

A new method for sequencing linear oligosaccharides on gels using charged, fluorescent conjugates*

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ABSTRACT

A new method is described for sequencing linear oligosaccharides on gels using charged, fluorescent conjugates. The reducing ends of various mono-, di-, tri-, and tetra-saccharides were conjugated with monopotassium 7-amino-1,3-naphthalenedisulfonate (a fluorescent and negatively charged compound) by reductive amination using sodium cyanoborohydride. The sugar conjugates were purified by preparative gradient polyacrylamide gel electrophoresis followed by a newly developed technique involving their semi-dry transfer to positively charged nylon membranes and elution with sodium chloride. The structures of a monosaccharide- and trisaccharide-conjugate were established by f.a.b.-m.s. and 2D n.m.r. Seven linear oligosaccharide-fluorescent conjugates were treated sequentially with exoglycosidases and with endoglycosidases. Analysis of the products by gel electrophoresis provided sequence information. These methods may be useful for sequencing oligosaccharides that are chemically or enzymically (endoglycosidase) released from glycoproteins, glycolipids, and proteoglycans.

INTRODUCTION

Many biological roles of carbohydrate units in glycoproteins have been reported¹. These include: protection of the polypeptide component against uncontrolled proteolytic attack^{2,3}, facilitation of the secretion of certain proteins or their mobilization to the cell surface⁴, maintenance of glycoprotein conformation in a biologically active form⁵, clearance of glycoproteins from plasma⁶, direction of the immune response by acting as immune decoys^{7,8}, and their importance as antigenic determinants in differentiation and development⁹. Therefore, information about glycoprotein sugar composition, and more importantly their sequence is required to establish structure–function relationships. However, glycoproteins are usually available in only limited quantities (typically 1–100 μg), making it difficult to determine the sequence, position, and anomeric configurations of glycosidic linkages in their carbohydrate chains¹.

Highly sensitive detection methods have been reported for oligosaccharides, such as tritium labeling at the reducing end of sugars by sodium [³H]borohydride reduction^{10–12} and fluorescent labeling by reductive amination. Fluorophores, including 2-aminopyridine^{13–15}, 7-amino-4-methylcoumarin¹⁶, monodansylethylenediamine¹⁷, dan-

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syldiazine¹⁸, and fluoresceinamine¹⁹ have been conjugated to a variety of reducing sugars. Additionally, aniline, *p*-aminoacetophenone, and ethyl *p*-aminobenzoate have been used to attach chromophores for u.v. detection^{20,21}. These analytical approaches have used thin-layer chromatography¹⁶ high-pressure liquid chromatography^{20,21} or mass-spectrometric methods²⁰ for the analysis of derivatized neutral oligosaccharides*.

As a routine, versatile and powerful tool for sequencing nucleic acids, gel electrophoresis was introduced by Maxam and Gilbert²² in 1977. Borate complexes of neutral sugars, including 2-aminopyridine derivatives, have recently been analyzed by electrophoresis²³. The presence of a strongly charged group, such as sulfate, permits the use of a variety of buffers through a wide pH range and may also improve resolution. Derivatization of neutral oligosaccharides with a fluorescent, negatively charged molecule, such as monopotassium 7-amino-1,3-naphthalenedisulfonate, should facilitate the application of gel electrophoresis to their sequencing. Reductive amination with this charged fluorescent probe would place it at the reducing terminus of the carbohydrate. Subsequent treatment with specific exoglycosidases acting at the non-reducing terminus would result in information on the oligosaccharide's sequence.

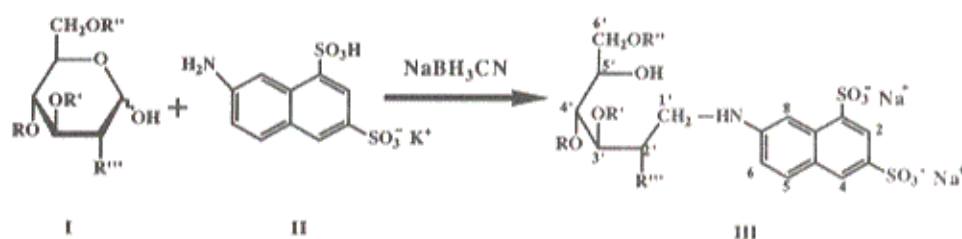
A second major problem associated with the gel electrophoresis-based sequencing of derivatized oligosaccharides involves the purification of the oligosaccharide-fluorescent conjugate to be sequenced. Typically, glycoproteins have several different types of attached oligosaccharides²⁴. Enzymic release of these oligosaccharides with endoglycosidases would be expected to give a mixture that would have to be resolved prior to sequencing. In addition, derivatization reactions are seldom quantitative and thus would be expected to further complicate the purification of a single oligosaccharide for sequencing. Standard separation methods, including low-pressure size-exclusion chromatography^{25,26}, thin-layer chromatography^{13,20}, high-voltage paper electrophoresis²⁷, and h.p.l.c.²⁸ are often unable to resolve such complex mixtures. This paper also describes the application of preparative gradient polyacrylamide gel electrophoresis²⁹ to fractionate oligosaccharides following their derivatization. Semi-dry electro-transfer³⁰ is then used to recover a single derivatized oligosaccharide for sequencing. A major advantage of all of these methods are that they do not require any specialized expensive equipment, permitting their use in most carbohydrate research laboratories.

