Purification and Properties of a Bacteriophage-induced Endo-N-acetyneuraminidase Specific for Poly-α-2,8-sialosyl Carbohydrate Units*

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The soluble form of a bacteriophage-induced endo-
N-acetyneuraminidase (Endo-N) specific for hydro-
yzing oligo- or poly-α-2,8-linked sialosyl units in sources as disparate as bacterial and neural membrane
glycoconjugates was purified approximately 10,000-
fold and characterized. The enzyme appears homoge-
nous by sodium dodecyl sulfate-polyacrylamide gel
electrophoresis and has a subunit $M$, 105,000. This
corresponds to one of the higher $M$, phage proteins
which comprises 7.5% (by weight) of the total phage
protein. The holoenzyme is active at neutral pH and
has a $M$, by gel filtration of 328,000, suggesting that
the active enzyme is a trimer. Endo-N requires a min-
imum of 5 sialyl residues (DP5, where DP represents
degree of polymerization) for activity. The limit digest
products from the α-2,8-linked polysialic acid capsule
of Escherichia coli K1 are DP4 with some DP3 and
dP1,2. DP2–4 do not appear to inhibit depolymeriza-
tion of polysialic acid. Endo-N digestion of the polysia-
losyl moiety on neural cell adhesion molecules yields
sialyl oligomers with DP3 and DP4. The presence of a
terminal sialyl changes both the distribution of limit
digestion products and the apparent minimum sub-
strate size. Higher $M$, α-2,8-linked sialyl polymers
(~DP200) are better substrates ($K$, 50–70 μM) than
sialyl oligomers of ~DP10–20 ($K$, 1.2 mM). Endo-N
activity is inhibited by DNA and several other polya-
nonies tested. An examination of the distribution of intermediate products shows that Endo-N binds and
and cleaves at random sites on the polysialosyl chains, in
contrast to initiating cleavage at one end and depo-
ymerizing processively. Endo-N can be used as a specific
molecular probe to detect and selectively modify poly-
α-2,8-sialosyl carbohydrate units which have been im-
plicated in bacterial meningitis and neural cell adhe-
sion.

Many bacteria are capable of synthesizing capsular poly-
saccharides containing a wide variety of sugar moieties joined
together in a diversity of linkages (3–6). The α-2,8-linked
polysialic acid capsule of invasive Escherichia coli K1 sero-
group B is a well-characterized, highly specific antigen
that is present on the cell surface of a number of bac-
terial species. The α-2,8-sialosyl epitope on N-CAM has
been shown to be involved in neural cell adhesion and
medication of cell-cell interactions (12). Endo-N can be
used as a tool to study the role of polysialic acid in these
and other processes and on the molecular characteristics of poly-
sialic acid biosynthesis will be facilitated by the use of specific,
well-characterized reagents that permit detection and selec-
tive modification of the polysialosyl moiety. Several endo-
exoglycosidases have shown to be highly useful reagents
for structural analysis of glycoconjugates (25–28). One such
enzyme is an endo-N-acetyneuraminidase (Endo-N) asso-
ciated with bacteriophages that specifically recognize the
polysialic acid capsule of E. coli K1 as a receptor (13). Initial
studies utilizing Endo-N and some of its properties have been
reported (13, 24, 29, 30). Whereas the enzyme used appeared
to be free of proteolytic and exoglycosidase activity, fur-
on some of the properties of a bacteriophage-bound endo-
neuraminidase have appeared (31, 32); however, a purified, soluble endo-neuraminidase may have a distinct advantage, particularly for in vivo microinjection experiments to study the functional significance of polysialosyl units on N-CAM. The purification of a soluble endo-neuraminidase and its speci-
city for various substrates containing sialic acid were recently reported (33), but no information was presented as to the size of the limit digestion products or the minimal sub-
strate size.

