human immunoglobulins incubated with the culture supernatant of a group A streptococcus. This streptococcal strain was isolated from a patient with poststreptococcal glomerulonephritis. In subsequent studies, these investigators found that the immunoglobulin so altered had the characteristics of a cryoglobulin and the potential for producing nephritis in experimental animals (14, 22, 23). Because of these findings, the present study was undertaken to determine the production of neuraminidase by group A streptococci and to characterize some of the biochemical properties of this streptococcal extracellular enzyme.

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MATERIALS AND METHODS

Streptococcal strains. Group A beta-hemolytic streptococci isolated in our laboratory from a variety of sources were studied. These strains included routine throat, skin, and blood isolates from our microbiology laboratory. All isolates were lyophilized from blood broth cultures of the original blood agar plates and stored in the lyophilized state until used.

Identification of the isolates as to serological group was done by the Lancefield technique (19). M typing and T typing of the group A streptococci were performed by the technique reported by Moody et al. (25, 26), with antisera kindly supplied by Richard Facklam of the Streptococcal Bacteriology Unit at the National Center for Disease Control, Atlanta, Ga.

Culture techniques. Lyophilized organisms were cultured in blood broth and then grown for 18 to 20 h at 37°C in either Todd-Hewitt broth or Pfanstiehl dialysate medium (29). The optimal glucose concentration required for production of neuraminidase in both of these media was examined by growing organisms in 0.2, 0.4, and 0.6% glucose. As reported by Hayano and Tanaka (15), maximal enzyme production was achieved in media containing 0.2% glucose. There was a five- to sixfold drop in supernatant enzyme activity when the organisms were grown in Todd-Hewitt broth containing 0.4 and 0.6% glucose, but only a 30 to 40% decrease in enzyme activity when the organisms were grown in Pfanstiehl dialysate medium containing 0.4 and 0.6% glucose. Studies on the partial purification and biochemical characterization of extracellular neuraminidase were carried out from cultures in Pfanstiehl dialysate medium.

Assay for neuraminidase activity. Neuramini-

dase activity was determined by the release of sialic acid from a variety of substrates. Portions of 0.1 to 0.2 ml of culture supernatant or the partially purified enzyme preparation were incubated with 0.1 ml of a solution of the substrate containing 60 to 70 μg of bound sialic acid. The final volume of the reaction mixture was brought to 0.5 ml with 0.1 M sodium acetate buffer, pH 5.5, containing 0.01 to 0.02 M calcium chloride. Incubation time for screening culture supernatants was 16 h, whereas the incubation time for testing the activity of the partially purified, concentrated enzyme was 20 min.

Substrates. A 10-mg/ml solution of bovine submaxillary mucin (Sigma Chemical Co., St. Louis, Mo.) dissolved in deionized, distilled water and containing 7.0% by weight bound sialic acid was utilized as substrate for screening a number of culture supernatants and for the biochemical characterization of streptococcal neuraminidase.

Other substrates tested for susceptibility to hydrolysis by streptococcal neuraminidase included porcine submaxillary mucin, alkali-labile reduced oligosaccharides from porcine submaxillary mucin, sialyl-lactose (Sigma Chemical Co.), and orosomucoid (Calbiochem, La Jolla, Calif.). These substrates contained 7.8, 7.4, 48.9, and 6.2% of bound sialic acid, respectively. Porcine submaxillary mucin was purified in our laboratory by the method of Aminoff et al. (2). Alkali-labile reduced oligosaccharides from porcine submaxillary mucin were prepared by the method of Carlson (8) as described by Baig and Aminoff (5). Orosomucoid was kindly supplied by Thomas W. Milligan, University of Texas, San Antonio.

Assay for free and total sialic acid. The amount of free sialic acid in the assay mixture was determined by the thiobarbituric acid method of Aminoff (1). Total sialic acid content of the substrates was assayed by the resorcinol method described by Svennerholm (28) with *N*-acetyl neuraminic acid as the standard.