RESULTS

The preparation of sugar-AGA conjugates by reductive amination is shown in Scheme I. The molar ratio of sugar (I), AGA (II), sodium cyanoborohydride and the

* Abbreviations: h.p.l.c., high pressure liquid chromatography; s.a.x., strong anion exchange; f.a.b., fast atom bombardment; m.s., mass spectrometry; n.m.r., nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; D-Glc, D-glucopyranose; D-Gal, D-galactopyranose; D-Man, D-mannopyranose; L-Ara, L-arabinopyranose; L-Fuc, 6-deoxy-L-galactopyranose; D-GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; D-GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; Neu5Ac, 5-acetamido-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid; SDS, sodium dodecyl sulfate; AGA, monopotassium 7-amino-1,3-naphthalenedisulfonate; TEMED, *N,N,N',N'*-tetramethylethylenediamine; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

reaction time, temperature and pH were optimized by using D-Glc and D-GlcNAc. The reaction was monitored by running each sample on the gradient PAGE and visualizing the products in the u.v. light chamber at 366 nm or by using s.a.x.-h.p.l.c. with detection at 247 nm. Hexoses, including D-Glc, D-Gal, D-Man, and D-Ara, were readily conjugated to AGA in high yields (70–90%), whereas acetamido hexoses, including D-GlcNAc and D-GalNAc, gave AGA conjugates in somewhat lower yields (60–80%). Di-, tri-, tetra-, and higher oligo-saccharides (a malto-oligosaccharide mixture containing tetrasaccharide through decasaccharides) gave yields of 50–80%. Recovered yields based on oligosaccharide starting material compared well with yields estimated by using gradient PAGE or s.a.x.-h.p.l.c.



Scheme I.

Under optimized reaction conditions, only trace quantities of side-products were observed as additional fluorescent bands or peaks on s.a.x.-h.p.l.c. The concentration of sodium cyanoborohydride did not play a significant role in forming any by-products. To drive the reaction to completion, however, it was necessary to use an excess of AGA. Thus a purification step to remove AGA was necessary to obtain sugar-AGA conjugate of sufficient purity for analysis and sequencing.

The crude reaction product obtained in the reductive amination of β -D-Gal-(1 \rightarrow 4)- β -D-Gal-(1 \rightarrow 4)-D-GlcNAc (**Ic**) was analyzed by s.a.x.-h.p.l.c. (Fig. 1A). In addition to the desired trisaccharide-AGA conjugate (**IIIc**) and the excess of AGA (**II**), some unidentified side-products were observed. This crude product mixture was loaded directly on gradient gel and fractionated by preparative electrophoresis. Trisaccharide-AGA conjugate (**IIIc**) appeared as a strongly fluorescent band under u.v. light well resolved from the unreacted AGA (Fig. 2A, lane c). The desired trisaccharide-AGA product (**IIIc**) was recovered from the gel by electro-transfer. Analysis of the purified product **IIIc** by s.a.x.-h.p.l.c. is shown in Fig. 1B. The u.v. and fluorescence spectra of the sugar-AGA conjugates as purified by preparative gradient PAGE were determined. The u.v. spectrum of AGA (**II**) exhibits a maximum at 247 nm ($\epsilon = 3.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and the sugar-AGA conjugates (**IIIa-i**) show maxima between 255 and 257 nm.

The fluorescence spectrum of AGA (**II**) shows an emission maximum at 447 nm and an excitation maximum at 343 nm. Excitation and emission spectra of all of the sugar-AGA conjugates (**IIIa-i**) are very similar. Sugar-AGA conjugates show emission maxima at 452 nm and excitation maxima at 314 nm. Sugar-AGA conjugates may be detected in a fluorimeter at femtomolar concentrations and in the u.v. light chamber at

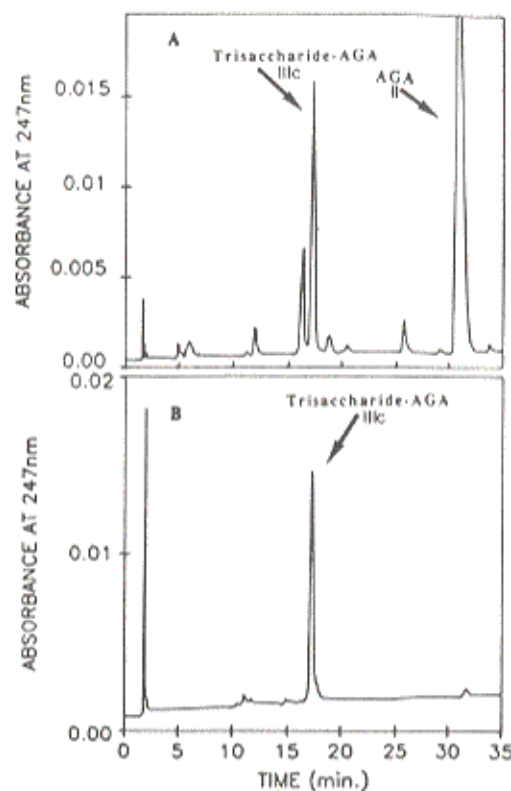


Fig. 1. S.a.x.-h.p.l.c. analysis of trisaccharide-AGA conjugate **IIIc**. A. Crude reaction mixture from the coupling of **Ic** and **II** (Scheme I). B. Trisaccharide-AGA conjugate (**IIIc**) that had been purified using gradient PAGE.