This report describes the purification and properties of the soluble form of a bacteriophage-induced endo-N-acetyl-
neuraminidase that is specific for degrading at neutral pH oligo- or poly-\(\alpha\)-2,8-sialic acid units in prokaryotic and eucaryotic glycoconjugates. Availability of the purified enzyme provides a valuable molecular probe to detect, modify selec-
tively, and study the structure, synthesis, and function of poly-\(\alpha\)-2,8-sialyl carbohydrate units. These results are dis-
cussed in relation to the properties of the previously described forms of endo-neuraminidases (31-33) and the minimum sialyl oligomer size required to interact with the substrate-binding site of the enzyme.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

**Characterization of Bacteriophage K1F Proteins**

The CsCl-purified phage particles isolated as described under "Experimental Procedures" were analyzed by SDS-
PAGE to characterize the molecular weights and relative abundance of the phage-associated proteins (Table I). The K1F phage banded at a density between 1.3 and 1.6 \(g/cm^3\) in the CsCl gradient, indicating that it was similar to the endo-
neuraminidase-containing phages studied by Kwiatkowski et al. (31) and Finne and Makela (32). These phage particles were reported to have a buoyant density of 1.47 \(g/cm^3\), but in neither case were the proteins characterized. The K1F phage isolated here appeared homogenous in that only a single, high molecular weight DNA band was seen upon agarose electro-
phoresis after phenol/chloroform extraction and ethanol pre-
peparation. As shown in Table I, the molecular weights of the major K1F-associated proteins were similar to other capsule-
specific bacteriophages previously studied. The three capsular depolymerases previously characterized were reported to be spike elements of the phage that existed as a high molecular weight complex of 313,000 (E. coli K11), 245,000 (E. coli \(\phi29\)), and 379,000 (Klebsiella aerogenes). The holoenzymes were reported to consist of non-identical subunits, as shown in Table I. In contrast, Endo-N is an oligomer (M, 328,000) composed of three identical subunits of M, 105,000 each. The purpose in purifying the phage and characterizing its proteins was 3-
fold. First, determination of the protein components of the phage served as a determinant of purity of the soluble depoly-
merase since Endo-N should consist of one or more of these proteins. Second, a comparison of the relative substrate specific-
ficities and other enzymological properties of the phage-
associated and soluble Endo-N depolymerase might reveal differences due to immobilization of the enzyme in the phage particle. Third, at least in some cases, it might prove more facile to purify the phage-bound enzyme to homogeneity since the enzyme is naturally enriched in the purified phage prepar-
ration, i.e. purification of the phage particle could serve as an "affinity" purification step.

Treatment of purified phage K1F with either 4 \(M\) guanidin-
ume CI or 0.1% sodium dodecyl sulfate destroyed Endo-N activity. However, two other procedures,\(^a\) either mild acid treatment, previously shown to be effective in solubilizing depolymerase-containing phages \(\phi29\) (42) and K11 (43), or treatment with 8 \(M\) urea, previously used to solubilize the *K. aerogenes* capsular polysaccharide depolymerase (45), were effective in quantitatively solubilizing Endo-N from K1F phage particles. However, substantial losses of activity accom-
pnied further attempts at purification, possibly due to varying degrees of reaggregation of the phage proteins during subsequent purification procedures. Therefore, the soluble form of the enzyme in K1F phage lysates of *E. coli* K1 was purified to homogeneity, as described below.

**Purification of Soluble Endo-N from K1F Phage Lysates**

The purification procedure described under "Experimental Procedures" and summarized in Table II allowed the purification to apparent homogeneity of about 4.4 \(mg\) of Endo-N in 3 days. Overnight incubation of purified Endo-N with a number of high and low molecular weight proteins and gly-
coproteins prior to SDS-PAGE showed no contaminating protease activity. The enzyme was purified greater than 10,000-fold, based on the lysate activity, which, however, might be underestimated. The 2-fold increase in total activity, and the apparent high degree of -fold purification are probably due to the removal of an inhibitor(s) of Endo-N at the final purification step. Various polyanions, including DNA, chondroitin sulfate, and poly-\(\gamma\)-D-glutamic acid, inhibit Endo-N, presumably through ionic interactions since Endo-N is bound to heparin-agarose (results not shown). At least part of the inhibition during purification appears to be due to the pres-
ence of DNA. The purified glycohydrolase (20 \(\mu g\) ml\(^{-1}\)) was inhibited >90% by *E. coli* DNA (0.75 mg ml\(^{-1}\)) and treatment of fractions from initial stages of the purification with DNase (-1 mg ml\(^{-1}\)) resulted in a subsequent 2-fold increase in Endo-
N activity. Thus, it is likely that it is the removal of DNA from the phage lysate by the hydroxylapatite column that is responsible for the increase in total activity and is therefore the most important purification step. The -fold purification shown in Table II may be an overestimation due to the unknown amount of inhibition of Endo-N activity in the lysate. However, we believe these results are the most accurate presentation of the data in the absence of detailed character-
ization of inhibitory factors that might be present in the phage lysate.

