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The Spectrum of Anionic Oligosaccharides Released by Endo-β-*N*-acetylglucosaminidase H from Glycoproteins

STRUCTURAL STUDIES AND INTERACTIONS WITH THE PHOSPHOMANNOSYL RECEPTOR*

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We have performed a detailed analysis of all the anionic oligosaccharides released by endo-β-*N*-acetylglucosaminidase H from the whole cell glycoproteins of P388D$_1$ mouse macrophage-like cells labeled for 14 h with [2-^{3}H]mannose. The major anionic species consisted of phosphorylated high mannose-type oligosaccharides containing one or two phosphomonoesters or phosphodiesters in several different positions. In addition we identified hybrid-type molecules containing one, two, or three sialic acid residues. A subset of the latter molecules also contained phosphodiesters or phosphomonoesters on another branch of the same oligosaccharide. Unlike previously reported hybrid-type molecules, these do not have a "bisecting" *N*-acetylglucosamine residue on the β-linked mannose. Some of these oligosaccharides contained an unidentified acid-labile group on the core *N*-acetylglucosamine or the β-linked mannose. The glycoproteins secreted by these cells were greatly enriched in hybrid oligosaccharides containing one sialic acid and one phosphomonoester.

The interaction of the isolated oligosaccharides with bovine liver phosphomannosyl receptor immobilized on Affigel was analyzed. Oligosaccharides with phosphomonoesters were the only species that interacted with high affinity with the receptor, and molecules with two phosphomonoesters showed the best binding. The location of the phosphomonoester on the oligosaccharide influenced the degree of interaction with the receptor. Removal of accesible nonphosphorylated mannose residues improved the binding in some cases. These findings indicate that the generation of the physiological phosphomannosyl ligand on lysosomal enzymes involves removal of the blocking *N*-acetylglucosamine residues, trimming of certain mannose residues, and correct positioning of the phosphate esters.

The phosphomannosyl recognition marker of lysosomal enzymes, which appears to be involved in the targeting of newly synthesized lysosomal enzymes to lysosomes (1, 2), is generated in two steps. First, *N*-acetylglucosamine 1-phosphate is transferred to the hydroxyl group at C-6 of a mannose residue on the high mannose-type oligosaccharides of lysosomal enzymes. This reaction is catalyzed by a specific *N*-acetylglucosaminylphosphotransferase (3–5). Deficiency of this enzyme (4, 6, 7) or of its capacity to recognize lysosomal enzymes (8) results in the failure of targeting of these hydrolases which characterizes the genetic human diseases, mucolipidosis II (I-Cell Disease) and mucolipidosis III (pseudo-Hurler polydystrophy). In the next step, the outer *N*-acetylglucosamine residues are removed by the action of an α-*N*-acetylglucosamine 1-phosphodiester *N*-acetylglucosaminidase (formerly called *N*-acetylglucosaminylphosphodiesterase)[1] (9–11). Several lines of evidence suggest that the phosphomonoester-containing oligosaccharides are responsible for high affinity binding of the hydrolase to a phosphomannosyl receptor involved in the targeting of lysosomal enzymes to the lysosomes (12–18).

In a previous study, we described the detailed structural features of phosphorylated high mannose-type oligosaccharides released by endo-β-*N*-acetylglucosaminidase H from the total cellular glycopeptides of [2-^{3}H]mannose-labeled mouse lymphoma cells (19). The similarity of these oligosaccharides to those found on newly synthesized β-glucuronidase (20, 21) and their absence in fibroblasts from patients with I-Cell Disease suggested that they represented the phosphorylated oligosaccharides of total cellular lysosomal enzymes. Subsequently, others have described similar oligosaccharides in lysosomal enzymes from other sources (22, 23).

In the course of our previous study we noted the existence of several unidentified anionic [2-^{3}H]mannose-labeled compounds that also appeared to have been released by endo H.[2] The behavior of these molecules could not be explained solely on the basis of the presence of phosphodiesters or phosphomonoesters. We report here a detailed structural analysis of all of the anionic oligosaccharides released by endo H from the [2-^{3}H]mannose-labeled glycoproteins of both the intracellular and the secreted pools of P388D$_1$ mouse macrophage cells.

Since a known physiological function of these oligosaccharides involves the targeting of lysosomal enzymes to lysosomes, we were interested in studying the interaction of the phosphomannosyl receptor with the numerous anionic species and isomers that we isolated. We describe here the isolation of this receptor from bovine liver using a new affinity method, its immobilization on Affigel, and an analysis of the structural

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[1] Varki, A., Sherman, W., and Kornfeld, S., (1983) *Arch. Biochem. Biophys.*, in press.

[2] The abbreviations used are: endo H, endo-β-*N*-acetylglucosaminidase H; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; S.A., sialic acid; HPLC, high performance liquid chromatography.

TABLE III

Types of anionic oligosaccharides present in each fraction

The origin of each fraction is indicated as follows: c = cellular, m = media. The capital letter indicates the QAE-Sephadex peak of origin (see Fig. 2). The Roman numeral I indicates failure to bind to concanavalin A, whereas II indicates binding.

Fraction	Source	QAE-Sephadex peak	Bound to ConA[a]	Fraction	Total ^3H	Anionic groups		
						Sialic acid	Phosphodiester	Phosphomonoester
				%	cpm × 10^{-3}			
cBI	Cells	B	No	99	68.2	1	0	0
cBII	Cells	B	Yes	62	194.0[b]	0	1	0
				38	124.6	1	0	0
mBI	Media	B	No	99	13.0	1	0	0
mBII	Media	B	Yes	58	15.0	1	0	0
				42	11.0[b]	0	1	0
cCII	Cells	C	Yes	99	218.0[b]	0	0	1
mCII	Media	C	Yes	99	124.0[b]	0	0	1
cDI	Cells	D	No	92	3.2	2	0	0
cDII	Cells	D	Yes	5	4.1	2	0	0
				15	12.2	1	1	0
				80	64.0[b]	0	2	0
cEII	Cells	E	Yes	28	6.2	3	0	0
				7	1.8	2	1	0
				55	12.1	1	0	1
				10	2.2	0	1	1
mEII	Media	E	Yes	95	140.0	1	0	1
				3	4.3	0	1	1
cFII	Cells	F	Yes	95	100.0[b]	0	0	2
mFII	Media	F	Yes	97	220.0[b]	0	0	2

[a] ConA, concanavalin A-Sepharose.

[b] Indicates species previously described by us (19).

determinants of the anionic oligosaccharides which influence binding to the receptor.

EXPERIMENTAL PROCEDURES and RESULTS[3]

Anionic Oligosaccharides Released by Endo H May Have Sialic Acids, Phosphodiesters, and Phosphomonoesters in a Variety of Combinations—Table III lists the origin and the actual recovery of radioactivity in each of the various oligosaccharides obtained from the original QAE-Sephadex gradients. The structural studies described in the Miniprint revealed that these oligosaccharides could be grouped into three major types of anionic species which are shown in composite form in Fig. 10.