Preliminary studies revealed that components of culture medium inhibited the development of the chromogen for determination of free sialic acid by the Aminoff method. This inhibitor was present in greater concentration in Pfanstiehl dialysate medium than in Todd-Hewitt broth and was associated with Casamino Acids. Further studies revealed that this inhibitor was of low molecular weight, approximately 300, and could be completely removed from the medium by dialysis against the assay buffer. Hence, all assays for neuraminidase activity in culture supernates were performed after overnight dialysis of the culture supernatant against the assay buffer.

Protein content of the samples was determined by the modification of the Lowry technique described by Campbell and Sargent (7) with bovine serum albumin as the standard.

RESULTS

Production of neuraminidase by group A streptococcal strains. Culture supernatants of 92 strains collected from our diagnostic laboratory and identified serologically as group A streptococci were screened for neuraminidase activity. The organisms were grown in Todd-Hewitt broth for 18 h, and 1 ml of the culture supernatant was dialyzed for 24 h at 4°C against 100 ml of acetate buffer containing 0.02 M calcium chloride. A 0.1-ml portion of the dialyzed supernate was then assayed for neuraminidase production with bovine submaxillary mucin as substrate and incubation for 18 h. Fourteen of the strains tested produced appreciable amounts of neuraminidase in the culture supernatant. Subsequent serological typing of the 92 strains studied yielded the distribution shown in Table 1. As can be seen, neuraminidase production was associated primarily with serotypes T-1, T-4, and T-12. M typing of these strains revealed that the serotype T-1 was M-1, that six of the seven T-4 strains belonged to the M-60 serotype, whereas one of the T-12 strains was an M-12 serotype; the other T-4 and T-12 strains were nontypable with the available M antisera.

Correlation of bacterial growth and production of neuraminidase. Studies were undertaken to correlate the growth of group A streptococci in culture and the appearance of neuraminidase activity in the culture supernate. Group A streptococci were grown in Pfanstiehl dialysate medium, at 37 and 22°C. At given intervals, a portion of culture supernatant was removed, and bacterial growth was estimated by absorbance at optical density at 650 nm in a Coleman Jr. spectrophotometer. A portion of the cultures supernate was dialyzed and assayed for enzyme activity. As can be seen in Fig. 1, the pattern obtained for production of neuraminidase in the culture medium paralleled closely the growth curve of the organism, both at 37 as well as 22°C. At 37°C, enzyme production rose

TABLE 1. *Neuraminidase production by 92 strains of group A streptococci*

T serotype	Neuraminidase production	
	No. tested	No. positive
1	2	1
4	11	7
5/27/44	6	0
6	8	1
11	5	0
12	12	4
Imp. 19	3	0
25	4	0
28	4	0
Other[a]	18	0
Nontypable	19	1

[a] Includes one strain of T types 1/12/13, 5/11/112/27/78, 5/12/27/44, 8/25, 9/13/, 9/13/28, 11/12, and 13/28/11 and two strains of T types 2, 9, 13, 14, and 131 B 3264.

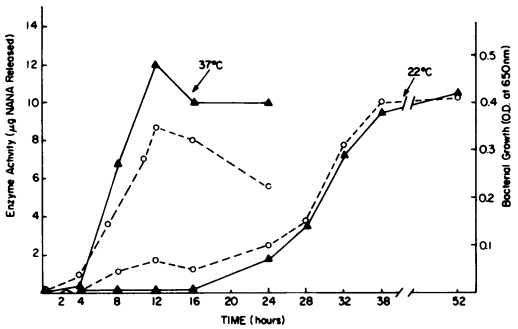

FIG. 1. *Correlation of extracellular production of neuraminidase* (O----O) *with bacterial growth* (▲——▲) *for group A streptococcus cultured at 22 and 37°C in Pfanstiehl dialysate medium.*

sharply during the phase of logarithmic growth of the organism and was maximal at the peak of this phase. A decline in enzyme activity was observed during the stationary phase and on further incubation of the culture at 37°C. Production of enzyme was also maximal during the logarithmic growth phase of the organism at 22°C. No decline in enzyme activity was observed during the stationary phase of the culture grown at 22°C.