366 nm, picomole amounts of sugar-fluorescent conjugate are readily detected by the human eye.

The negative-ion f.a.b. mass spectrum of the trisaccharide-AGA conjugate (**IIIc**) showed an ion at $853 [M - Na^+]^-$ (where M denotes the fully sodiated molecule) consistent with a molecular weight of 876. The fragment ion at m/z 691 is the result of the loss of one D-Gal residue at the non-reducing end. The ion corresponding to the loss of a second galactose residue was not observed in the spectrum, perhaps because of the preferential fragmentation of the linking chain. 1H -N.m.r. spectroscopy was used to further characterize the sugar-fluorescent conjugates. It was particularly important to confirm the structure of the linkage between the fluorescent label and the sugar. However, because there are two β -D-Gal residues in **IIIc**, severe overlap was seen in the 360-MHz spectrum.

Monosaccharide-AGA conjugate **IIIa** was prepared by reductive amination of D-GlcNAc and purified (Fig. 2A, lane a). Compound **IIIa** showed the expected ion at $529 [M - Na^+]^-$ in its f.a.b. mass spectrum, consistent with a molecular weight of 552. Compound **IIIa** was used to assist in the n.m.r. assignment of trisaccharide-fluorescent

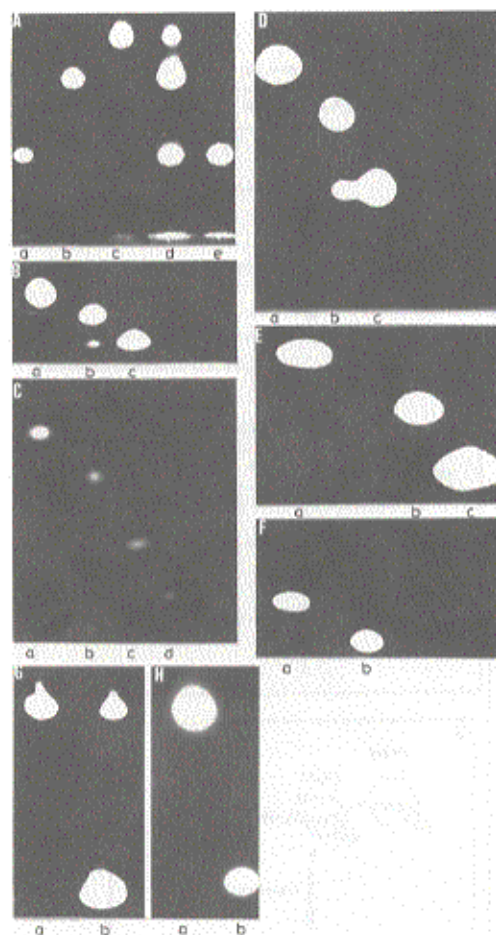


Fig. 2. Gradient PAGE analysis of oligosaccharide-AGA conjugates (**IIIa-i**, Scheme I, Table I) untreated and treated with various exoglycosidases and endoglycosidases. A. The analysis of **IIIa**, **IIIb** and **IIIc**: lane a, **IIIa**; lane b, **IIIb**; lane c, **IIIc**; lane d, **IIIa**, **IIIb**, and **IIIc**; lane e, **IIIc** treated with β -galactosidase (*E. coli*). The minor band running near the bottom of the gel in lanes c, d and e is AGA. B. The analysis of **IIId**: lane a, **IIId**; lane b, **IIId** treated with neuraminidase; lane c, **IIId** treated with neuraminidase and β -galactosidase (*E. coli*). C. The analysis of **IIIe**: lane a, **IIIe**; lane b, **IIIe** treated with β -galactosidase (bovine testes); lane c, **IIIe** treated with β -galactosidase (bovine testes) and β -*N*-acetylglucosaminidase; lane d, **IIIe** treated with β -galactosidase (bovine testes), β -*N*-acetylglucosaminidase and β -galactosidase (*E. coli*). D. The analysis of **IIIf**: lane a, **IIIf**; lane b, **IIIf** treated with β -galactosidase (*E. coli*); lane c, **IIIf** treated with β -galactosidase and β -*N*-acetylglucosaminidase. E. The analysis of **IIIg**: lane a, **IIIg**; lane b, **IIIg** treated with α -L-fucosidase; lane c, **IIIg** treated with α -L-fucosidase and β -galactosidase (*E. coli*). F. The analysis of **IIIh**: lane a, **IIIh**; lane b, **IIIh** treated with α -D-mannosidase. G. The analysis of **IIIi**: lane a, **IIIi**; lane b, **IIIi** treated incompletely with endo- β -galactosidase. H. The analysis of **IIIj**: lane a, **IIIj**; lane b, **IIIj** treated with chitinase.

conjugate **IIIe**. The sugar-AGA conjugates **IIIa** and **IIIc** showed a slight upfield shift of the H-6 and H-8 signals as compared to **II**, and the absence of an anomeric proton signal for the D-GlcNAc residue support their structure. The two-dimensional COSY spectrum at 300 MHz of **IIIa** provided the assignments necessary to firmly establish the