A major portion of the endo H-released material consisted of high mannose-type oligosaccharides containing one or two phosphate groups (*structure A*, Fig. 10). The phosphate groups were located predominantly on four different mannose residues, labeled *d*, *f*, *h*, and *i* in Fig. 10. The great majority of the molecules with two phosphates contained either two phosphodiesters or two phosphomonoesters. However, there was a small amount of oligosaccharide which contained one phosphodiester and one phosphomonoester (fraction $cEIIN_{100}$ in Table II). In addition to these oligosaccharide species which have been previously described (19, 23) we were able to identify several new anionic species.

One new oligosaccharide species consisted of hybrid molecules containing one, two, or three sialic acid residues (*structure C*, Fig. 10). Most of these molecules contained four or five mannose residues although a small fraction had three or six mannose residues. The sialic acids were located on the side of the molecules that contained the mannose residue linked α1,3 to the β-linked mannose. When a single sialic acid was present it was linked to a penultimate galactose which, in turn, was linked to an *N*-acetylglucosamine. The linkage of the additional sialic acid residues could not be elucidated. There was no evidence for the presence of a "bisecting" *N*-acetylglucosamine on the β-linked mannose or for branching on the mannose residue linked α1,3 to the β-linked mannose.

In addition to these hybrid species, we identified molecules containing both sialic acid and phosphate residues (*structure B*, Fig. 10). The sialic acid residues were all located on the branch containing the mannose residue linked α1,3 to the β-linked mannose. The phosphate groups in the form of phosphodiesters or phosphomonoesters were located exclusively on mannose residues *h* or *i*. Mannose residue *f*, which can be phosphorylated in the usual high mannose oligosaccharides, was not phosphorylated in any of these hybrid molecules. The number of mannose residues varied but tended to be greater than in the hybrids that only contain sialic acid. Once again, there was no evidence for a "bisecting" *N*-acetylglucosamine linked to the β-linked mannose.

Interaction of the Anionic Oligosaccharides with the Phosphomannosyl Receptor—The availability of these various phosphorylated oligosaccharides in a purified state allowed us to study their interaction with the phosphomannosyl receptor. To do this, each oligosaccharide was passed over a column of bovine liver phosphomannosyl receptor which was coupled to Affigel as described under "Experimental Procedures." In order to detect subtle interactions, the ^3H-labeled oligosaccharides were premixed with [^{14}C]maltose which served as an internal marker that did not interact with the receptor.

Molecules carrying one or two phosphodiesters eluted with the [^{14}C]maltose marker, indicating a lack of significant interaction with the receptor (Fig. 11*A* and Fig. 12*A*, respectively). Similarly, hybrid-type molecules with one sialic acid as well as neutral high mannose-type oligosaccharides failed to be retarded on the column (data not shown). However, oligosaccharides with one phosphomonoester were significantly retarded in their elution from the column (Fig. 13, *upper*) and molecules with two phosphomonoesters bound to the column

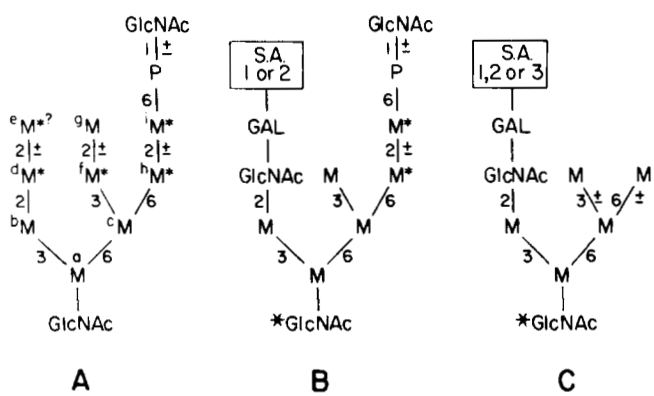

FIG. 10. **Summary of proposed structures of Endo H-released anionic oligosaccharides.** *M*, mannose; *S.A.*, sialic acid. Each mannose residue in *structure A* is assigned a *letter* so that they can be referred to individually in the text. The residues marked with the * are phosphorylated in one or more of the phosphorylated oligosaccharide species. The ★ indicates that the core Manβ1-4GlcNAc can carry the unexplained acid-labile substitution (see text for discussion).

[3] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1–9, and Tables I and II) 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. 82M-2530, cite the authors, and include a check or money order for $13.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

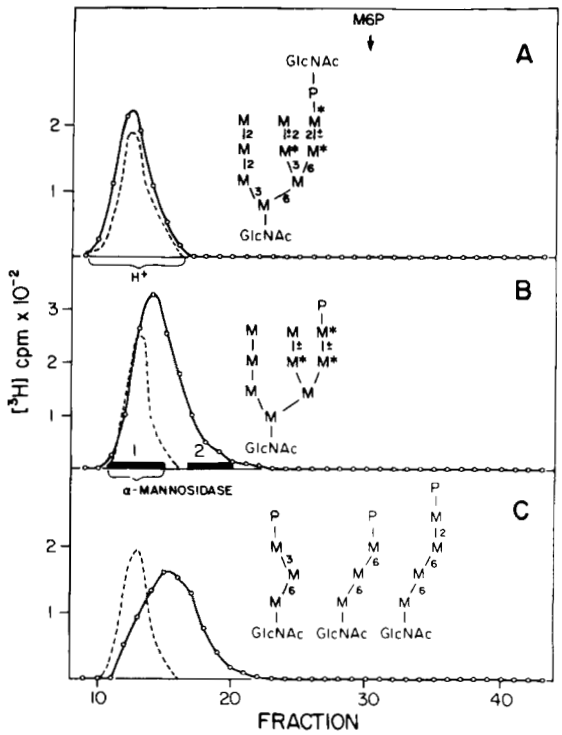

FIG. 11. **Phosphomannosyl receptor-Affigel affinity chromatography.** The various fractions were chromatographed on the column and then eluted with 5 mM mannose 6-phosphate as described under "Experimental Procedures." *M6P* shows the position where mannose 6-phosphate first elutes from the column. The position of the [^{14}C]maltose marker is indicated by the *dashed line*. *A*, molecules carrying one phosphodiester each (purified by neuraminidase treatment of fraction cBII—see Fig. 3*C*) were applied to the column; *B*, the same fraction was treated with mild acid to remove the covering *N*-acetylglucosamine residues and then applied to the column. Fractions 1 and 2 were pooled as shown and desalted on Bio-Gel P2; *C*, fraction 1 was treated with jack bean α-mannosidase and the charged fragments recovered by desalting on Bio-Gel P2. The charged fragments were then passed over the column. The * indicates the positions of the phosphates in the various isomers.

and required Man-6-P for elution (Fig. 14*D*). Since these samples contain a number of different isomers, all of the isomers must be capable of interacting with the receptor, although not necessarily to the same extent (see below).