Partial purification of neuraminidase. Streptococcal strain UF 245, group A, serotype T-4:M-60, which consistently produced the greatest amount of neuraminidase among the strains screened, was selected for partial purification and biochemical characterization of the enzyme. The organism was subcultured into blood broth, and 10 ml of an overnight growth was inoculated into 2 liters of Pfanstiehl dialysate medium. After incubation for 18 h at 37°C, the culture supernatant was separated by centrifugation at 6,000 × *g* for 30 min at 4°C. The supernatant was then filtered through a filter (0.45 μm, Millipore Corp.) previously soaked in a solution containing 1 mg of bovine serum albumin per ml.

The filtrate was brought to 80% saturation with ammonium sulfate and allowed to stand at 4°C for 3 days. The precipitate was collected by centrifugation at 10,000 × *g* for 1 h at 4°C. The supernatant which was devoid of neuraminidase activity was discarded. The precipitate containing neuraminidase was dissolved in 15 ml of 0.15 M NaCl containing 0.01 M $CaCl_2$, and the dissolved precipitate was exhaustively dialyzed against the same saline. A portion of 1 ml was further dialyzed overnight against 0.01 M sodium acetate buffer (pH 5.5) containing 0.01 M $CaCl_2$ and then applied on a column of Sephadex G-200 (1.5 by 90 cm) equilibrated with the same buffer and calibrated with proteins and glycoproteins of known molecular weight. Elution was carried out with the same buffer, and neuraminidase activity was determined on each fraction.

The results of these fractionation procedures are summarized on Table 2 and Fig. 2. As shown in Table 2, 80% ammonium sulfate resulted in complete precipitation of neuraminidase activity and approximately fourfold increase in specific activity. Filtration of this dialyzed precipitate through G-200 Sephadex resulted in recovery of 70% of the enzyme applied on the column. The enzyme eluted as a single peak, Fig. 2, with an additional threefold enrichment of its activity. The molecular weight of the recovered enzyme is estimated by Sephadex fractionation to be approximately 90,000.

pH optimum. The pH optimum was determined for the partially purified enzyme recovered from G-200 Sephadex fractionation with bovine submaxillary mucin as substrate. As shown in Fig. 3, a peak of maximal activity occurs at pH 5.5 to 5.7 in 0.1 M acetate containing 0.02 M $CaCl_2$.

Cation requirement. The partially purified enzyme was dialyzed exhaustively against 0.1 M acetate buffer pH 5.5 and activity was tested in various molar concentrations of calcium, magnesium, and manganese. Manganese appeared to inhibit enzyme activity. The results of enzymatic activity in presence of calcium and magnesium either singly or in combination, are shown in Table 3. Maximal activity occurred with 0.01 to 0.03 M calcium in the presence of 0.001 to 0.01 M magnesium.

Substrate specificity. The activity of the partially purified enzyme against a variety of sialic acid-containing substrates was tested under optimum pH and cation concentrations. No release of sialic acid was effected when the enzyme was incubated with sialyl-lactose, porcine submaxillary mucin, alkali-labile reduced oligosaccharides of porcine submaxillary mucin, or human orosomucoid. Incubation of enzyme with bovine submaxillary mucin with increasing

TABLE 2. *Partial purification of streptococcal neuraminidase*

	Enzyme activity[b]	Purification factor	% Recovery
Culture supernatant[a]	362	1	100
80% Ammonium sulfate precipitate	1,514	4.18	100
G-200, neuraminidase peak	4,476	12.36	70

[a] Values refer to dialysed culture supernate. For details see text.

[b] Enzyme activity is defined as millimoles of sialic acid released per milligram of protein, upon incubation of enzyme sample with bovine submaxillary mucin for a period of 20 min at 37°C.