The ammonium sulfate pellet (Table II, Step 2) was re-
extracted with buffer to enhance recovery of Endo-N activity entrained in the mass of insoluble debris. The main purpose of the high speed centrifugation (Step 3) was to recover K1F phage, which is why both the total activity and the specific activity decreased at this stage. This step can be eliminated

\(^a\) Portions of this paper (including "Experimental Procedures," Figs. 1, 6, and 7, and Tables I, II, and IV) 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. 56M-3027, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
tracking dye had reached the bottom of the tube, the gel was removed and cut into 2-mm slices. The slices were eluted for 2 h with 50 mM Tris buffer (pH 7.5) and then assayed for Endo-N activity. The only gel slice that showed Endo-N activity contained only the M, 105,000 protein, as shown by subsequent SDS-PAGE. Thus, the purification procedure reported here allows the facile purification to homogeneity of sufficient quantities of Endo-N for further enzymological studies and to specifically detect, modify selectively, and study the function of poly-α-2,8-sialosyl units in a variety of biological systems. In the only previous report of the purification of a soluble endonemuidase (33), the enzyme was reported to consist of two subunits (M, 74,000 and 38,500). This difference in size and number of subunits may be due to different phases being used. That enzyme had been purified 238-fold and had a specific activity of 0.95 (μmol of sialic acid released per min/mg of protein). In contrast, the Endo-N reported here was purified ~10,000-fold and is markedly more efficient (~4,200 μmol min⁻¹ mg⁻¹). It is thus possible that an inhibitor was present and that one of the two reported subunits could be a contaminant or a proteolytic cleavage product.

Properties of Endo-N-acetyleneuramidase

Kinetic Constants—Relatively little is known about the kinetic constants and enzyme mechanisms of endoglycosidases, in particular, endo-N-acetyleneuramidases. Here the apparent Kₘ and V_max for both the phage-bound and soluble form of Endo-N were determined for several oligo- and polysialosyl substrates, as summarized in Table III. No significant difference in apparent Kₘ values was found between the K1F phage-bound and soluble form of the enzyme for either the low or high molecular weight α-2,8-linked substrates or the α-2,8-α-2,9-linked mixed linkage polymer (Table III). The high molecular weight α-2,8-linked sialyl polymer (~DP150–200) appeared to be a substantially better substrate (apparent Kₘ, 50–70 μM) than the shorter (DP10–20) sialyl oligomers (apparent Kₘ~1.2–1.6 mM). The large difference in Kₘ values found here for Endo-N action on long polymers and short oligomers of sialic acid suggests that a different enzyme mechanism might be involved in processing these two different substrates. However, since the total number of

No evidence for multiple forms (e.g. partially assembled viral base plates) of Endo-N activity was found during this or other purification steps, although the Endo-N holoenzyme (M, 320,000) may have been dissociated from other phage components by the heat treatment.

### Table III

| Substrate | DP | Soluble Endo-N | Phage-bound Endo-N |
|-----------|----|----------------|-------------------|
|           |     | Kₘ (μM) | V_max (μmol/min/mg) | Kₘ (μM) | V_max (μmol/min/mg) |
| Poly-α-2,8 | 150–200 | 51 | 36 | 71 | 19 |
| Oligo-α-2,8 | 10–20 | 1.6 | 72 | 1.2 | 18 |
| Poly-α-2,8 | 6.6 | 25 | 7.1 | 13 |

*Endo-N activity was measured by product formation, as described under "Experimental Procedures," which yields initial reaction rates with all the substrates used. Thus, V_max is expressed as micromoles of NeuNAc-reducing equivalents formed min⁻¹ mg⁻¹ of protein⁻¹.