Removal of Blocking N-Acetylglucosamine Residues Is Not Sufficient for Receptor Binding in All Cases—As shown in Fig. 11*B*, when oligosaccharides containing a single phosphodiester were treated with mild acid to remove the blocking *N*-acetylglucosamine residue and then chromatographed on the receptor column, most of the molecules continued to interact poorly with the receptor (fraction 1), while a small portion was slightly retarded (fraction 2). This is in contrast to the naturally occurring molecules with one phosphomonoester which all showed some retardation on the receptor column (Fig. 13, *upper*). In order to ascertain the reason for this difference we compared the structures present in fractions 1 and 2. The most striking difference was in the size of the underlying oligosaccharide. Fraction 1 contained $Man_9GlcNAc_1$, $Man_8GlcNAc_1$, $Man_7GlcNAc_1$, $Man_6GlcNAc_1$, and $Man_5GlcNAc_1$ in the ratio of 24:38:26:10:2, whereas fraction 2 had the same molecules in the ratio of 4:30:27:19:20. Thus an increased size of the underlying oligosaccharide appeared to be contributing to the decreased binding. We therefore treated a portion of fraction 1 with jack bean α-mannosidase and chromatographed the resulting fragments on the receptor column. As shown in Fig. 11*C*, the fragments were now retarded on the column, demonstrating that the removal of the accessible mannose residues enhanced the interaction of the residual fragments with the receptor. On the other hand, the underlying mannose residues of the fragments must enhance the interaction with the receptor since Man-6-P alone is not retarded on the column (data not shown).

When the blocking *N*-acetylglucosamine residues were removed from the oligosaccharides with two phosphodiesters, the majority of the molecules now bound to the receptor column and required Man-6-P for elution (Fig. 12*B*). However, a significant fraction of the molecules were only retarded on the column (fractions 1 and 2 in Fig. 12*B*). Treatment of this partially retarded material with α-mannosidase caused a major portion to bind with a higher affinity to the receptor column and require Man-6-P for elution (Fig. 12*C*). Thus some of the oligosaccharides require removal of both the blocking *N*-acetylglucosamine residues and certain mannose residues for high affinity interaction with the receptor.

The Position of the Phosphate Residue on the Oligosaccharide Affects the Interaction with the Receptor—The naturally occurring oligosaccharides with one phosphomonoester were retarded to different extents on the receptor column.

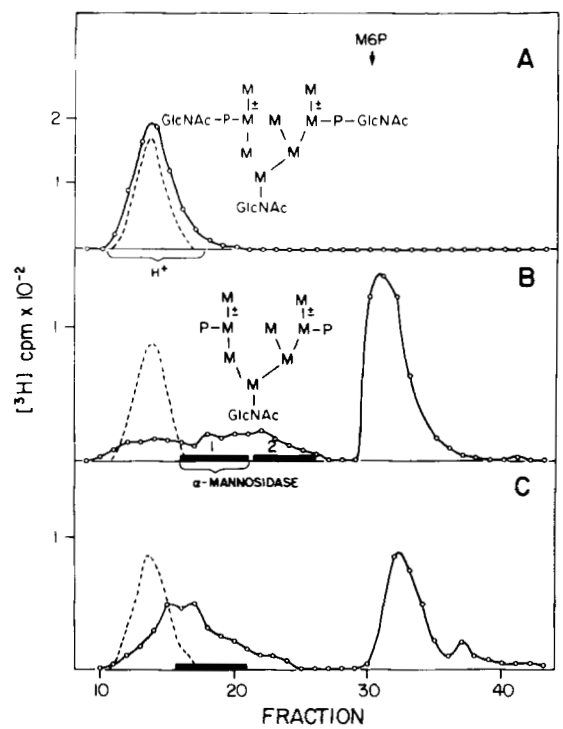

FIG. 12. **Phosphomannosyl receptor-Affigel affinity chromatography.** The various fractions were chromatographed on the column and then eluted with 5 mM mannose 6-phosphate as described under "Experimental Procedures." *M6-P* shows the position where mannose 6-phosphate first elutes from the column. The position of the [^{14}C]maltose marker is indicated by the *dashed line*. *A*, molecules with 2 phosphodiesters each were purified from fraction cDII by neuraminidase treatment (see Fig. 4*C* and Table II) and applied to the column directly; *B*, another aliquot was treated with mild acid to remove the outer GlcNAc residues and then applied to the column. Fractions 1 and 2 were pooled as shown. Each was then desalted on Bio-Gel P2, treated with jack bean α-mannosidase, and then desalted again on Bio-Gel P2; *C*, the charged molecules from fraction 1 reisolated after α-mannosidase treatment were applied to the column. Similar results were obtained with fraction 2. The *inset structures* only show the most common isomer of molecules with two phosphates, for convenience (see Fig. 10, *structure A* for other possibilities).