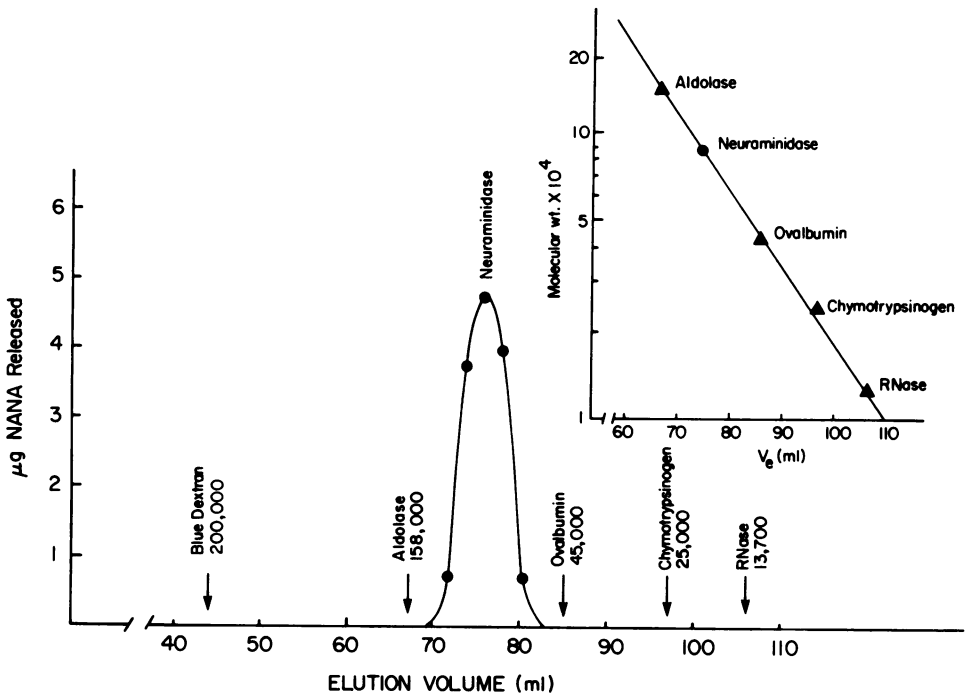

FIG. 2. *Elution of group A streptococcal neuraminidase from a G-200 Sephadex column (1.5 by 90 cm). The organism was grown in Pfanstiehl dialysate medium, and the neuraminidase was precipitated from the culture supernatant with 80% ammonium sulfate. The elution buffer was 0.01 M acetate (pH 5.5) containing 0.01 M $CaCl_2$. Calibration of column with blue dextran, protein, and glycoproteins of known molecular weight was performed in same buffer.*

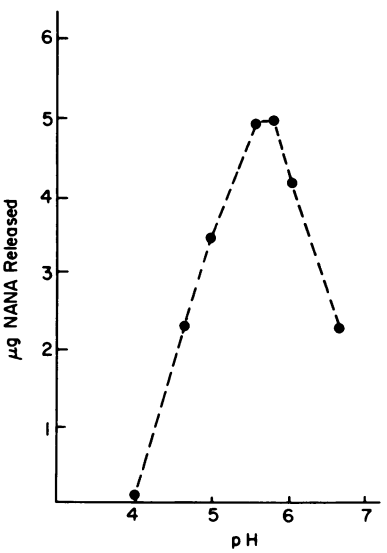

FIG. 3. *pH optimum of neuraminidase eluted from G-200 Sephadex in 0.1 M acetate containing 0.02 M $CaCl_2$.*

amounts of enzyme effected release of sialic acid to a maximum of 40% of the total sialic acid content of the substrate.

Previous studies have shown that the intensity of chromogen formed by the thiobarbituric acid assay procedure of Aminoff (1) varies with the nature of the free neuraminic acid. Bovine submaxillary mucin contains a variety of neuraminic acids which include *N*-glycoloyl neuraminic acid (17%), *N*-acetyl neuraminic acid (37%), and 9-*O*-diacetyl neuraminic acid (33%) (6, 10). The possibility that these sialic acids were being released by the enzyme but not detected by the assay procedure used was entertained. Bovine submaxillary mucin was incubated with enzyme and then exhaustively dialyzed to remove any free neuraminic acid from the incubation mixture. The dialyzed material was then assayed for total neuraminic acid content by the Svennerholm method (28). The results obtained showed that the residual neuraminic acid which was not cleaved by the enzyme from the substrate could be accounted for as bound neuraminic acid.

Kinetics of enzyme activity. With bovine submaxillary mucin as substrate, the kinetics of the partially purified neuraminidase were studied. The results are shown in Fig. 4. As determined from the Lineweaver-Burk plot (20), the K_m value of this enzyme is 3.6×10^{-4} M.

