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A Neuraminidase from *Streptococcus sanguis* That Can Release *O*-acetylated Sialic Acids*

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The naturally occurring sialic acids can have different types of *N*- and *O*-substitutions, resulting in more than 20 known isomers and compounds. Most methods for the detailed study of these various sialic acids require that the molecules be first released from their α-glycosidic linkage. When mild acid hydrolysis is used for this purpose, significant destruction of *O*-substituent groups occur. On the other hand, the presence of *O*-substituent groups renders the sialic acid molecule partially or completely resistant to the action of the currently known neuraminidases. To circumvent this problem, we searched for a neuraminidase whose activity is not affected by *O*-substitution. We reasoned that because *Streptococcus sanguis* from the human oral cavity is continually exposed to *O*-substituted sialic acids, its extracellular neuraminidase might not be blocked by *O*-substitution. We therefore purified this enzyme 3100-fold (56% yield) using ammonium sulfate precipitation, *N*-(*p*-aminophenyl)oxamic acid-agarose affinity chromatography, and chromatography on quaternary aminoethyl (QAE)-Sephadex, sulfopropyl (SP)-Sephadex, and Sephacryl S-200. The purified preparation is free of other significant glycosidase activities and proteolytic activities. It is capable of quantitatively releasing all the *O*-acetylated sialic acids that we studied with the single exception of the 4-*O*-acetylated sialic acid of equine submaxillary mucin. The activity of the enzyme is also not restricted by the type pf sialic acid linkage or the nature of the underlying oligosaccharide. However, it has maximal activity on gangliosides only in the presence of detergents. The general properties of this enzyme are described and its substrate specificities are contrasted with those of the commonly used neuraminidase from *Vibrio cholerae*.

The sialic acids are a family of *N*- and *O*-substituted derivatives of neuraminic acid, a nine-carbon polyhydroxyaminoketoacid sugar (5-amino-3,5-dideoxy-D-glycero-D-galactononulosonic acid). They have a restricted distribution in nature, being present in higher animals and certain bacteria, but not in plants or lower invertebrates (1, 2). In animals they are found in α-glycosidic linkage as the outermost sugar of glycoproteins and glycolipids, except in the case of the polysialosyl linkage (3–6), and certain other rare exceptions (7). Many different functions have been attributed to the sialic acids, including the binding of hormones, toxins, and viruses, the masking of surface antigens, the modification of the circulating half-lives of red cells and glycoproteins, the maintenance of surface negative charge, and contribution to the viscosity of the mucins, etc. (8–11). Much has also been written about differences between the sialylation of normal cells and their malignant counterparts (12–14) and the possible relationship of these differences to certain properties of tumors, such as metastatic potential (12, 15–18).

Many of these studies do not take into account the fact that the two principal *N*-substituted sialic acids, Neu5Ac[1] and Neu5Gc can be *o*-substituted at the 4, 7, 8, and 9 positions giving rise to a great variety of possible compounds and isomers (see Fig. 1) (11, 19). These substitutions clearly affect the physicochemical nature of the parent molecule (11, 20, 21) and also interfere considerably with many of the standard methods used for the analysis of sialic acids (11, 19, 22). The elegant studies of Schauer and others have to date resulted in the identification of over 20 different kinds of sialic acids in nature, including specific types of *O*-substitutions in many human (and other mammalian) tissues such as brain, colon, salivary and gastric mucins, and peripheral blood cells (11). However, very little is known about their biological significance.

One effect of *O*-substitution is that it can partially or completely block the action of bacterial and viral neuraminidases (11, 19, 23). While this may have biological relevance to protection from bacterial and viral attack, it also poses practical problems in the use of purified neuraminidases for analysis of the sialic acids. Most reliable methods for the detailed study of the various types of sialic acids require that the molecules be first released from their α-glycosidic linkage (11, 19). When mild acid and heat are used for this purpose, significant destruction and the *O*-substituents will occur even

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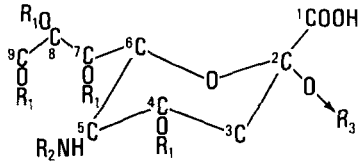
[1] The abbreviations used are: Several different systems of nomenclature for the sialic acids are in use (1, 65, 66). We have chosen to use the system recommended by Schauer and others (65). The various sialic acids are designated by combinations of Neu = neuraminic acid; Ac = acetyl; Gc = glycolyl; and Lac = lactyl. The amino group at the 5 position is always substituted with an acetyl (Ac) or a glycolyl (Gc) group. Other substitution positions are indicated by the numerals. For example, *N*-acetyl-9-mono-*O*-acetylneuraminic acid may be written as Neu5,9Ac_2 and *N*-glycolyl-7,8,9,-tri-*O*-acetyl neuraminic acid as Neu7,8,9$Ac_3$5Gc. Other abbreviations are Neu2en5Ac, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; SA, sialic acid; *O*-Ac, *O*-acetyl group; BSM, bovine submaxillary mucin; ESM, equine submaxillary mucin; 4MU-Neu5Ac, 2′(4-methylumbelliferyl-)α-D-*N*-acetylneuraminic acid; GLC, gas-liquid chromatography; TLC, thin layer chromatography; SDS, sodium dodecyl sulfate.



R_1 = H, ACETYL (4,7,8,9), LACTYL (9), METHYL (8), or SULFATE (8).
R_2 = ACETYL or GLYCOLYL
R_3 = Gal, GalNAc, GlcNAc or S.A.

FIG. 1. **The sialic acids.** The parent molecule neuraminic acid is shown in partially stylized form in the chair conformation. The individual carbon atoms are numbered from 1 through 9. R_1 indicates possible glycosidic linkages, R_2 indicates substitutions of the N group, and R_3 indicates O-substitutions. The known types of substituents are indicated on the figure for each case.

under the mildest possible conditions (11, 19, 22). Also, O-substitution renders the molecule relatively more resistant to release by acid hydrolysis (24, 25), resulting in selective release of the nonsubstituted species. On the other hand, for the reasons mentioned above, quantitative release is not obtained by enzymatic means. Therefore, precise measurement of the degree and type of O-substitution of glycosidically bound sialic acids in a given biological specimen has not been possible (11, 22).