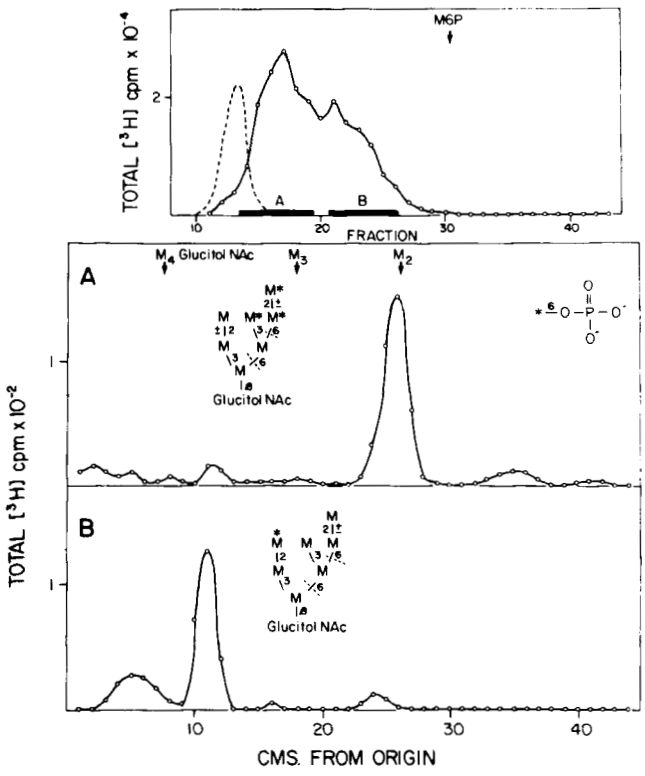

FIG. 13. **Phosphomannosyl receptor-Affigel chromatography.** Molecules with one phosphomonoester each (Fraction cCII—see Table III) were applied to the column and eluted as described under "Experimental Procedures." The *dashed line* shows the position of elution of [^{14}C]maltose. *Peaks A* and *B* were pooled as shown and desalted on Bio-Gel P2. Aliquots of fractions A and B were reduced, fragmented by acetolysis, and fractionated on QAE-Sephadex as described under "Experimental Procedures." The negatively charged fragments from each fraction were dephosphorylated with alkaline phosphatase, desalted on Amberlite MB-3, and spotted on Whatman No. 1 paper. *A* and *B* show paper chromatography in Solvent A for 14 h. The standards are: M_2, mannobiose; M_3, mannotriose; M_4 GlucitolNAc, Man$_4$GlucitolNAc. The *inset* structures show the acetolysis fragmentation pattern (*dashed lines*) and location of the phosphomonoesters (*) in fractions A (*A*) and B (*B*). *M6P*, mannose-6-P.

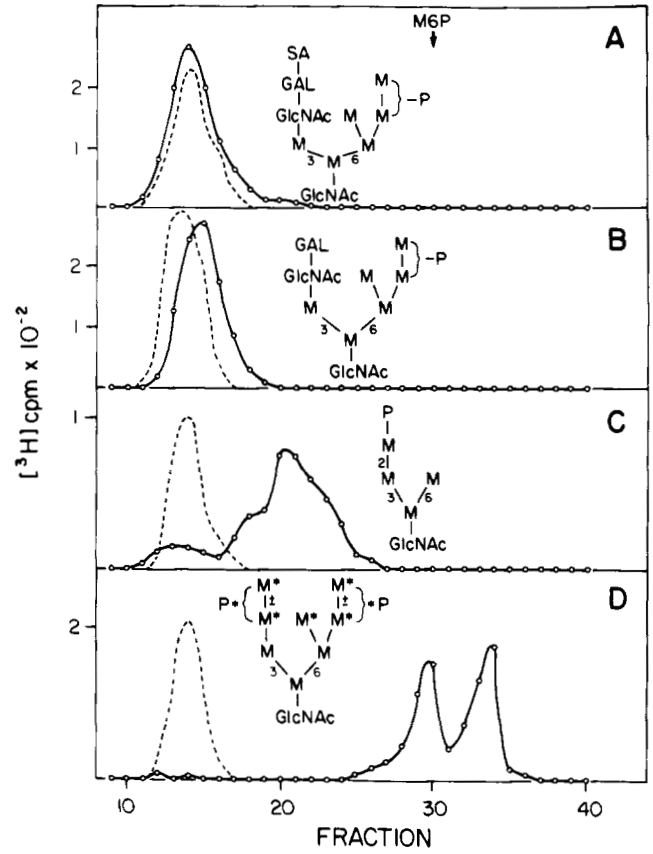

FIG. 14. **Phosphomannosyl receptor-Affigel affinity chromatography.** The various fractions were chromatographed on the column and then eluted with 5 mM mannose 6-phosphate, except in *D* when the elution was made with 0.5 mM mannose 6-phosphate. *M6P* shows the position where mannose 6-phosphate first elutes from the column. The position of the [^{14}C]maltose marker is indicated by the *dashed line*. *A*, chromatography of molecules with one sialic acid and one phosphomonoester (Fraction mEII, see Table III); *B*, chromatography of the same fraction after removal of the sialic acid with neuraminidase; *C*, chromatography of the phosphorylated oligosaccharide from the Thy-1$^-$ mutant (see Ref. 43). This fraction was approximately 85% pure. *D*, chromatography of molecules with two phosphomonoesters (fraction cFII, see Table III). The *asterisks* mark the possible positions of phosphates on the various isomers.

These molecules were separated into two pools (*A* and *B*) as shown in Fig. 13, *upper*. We have previously shown that such molecules contain a mixture of isomers, with the phosphomonoesters being present predominantly at four different positions (residues *d*, *f*, *h*, and *i* of Fig. 10, *structure A*) (18). In order to ascertain if the differences in affinity were related to the position of the phosphomonoester on the oligosaccharides, portions of *pools A* and *B* were subjected to acetolysis and the resulting fragments separated into neutral and anionic species on QAE-Sephadex as described under "Experimental Procedures." The charged fragments were dephosphorylated and analyzed by paper chromatography. The material from *pool A* gave rise to mannobiose almost exclusively, whereas the material from *pool B* gave rise to a fragment that migrated in the position expected for Man$_3$GlucitolNAc (Fig. 13, *A* and *B*, respectively). This difference in the fragmentation pattern demonstrates that the phosphate residues are located at very different positions in the two groups of molecules. As shown in the *insets* on Fig. 13, the molecules with the highest affinity for the receptor have the phosphomonoester on the branch linked α1,3 to the β-linked mannose whereas the molecules with the lowest affinity have the phosphomonoester on the branch linked α1,6 to the β-linked mannose. This corresponds well with the observation that the naturally occurring molecules with one phosphodiester behave like the *pool A* material after undergoing conversion by acid treatment to the phosphomonoester state. Previous studies have demonstrated that these molecules have their phosphate residues predominantly on the branch linked α1,6 to the α-linked mannose (19).

When aliquots of *pools A* and *B* were treated with α-mannosidase and the residual charged fragment rechromatographed on the receptor column, there was no change in the elution position. This demonstrates that in the case of the molecules with one phosphomonoester, the mannose residues on the other branches do not have a significant effect on the binding to the receptor. This conclusion was confirmed by the finding that the phosphorylated oligosaccharide isolated from the Thy-1$^-$ class E mutant cell line (43) eluted from the receptor column in the same position as the *pool B* material even though it has only four mannose residues (Fig. 14 *C*). In this molecule, as in the *pool B* material, the phosphomonoester is on the branch linked α1,3 to the β-linked mannose.

Sialylated, Phosphorylated Hybrid Molecules Interact Poorly with the Mannose 6-Phosphate Receptor—The hybrid molecules with one sialic acid residue and one phosphomonoester displayed no detectable binding to the receptor column (Fig. 14*A*). Upon removal of the sialic acid the molecules

migrated in the position expected of species with one phosphomonoester on the branch linked α1,6 to the β-linked mannose (Fig. 14*B*). In this case, the sialic acid present on another branch of the oligosaccharide appears to interfere with the binding to the receptor.