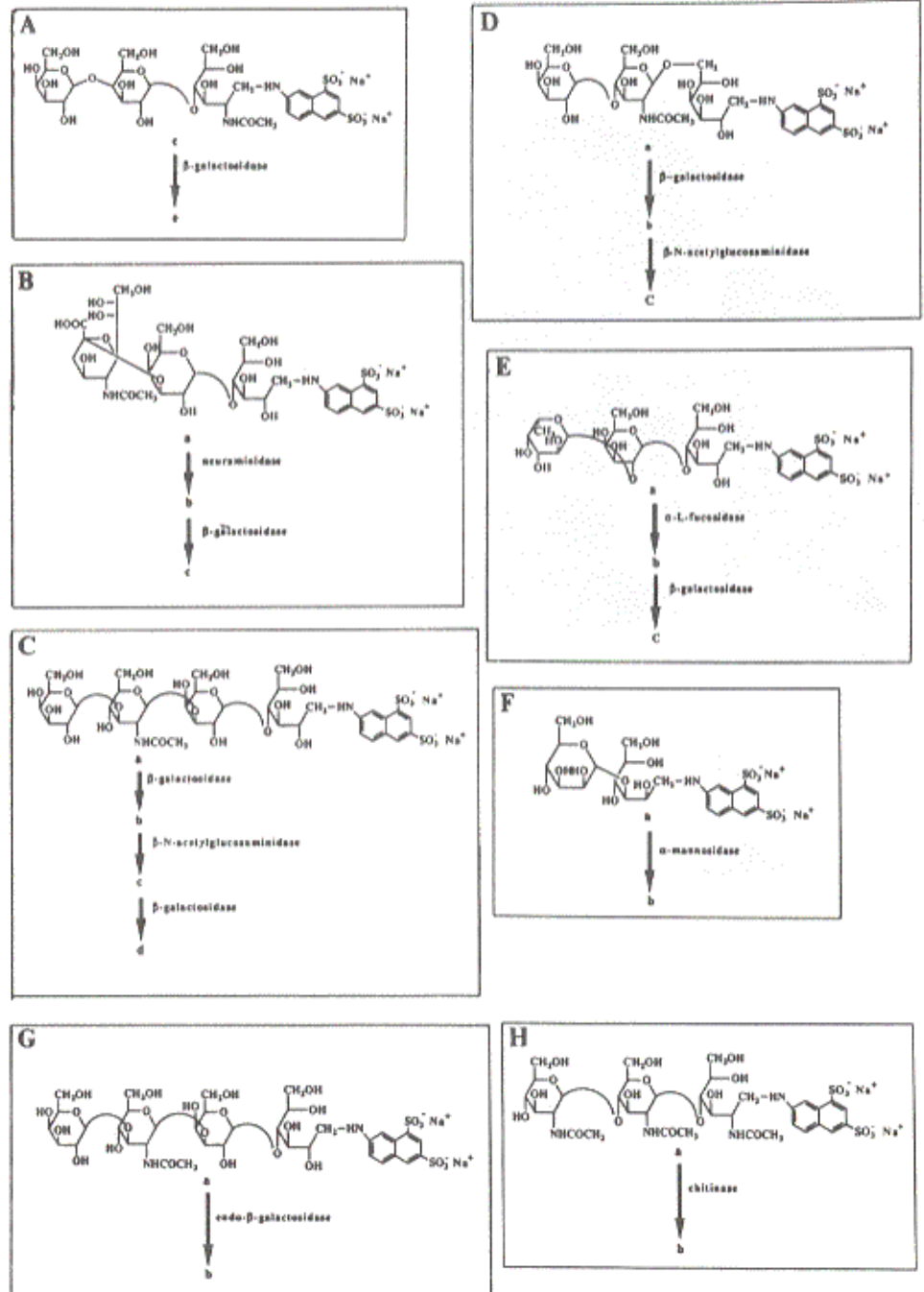


Fig. 3. Structures of oligosaccharide-AGA conjugates (IIIc-i) and enzymic treatments confirming their sequence. Panels A-H correspond to the gradient PAGE gels in Fig. 2A-H. The lower-case letters in panels A-H indicate the product analyzed in the corresponding lanes of each gel (Fig. 2A-H).

structure of linkage between D-GlcNAc and AGA (Scheme 1, **IIIa**). The assignments were as follows (in p.p.m.): 1.90 (COCH₃), 3.47 (t, *J* 9.5 Hz, H-3'), 3.60 (H₂-1'), 3.76 (H-2'), 3.82 (H-4'), 7.17 (dd, *J* 2.0 and 9.0 Hz, H-6), 7.52 (d, *J* 2.0 Hz, H-8), 7.88 (d, *J* 9.0 Hz, H-5), 8.25 and 8.30 (d, *J* 1.7 Hz, each, H-2 and H-4, interchangeable).

Sugar-AGA conjugates (**IIIa-i**) were used in sequencing studies by examining the products formed on exoglycosidase and endoglycosidase treatment by gel electrophoresis. For this sequencing strategy to be successful, the sugar-AGA conjugates needed to retain their sensitivity towards these glycosidases. To test this, oligosaccharide-AGA conjugates were prepared and purified and are shown in Fig. 3A-H. Each was treated sequentially with appropriate enzymes. The product resulting from each glycosidase treatment was analyzed directly by gradient PAGE (Fig. 2A-H) to confirm the known sequences.