A long term goal of this laboratory is to study the biosynthesis, regulation, and biological significance of O-substitutions of the sialic acids. Therefore, we felt it was necessary to solve the practical problem described above. We reasoned that a bacterial organism that was normally exposed to O-substituted sialic acids in its environment might produce a neuraminidase whose activity was not blocked by O-substitution. We also noted that the original studies concerning the existence of neuraminidases in *Streptococci* suggested that bovine submaxillary mucin (which contains many types of O-acetylated sialic acids) was a superior substrate to sialyl-lactose (26, 27). We therefore chose to study the extracellular neuraminidase of *Streptococcus sanguis*. This organism is a normal commensal of the human mouth, in which the salivary mucins contain O-acetylated and O-lactylated sialic acids (28).

EXPERIMENTAL PROCEDURES[2]

RESULTS

Spectrum of Activity of S. sanguis Neuraminidase—Our major purpose in purifying this enzyme was to obtain a practical tool for quantitative release of O-substituted and unsubstituted sialic acids from any biological source. Previous studies have shown that the activity of a neuraminidase can be limited by the presence of O-substitution (11, 19, 23), the type of N-substitution (45), the type of glycosidic linkage (11, 23, 45–47), and by the nature of the molecule to which the sialic acid is linked (11, 23, 48–50). Esterification of the carboxyl group also results in resistance (11), but this has never been found in nature (1). We therefore studied the activity of this enzyme toward a wide variety of substrates, containing many different types of sialic acids, in different types of linkages, on different types of underlying molecules. Each of these substrates (unlike the synthetic substrate 4MU-Neu-5Ac, see miniprint) contained a mixture of different types of sialic acids and/or different types of glycosidic linkage. Therefore, we did not feel it was worthwhile to study the kinetic parameters of the *initial rates* of release of sialic acids from these substrates. Instead, we asked the following practical questions. Can all of the sialic acids be released from each of these substrates upon incubation with the enzyme? Are there sialic acid residues (especially O-substituted ones) in these substrates that are resistant to the commercially available *Vibrio cholerae* neuraminidase, which are sensitive to the *Streptococcus sanguis* neuraminidase? The results of the studies aimed at answering these questions are summarized in Table II, which compares the ction of the two enzymes on a variety of substrates. In these experiments, prolonged incubations were carried out, with repeated addition of enzyme, until no more sialic acids were released from the substrate. For each substrate, the maximum possible release was also determined by the 2-thiobarbituric acid assay following de-O-acetylation and acid hydrolysis. The results with each substrate are therefore expressed as a per cent of the maximum possible release that could be obtained with the enzyme in question. It can be seen that both enzymes were capable of cleaving unsubstituted sialic acids from glycoproteins, gangliosides, and red cell membranes in all the common types of glycosidic linkage (α2→8SA, α2→6Gal, α2→3Gal, α2→6GalNAc, α2→3GalNAc, and Galβ1→3(Neu5Acα2→6)GlcNAc). We are not certain why the sialic acids of bovine prothrombin were not fully released by the *Vibrio* enzyme. It is possible that the Galβ1→3(Neu5Acα2→6)GlcNAc linkage is relatively resistant to this enzyme. Alternatively, a portion of the sialic acids may be O-acetylated (see below). The maximal release of sialic acids from mixed brain gangliosides by the *Streptococcus* enzyme required the presence of detergents. The "internal" sialic acid residue of G_{M_1} ganglioside was resistant to both enzymes, as it is to all other known neuraminidases, except that of *Arthrobacter ureafaciens* (49). We did not study certain other rare neuraminidase-resistant linkages that have been reported in the literature (50) or the uncommon Neu5Acα2→4Gal linkage (11).

As model substrates containing O-substituted sialic acids, we chose to use bovine and equine submaxillary mucins. This is because these two substrates together contain most of the known kinds of substituted sialic acids with O-acetyl substitutions (11). Bovine submaxillary mucin contains, in addition to some unsubstituted Neu5Ac and Neu5Gc, the 7-, 8-, and 9-mono-O-acetylated species, smaller amounts of the di-O-acetylated molecules and traces of tri-O-acetylated species (11). As shown in Table II, the *S. sanguis* neuraminidase was capable of releasing all of the sialic acids from BSM. On the other hand, even with prolonged incubations and repeated additions of enzyme, *V. cholerae* neuraminidase was only capable of releasing 59% of the sialic acids from this substrate. As expected, the combination of the two enzymes also released all of the sialic acids from BSM.

To further detail the differences in the activities of the two enzymes toward BSM sialic acids, we carried out a time course experiment that is shown in Fig. 3. Under identical conditions of enzyme and substrate concentration, it can be seen that the *Streptococcus* enzyme releases a greater proportion of the sialic acids at all time points. With further addition of the *Vibrio* enzyme and prolonged incubation, a very small amount of further release was obtained and a significant proportion of the available sialic acid remains resistant. Next, we wished to determine the nature of the sialic acids that were released by the two enzymes, especially those that were released by the *Streptococcus* enzyme but not by the *Vibrio* enzyme. We

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Table I, and Fig. 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83 M-1481, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE II

Maximal release of sialic acids from various substrates by neuraminidases

Substrates 2–8 were first dialyzed 3 times to remove all traces of free sialic acids. Each reaction mixture contained 200 nmol of bound sialic acids, 7.25 milliunits of *S. sanguis* neuraminidase (SSN) and/or *V. cholerae* neuraminidase (VCN), 150 mM NaCl, 50 mM sodium acetate, 2 mM $CaCl_2$, pH 5.5, in a final reaction volume of 200 μl. The reaction mixtures were incubated for 18 h under a toluene atmosphere at 37 °C. Aliquots (10 μl) were removed, treated with 0.1 N NaOH at 4 °C for 45 min to eliminate interference by *O*-acetyl groups, and the total released sialic acids were measured by the 2-thiobarbituric acid method. Maximum possible (100%) release was determined by the α-thiobarbituric acid method after treating similar aliquots with base followed by 0.1 N H_2SO_4 at 80 °C for 1 h. If >95% release was not obtained, additional enzyme was added and the incubation was continued. This procedure was repeated until no further release could be obtained after repeated addition of enzyme.