Temperature stability of neuraminidase. The stability of the crude enzyme in Pfanstiehl

TABLE 3. *Effect of cations on neuraminidase activity*

Mg concn (M)	NANA[a] released (μg) with Ca concn:				
	0 M	10^{-3} M	3×10^{-3} M	10^{-2} M	3×10^{-2} M
0	2	3	4	7	9
10^{-3}	4	5	8	10	13
3×10^{-3}	5	7	8	12	12
10^{-2}	8	10	10	11	11
3×10^{-2}	10	9	9	10	9

[a] NANA, *N*-acetyl neuraminic acid released from bovine submaxillary mucin after incubation in 0.1 M acetate buffer (pH 5.5) at 37°C for 20 min.

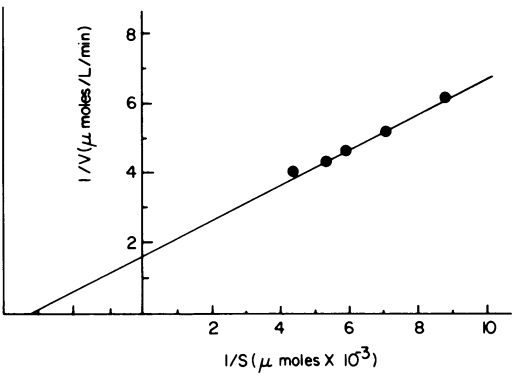

FIG. 4. *Lineweaver-Burk plot for hydrolysis of bovine submaxillary mucin by partially purified neuraminidase. The enzyme was incubated with substrate for 30 min in 0.1 M sodium acetate (pH 5.7) containing 0.02 M $CaCl_2$.*

dialysate medium was assessed after incubation at various temperatures in the presence or absence of NaCl (Table 4). Preincubation of the enzyme at 4 to 22°C for 10 to 30 min before assay did not appear to alter significantly the activity of the enzyme. Although there was some decrease in enzyme activity after incubation at 37°C in the presence of 0.15 M NaCl, an appreciable drop in enzyme activity was observed after preincubation at 37°C for 30 min in the absence of NaCl. Almost total loss of enzyme activity occurred after incubation periods of 10 min at 50 and 65°C.

DISCUSSION

Although several investigators have reported the production of neuraminidase by β-hemolytic streptococci (15–18, 24, 27), there are no definitive reports regarding production of this enzyme by group A streptococci. Hayano and Tanaka (15) first suggested that group A streptococci of serotypes 3, 12, and 42 produced slight decomposition of submaxillary mucin included in the culture medium. However, they could not find evidence for extracellular production of neuraminidase similar to that observed for a group K streptococcal strain. In subsequent studies (16, 17) these investigators described the production of a "sialidase-like" enzyme by several beta-hemolytic streptococcal organisms belonging to serogroups A, B, C, G, and L. This enzyme, unlike other bacterial neuraminidases, was active on a sialomucoid prepared by acid treatment of bovine submaxillary mucin. The enzyme had no activity on bovine *N*-acetyl-neuraminyl lactose or alkali extracted bovine sialomucoid, substrates that were quite susceptible to degradation by the group K streptococcal sialidase and *Vibrio cholera* sialidase. Of the many group A streptococcal serotypes tested, M types 4 and 22 produced appreciable amounts of this enzyme (17).

Our studies have confirmed the reports by Hayano and Tanaka (16, 17) that a neuraminidase which hydrolyzes bovine submaxillary mucin is produced in culture supernatants of a limited number of serotypes of group A streptococci. The serotypes that produced appreciable amounts of the enzyme included T-1:M-1, T-4:M-60, and T-12:M-12. No pretreatment of submaxillary mucin was needed to demonstrate neuraminidase activity in these supernatants. However, like the St-sialidase reported by Hayano and Tanaka, this neuraminidase showed no hydrolysis of sialic acid from neuraminyl-lactose, or from the alkali-labile reduced oligosaccharides of porcine submaxillary mucin or from human orosomucoid. That this difference in substrate specificity was not due to the release of glycoloyl, diacetyl, or triacetyl neuraminic acids which are only partially chromogenic with thiobarbituric acid was excluded by the determination of total sialic acid in the bovine submaxillary mucin substrate after incubation with the enzyme preparations. A similar heterogeneity in