Polysialic acid (colominic acid) isolated from E.coli K1.

Oligosialic acid (colominic acid) isolated from E.coli K1.

Poly-α capsule isolated from E.coli N67.

![Fig. 2. SDS-PAGE analysis of purification of Endo-N and K1F phage proteins. Various fractions obtained during the purification of the soluble form of Endo-N were analyzed by SDS-PAGE (5–15%) (46). Lane 1, M, markers; lane 2, K1F phage; lane 3, crude phage lysate; lane 4, resuspended 50% (NH₄)₂SO₄, pellet; lane 5, high speed supernatant; lane 6, heat treatment; lane 7, resuspended 40% (NH₄)₂SO₄, pellet; lane 8, octyl-Sepharose fraction; lane 9, purified Endo-N (active fraction from hydroxylapatite, DEAE chromatography); lane 10, K1F phage. Lanes 3–8, ~20 μg of protein; lane 9, ~5 μg of protein. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.*
polymers of sialic acid were examined. As shown in Fig. 3a, neuraminidase of the 41.2 phage has been reported to digest partially the poly-a-2,8-(α-2,9-sialic acid capsule of units obtained by the partial and random hydrolysis of poly-
the mixed linkage polymer, producing only oligomers containing an even number of sialyl units. This is in contrast to the spectrum of sialyl oligomers with an even and odd number of units obtained by the partial and random hydrolysis of poly-
α-2,8-linked sialic acid (see below; Fig. 5 and 7). The endo-
neuraminidase of the α1.2 phage has been reported to digest partially the poly-α-2,8-α-2,9-sialic acid capsule of E. coli K92;
but in this case, the mixed linkage polymer appeared to be a poor substrate, and the digestion products were reported to be DP3,4,6,7, and higher residues, indicating that some cleavage of α-2,9 linkages may have occurred (49). As stated above, the primary hydrolysis product of the poly-α-2,8-α-2,9 capsule by Endo-N was DP8. In contrast, the primary digestion product of U-14C-labeled, high molecular weight poly-α-2,8-sialic acid was DP4 with a smaller amount of DP3 and DP1,2 (Fig. 3b). These results further confirm that Endo-N was specific for the poly-α-2,8 linkage in the poly-α-2,8-α-2,9 mixed linkage polymer. The molar distribution of products shown in Fig. 3b is consistent with what can be calculated for the random cleavage of a polymer with a minimum substrate size of DP5. Unexpectedly, the major digestion product from colominic acid (DP10-20) that had been reduced with NaB₃H₄ was DP3 and not DP4 (Fig. 3c), although smaller amounts of DP4 and DP1,2 were present. These results show that sialyl oligomers terminating in sialitol perturbed Endo-N catalysis and yielded different reaction products than oligomers terminating in sialic acid. As described below, reduction also changes the minimum substrate size from DP5 to DP6. No significant difference was seen between K1F phage-bound and -soluble Endo-N with respect to the products of limit digestion (results not shown).

**Products of Endo-N Limit Digestion**—The products of prolonged digestion (limit digestion) of various oligomers and polymers of sialic acid were examined. As shown in Fig. 3a, the primary digestion product of U-14C-labeled, high molecular weight poly-α-2,8-α-2,9-sialic acid was DP8 with a smaller amount of DP4,6,10,12,14. Since Endo-N does not hydrolyze α-2,9-linked polyasialic acid (results not shown), this pattern was due to the specific cleavage of the α-2,8 linkages within the mixed linkage polymer, producing only oligomers containing an even number of sialyl units. This is in contrast to the spectrum of sialyl oligomers with an even and odd number of units obtained by the partial and random hydrolysis of poly-
α-2,8-linked sialic acid (see below; Fig. 5 and 7). The endo-

![Fig. 3. Endo-N limit digestion products. U-14C-Labeled poly-α-2,8-α-2,9-sialic acid (DP150-200; a), U-14C-labeled poly-α-2,8-α-2,9-sialic acid (DP150-200; b), and oligo-α-2,8-sialic acid (-DP10-20) labeled in the reducing termini after reduction with NaB₃H₄ (c) were subjected to overnight digestion by Endo-N, and the products were identified by HPLC as described under "Experimental Procedures."](image-url)