Substrate	*O*-substitution of sialic acid		Type of linkage of sialic acid	Type of oligosaccharide	% of total sialic acids released by		
	Type	%[a]			SSN	VCN	SSN and VCN
Sialyllactose	None		α2→3 Gal (85%) α2→6 Gal (15%)	Free oligosaccharide	>95	>95	ND[b]
Colominic acid	None		α2→8 Neu5Ac	Polymer	92	95	ND
Collocalia mucoid	None		?	*O*-linked (?)	>95	>95	ND
Fetuin	None[c]		α2→3 Galβ1→3 GalNAc (6, 2) α2→6 Gal (α)	*O*-linked *N*-linked	>95	>95	ND
α_1-Acid glycoprotein	None[c]		α2→3 Gal α2→6 Gal	*N*-linked	>95	>95	ND
Bovine prothrombin	None[c]		α2→3 Galβ1→3 GlcNAc (6, 2) α	*N*-linked	>95	78	>95
Bovine submaxillary mucin	±9-OAc, ±8-OAc	≃80	α2→6 GalNAc (1, ±3) GlcNAc	*O*-linked	>95	55	>95
	±7-OAc	≃50					
Equine submaxillary mucin	±4-OAc	≃80	?	*O*-linked	18	20	27
G_{M_1} ganglioside (+)/(−) 5 mM sodium taurocholate	None[c]		Galβ1→3 GalNAcβ1→4 Gal (3, 2) α	Ganglioside	3.1(−) 3.0(+)	2.4(−) 3.1(+)	ND ND
Mixed brain gangliosides (+)/(−) 5 mM sodium taurocholate	None[c]		α2→8 Neu5Ac α2→3 Gal and G_{M_1} linkage	Gangliosides	8(−) 92(+)	88(−) ND(+)	ND ND
Human red blood cell ghosts	None		α2→3 / α2→6 Gal or GalNAc	*O*-linked + *N*-linked + gangliosides (membrane-bound)	>95	>95	ND

[a] The % of *O*-acetylation of the sialic acids in the intact substrate was estimated by the periodate/formaldehyde method (9/8 *O*-acetylation) (67) or by analysis of acid-released purified sialic acids (7 or 4 *O*-acetylation).

[b] ND, not done; ± indicates that the *O*-acetyl groups may be present or absent on a given sialic acid molecule, in these substrates.

[c] The literature does not report *O*-acetylation of sialic acids in these substrates. However, none of these structural studies mentions whether *O*-acetylation was specifically looked for.

therefore purified the released sialic acids from several points along this time course (see Fig. 3) in order to analyze them by gas-liquid chromatography.

Certain factors that complicate this analysis must be mentioned at this point. The conventional method for purification of free sialic acids is known to cause considerable and variable (30–50%) loss of *O*-acetyl groups. This would obviously confound the study of the enzymatically released sialic acids by GLC. We have therefore modified the purification procedure to reduce this loss considerably (less than 10%). However, in addition to this problem, we[3] and others (11) have noted that variable degrees of migration of *O*-acetyl groups can also occur during the purification, apparently from the 7/8 positions to the 9 position. With our current method of purification, such migration occurs at a low rate if the original sample originates from an acid hydrolysis reaction. However, the presence of the acetate buffer seems to potentiate the problem, so that migration is essentially complete by the end of purification from an enzyme-released sample.[3] Therefore, although the bovine submaxillary mucin contains a significant fraction of $Neu5,7Ac_2$ when studied by acid hydrolysis, the purified products of the enzyme reactions contain mainly $Neu5,9,Ac_2$, a portion of which must be derived from $Neu5,7Ac_2$ during the purification and/or during the enzyme incubation itself. This fact has to be kept in mind in interpreting the results obtained with GLC of the enzyme-released purified sialic acids.

Fig. 4 shows some representative tracings obtained from GLC analysis of the enzyme-released sialic acids. It can be seen that early in the course of the reaction of *Vibrio* enzyme

[3] A. Varki and S. Diaz, unpublished observations.

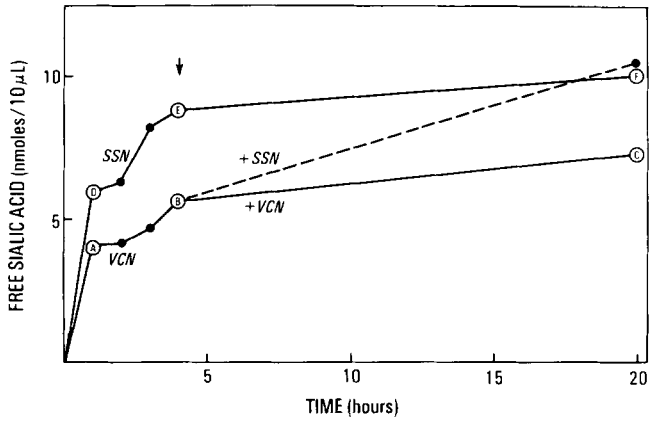


FIG. 3. **Time course of release of sialic acids from bovine submaxillary mucin by neuraminidases.** Two identical reaction mixtures were prepared, each containing bovine submaxillary mucin (final concentration of bound sialic acids, 1.1 mM), sodium acetate (final, 50 mM), pH 6.0, and calcium chloride (final, 2 mM), and either *V. cholerae* neuraminidase (*VCN*) or *S. sanguis* neuraminidase (*SSN*). The final concentration of enzyme in each case was 86 milliunits/ml. (The activities of the two enzymes were standardized immediately prior to use by their activity against Collocalia mucoid, as described under "Experimental Procedures." The reaction mixtures were incubated at 37 °C; at the various time points indicated, 10-μl aliquots were assayed for total free sialic acid by the 2-thiobarbituric acid method after de-*O*-acetylation with base. Each *point* represents the mean of duplicate determinations, with subtraction of an identically incubated enzyme-free blank. (The blank never exceeded 1 nmol, even after prolonged incubation.) At the time points indicated, larger aliquots were also removed from the *Vibrio* enzyme incubation (*A*, *B*, and *C*) and the *Streptococcus* enzyme incubation (*D*, *E*, and *F*) and saved for analysis of the released sialic acids by GLC (see Fig. 4). At the time point indicated by the *arrow*, the first incubation was split into two portions. To one, additional *V. cholerae* neuraminidase was added (*solid line*) and to the other, *S. sanguis* neuraminidase was added (*dotted line*). In each case, the amount of enzyme added was sufficient to increase the concentration by 86 milliunits/ml). The aliquots subsequently assayed for free sialic acid were appropriately corrected for the change in volume. The reaction mixture that originally started with *Streptococcus* enzyme alone was allowed to continue without any further additions.