DISCUSSION

In our previous study of endo H-released oligosaccharides from mouse BW5147 cells, we found that the major anionic fractions contained high mannose-type oligosaccharides that had one, two, or possibly three 6-phosphomannosyl residues present as phosphomonoesters or as phosphodiesters with outer *N*-acetylglucosamine residues (19). We noted, however, the existence of several unexplained anionic species in the various fractions. The major goal of the current study was to isolate and characterize these species. To accomplish this we made several changes in the purification scheme used for the previous study. The most significant change was to release the oligosaccharides directly from the intact glycoproteins (by incubating with endo H in the presence of 0.2% SDS) rather than from pronase-derived glycopeptides. The released oligosaccharides could then be more readily separated from the residual material. This is a problem when dealing with glycopeptides since highly anionic oligosaccharides are difficult to separate from small glycopeptides by gel filtration. The other major change in the fractionation scheme was to separate the various anionic oligosaccharides on QAE-Sephadex using a gradient elution with ammonium acetate, pH 5.3, as buffer. This proved to be a more effective way to separate the different species then the stepwise elution scheme used previously.

We found that all of the anionic charges on the endo H-released oligosaccharides from the cell types analyzed could be accounted for by some combination of sialic acids, phosphodiesters, or phosphomonoesters. The molecules were of three basic types: high mannose-type oligosaccharides with phosphomannosyl residues in the form of phosphodiesters or phosphomonoesters; hybrid-type molecules containing sialic acid, galactose, and *N*-acetylglucosamine in one outer branch and mannose residues in the other branch; and hybrid-type molecules containing sialic acid, galactose, and *N*-acetylglucosamine residues as well as phosphomannosyl residues (see composite structures in Fig. 10).

The hybrid-type molecules differed from those previously described (39, 40) in several ways. First, the molecules were sialylated, containing up to three sialic acid residues. All of the sialic acid residues were on the branch of the molecule which contains the mannose linked α1,3 to the β-linked mannose and one of the sialic acid residues was linked to a penultimate galactose. The location of the other sialic acids could not be determined with the amount of available material. Since there was no evidence for more than one *N*-acetylglucosamine residue substituting the mannose residue linked α1,3 to the β-linked mannose, the additional sialic acid residues could have been present in the form of a polysialosyl sequence (41) or directly linked to the galactose-substituted *N*-acetylglucosamine residue (42). Another novel feature of these hybrid-type molecules is that they did not have a "bisecting" *N*-acetylglucosamine residue linked to the β-linked mannose. Thus the suggestion that hybrid molecules are generated because the "bisecting" *N*-acetylglucosamine residue impedes the action of α-mannosidase II (44) is not applicable in all cases. In those instances where the sialylated hybrid-type molecules also contained a phosphomannosyl residue, the origin of the hybrid species can be readily explained since the phosphate residues are known to block the action of the α-mannosidases (19). Because of the location of the blocking phosphate groups, some of the molecules contained more than the five mannose residues usually found in hybrid-type oligosaccharides (39, 40). This indicates that *N*-acetylglucosaminyltransferase I, the enzyme that transfers the *N*-acetylglucosamine residue to the mannose residue linked α1,3 to the β-linked mannose, does not have an absolute requirement for the typical $Man_5GlcNAc_2$ oligosaccharide as its substrate. The existence of the sialylated, phosphorylated hybrid species indicates that at least some of the newly synthesized lysosomal enzymes must pass through the region of the Golgi apparatus where terminal glycosylation takes place. Finally, some of the hybrid-type molecules contained an unidentified acid-labile substitution, most likely on the outer *N*-acetylglucosamine residue of the core or on the β-linked mannose. This acid-labile moiety was not found on any of the high mannose-type oligosaccharides.

A scheme that relates the various events in the biosynthesis of the species identified in this study and integrates them with previous data concerning *N*-linked oligosaccharide processing (45) is shown in Fig. 15. Starting with the neutral high mannose-type oligosaccharide precursor (*structure A*), it is possible to generate all of the molecules that have been identified. The small amount of *structure E*, with one phosphodiester and one phosphomonoester, relative to *structure F* with two phosphomonoesters (6.5×10^3 cpm *versus* 320×10^3 cpm) indicates that removal of the blocking *N*-acetylglucosamine from this species is quite efficient.

The studies of the interaction of the various anionic oligosaccharides with the immobilized bovine liver Man-6-P receptor have revealed that binding to the receptor is influenced by a number of the structural features of these oligosaccharides. The state of the phosphate is of primary importance. Thus the molecules that interacted with highest affinity with the receptor were those with phosphomonoesters, and molecules with two phosphomonoesters interacted better than molecules with one phosphomonoester (Fig. 15). However, within these two classes of molecules there was further heterogeneity in the extent of interaction with the receptor. In the case of molecules with a single phosphomonoester, those with the phosphomonoester on the branch linked α1,3 to the β-linked mannose (position *d*) bound more tightly than the isomers with the phosphomonoester on one of the other two branches (positions *f*, *h*, or *i*). This difference persisted after the various molecules were degraded to smaller fragments with jack bean α-mannosidase. In addition, all of the fragments generated by α-mannosidase interacted with the receptor better than mannose 6-phosphate interacted. This indicates that the linkage of the Man-6-P to the underlying sugars is important for high affinity binding. The fragment with the sequence P-Manα1-2Manα1-3Manβ1-4GlcNAc interacts best with the receptor.

If molecules with one or two phosphodiesters interact with the receptor, these interactions are too weak to be detected in our assay system. Even after removal of the blocking *N*-acetylglucosamine residues, a significant portion of the molecules did not bind as well as the naturally occurring species with phosphomonoesters in the same locations. Since the naturally occurring molecules have fewer mannose residues, we treated the artificially uncovered molecules that had a lower than expected affinity for the receptor with α-mannosidase. This treatment caused most of these molecules to achieve the expected level of binding for the number and location of the phosphate residues. The most likely explanation for this effect is that α-mannosidase is removing mannose residues which are linked α1,2 to penultimate phosphomannosyl moieties (as occurs at residues *d* and *h* in species with phosphodiesters). Since Man-6-P interacts with the receptor much better than glucose 6-phosphate, its 2-epimer (12), it is