DISCUSSION

Reductive amination has long been an accepted method of labeling sugars with probes to permit their detection and facilitate their separation. Yamamoto and co-workers¹⁵ have recently optimized the labeling of sugars with 2-aminopyridine by reductive amination, and have used this chemistry in an effort to develop an h.p.l.c.-based method for sequencing oligosaccharides. This paper describes a similar method of labeling sugars but instead focuses on a visibly fluorescent probe, with a fixed negative charge, to facilitate its sequencing by electrophoresis. Although it is possible to introduce charge into sugar-fluorescent conjugates by other methods, as through borate complexation²³, a covalently fixed charge such as that present in AGA offers a better approach towards optimizing such charged-based separations as electrophoresis.

One problem associated with the preparation of oligosaccharide derivatives for sequencing is their purification. The sugar-fluorescent conjugate **IIIa-i** is contaminated with excess fluorescent tag used to drive the reaction to completion and also with minor reaction side-products. The oligosaccharide starting material **I** is often not absolutely pure, leading to the formation of other minor products during reductive amination. Important targets for sequencing, such as glycoproteins and glycopeptides, may have different isoforms, giving rise to multiple oligosaccharides for fluorescent tagging. Such cases make a separation step necessary, even using the best coupling chemistry. Yields of 50-80% obtained in this study were not optimized and some improvement might be possible by using alternative reducing agents³¹ or organic solvents that favor Schiff-base formation.

Gradient PAGE is used as a rapid, convenient, high-resolution method to purify sugar-fluorescent conjugates (**IIIa-i**) from the crude reaction mixture. Preparative gradient PAGE on 1.5-mm gels permits purification of 100 mg of sugar-AGA conjugate from the crude reaction mixture, and 3-mm thick gels permit the loading of up to 1 g of **IIIa-i**. Transfer from the gel onto positively charged nylon membranes and recovery of **IIIa-i** from the membrane by washing with salt are nearly quantitative³⁰.

Sequential treatment with exoglycosidases has been widely used to establish saccharide sequence³². Seven linear-chain oligosaccharide-AGA conjugates were prepared and purified for sequencing studies using specific exoglycosidases and endoglycosidases. Each enzyme behaved in a predictable fashion towards these fluorescently tagged substrates.

In the simplest example, the trisaccharide-AGA conjugate **IIIc** was treated with β -D-galactosidase (*Escherichia coli* or jack beans) resulting in the release of both β -D-Gal residues and the formation of monosaccharide-AGA conjugate **IIIa** (Fig. 2A and 3A). Partial enzymic treatment resulted in a mixture of unreacted trisaccharide-AGA (**IIIc**), disaccharide-AGA (**IIIb**), and monosaccharide-AGA (**IIIa**). This example illustrates how a structurally complex oligosaccharide having two enzyme-susceptible linkages might be sequenced. Trisaccharide **Id** was coupled with AGA at a slightly elevated pH to prevent hydrolysis of the sensitive α -Neu5Ac(2 \rightarrow 3)- β -D-Gal linkage³³. This increased pH only caused a minor decrease of product yield. Trisaccharide-AGA (**IIIId**) (Fig. 2B and 3B), containing two different glycosidic linkages, was sequenced by treating first with neuraminidase followed by β -galactosidase (*E. coli*). The neuraminidase contained a minor amount of β -D-galactosidase contaminant (indicated by the low intensity, rapidly migrating band in Fig. 2B, lane b). The sequencing of a tetrasaccharide-AGA conjugate (**IIIe**) required β -D-galactosidase (bovine testes), β -N-acetylglucosaminidase, and β -D-galactosidase (*E. coli*) (Figs. 2C and 3C). The two different β -galactosidases are used to exploit differences in their reactivity towards 1 \rightarrow 3 and 1 \rightarrow 4 galactosidic linkages¹. Trisaccharide-AGA conjugate **IIIIf** was sequenced by using β -galactosidase (*E. coli*) and β -N-acetylglucosaminidase. Contaminating enzymatic activity was again detected by the presence of a second, lower intensity, rapidly migrating band (Fig. 2D, lane b). Oligosaccharides containing α -L-Fuc and α -D-Man were also sequenced (Figs. 2 and 3, E and F), demonstrating the wide applicability of this method.

Endoglycosidases may also be useful in sequencing oligosaccharides, particularly larger oligosaccharides or ones that are branched. The behavior of endo- β -galactosidase and chitinase towards two oligosaccharide-AGA conjugates were examined (Figs. 2G and 2H). Endo- β -galactosidase acted on the internal β -galactosidic linkage, even though it was linked to the ring-opened sugar bearing the AGA label (Fig. 3G). Partial treatment with endo- β -galactosidase resulted in the breakdown of tetrasaccharide-AGA conjugate **IIIe** directly to monosaccharide-AGA product (Fig. 2G). No band corresponding to trisaccharide-AGA could be seen, suggesting that no exolytic cleavage had taken place. Chitinase acted similarly, converting trisaccharide-AGA conjugate **IIIi** into monosaccharide-AGA.