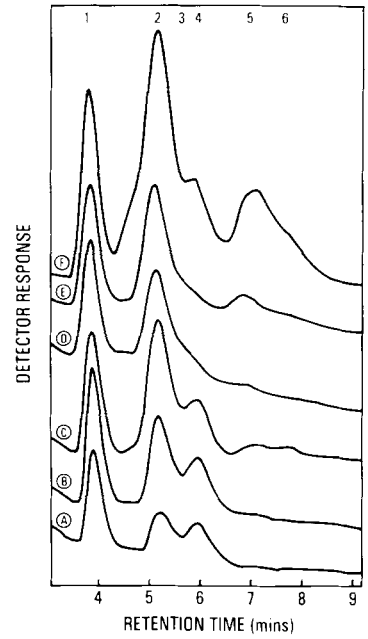


FIG. 4. **Gas-liquid chromatography of sialic acids released by neuraminidases from bovine submaxillary mucin.** Aliquots of the enzyme reactions described in Fig. 3 were removed at the times indicated and quenched by addition of 1/10 the volume of 2 M formic acid. After centrifugation to remove insoluble materials, the free sialic acids were purified and then separated by gas-liquid chromatography on OV-17 (3%) as their trimethylsilyl esters, trimethylsilyl ethers as described under "Experimental Procedures." Six separate tracings are superimposed in this figure for convenience. The *capital letters* indicate the origin of the sialic acids (see Fig. 3). *A*, *B*, and *C* represent samples removed from the *V. cholerae* neuraminidase reactions at 1, 4, and 20 h, respectively, and *D*, *E*, and *F* are samples removed from the *S. sanguis* neuraminidase reaction at the same time points. The *numbers* indicate the elution times of *1*, Neu5Ac; *2*, Neu5,9Ac_2; *3*, Neu5,7Ac_2; *4*, Neu5Gc; *5*, Neu5,7(8),9Ac_3; and *6*, Neu5Gc9Ac and/or Neu5,7,8,9,Ac_4. The elution times of the first four are based on known standards and those of the last two are based on the literature (11, 19). In addition, the *O*-acetylated sialic acids were identified by the fact that these peaks were eliminated by prior treatment with mild alkali (2 M NH_4OH, at 60 °C for 60 min), with corresponding increase in the amount of the Neu5Ac peak (not shown).

releases primarily Neu5Ac and small amounts of Neu5Gc and Neu5,9Ac_2. At the same time point (1 h) (also see Fig. 3), the *Streptococcus* enzyme appears to be releasing Neu5Ac and Neu5,9Ac_2 at approximately an equal rate (note that the acetylated sialic acids are known to have a decreased relative detector response compared with the non-*O*-acetylated species (19), thereby causing underestimation of the *O*-acetylated species). With further incubation, the *Vibrio* enzyme continues to release Neu5,9Ac_2 slowly, but even with incubation for 20 h and use of additional enzyme, complete release is not obtained. On the other hand, most of the sialic acids are released by the *Streptococcus* enzyme within 4 h including a significant amount of di-*O*-acetylated species. However, the rate of release of these di-*O*-acetylated species and of Neu5Gc by the *Streptococcus* enzyme is relatively slow compared to the release of Neu5Ac and Neu5,9Ac_2, and prolonged incubation is necessary to release all of these species. In summary, this analysis shows the following (*a*) *Vibrio cholerae* neuraminidase is superior to *Streptococcus sanguis* neuraminidase in only one respect: it appears to work *relatively* better on the Neu5Gc. (*b*) The *Streptococcal* enzyme is superior to the *Vibrio* enzyme in that it releases all of the Neu5,9Ac_2 (and presumably all of the Neu5,7Ac_2) in a short time, and also releases the di-*O*-acetylated species (presumably originally Neu5,7,9Ac_3 and Neu5,8,9Ac_3) against which the *Vibrio* enzyme has very little if any activity. Since the enzyme reactions were carried out at pH 6.0, it is possible that some degree of migration of *O*-acetyl groups from the 7 to the 9 position occurred during the incubation itself. (The migration phenomenon seems to occur mainly above pH 7 (11), but can also occur at lower pH values, at a slow rate.[3]) Therefore, it is possible that the bound Neu5,7Ac_2 may be completely resistant to the *Vibrio* enzyme and gradually becomes available for (slow) release as Neu5,9Ac_2, during the prolonged incubations. This could explain the very slow late phase of release by the *Vibrio* enzyme (see Fig. 3). In the case of the *Streptococcus* enzyme, the kinetics suggests that the late phase of release is mainly accounted for by the appearance of Neu5Gc and the di-*O*-acetylated species. Since the quantity of the tri-*O*-acetylated species (Neu5,7,8,9,Ac_4) in BSM is very low, we could not be certain whether or not it was released by the *Streptococcus* enzyme.

Equine submaxillary mucin has sialic acids that are predominantly *O*-acetylated at the 4 position, in addition to some acetylation (and lactylation) at the 9 position (51). As shown in Table II, most of the sialic acids of ESM were resistant to the action of both enzymes. Thus, the presence of an *O*-acetyl group at the 4 position renders the sialic acid molecule resistant to all known neuraminidases to date, except for certain viral neuraminidases that have a very low rate of activity against this molecule (11, 52). It should be noted that we have studied the activity of the enzymes toward most of the more

common *O*-acetylated sialic acid species. We have not studied substrates with the rare *O*-methylated (53), *O*-sulfated (54, 55), and *O*-lactylated (28, 46) sialic acids.

DISCUSSION

The neuraminidases (sialidases) have long been used as tools for the structural analysis of glycoconjugates. Commercial preparations of these enzymes from several bacterial sources are now available and are widely used to study the structure and biological roles of the sialic acids. However, many such studies do not take into account the fact that *O*-substitutions at the 4, 7, 8, and 9 positions are present in many biological samples, which can render the sialic acid molecules partially or completely resistant to the sialidases (11, 19, 23). This problem limits the utility of these enzymes in quantitative and qualitative studies of sialic acids. On the other hand, when mild acid hydrolysis is used to release sialic acids for analysis, considerable destruction of *O*-acetyl groups occurs.