To determine directly the minimum number of sialyl units required to serve as a substrate, U-14C-labeled sialyl oligomers and sialyl oligomers terminating in H-labeled sialitol (labeled by reduction with NaB₃H₄) were purified as described under "Experimental Procedures." Each oligomer of defined length was then incubated overnight at 37 °C with an excess of Endo-N, and the reaction products were analyzed by HPLC. Minus enzyme controls showed no degradation. Under these conditions, reduced DP5-03H ((NeuNAc)₅-NeuNAc-03H) (Fig. 4a) was not hydrolyzed by Endo-N, whereas reduced DP6-03H and D7-03H were (Fig. 4, b and c), again giving DP3-03H as the major digestion product. However, when nonreduced U-14C-labeled oligomers were used, different results were obtained. As shown in Fig. 4d, DP4 was not cleaved by Endo-N, but DP5 was, yielding DP4 and DP1 (Fig. 4e). Whereas the Kₘ for DP5 was not determined, the results shown in Fig. 4e suggest that it is a relatively poor substrate since complete hydrolysis did not occur, even after an overnight incubation. Cleavage of DP10 (Fig. 4f) again gave close to the expected molar distribution of products with some DP6 remaining unhydrolyzed. Analysis of the digestion products of DP6-8 gave similar results (not shown). We conclude from these results that the minimum substrate size for Endo-N is DP5. DP5 and higher oligomers were cleaved by Endo-N to give DP4 and sialic acid as major products (Fig. 4e). In contrast, reduced DP5 (DP5-03H) was not cleaved by Endo-N (Fig. 4a), but DP6-03H and higher reduced oligomers were hydrolyzed to DP3-03H (Fig. 4, b and c). Thus, the presence of a terminal sialitol changed the minimum substrate size from DP5 to DP6 and also appeared to alter the Endo-N cleavage pattern. Confirmation of this conclusion was provided as shown in Fig. 4 (g-i). In these
Poly-α-2,8-sialosyl Endo-N-acetylneuraminidase

The ability of various sialyl oligomers of defined length to serve as substrates for Endo-N was examined. The substrates used were as follows: a-c, oligomers reduced by NaB₃H₄, DP5-⁰⁻H, (NeuNAc)₅-NeqNAc-⁰⁻H, etc.; d-f, oligomers isolated from U⁻¹⁴C-labeled polysialic acid, [¹⁴C]DP5, [U⁻¹⁴C](NeuNAc)₅, etc.; g-i, oligomers isolated from U⁻¹⁴C-labeled polysialic acid and then reduced by NaBH₄, [¹⁴C]DP5-⁰⁻OH, [U⁻¹⁴C](NeuNAc)₅-NeqNAc-⁰⁻OH, etc.

Experiments, ¹⁴C-labeled DP5, which served as a substrate (Fig. 4e), was reduced with NaBH₄ to form [¹⁴C]DP5-⁰⁻OH and tested for its ability to serve as a substrate for Endo-N. As shown in Fig. 4g, this reduced oligomer no longer served as a substrate. Reduced DP6 ([¹⁴C]DP6-⁰⁻OH) and DP7 ([¹⁴C]DP7-⁰⁻OH) both served as substrates (Fig. 4, h and i). Interestingly, the proportion of product found as DP3 with both [¹⁴C]DP6-⁰⁻OH and [¹⁴C]DP7-⁰⁻OH was again markedly increased over the nonreduced oligomers. Therefore, these results demonstrate that a terminal sialitol residue not only changes the minimum substrate size but also changes the cleavage pattern from that expected for completely random, as seen with nonreduced substrates, to one that is biased toward producing DP3-⁰⁻OH.