Gradient PAGE analysis of oligosaccharide-AGA conjugates is comparable to SDS-PAGE of proteins in that separation is based primarily on the molecular size of a molecule. The resolution of gradient PAGE was sufficient to separate oligosaccharides having a different number of saccharide units. In contrast to some h.p.l.c. methods, the separation of oligosaccharides having the same number of oligosaccharides is not yet possible. Some oligosaccharides containing monosaccharides of decreased steric size (such as L-Fuc), or ones having an additional negative charge (such as Neu5Ac), migrate

slightly ahead of other oligosaccharides containing only hexose or *N*-acetylhexosamines. Fig. 2A, lane d, illustrates that baseline separation is obtained between AGA (**II**), monosaccharide-AGA (**IIIa**), disaccharide-AGA (**IIIb**), and trisaccharide-AGA (**IIIc**). By decreasing the sample loading on the gel (Fig. 2C) the resolving power of gradient PAGE is more clearly demonstrated, with the enhanced resolution of tetra-, tri-, di-, and mono-saccharide-AGA conjugates. Although this separation is suitable for small linear oligosaccharides, future studies must be directed towards enhancing resolution. One interesting aspect of this separation is the 'rounding' of bands. This effect may be diminished by running the sample further into the gel (closer to its pore-exclusion limit²⁹), as demonstrated by the more-focused, narrow lower band in Fig. 2A, (lanes c, d, and e) corresponding to residual AGA. Much of the rounding effect may also be associated with the method used to prepare Fig. 2. These photographs were of a wet gel (between two glass plates) over which a u.v. light was held. Transfer onto a nylon membrane results in more-intense, narrower bands, possibly because of a decrease in the reflectance of the light from the fluorescing bands. Band rounding is also caused by diffusion, and thus a decrease in electrophoresis time as well as the time between terminating the experiment and photographing the gel results in sharper bands.

Preliminary studies have demonstrated that it is possible to prepare AGA conjugates of branched bianternary and triaternary oligosaccharides obtained from glycoproteins, but with greatly decreased coupling yields (~10%). There are major difficulties that must be overcome with sequencing such complicated structures, including distinguishing between non-equivalent branch arms. Some of the strategies developed on simpler linear oligosaccharides, such as the partial enzymic treatment or the use of endoglycosidases, might be valuable in examining these more-complicated structures.

The results presented on linear oligosaccharides suggest that it may be possible to use this approach to sequence more-complex branched oligosaccharides. The strategy would involve: (1) release of oligosaccharides from glycoproteins using an endoglycosidase such as *N*-glycanase; (2) conjugation of the released oligosaccharides to a fluorescent compound; (3) fractionation and purification of each oligosaccharide-fluorescent conjugate; (4) sequential treatment of each purified oligosaccharide-fluorescent conjugate with specific exoglycosidases and possibly endoglycosidases; and (5) analysis by analytical PAGE and reading of the sequence from the observed banding pattern. Work using this approach on structurally complex glycoprotein-derived oligosaccharides is currently underway.

EXPERIMENTAL

Materials. — *Chemicals.* D-Glc, D-Gal, D-Man, L-Ara, D-GlcNAc, D-GalNAc (**Ia**, Scheme I, Table I), [4→)-β-D-Glc-(1→)]₄₋₁₀ (maltooligosaccharides), β-D-Gal-(1→4)-D-GlcNAc (**Ib**), β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-GlcNAc (**Ic**), α-Neu5Ac-(2→3)-β-D-Gal-(1→4)-D-Glc (**Id**), β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc (**Ie**), β-D-Gal-(1→4)-β-D-GlcNAc-(1→6)-D-Gal (**If**), α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc

(Ig), α -D-Man-(1 \rightarrow 3)-D-Man (Ih), β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4)-D-GlcNAc (Ii), sodium cyanoborohydride, β -galactosidase (jack beans), β -galactosidase (*Escherichia coli*), β -galactosidase (*Aspergillus niger*), neuraminidase (*Clostridium perfringens*), β -N-acetylglucosaminidase (jack beans), α -mannosidase (jack beans), α -L-fucosidase (bovine kidney) and chitinase (*Streptomyces griseus*) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Endo- β -galactosidase (*Escherichia freundii*) was from Seikagaku America, St. Petersburg, FL, U.S.A. and β -D-galactosidase (bovine testes) was from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Amido-G-acid [AGA, monopotassium 7-amino-1,3-naphthalenedisulfonate (II)] and $^2\text{H}_2\text{O}$ (99.996%) were purchased from Aldrich, Milwaukee, WI, U.S.A. Spectrapore dialysis tubing (M_r cut-off 100 and 500) was purchased from Spectrum Medical, Los Angeles, CA, U.S.A. Bio-Gel P-2 was from Bio-Rad, Richmond, CA, U.S.A. Acrylamide (ultrapure), Tris, Alcian Blue dye, Bromophenol Blue dye, and ammonium peroxydisulfate were obtained from Boehringer Mannheim. Glycine hydrochloride, disodium EDTA, boric acid, sucrose, *N,N'*-methylenebis(acrylamide) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were from Fisher Chemical Company, Fair Lawn, NJ, U.S.A. Biotrace RP nylon membrane was obtained from Gelman Science Inc., Ann Arbor, MI, U.S.A. and 3MM paper, from Whatman, Hillsboro, OR, U.S.A. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was obtained from Merck Sharp & Dohme, Kirkland, Quebec, Canada. All other chemicals were reagent-grade.