In this paper, we describe a neuraminidase from *Streptococcus sanguis* whose activity is not significantly limited by *O*-acetylation of the exocyclic hydroxyl residues at the 7, 8, and 9 positions. Since its activity is also not affected by the type of glycosidic linkage, it should prove to be a useful tool for the complete release of sialic acids. However, like all other known neuraminidases, its activity is still blocked by the presence of an *O*-acetyl group at the C-4 position. Fortunately, this type of sialic acid has so far only been described in equine species and in Australian monotremes (11). We are currently studying the neuraminidases from the streptococci that are normal commensals of the horse's mouth, to see if any of them will cleave this sialic acid.

There is evidence that oral α-hemolytic streptococci such as *S. sanguis* and *S. mutans* may play an important role in the pathogenesis of dental caries (cavities) (56, 57). The proposed events include synthesis of dextrans from sucrose that permit adhesion of the organisms to the teeth (56) and a lectin-mediated binding of the organisms to specific sialyl-oligosaccharide sequences (58, 59). The sialic acids of the salivary mucins from many species are *O*-substituted (11); perhaps this may serve as a protective mechanism against attack by bacterial neuraminidases. The capacity of the *S. sanguis* neuraminidase to cleave *O*-acetylated sialic acids could therefore be of importance in the pathogenesis of caries.

Several other streptococcal strains have been noted to produce neuraminidases (26, 27, 36, 37, 60–62). In most cases, detailed studies of the substrate specificities of these enzymes were not reported, particularly with regard to the effects of *O*-acetylation. The neuraminidase produced by Type III Group B *Streptococcus* was reported to show some activity against bovine submaxillary mucin but was inactive against colominic acid or sialyl-lactose (37). The neuraminidase from a Group A *Streptococcus* hydrolyzed 40% of the sialic acids from BSM but was inactive against sialyl-lactose, porcine submaxillary mucin, and human orosomucoid (62). This suggests that the neuraminidases from other streptococcal strains may have a more limited range of action than that reported here for the enzyme from *S. sanguis*.

This enzyme also has other advantages. The crude extracellular starting material is already essentially free of proteolytic activities, several glycosidase activities (notably β-galactosidase), and acylneuraminate pyruvate-lyase, which are usually found in the culture filtrates of other organisms such as *Streptococcus pneumoniae* and *Clostridium perfringens* (39, 61). The reasons for this are not clear; perhaps it could relate to the fact that this organism is a normal commensal and only occasionally causes systemic invasive disease (63). Regardless of the reason, it becomes much less likely that a final preparation will contain contaminating activities that can confound the use of the neuraminidase in the structural study of glycoproteins. Unlike some other neuraminidases, this enzyme also does not seem to require the addition of divalent cations for maximal activity. Since traces of divalent cations can markedly interfere with the subsequent analysis of sialic acids by TLC and GLC (19), this has obvious advantages.

A relative disadvantage of this enzyme is that the amount of activity produced is lower than that produced by organisms such as *Diplococcus pneumoniae* (about 5-fold less). However, there is still enough produced to make it feasible to prepare practically useful quantities. It should be noted that some strains of *S. sanguis* may not produce significant levels of neuraminidase; fresh clinical isolates are more likely to be active secretors of the enzyme and this capacity may be lost on serial passage *in vitro* (63, 64). Another relative disadvantage of this enzyme is its somewhat lower rate of activity against Neu5Gc and ganglioside-bound sialic acids in the absence of detergent. Since *Vibrio cholerae* neuraminidase does not have this disadvantage, mixtures of the two enzymes can be used to maximize release of essentially all 7-, 8-, and 9-*O*-acetylated sialic acids in biological samples. We are currently using such a combination of enzymes in our studies of the biosynthesis and regulation of *O*-substituted sialic acids.

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SUPPLEMENTARY MATERIAL

TO

A Neuraminidase from Streptococcus sanguis that can release

O-acetylated sialic acids

Ajit Varki and Sandra Diaz

EXPERIMENTAL PROCEDURES

Materials

The following materials were obtained from the sources indicated: Dehydrated Todd-Hewitt Broth, DIFCO; Sephacryl S-200, Pharmacia Fine Chemicals; Trimethylsilylimidazole (TSMI), Pierce Chemical Company; Bovine serum albumin, Polyethylene Glycol (PEG-8000), Ammonium Sulfate, QAE-Sephadex (Q 25-120), SP-Sephadex and N-(p-aminophenyl) oxamic acid-agarose (Glycyl-L-Leucyl-L-Tyrosine spacer), Sigma Chemical Company; Dowex 1 AG(1x8)(formate form) and Dowex 50 AG (1x2) (Hydrogen form), Biorad; NADH, Boehringer-Mannheim Chemicals; 3% OV-17 on Gas Chrom Q (100/120 mesh), Applied Sciences Laboratories. Sodium Taurocholate was kindly provided by Dr. M. S. Anwer, Division of Gastroenterology, University of California at San Diego. All other chemicals were of reagent grade and were purchased from commercial sources.

Standards, Inhibitors and Substrates.