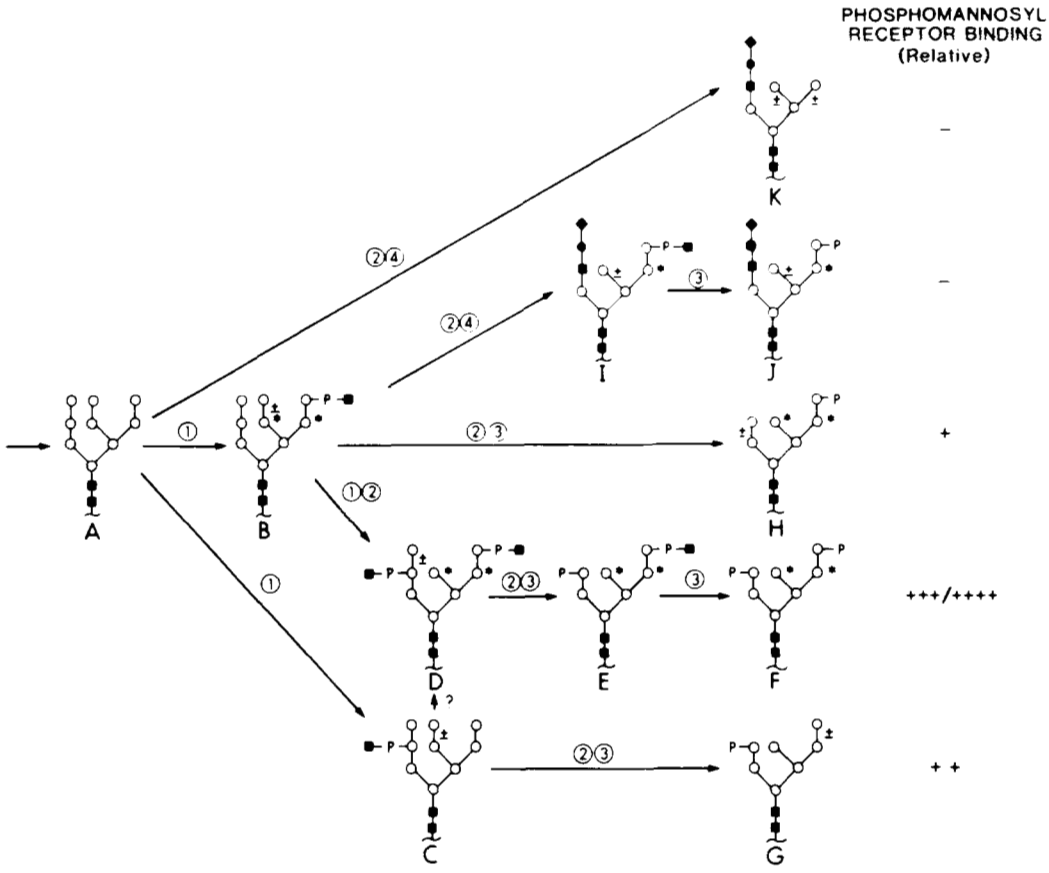

FIG. 15. **Proposed steps in the generation of the anionic oligosaccharides described in this study.** The high mannose-type oligosaccharide A on a protein backbone arises from trimming of the three glucose residues of the oligosaccharide originally transferred from the lipid carrier. The subsequent steps are indicated by *arrows*, along with the enzymatic activities involved at each step. *1*, *N*-acetylglucosaminylphosphotransferase; *2*, α1-2-specific mannosidase; *3*, *N*-acetylglucosamine 1-phosphodiester glycosidase; *4*, *N*-acetylglucosaminyltransferase I, galactosyltransferase, and sialyltransferase with or without the α1-3/1-6-specific mannosidase. The * indicate alternate positions of the phosphate groups; the individual sugars are depicted by the following: ○, mannose; ■, *N*-acetylglucosamine; ●, galactose; ♦, sialic acid. The relative degree of binding of the oligosaccharides *J*, *H*, *F*, and *G* are indicated on a scale of − to ++++.

not surprising that oligosaccharides with 6-phosphomannosyl residues that are substituted at C-2 do not interact well with the receptor until the outer mannose residues are removed. We conclude from these data that the generation of the physiologically important ligand for the phosphomannosyl receptor involves three steps: the transfer of GlcNAc 1-phosphate to mannose residues, the removal of the blocking GlcNAc residues, and the trimming of outer mannose residues which are linked α1,2 to penultimate phosphomannosyl residues. This triad of events is, in fact, what occurs during the synthesis of lysosomal enzymes (21).

These studies of the interaction of isolated oligosaccharides with the Man-6-P receptor do not reveal the minimal requirements for binding of a lysosomal enzyme which contains multiple oligosaccharide units. In this regard we have found that lysosomal enzymes of the BW5147 Thy-1⁻ E murine lymphoma cell line are capable of high affinity binding to the Man-6-P receptor and subsequent adsorptive endocytosis (43). These enzymes contain truncated oligosaccharides with single phosphomannosyl residues (as shown in Fig. 14*C*) demonstrating that the presence of monophosphorylated oligosaccharides on a lysosomal enzyme is sufficient for recognition and translocation by the Man-6-P receptor.

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SUPPLEMENTARY MATERIAL

TO

The Spectrum of Anionic Oligosaccharides Released by Endo-β-N-acetylglucosaminidase H from Glycoproteins: Structural Studies and Interactions with the Phosphomannosyl Receptor

Ajit Varki and Stuart Kornfeld

Materials - D-[2-^{3}H]Mannose (10 Ci/mmol) and ^{14}C maltose were from New England Nuclear. Affigel-10 and AG 50W (1-X2) were from BioRad Laboratories. Amberlite MB-3 ion exchange resin was from Mallinckrodt. QAE-Sephadex (Q-25-120), Sephadex G-25-80, Sephadex G-15-80, Sephadex G-50-80, bovine liver acetone powder, γ-D-galactonolactone, sodium borohydride, and mannose 6-phosphate were from Sigma Chemical Co. Con A-Sepharose was from Pharmacia; Triton X-100 and the 3a70 scintillation counting cocktail were from RPI. DE-52 (DEAE cellulose) was from Whatman. GLC-grade pyridine was from Pierce. The $Man_9GlcNAc$, $Man_8GlcNAc$, $Man_7GlcNAc$, $Man_6GlcNAc$, and $Man_5GlcNAc$ standards were isolated and characterized as previously described (24). Manα1→2Mannitol and Manα1→3Mannitol were prepared as previously described (25). Methylated [2-^{3}H]mannose standards were prepared as previously described (26) 2-acetamido-2-deoxygluconolactone was kindly provided by Dr. J. Baenziger. All other chemicals were of reagent grade.

Enzymes - Streptomyces griseus endo-β-N-acetylglucosaminidase H was from Miles. β-Galactosidase, β-hexosaminidase and α-mannosidase from jack bean meal were prepared as described by Li and Li (27). Homogeneous Escherichia coli alkaline phosphatase was a gift of Dr. M. Schlesinger, Washington University. Vibrio cholerae neuraminidase was from Calbiochem. The α-N-acetylglucosamine 1-phosphodiester glycosidase was purified 1800-fold from rat liver as previously described (11).

Buffers - The following buffers were used in various procedures described below. Buffer A: 25 mM potassium phosphate pH 6.5; Buffer B: Citrate phosphate pH 6.0, 75 mM NaCl, 0.05% Triton X-100, with 0.02% sodium azide, Buffer C: 0.2M NaCl, 10 mM EDTA, 0.05M sodium acetate pH 5.0; Buffer D: 0.4M KCl, 50 mM Imidazole pH 7.0, with 1% Triton X-100. Buffer E: 10 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% sodium azide.