TABLE I

Synthesis of AGA-oligosaccharide conjugate (III)

Oligosaccharide	R, R', R'', R'''
Ia	R = R' = R'' = H, R''' = NHCOCH ₃
Ib	R = β -D-Gal-(1 \rightarrow 4), R' = R'' = H, R''' = NHCOCH ₃
Ic	R = β -D-Gal-(1 \rightarrow 4)- β -D-Gal-(1 \rightarrow 4), R' = R'' = H, R''' = NHCOCH ₃
Id	R = α -Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 4), R' = R'' = H, R''' = OH
Ie	R = β -D-Gal-(1 \rightarrow 3)- β -D-GlcNAc-(1 \rightarrow 3)- β -D-Gal-(1 \rightarrow 4), R' = R'' = H, R''' = OH
If	R' = β -D-Gal-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 6), R = R'' = H, R''' = OH, reducing end = D-Gal
Ig	R = α -L-Fuc-(1 \rightarrow 2)- β -D-Gal-(1 \rightarrow 4), R' = R'' = H, R''' = OH
Ih	R' = α -D-Man-(1 \rightarrow 3), R = R'' = H, R''' = OH, reducing end = D-Man
Ii	R = β -D-GlcNAc-(1 \rightarrow 4)- β -D-GlcNAc-(1 \rightarrow 4), R' = R'' = H, R''' = NHCOCH ₃

Equipment. Strong-anion-exchange high-performance liquid chromatography (s.a.x.-h.p.l.c.) was performed by using two face-programmable, titanium-based, Shimadzu Bio Liquid Chromatograph LC-7A pumps (Kyoto, Japan). The system was equipped with a titanium-based, fixed-volume loop Rheodyne (Cotati, CA) No. 7125 injector and a 2141 variable-wavelength detector from Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, U.S.A. The data were processed using a Shimadzu Chromatopac

C-R3A integrating recorder. S.a.x.-h.p.l.c. was performed on a Spherisorb (5- μ m particle size) column of dimensions 4.6 mm \times 25 cm, with a 4.6 mm \times 5 cm guard column from Phase Separations, Norwalk, CT, U.S.A. A 32 \times 16 cm vertical slab-gel unit (SE 620), 250-mL SG500 linear gradient maker apparatus, and the TE70 semi-dry electrophoretic transfer unit were obtained from Hoefer Scientific Instruments, San Francisco, CA, U.S.A. An electrophoresis power unit model Bio-Rad, Richmond, CA, U.S.A. Sugar-fluorescent conjugates were made visible in an u.v. light chamber from Ultra-violet Products, Inc., San Gabriel, CA, U.S.A. Freeze-drying was done on a Virtis Freezemobile 6 freeze-drier. U.v. spectroscopy was performed with a Shimadzu UV-160 spectrophotometer and fluorescent spectroscopy with a Shimadzu RF-540 spectrofluorophotometer.

Methods.— *Preparation of fluorescently labeled sugars by reductive amination.* AGA (II) was used after recrystallization from deionized water³⁴. Sugar Ia-i (3.5 μ mol) was dissolved in 750 μ L of AGA (II) solution (50% w/v) in water adjusted with NaOH to pH 6.2 (pH 8.3 for compound Id). After being heated for 60 min at 80°, NaBH₃CN (16 μ mol) was added (the pH changed <0.1 unit). The mixture was heated for 12 h at 65° in an incubator shaker. After the reaction was complete the products were dialyzed and desalted overnight at 20° against two changes of 2 L of double-distilled deionized water in either 100 or 500 *M_r* cut-off, controlled-pore dialysis bags. The samples were freeze-dried and reconstituted in 100 μ L of distilled water before loading on the preparative gel.

Preparation and electrophoresis of gradient polyacrylamide gels. Gradient polyacrylamide resolving-gel was prepared from two different resolving-gel concentrations. The back chamber contained 11.5% (w/v) acrylamide, 0.5% (w/v) *N,N'*-bisacrylamide [total acrylamide 12% (w/v)], and 1% (w/v) sucrose in resolving buffer (lower buffer chamber) made from 0.1M boric acid, 0.1M Tris and 0.01M disodium EDTA, pH 8.3. The mixing chamber contained 20% (w/v) of acrylamide, 2% (w/v) *N,N'*-bisacrylamide [total acrylamide 22% (w/v)] and 15% (w/v) of sucrose, pH 8.3, in resolving buffer. Gradient gels (16 \times 32 cm) were poured vertically using 1.5-mm spacers by adding 35 mL of 12% solution (degassed) to the reservoir (back chamber) and 35 mL of 22% solution (degassed) to the mixing chamber. Ammonium peroxydisulfate (400 μ L of 10% solution) was added to the reservoir and 200 μ L to the mixing chamber, followed by addition of 30 μ L of TEMED to both chambers. Polyacrylamide solution from the mixing chamber passed by gravity into two channels leading to the top of the glass plates, forming a linear gradient from bottom to top. After polymerization, 10 mL of stacking gel was added to the top of the resolving gel. It was prepared from 4.75% (w/v) *N,N'*-acrylamide and 0.25% (w/v) *N,N'*-bisacrylamide in resolving buffer, adjusted to pH 6.3 with HCl and ammonium peroxydisulfate, 135 μ L of 10%, and 10 μ L of TEMED. A comb (well former) was inserted and, after polymerization, it was removed and the wells were washed with water and filled with a buffer made from 1.25M glycine and 0.2M Tris, pH 8.3. Samples combined with an equal volume of 50% sucrose solution containing trace quantities of Phenol Red and Bromophenol Blue were loaded carefully to the bottom corner of each well. Electrophoresis was performed for 18 h at 400 V (constant voltage) with cooling.