Chemically synthesized N-acetylneuraminic Acid (Neu5Ac) (>99% purity) was from Kantoishi Pharmaceutical Co. , Tokyo, Japan; N-glycolylneuraminic acid (Neu5Gc), sialyllactose (Type 1), colominic acid (sodium salt, from E. Coli) and the various p-nitrophenyl glycosides were purchased from Sigma; sialic acids from bovine submaxillary mucin (A mixture of Neu5Ac, Neu5Gc, Neu5,9Ac_2, Neu5,7Ac_2, Neu5,7(8),9Ac_3, and Neu5Gc9Ac) were prepared as previously described (22). Authentic Neu5,9Ac_2 was kindly provided by Professor Roland Schauer, Kiel, Federal Republic of Germany. 2,3 Dehydro-2-Deoxy-N-acetylneuraminic acid (Neu2en5Ac) was from Boehringer- Mannheim. Bovine submaxillary mucin was from US Biochemical Corporation; α_1-acid glycoprotein (99% purity) and Azocoll (a broad-spectrum protease substrate) were from Calbiochem; mixed bovine brain gangliosides from Sigma; GM_1 ganglioside from Supelco; fetuin (Spiro method), from GIBCO. 2'-(4-methylumbelliferyl)-α-D-N-acetyneuraminic acid (4MU-Neu5Ac) was kindly provided by Dr. Tom Warner, Dept. of Neurosciences, University of California at San Diego (29). Pure bovine prothrombin was the generous gift of Dr. Paul Bajaj, Dept. of Medicine, University of California at San Diego. Human RBC Ghosts were prepared as previously described (22). Edible bird's nest (Wai Tai Hong, Hong Kong) was purchased from a chinese grocery store, and Collocalia mucoid extracted from it as previously described (30). Equine submaxillary glands were kindly provided by Dr. Olander of the University of California at Davis School of Veterinary Medicine. The glands were removed from a fresh corpse and shipped unfrozen on ice the same day. Equine submaxillary mucin (ESM) was prepared from these glands as follows. After removal of surrounding fat and nodes, the glands were minced into small pieces, and homogenized in three volumes of water with several bursts of a Waring Blender. The homogenate was centrifuged at 15,000 x G for 20 min. The pellet was homogenized again and centrifuged as above. The pooled supernates were passed through a glass wool plug to remove macroscopic lipid material. The material was then slowly acidified with dropwise addition of 2M formic acid, until the mucin precipitated out between pH 4.0–3.5. The precipitated mucin was collected by centrifugation, and dissolved in saturated $CaCl_2$. The mucin was then reprecipitated by addition of 4 volumes of ice-cold ethanol. The precipitate was collected, lyophyllized, and then brought up in water, with mild sonication. All macromolecular substrates were dialysed to remove any traces of free sialic acids before they were used as substrates.

Buffers. The following buffers were used as indicated in the text: Buffer A, 20mM TrisHCl, 80mM NaCl, 2mM $CaCl_2$, pH 7.5; Buffer B, 20mM Tris HCl, 500mM NaCl, 2mM $CaCl_2$, pH 7.5; Buffer C: 50mM Sodium Acetate, 2mM $CaCl_2$, 0. 2mM EDTA, pH 5.5; Buffer D, 50mM $NaHCO_3/Na_2CO_3$, pH 9. 0; Buffer E, 5mM sodium acetate, 2mM $CaCl_2$, pH 5.5. ; Buffer F, 100mM NaCl, 10mM sodium acetate, 2mM $CaCl_2$, pH 5.5.

Enzymes. Streptococcus sanguis neuraminidase was purified as described below. Vibrio cholerae neuraminidase was purchased from Calbiochem. Lactate Dehydrogenase and Acyl-Neuraminate pyruvate-lyase were from Boehringer-Mannheim.

Enzyme Assays Neuraminidase activities were routinely assayed (unless otherwise stated) by the release of Neu5Ac from Collacalia mucoid under the following conditions: The reaction mixture contained Collocalia mucoid (6mM final concentration of bound Neu5Ac), 0.1M Sodium Cacodylate, 2mM Calcium Chloride, pH 6.0, in a final reaction volume of 90 µl. The reaction was allowed to proceed for 10–30mins, and quenched by addition of 10 µl of 0.9N H_2SO_4. The released sialic acid was determined by the TBA assay of Warren (31). Under these conditions, the assay was linear with time and amount of protein added. Although 100% of the Neu5Ac in the substrate could be released by the enzyme, less than 5% was actually released under these assay conditions. One unit of neuraminidase activity is defined as the release of 1µmol of Neu5Ac per minute under these conditions. The release of sialic acids from most other substrates was also monitored by the TBA assay. However, in the case of substrates that contained O-substituted sialic acids, the reaction was quenched with an equal volume of 0.2N NaOH,the mixture placed on ice for 45 min, then acidified and assayed by the TBA method. The base treatment was done to eliminate the known interference caused by the O-substituents in the colorimetric assay (11, 19, 22, 32). The assays utilising 4MUNeu5Ac were carried out under similar conditions to those described above, with the various concentrations of the substrate as indicated. The reactions were quenched with 1ml. of 0.1M sodium bicarbonate buffer, pH 9.1 and the released 4-methylumbelliferone detected as previously described (29), using a Perkins-Elmer 650-10S Fluorescence Spectrophotometer. Glycosidase assays using the various p-nitrophenol glycoside substrates were carried out in 100 µl of sodium acetate buffer, pH 5.0, containing 2mM concentrations of the substrates, quenched by addition of 900 µl of 0.2M sodium carbonate, and the released p-nitrophenol detected by the change in A_{410}. The glycosidase activities are expressed in Units (1 µmol of para-nitrophenol released per min under the conditions described). Acyl-neuraminate pyruvate-lyase was assayed exactly as described (33). Non-specific proteolytic activity was assayed using the substrate Azocoll, exactly as described by the manufacturer, except that the conditions were those under which the neuraminidase was normally assayed. In all enzyme assays, the appropriate blanks without substrate or enzyme were incubated in parallel.

General Analytical Methods. Protein was assayed by the method of Lowry (34), using bovine serum albumin as a standard. In some cases the protein in the eluate from columns was monitored by measuring A_{280}.