Radiolabeling of Tissue Culture Cells - P388D_1 murine macrophage cells were maintained in suspension culture as previously described (21). A 150 mm diameter tissue culture dish was seeded with 2 x 10^6 cells which were allowed to attach and grow to confluence. The plate was washed once with complete α-minimal essential medium, and the labeling initiated with 50 ml of the same medium containing 10% fetal calf serum and 4 mCi of [2-^{3}H]mannose. We had previously found that under these conditions, the consumption of glucose by the cells is extremely rapid. We therefore monitored the concentration of glucose in the medium every two hours. At 8 hours, when the concentration had fallen from the initial level of 5 mM to 2.1 mM, an aliquot of sterile 1M glucose was added to bring the concentration back to 5 mM. At 14 hours, when the concentration of glucose had once again fallen to 2.4 mM, the media was removed, and the cells were harvested into 25 ml of ice cold complete media. The cells were pelleted at 600 x g and the pellet washed three times in the same ice-cold media.

Isolation of [2-^{3}H]Mannose-Labeled Glycoproteins from the Cellular Material and Media - The washed cell pellet (150 µl) was suspended in 830 µl of 20 mM Tris, pH 7.4, and sonicated three times, for 20 s each time, with a Biosonik IV probe set at 60. 20 µl of 10% SDS was added (for a final concentration of 0.2%) and the sonicate was boiled at 100°C for 10 min and transferred to a 1.5 ml Eppendorf tube. The tube was centrifuged at 10,000 x g for 5 min, and the supernatant removed. The pellet was re-extracted with 500 µl of 20 mM Tris, pH 7.4 containing 0.2% SDS, at 100°C for 5 min, and the supernatant pooled with the first one. The SDS extract, which contained greater than 90% of the cell-associated radioactivity, was applied to a column of Sephadex G-50-80 (0.9 x 90 cm) and the column was eluted with 20mM Tris pH 7.5, with 0.2% SDS. One ml fractions were collected and monitored for radioactivity. The high molecular weight [2-^{3}H]mannose-labeled glycoproteins eluting in the void volume of the column were pooled (see Figure 1), chilled on ice, and nine volumes of ice cold acetone added slowly, with frequent mixing. The mixture was then allowed to stand at -20°C for 10 minutes. Under these conditions, greater than 95% of the ^{3}H label was in the precipitate, which was collected at 12000 x g for 15 min. For isolation of glycoproteins from the media, the medium was clarified by centrifugation, concentrated to about 10 ml using an immersible CX-10 ultrafiltration unit (Millipore), and the proteins precipitated with cold 90% acetone as described above. The precipitate was redisolved in 20 mM Tris, pH 7.4, containing 0.2% SDS, applied to the Sephadex G-50 column, and the high molecular weight material reprecipitated with 90% acetone as described above. Because of the large amount of protein in the serum-containing media, it was necessary to run the column three times to fractionate all of the solubilized material.

Isolation and Fractionation of Endo H Releasable Oligosaccharides - The acetone precipitates were washed once in ice-cold 90% acetone, resuspended in 1 ml of citrate-phosphate buffer, pH 5.5, containing 0.2% SDS, and then heated for 5 min at 100°C. After cooling to room temperature, the solutions were treated with 2 mU of endo H at 37°C under a toluene atmosphere for 36 hours; an additional 1 mU of enzyme was added after 20 hours. The digest was boiled for 5 min, centrifuged at 10,000 x g for 5 min, and the supernatant fluid reapplied to the Sephadex G-50 column. The released radioactivity, which was included by the column, (see Figure 1) was pooled. To remove the SDS, 2M KCl was added for a final concentration of 20 mM, and the mixture was chilled on ice for 30 minutes. The resulting precipitate was removed by centrifugation, and the supernatant was lyophilized and desalted on a Sephadex G-15 column (0.9 x 50 cm) in H_2O. The material eluting in the void of the G-15 column, which represented all of the labeled endo H releasable oligosaccharides in the starting glycoproteins, was taken to dryness. The anionic species present in this mixture of oligosaccharides were isolated and fractionated by gradient elution from QAE-Sephadex, exactly as previously described (20), except that the buffer used was ammonium acetate, pH 5.3, and the gradient was run from 2 to 350 mM acetate over 200 ml.

Analysis of Anionic Oligosaccharides on QAE-Sephadex - The number of negative charges on the endo H-released oligosaccharides was determined prior to, or following, various treatments by batch elution from QAE-Sephadex in a manner similar to that previously described (18). The oligosaccharides were dissolved in 2 mM Tris base, passed over 1 ml columns of QAE-Sephadex equilibrated in 2 mM Tris base and eluted with increasing concentrations of NaCl in 2 mM Tris base (0.75 ml x 6 for each concentration). We have found that, under these conditions, endo H-released oligosaccharides with 1,2,3 or 4 net negative charges elute at 20 mM, 70 mM, 100 mM, and 140 mM NaCl respectively. A final elution with 1M NaCl in 0.1N HCl (0.75 ml x 2) was done. 1.5 ml fractions were collected, and the radioactivity monitored.

Enzyme Digestion of Oligosaccharides - All digestions were carried out at 37°C in a toluene atmosphere. α-GlcNAc 1-phosphodiester glycosidase, E. coli alkaline phosphatase, jack bean α-mannosidase, jack bean β-hexosaminidase, jack bean β-galactosidase and V. cholerae neuraminidase digestions were carried out as described elsewhere (11,19,28). In addition, the β-hexosaminidase incubations contained γ-galactonolactone (to inhibit any traces of β-galactosidase) and the β-galactosidase digestions contained 2-acetamido-2-deoxygluconolactone, (to inhibit any traces of β-hexosaminidase) (29). The α-mannosidase digestions contained both inhibitors.

Mild Acid Hydrolysis - Oligosaccharides in 0.3 ml of 0.01 N HCl were heated at 100°C for 30 min. The samples were then frozen, lyophilized, and brought to 2 ml with 2 mM Tris base in preparation for QAE-Sephadex fractionation.

Paper Chromatography - Descending paper chromatography was performed on Whatman No. 1 paper in ethyl acetate/pyridine/acetic acid/water (5:5:1:3) (solvent A), or in ethyl acetate:pyridine:H_2O (8:2:1) (solvent B). Strips (1 cm) were cut, soaked in 0.4 ml of water, and the ^{3}H radioactivity determined.

High-Voltage Paper Electrophoresis - Manα1→2mannitol and Manα1→3mannitol were separated by high voltage paper electrophoresis according to the method of Verma, et al. (30).

Methylation Analysis of Oligosaccharides - The methylation procedure was carried out exactly as previously described (26).