Semi-dry electro-transfer. The resolving gel was made visible using u.v. light at 366 nm and the desired fluorescent band was located. The band was removed from the gel by carefully cutting the gel. This slice was then soaked in transfer buffer consisting of Tris base (5.82 g), glycine HCl (4.35 g), and MeOH (200 mL) made up in 1 L with double-distilled deionized water. Several layers of blotting paper and positively charged transfer nylon membranes were cut to the same size as the gel slice and soaked in transfer buffer. Two pieces of transfer buffer-saturated blotting papers (3 mm) were placed on the top of the mylar mask, centering them over the opening. Multiple layers of saturated nylon membranes (depending on the concentration of sample in the gel) were placed on the top of the blotting papers. The soaked gel slice was placed directly on the nylon membranes followed by 3 layers of blotting paper, thus constructing a transfer sandwich. The cover of the semi-dry transfer unit was placed over the transfer sandwich and electro-transfer was performed at 7–10 V for 1 h. Completion of the transfer process was ensured by examining the gel under u.v. light and making sure that no material was left behind.

Elution and recovery of membrane-bound sugar-fluorescent conjugates. Bands on the nylon membrane were located using u.v. light at 366 nm. Nylon membrane containing conjugate were cut into small pieces and immersed in test tubes containing 3 mL of 2.0M NaCl and placed on a shaker for several h at room temperature. The salt solution containing recovered materials were dialyzed exhaustively against double-distilled deionized water. Desalting was also performed on a 2.5 × 75 cm Bio-gel P2 low-pressure column. The salt-free sample solution was concentrated by freeze-drying.

Spectroscopic methods. AGA (II) and purified sugar-fluorescent conjugates (IIIa-i) were prepared in double-distilled deionized water at various concentrations by serial dilution. Measurements were performed in 1.0 mL (u.v.) or 3.0 mL (fluorescence) quartz cuvettes at room temperature. Fluorescence spectra were recorded using excitation and emission slits of 10 nm. One- and two-dimensional n.m.r spectroscopy was performed on a Bruker WM-360 or MSL-300 spectrometer operating under ASPECT 2000 or 3000 control. Samples were prepared in $^2\text{H}_2\text{O}$ (>99.996%) containing DSS as the internal standard at room temperature. The two-dimensional COSY-45 experiment was run using standard Bruker software. Mass spectrometry was performed with a VG ZAB-HF spectrometer in the fast-atom-bombardment (f.a.b.) ionization mode. Negative-ion f.a.b. spectra were obtained using triethanolamine as the matrix¹⁵.

S.a.x.-h.p.l.c. analysis. S.a.x.-h.p.l.c. was performed to monitor the derivatization reaction and to determine the purity of sugar-fluorescent conjugates obtained using preparative gradient-gel electrophoresis. The reaction yield is based on the percent of starting sugar (determined by weight) converted into sugar-AGA conjugate (determined by absorbance at 247 nm from the ϵ_M of purified conjugate). The column was eluted with a linear gradient (0.2 to 2M over 60 min) of NaCl adjusted to pH 3.5 with HCl and a flow rate of 1.0 mL/min³⁶. The elution profile was monitored by absorbance at 247 nm at 0.02 absorbance units full scale (AUFS).

Enzymic digestions. Approximately 1 μg of sugar-fluorescent conjugate (IIIa-i) was treated for 12 h: with 100 mU of jack bean β -galactosidase at 25° in 100 μL of 0.1M

sodium citrate buffer, pH 4; with 100 mU of *E. coli* β -galactosidase at 37° in 100 μ L of 0.1M sodium phosphate buffer, pH 7.3; with 100 mU of *A. niger* β -galactosidase at 25° in 10 μ L of 50mM sodium acetate buffer, pH 5.2; with 100 mU of bovine testes β -galactosidase at 37° in 100 μ L of 0.1M sodium phosphate buffer, pH 7; with 1 U of *C. perfringens* neuraminidase at 37° in 10 μ L of 50mM sodium acetate buffer, pH 5; with 10 mU of jack bean *N*-acetylglucosaminidase in 10 μ L of 50 mM sodium phosphate buffer, pH 6; with 10 mU of jack bean α -mannosidase at 25° in 10 μ L of 50mM sodium phosphate buffer, pH 7.5; with 100 mU of α -L-fucosidase at 25° in 10 μ L of 10 mM sodium phosphate buffer, pH 6.0; with 10 mU of *E. freundii* endo- β -galactosidase at 50° in 1 μ L of 0.1M sodium acetate buffer, pH 7; and with 1 mU of *S. griseus* chitinase at 25° in 10 μ L of 0.1M sodium phosphate buffer, pH 6. After each enzymic treatment the sample was heated for 1 min at 100° to inactivate the enzyme thermally. On sequential enzyme treatments, the second enzyme dissolved in its buffer was added directly to the first, thermally inactivated enzyme dissolved in its buffer. Although this procedure resulted in the use of certain glycosidases outside their optimal pH range, sufficient activity was present to cleave the small amount of sugar-fluorescent conjugate being sequenced³⁷.

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