Purification of Sialic acids. Sialic acids released from glycosidic linkage by enzymatic or acid hydrolysis were purified by the method of Schauer (11, 19), with several modifications, to minimize the loss of O-acetyl groups. The reactions mixtures were quenched with 1/10 volume of 2M formic acid, clarified by centrifugation, and the released sialic acids were separated from macromolecular material by vacuum reverse dialysis with a Millipore CX-10 device. The level of fluid was maintained using 10mM formic acid. The collected filtrates were taken to dryness using a Buchler Shaker-Evaporator (water bath at 35°C), brought up in 1ml of 10mM formic acid, and extracted three times with five volumes of ether to remove lipids. The aqueous phase was taken to dryness again, brought up in 0.5ml of water and immediately applied to a 1ml column of Dowex 50 AG (Hydrogen form) that was well washed in water. The column was washed with 4ml of water and the eluates collected into a tube containing 20µl of 2M formic acid. The combined acidified eluates were taken to dryness as above, brought up in 0.5ml of 50mM sodium acetate, pH 5.5 and applied to a 1ml column of Dowex1 AG 1x8 (formate form) equilibrated in 10mM sodium formate, pH 5.5. The column was immediately washed with 5ml of 10mM formic acid. The sialic acids were then eluted with 6ml of 2M formic acid, and the eluate taken to dryness as described above. The sample was then brought up in 0.5 ml of water and immediately applied to a 1ml column of Dowex50 AG 1x2 (Hydrogen form) in water. The column was washed with 4ml of water, and the washings collected directly into a tube containing 20µl of 2M formic acid. The acidified washings were taken to dryness; the purified sialic acids were then ready for analysis by gas-liquid chromatography. All steps in this procedure were carried out at room temperature, with the sole exception of the anion exchange step, which is performed at 4°C. The rationale for the various modifications in the conventional purification procedure will be described in detail elsewhere. This procedure results in > 90% recovery with no detectable loss of O-acetyl groups. However, as discussed in the text, migration of O-acetyl groups from the 7 to the 9 position did occur during the purification.

Analysis of Sialic Acids. For general purposes, the ferric-orcinol and 2-thiobarbituric acid (TBA) assays were used to assay total and free sialic acids, respectively (19, 31). In the case of the TBA assay, analytical de-O-acetylation was always carried out to quantify the interference caused by the presence of O-acetyl substituents at the 7,8, or 9 positions (22, 32, 35). Purified sialic acids were derivatized with Trimethylsilylimidazole and analysed by gas-liquid chromatography on OV-17 (3%) exactly as described by Schauer (19), except that the N_2 flow rate was 60ml/min. and the temperature program began at 190°C and proceeded at 1.5°/min.

Bacterial Organism: a clinical isolate of Streptococcus sanguis, originally obtained from the California Public Health Service was kindly provided by Dr. Charles Davis, Department of Pathology, University of California at San Diego. The organism was maintained on an agar slant, and further cultured in Todd-Hewitt Broth that was adjusted to pH 7.7 before use. These conditions gave maximum enzyme production in the case of other streptococcal neuraminidases (36, 37).

Purification of the extracellular neuraminidase from Streptococcus sanguis

1. Culture Supernatant: two 12L carboys of Todd-Hewitt broth were inoculated with 200ml spent-phase cultures of Streptococcus sanguis that had been grown in the same medium. The cultures were placed at 37°C without stirring for 18 hours, and then placed in a cold room at 4°C. All further operations were carried out at 0-5°C, and all utensils used were made of plastic. The bacteria were removed by centrifugation at 10,000 x G for 15 mins, and the supernate passed over a plug of siliconized glass wool in a plastic funnel, into plastic pails. 18L of the culture supernate thus obtained was used for the subsequent purification steps.

2. Ammonium Sulfate Precipitation: Solid ammonium sulfate was added slowly with stirring, for a final 80% saturation. The mixture was allowed to stand overnight without stirring, and the precipitate collected by centrifugation at 14,500 x G for 20 min. The combined pellets were dissolved into a total volume of 310 ml with distilled water, and 35 G of solid ammonium sulfate was then added while stirring. After 1h, the precipitate was collected as described above.

3. QAE-Sephadex chromatography. The ammonium sulfate pellet was brought up in 200ml of Buffer A, and dialysed 3 times for 12 hours each against a 10-fold volume of the same buffer. The precipitate that formed during the dialysis was removed by centrifugation at 7000 x G for 15 min, and the supernate was applied to a column of QAE-Sephadex (220ml total volume) equilibrated in Buffer A. The column was washed with two column volumes of the same buffer and then eluted with a linear gradient of NaCl using 200ml each of Buffer A and Buffer B. Fractions(6.5ml) were collected and monitored for protein and neuraminidase activity. Under these conditions, all of the enzyme bound to the column and eluted in a single peak (not shown).

4. N-(p-Amino-Phenyl) Oxamic Acid-Agarose Affinity Chromatography.The pooled material from the previous step was dialysed against two liters of Buffer C., and loaded onto a 10ml. column of the N-(p-amino-phenyl) oxamic acid-agarose that had been washed well with the same buffer. The column was then washed with 40ml of Buffer C., and then eluted with Buffer D. Fractions (0.5ml) were collected directly into tubes containing 50µL of 0.5M sodium acetate pH 5.5, and monitored for neuraminidase activity. Under these conditions all of the enzyme bound to the column and eluted with Buffer D. The fractions containing the activity were pooled and dialysed against Buffer E.

5. SP-Sephadex Chromatography The dialysed material from the previous step was applied to a 2ml column of SP-Sephadex equilibrated in Buffer E. Under these conditions, all of the activity ran thru the column while almost half of the total protein remained bound.

6. Sephacryl S-200 Chromatography. The material running through the SP-Sephadex column was adjusted to 100mM NaCl by addition of 4M NaCl, concentrated to 3ml by reverse dialysis against PEG-8000, and then applied to a column of Sephacryl S-200 (1.5cms x 46cms.) equilibrated in Buffer F. The column was eluted with the same buffer, 1ml fractions were collected and monitored for protein (A_{280}) and neuraminidase activity. The enzymatic activity eluted as a single peak close to the void volume of the column.

RESULTS

Purification of the extra-cellular neuraminidase from S. sanguis.

A representative purification is shown in Table 1. The enzyme was purified about 3100-fold, with an apparent 56% yield of the starting activity. The slight over-recovery at the second step may have resulted from removal of inhibitor(s), making the estimation of the final yield less accurate. In keeping with previous observations (38-41), the N-(p-aminophenyl) oxamic acid-agarose affinity step was not highly specific for the neuraminidase, but nevertheless resulted in useful purification. At the final step (Sephacryl S-200 chromatography) the activity profile did not coincide exactly with the protein profile. This suggests that the final preparation is not homogeneous. At this step the activity eluted close to, but not at the void volume of the column of the column.