The results of Endo-N digestion of different oligomers of polysialic acid clearly show that the minimum requirement for cleavage is α-2,8-(NeuNAc)₅. This is in contrast to previously described phage-bound endoneuraminidases that were reported to require a minimum of eight α-2,8-linked sialic acid units (31, 32). In the present study, comparison of reduced and nonreduced oligomers of sialic acid has demonstrated that reduction of the reducing terminus to sialitol changes the apparent substrate specificity of Endo-N. In a previous study (32), reduced oligomers of α-2,8-linked sialic acid were used to define the substrate specificity of a bacteriophage (PK1A)-bound endoneuraminidase. The possible influence of reduction of the substrates on enzyme specificity and activity was not addressed. The previous study by Finne and Makela (32) reported that their enzyme required a minimum of 8 sialyl residues and that cleavage produced a DP3, derived from the nonreducing terminus, and a DP5, derived from the reducing end. It was therefore concluded that digestion of brain polysialosyl glycopeptides by endoneuraminidase would leave 5 sialyl residues attached to the glycopeptide moiety (32). However, this conclusion may have to be reconsidered in light of the results presented here.

Kinetics of Endo-N Digestion of [U⁻¹⁴C]Poly-α-2,8-sialic Acid—Examination of the products produced by the action of a glycohydrolase as a function of time can yield information about the mode of enzymatic action. At low Endo-N to substrate ([U⁻¹⁴C]poly-α-2,8-sialic acid, DP150–200) ratios, an

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**Fig. 4.** Minimum substrate size for Endo-N catalysis. The ability of various sialyl oligomers of defined length to serve as substrates for Endo-N was examined. The substrates used were as follows: a-c, oligomers reduced by NaB₃H₄, DP5-⁰⁻H, (NeuNAc)₅-NeqNAc-⁰⁻H, etc.; d-f, oligomers isolated from U⁻¹⁴C-labeled polysialic acid, [¹⁴C]DP5, [U⁻¹⁴C](NeuNAc)₅, etc.; g-i, oligomers isolated from U⁻¹⁴C-labeled polysialic acid and then reduced by NaBH₄, [¹⁴C]DP5-⁰⁻OH, [U⁻¹⁴C](NeuNAc)₅-NeqNAc-⁰⁻OH, etc.
apparent lag in the formation of limit digest products (DP2-4) was seen when the reaction products were examined by paper chromatography and radiochromatographic scanning (results not shown). Under these conditions, the increase in number of free reducing termini, measured by thiobarbituric acid, was linear, suggesting that the initial products of Endo-N action were intermediate size oligomers (10 < DP > 100) that were not resolved by paper chromatography. This would imply that the mechanism of Endo-N hydrolysis of polysialic acid was random since a processive mechanism (repetitive hydrolysis from one end) would produce limit digest products during the initial phase of the reaction. To examine the mechanism of Endo-N catalysis, a detailed kinetic analysis of the products of Endo-N hydrolysis of [U-14C]poly-α-2,8-sialic acid was carried out (Fig. 5). When Endo-N digestion of substrate was 75% complete (4 min, Fig. 5a), only oligomers of DP3-8 were present. This suggested either that intermediate size oligomers were preferred substrates or that Endo-N catalysis might be processive with intermediate length oligomers (10 < DP > 50). An examination of the relative rates of disappearance of DP5-8 (Fig. 5, b-e) supports the earlier conclusion that the smaller oligomers were poorer substrates. These results also confirm the conclusion that DP5 was the minimum substrate size for Endo-N since the major product of limit digestion was DP4 (Fig. 5, d and e).

Partial Digestion Products of Different Length Oligomers of (NeuNAc)\textsubscript{2}-NeuNAc-O\textsubscript{2}H—To differentiate whether the products of partial digestion of [U-14C]poly-sialic acid noted above were due to a processive mechanism or to preferential hydrolysis of intermediate oligomers, the products of partial Endo-N digestion of DP7-OH to DP14-OH were examined (Fig. 6). DP7-OH and DP8-OH were degraded directly to DP3-OH without the formation of intermediate species. However, the formation of intermediates became more apparent with the longer chain oligomers (DP10-OH to DP14-OH). This shows that for the intermediate size oligomers, the mechanism of Endo-N hydrolysis is random and not processive. Moreover, for the longer oligomers (DP10-OH to DP14-OH), cleavage is primarily on the distal (nonreducing end) of the terminal sialitol, in contrast to the preferential cleavage on the proximal (reducing end) of the terminal sialitol observed with DP7-OH to DP9-OH. This is additional evidence consistent with the conclusion that conformational differences may exist between sialyl oligomers when their size approaches a DP9,10. The presence of a terminal sialitol influences Endo-N action even with intermediate length oligomers where presumably binding and hydrolysis near the reducing terminus is hindered due to the presence of sialitol. However, the apparent random mechanism of Endo-N hydrolysis under these conditions is not due to the terminal sialitol since partial Endo-N digestion of nonreduced [14C]DP12 also formed intermediate sized oligomers (Fig. 7).