TABLE 4. *Stability of streptococcal neuraminidase after preincubation at various temperatures*

Preincubation temp (°C)	NANA released[a] (μg)		
	10-min preincubation (0.1 M acetate)	30-min preincubation	
		0.1 M acetate	0.1 M acetate–0.15 NaCl
4	21.5	20.0	22.6
22	22.0	19.5	20.2
37	23.2	15.5	19.3
50	1.1	0.6	0.8
65	0.8	0.8	1.0

[a] NANA, *N*-acetyl neuraminic acid released from bovine submaxillary mucin after incubation in 0.1 M acetate (pH 5.5) with 0.02 M $CaCl_2$, with or without 0.15 M NaCl, at 37°C for 2 h.

substrate specificity has been encountered for *V. cholera* and myxovirus neuraminidases (9, 10). Structural studies on the carbohydrate moiety of bovine submaxillary mucin have shown that neuraminic acid is covalently linked to *N*-acetylgalactosamine by an α,2-6 linkage (10). The same linkage is present in porcine submaxillary mucin, whereas in sialyl-lactose at least 15% of the sialic acid is attached to D-galactose via an α,2-6 linkage (5, 8). Yet the sialic acid of these latter substrates are not hydrolyzed by the group A streptococcal neuraminidase. It would thus appear that specificity is not directed at the linkage between the neuraminic acid and the terminal oligosaccharide.

Although the biochemical characteristics of the enzyme produced by the group A streptococcal serotype T-4:M-60 were in general similar to those reported by Hayano and Tanaka for the M-4 serotype (16), certain differences were encountered between the two enzymes. The optimal pH range of the T-4:M-60 enzyme was narrower, and its K_m was approximately one order of magnitude lower than that of the M-4 enzyme. These differences may be ascribed to our use of the partially purified enzyme for determining the biochemical characteristics or may be due to the difference in substrate used in these studies. However, striking similarities were observed between the T-4:M-60 neuraminidase and that produced by the group K streptococcus (18). The molecular weight of the latter enzyme is 110,000, the pH optimum is 5 to 7, and it requires cations at similar concentrations for maximal activity. It has a K_m of 3.6×10^{-4} for submaxillary mucin and is labile at temperatures of 50°C and above. Yet as discussed above, the substrate specificities of the two enzymes are strikingly different.

Extracellular neuraminidase production by the group A streptococcus paralleled the growth of the organism. As with group B streptococcus (24), maximal activity was present during the late exponential phase of growth. The decline in supernatant activity that was encountered during the stationary phase at 37°C is probably related to instability of the enzyme at various temperatures, or inactivation by extracellular group A streptococcal proteinase (4, 11). Another factor that may mask enzyme activity is the dialyzable inhibitor of the chromogen formed by thiobarbituric acid in the assay procedure described by Aminoff (1). This substance may obscure sialidase activity in undialyzed culture supernatants, a phenomenon also reported by Pinter and his co-workers (27).

Although the survey for neuraminidase production by group A streptococcal serotypes in this study was not exhaustive, the fact that strains belonging to T types 1, 4, and 12 account for 12 of the 14 strains found to produce appreciable amounts of this enzyme may be of particular interest. As reported by Ferrieri (12), strains belonging to these serotypes are among those that have shown a strong association with acute glomerulonephritis. The implication of this finding is emphasized by the reports of McIntosh and his co-workers (14, 21-23) on the formation of cryoprecipitates that possess nephritogenic activity after incubation of immunoglobulins with either the culture supernatant of a nephritogenic streptococcal strain or with neuraminidase of *Clostridium perfringens*. In addition, the recent report by Fillit and his co-workers (13) in which cell-mediated immunity by lymphocytes of patients with nephritis was demonstrated to glomerular basement membrane treated with neuraminidase and glycosidase, but not to membrane not treated with these enzymes, further emphasizes the significance of these results. Because the strains included in this report were not isolated from patients with nephritis, studies will be performed to correlate the production of neuraminidase with documented nephritogenic streptococcal strains and to determine the factors that influence the in vitro and in vivo production of this enzyme by these strains.

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