Since our primary purpose was to use this enzyme preparation for structural studies of oligosaccharides, it was important to look for the presence of other contaminating glycosidases. The starting material after the initial ammonium sulphate precipitation contained a total of 17.8 U of β-hexosaminidase, 3.1 U of α-galactosidase, and 1.12U of α-fucosidase, but had no detectable α-mannosidase, β-mannosidase, β-galactosidase, or Acyl-neuraminate pyruvate-lyase. The final preparation was free of the α-fucosidase, but contained traces of β-hexosaminidase (0.2U total) and α-galactosidase (0.14U total). These trace contaminating activities could only be detected by the use of large volumes of the final preparation. Therefore we felt that their presence should not pose a practical problem in the use of the enzyme preparation for structural studies. If necessary the β-hexosaminidase could be easily inhibited by the addition of 2-acetamido-2-deoxygluconolactone (42). Non-specific proteolytic activity assayed with Azocoll was undetectable in both the starting material and the final preparation.

Storage and Stability. The purified enzyme gradually lost approximately 50% of its original activity when stored at 4°C for over 90 days. Although a single cycle of freezing and thawing caused a loss of about 30% of the activity, the remaining activity remained stable to storage for over 90 days at -80°C. The activity was also stable to lyophylization. Most of the the preparation was therefore frozen, lyophylized and stored at -80°C for future use. We also found that the enzyme lost a considerable amount of activity when it was diluted to a protein concentration of less than 1µg/ml. This loss of activity upon excessive dilution could not be corrected by addition of bovine serum albumin at 100µg/ml. The enzyme also rapidly lost activity when maintained at a pH less than 5 for any length of time.

General Properties. The pH/activity profile of the enzyme is demonstrated in figure 2. Activity was maximal in a narrow range between 5.5 and 7.0. The effect of addition of various ions was studied. Except for a slight stimulation of activity at high concentrations of calcium, none of the divalent cations had any positive effects. On the other hand, zinc, copper and cobalt ions caused considerable loss of activity. The addition of sodium chloride at concentrations upto 500mM had no effect upon the activity. Chelating agents such as EDTA had no effect on the enzyme activity in concentrations as high as 10mM. The enzyme retained greater than 80% of its activity in 0.1% SDS, but lost activity rapidly at higher concentrations of the detergent.

Kinetic properties of the neuraminidase against 2'(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid. (4MU-Neu5Ac). Most biological substrates contain more than one type of sialosyl linkage, and often more than one type of sialic acid. They are therefore not suitable for precise study of the kinetic properties of the enzyme. We therefore used the synthetic substrate (4-MU-Neu5Ac), against which the enzyme was active. The initial rate of release of the fluorescent product, 4-methylumbelliferone was determined under varying substrate concentrations, from 25 to 600µM, in 0.1M sodium cacodylate buffer, pH 6.0. All assays were carried out in duplicate with appropriate blanks and controls. Michelis-Menten constants were derived from double reciprocal plots ([1/V] versus [1/S]) of these multiple data points. Under these conditions, the apparent Km was 4.7×10^{-5}M. The V_{max} for this preparation of enzyme was 749 nmoles/min/mg protein.

Inhibition of activity. The effect of the neuraminidase inhibitor 2-deoxy 2,3-dehydro-N-acetyineuraminic acid (Neu2en5Ac) on the activity of the enzyme was studied. The activity of the enzyme against 4MU-Neu5Ac was assayed with the substrate at various concentrations with freshly prepared inhibitor solutions added for final concentrations in the range of 0-30µM. The data was plotted in the form of a Dixon plot ([1/V] versus [I]). The compound behaved as a competitive inhibitor, with a K_i of 7×10^{-6}M. This is within the range reported for the inhibition of other neuraminidases (43, 44).

TABLE 1

PURIFICATION OF STREPTOCOCCUS SANGUIS NEURAMINIDASE

STEP	VOLUME (ml)	PROTEIN (mg)	TOTAL ACTIVITY (mU)	SPECIFIC ACTIVITY (mU/mg)	APPARENT YIELD (%)	PURITY (-fold)
1. Ammonium Sulfate	374	10520	9044	0.86	100	1
2. QAE-Sephadex	90	594	9675	16.3	107	19
3. N-(p-aminophenyl) Oxamic Acid-Agarose	13.5	26.1	8168	313	90	364
4. SP-Sephadex	20	15.7	6950	442	77	514
5. Sephacryl S-200	7	1.9	5075	2685	56	3122

Experimental details are as described under "Experimental Procedures".

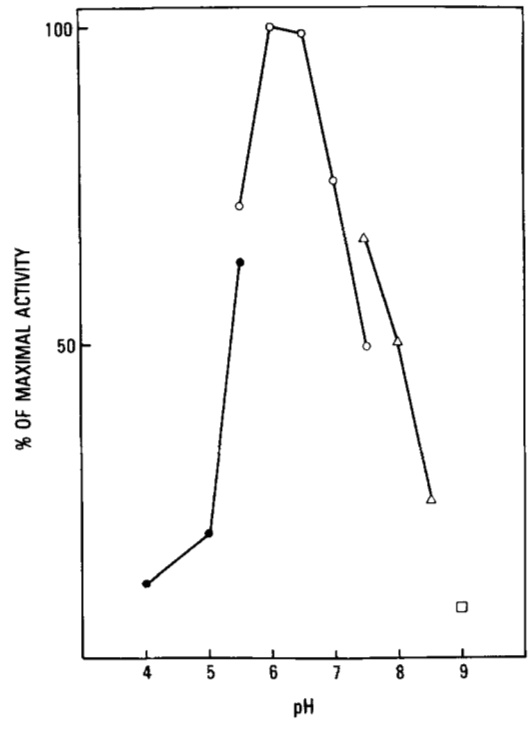

Figure 2: Effects of pH upon S.Sanguis neuraminidase activity. Reaction mixtures (90 µl) contained 1.4mUnits of enzyme, Collocalia mucoid as substrate (final sialic acid concentration 6mM), and the appropriate buffer as indicated: ● , 0.1M Sodium acetate; ○ ,0.1M Sodium cacodylate; Δ ,0.1M Tris HCl; □ , 0.1M sodium carbonate/bicarbonate. The reaction mixtures were incubated at 37°C for 20min, quenched by addition of 10 µl of 1N H_2SO_4, and the released sialic acid assayed by the TBA method.

A neuraminidase from Streptococcus sanguis that can release O-acetylated sialic acids.

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