Model of Endo-N Catalysis—Several models have been proposed to account for the binding and subsequent hydrolysis of oligomeric substrates by depolymerases (50-57). In these models, the active site of the enzyme is postulated to consist of a series of subsites, each of which is capable of binding a single carbohydrate moiety. These models can be used to predict time-dependent changes in the distribution of products, for example, amylase action (58, 59), when the subsite binding affinities have been determined by other methods or the subsite binding affinities can be determined by measuring product distribution as a function of time (60, 61). Whereas a detailed, quantitative treatment of Endo-N action in this manner is beyond the scope of this study, a schematic model of Endo-N action (Fig. 8) is instructive and allows some qualitative assessments to be made.

In the case of the nonreduced substrate (Fig. 8a), interaction of sialic acid residues with enzyme-binding subsites is approximately equipotential, although occupancy of subsite 1 is preferred to subsite 2 since cleavage of [14C]DP5 gives primarily DP4 and sialic acid (Fig. 4e). Additional, long range interactions are probably also important since sialic acid oligomers of intermediate length appear to be better substrates than shorter oligomers (Table III). In the case of oligomers up to DP9 with a terminal sialitol (Fig. 8b), the presence of the alcohol causes preferential binding of the terminal sialitol at subsite 7, favoring cleavage predominantly at (NeuNAc)\textsubscript{2}-(NeuNAc)\textsubscript{2}-NeuNAc-OH. Since DP6-OH is cleaved, this could indicate that occupancy of subsite 2, al-

![Fig. 5. Kinetics of Endo-N digestion of (U-14C]poly-α-2,8-sialic acid. [U-14C]Poly-α-2,8-sialic acid and purified Endo-N in Tris buffer (pH 7.5) were incubated at 37°C. Aliquots were removed at the indicated times, and sialyl oligomers with <DP16 were resolved by HPLC.](image-url)
though not preferred, is sufficient to promote catalysis, at least with substrates altered at their reducing termini.

Conclusions

The purification procedure reported here provides sufficient quantities of highly purified (homogenous) soluble Endo-N, free of proteases and exonucleamidase activities, suitable for future studies on the biosynthesis and biological function of poly-α-2,8-sialosyl-containing glycopolymers. The following considerations are relevant to the future use of Endo-N as a probe for polysialic acid. Since Endo-N catalysis is random and long polymers are substantially better substrates than short ones, high enzyme concentrations and long incubation periods may be necessary to achieve complete digestion of oligomers that are only slightly larger than the minimum substrate size (DP5). Since the minimum substrate size is DP5, the release of sialic acid from a homooligomer by Endo-N would indicate the presence of an oligomer of >DP4. However, the lack of release of sialic acid does not necessarily indicate a polymer of <DP5 since Endo-N could be sterically hindered due to an attached reducing end. Indeed, here it was found that merely the presence of a terminal sialitol changes both the apparent minimum substrate size and the cleavage pattern obtained. Experiments using Endo-N to study the biosynthesis of polysialyl units in neural cell adhesion molecules and to probe the function of polysialic acid in a variety of biological systems are currently in progress in a number of laboratories.

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Polycationic surfactant systems have been employed in the study of biological membranes and their interactions with charged molecules. The polycationic surfactant systems have been found to induce aggregation and phase separation in biological membranes. The aggregation and phase separation phenomena have been studied using various techniques such as optical microscopy, fluorescence spectroscopy, and dynamic light scattering. These studies have provided insights into the mechanisms of membrane aggregation and phase separation, which are important for understanding the behavior of biological membranes in various physiological and pathological conditions.
TABLE II
PURIFICATION OF SOLUBLE ENDO-NEURAMINIDASE

| Step | Total Activity (U/ml) | Yield (%) | Total Protein (mg) | Specific Activity (units/mg) | Purification Factor |
|------|----------------------|-----------|-------------------|-----------------------------|-------------------|
| 1.   | Phage lysate          | 9,450     | 100               | 21,500                      | 0.6               | 15               |
| 2.   | 30% (NH₄)₂SO₄   | 5,852     | 42                | 2,790                       | 2.5               | 4                |
| 3.   | High Speed Centrifugation | 3,955 | 26                | 2,040                       | 1.4               | 3.4              |
| 4.   | Heat Treatment (40°C, 20 min) | 3,058 | 33                | 456                         | 7.1               | 17.5             |
| 5.   | 40% (NH₄)₂SO₄   | 3,108     | 33                | 456                         | 7.1               | 17.3             |
| 6.   | Octyl-Sepharose Column | 2,712 | 29                | 235                         | 11.3              | 28               |
| 7.   | Bioxypermtein-DEAE Chromatography | 18,337 | 195               | 4,44                         | 42.9              | 10,290           |

Notes:
- The data shown are for a representative purification. Due to limitations in the substrate depletion assay, the absolute values in any purification may vary from purification to purification.
- Endo-N activity was determined by the substrate depletion assay by measuring the depolymerization of radiolabeled poly saccharide as described under "Experimental Procedures." One unit is defined as the amount of enzyme required to mobilize 10,000 cpm of sialic oligomers per min at 37°C. This is equivalent to the product of about 1 unit of sialic acid per minute.

TABLE IV
QUANTITATIVE ANALYSIS OF THE PRODUCTS OF LIMIT DIGESTION OF OLIGOSACCHARIDES BY ENDO-N

| Substrate | LIMIT DIGEST PRODUCTS | DP6-03H | DP7-03H | DP8-03H |
|-----------|----------------------|---------|---------|---------|
| DP1-03H   | DP2-03H             | 93.6    | 4.6     | 3.8     |
| DP2-03H   | DP3-03H             | 92.6    | 3.1     | 2.3     |
| DP3-03H   | DP4-03H             | 72.5    | 11.5    | 2.2     |
| DP4-03H   | DP5-03H             | 83.7    | 7.6     | 3.2     |
| DP5-03H   | DP6-03H             | 93      | 7       |         |
| DP6-03H   | DP7-03H             | 87.3    | 10.3    | 3.2     |
| DP7-03H   | DP8-03H             | 84      | 16      |         |
| DP8-03H   | DP9-03H             | 89      | 11      | 2.2     |
| DP9-03H   | DP10-03H            | 88      | 12      |         |
| DP10-03H  | DP11-03H            | 88.9    | 11.1    |         |
| DP11-03H  | DP12-03H            | 89.3    | 10.3    |         |

Notes:
- Results are given as percent of total radioactivity recovered in that fraction.
- Sialic oligomers were labeled in the reducing terminal by reduction with NaBH₄ (12). Then, DP5-03H, (Neu5Ac₂₄₂Neu5Ac₄) was used.

FIGURE 4. PARTIAL ENDO-N DIGESTION OF OLIGOSACCHARIDES OF ISO-SACCHARIDES WITH TERMINAL 2-O-ACETYLGLUCOSAMINE. Various oligomers of defined length (DP6-03H to DP8-03H) were partially digested with purified Endo-N in Tris-Cl buffer, pH 7.5 at 37°C, and the products analyzed by HPLC. The digestion products of DP9-03H were analyzed as described under "Experimental Procedures." The digestion products of DP10-03H were analyzed by a modified linear NaCl gradient where the final concentration at 30 min was 10 and Tris-Cl buffer, pH 7.5 at 23°C (600 and NaCl (65)).

FIGURE 5. PARTIAL DIGESTION PRODUCTS OF 14C-DP12. Purified 14C-DP12 was incubated with purified Endo-N in Tris-Cl buffer, pH 7.5 at 37°C, and the digestion products analyzed by HPLC (62).