Reduction of Endo H-Released Oligosaccharides - The desalted oligosaccharides were reduced in 200 µl of 0.2 M borate pH 9.8., containing 0.1M $NaBH_4$ for 2 hours at 37°C (31). The borates were then removed by repeated evaporation from methanol containing a drop of glacial acetic acid. The sample was desalted on a Sephadex G-25 column (for anionic oligossaccharides) or on a 1ml Amberlite MB-3 column (for neutral oligosaccharides).

High-Pressure Liquid Chromatography for Separation of Reduced Oligosaccharides - This was performed using an AX-5 (micropak) column on a Varian 5000 High Pressure Liquid Chromatograph, exactly as described by Mellis and Baenziger (31) except that the program was started at 25% water in acetonitrile. All samples were first desalted by passage over a small column of Amberlite MB-3 (1.5 ml)in H_2O.

Acetolysis of Reduced Oligosaccharides - The procedure incorporated several modifications of previously described methods (19,26,32-34). The reduced, desalted, oligosaccharides were lyophilized in a reactivial with a tight-fitting teflon cap. 100 µl of dry pyridine: acetic anhydride (1:1) was added, the sample vortexed well, and placed in a dessicator in a dark place at room temperature for 2 days. The acetylation was then completed by heating at 100°C for 6 hours, when a yellow color developed. The reagents were then removed by drying under a stream of nitrogen, followed by lyophilization for a few hours. 100 µl of the acetolysis mixture [(11:10:1) glacial acetic acid:acetic anhydride:concentrated sulfuric acid (all dry)] was added, the vial capped tightly, vortexed well, and placed at 37°C in a tissue culture warm room for 8 to 10 hours. The reaction was quenched by transferring the contents to an Eppendorf microcentrifuge tube containing sufficient 1M barium acetate (85 µl) to neutralize all of the sulfuric acid. The reactivial was then washed with 0.4 ml x 3 of 70% methanol, and the washings added to the reaction. The barium sulfate was removed by centrifugation, and the pellet extracted once with 70% methanol. The pooled supernatants were taken to dryness three times with 70% methanol to remove the acetic acid. The acetolysis fragments were deacetylated by incubation in 0.08 ml of 0.2 M NaOH at R.T. for 45 min (a white precipitate of barium hydroxide is seen). In the case of neutral oligosaccharides, the fragments were desalted by passage over a column of Amberlite MB-3 mixed bed resin (1.5 ml) in water. In the case of the anionic oligosaccharides, the mixture was diluted to 8 ml and the charged fragments were recovered on a column of QAE-Sephadex, as previously described (19).

Preparation of Phosphomannosyl Receptor-Sepharose Column

1. Preparation of Affinity Column - We reasoned that since the slime mold Dictyostelium discoideum secretes large quantitites of "high uptake" lysosomal enzymes (35), an affinity column could be prepared from the total secretions. 2 x 10^9 vegetative cells of the axenic strain A-3 maintained in HL-5 medium were washed and transferred to differentiation medium (15 mM PO_4 buffer, pH 6.0) and incubated at R.T. for 12 hours. The cells were then removed by centrifugation, and the medium containing the secreted material further clarified by centrifugation at 10,000 x g for 10 min. The supernatant fluid was passed over a column of DE-52 (2.7 x 10 cm) equilibrated in Buffer A. The column was washed with 1200 ml of the same buffer, and eluted with 100 ml of 1M NaCl in this buffer. The eluted material was dialyzed twice, for 10 hours each, against a 20-fold excess of Buffer A. 1M MES pH 6.5 was added for a final concentration of 0.1M and the protein coupled to 25 ml of Affigel-10 using the manufacturer's recommended procedure. After 15 hours, 2.5 ml of 1M ethanolamine HCl, pH 7.8, was added and the gel mixed for 2 hours. β-Hexosaminidase activity was followed throughout these steps as a representative of the lysosomal enzymes, and protein was monitored by the method of Lowry (36). From the starting medium containing 184 mg of protein and 2443 units of β-hexosaminidase, approximately 50 mg of protein and 2342 units of enzyme were coupled to the 25 ml of gel. The gel was poured into a column and equilibrated in Buffer B.

2. Purification of Bovine Liver Phosphomannosyl Receptor - The procedure used was similar to that of Sahagian, et al. (17,37), except that the affinity column described above was utilized. 100 g of bovine liver acetone powder was suspended in 2000 ml of Buffer C, and stirred for 15 min on ice. The pH, which was 5.8, was adjusted to 5.0 briefly with glacial acetic acid (added dropwise) and the pH was then brought back up to 6.0 with addition of 1N NaOH. The mixture was centrifuged at 10,000 x g for 15 min and the supernatant fluid discarded. The pellet was resuspended in 2600 ml of distilled water, dispersed with three quick bursts in a Waring blender, and the solid material collected again by centrifugation. The pellet was similarly resuspended in Buffer D, and stirred at 4°C for 45 min. The material was centrifuged at 35,000 x g for 15 min and the supernatant fluid passed over a plug of glass wool. The clear extract was passed over the affinity column at the rate of 3 ml/min. The column was washed with 2000 ml of Buffer B then eluted with Buffer B containing 5 mM mannose 6-phosphate. After one column volume of the buffer containing mannose 6-phosphate, the flow was stopped for 1 hour, and then resumed. Protein was monitored in the various fractions by the method of Lowry, et al. (36). 2 mg of protein was eluted by the Man 6-P. When 2-10 µg of this protein was subjected to SDS-PAGE analysis, it showed a single Coomasie blue-staining band of M_r 210,000 which corresponds well with the previously reported molecular weight of the phosphomannosyl receptor from bovine liver (17,37). The receptor preparation was concentrated 10-fold using an Amicon concentrator and a YM-10 membrane.

3. Coupling of the Phosphomannosyl-Receptor to Affigel - 1 mg of the receptor preparation in 3.3 ml was mixed with 0.7 ml of 1M HEPES for a final pH of 7.8. 4 ml of Affigel-10 was prepared as recommended by the manufacturer, washed with 20 ml of Buffer B, added to the protein solution, and mixed end-over-end for 14 hours at 4°C. 0.5 ml of ethanolamine HCl, pH 7.8, was then added, and the suspension mixed for an additional 2 h. The coupling efficiency was estimated to be greater than 80%. The gel was degassed and poured into a column (0.7 cm x 18 cm) and equilibrated in Buffer B before use.

Phosphomannosyl-Receptor Affinity Chromatography - All operations were carried out at 4°C. Desalted [2-^{3}H]mannose-labeled oligosaccharides or glycopeptides were taken to dryness, and then dissolved in 200 µl of Buffer B, containing 400 cpm of ^{14}C Maltose (as an internal marker). The sample was applied to the column, washed in with 200 µl of Buffer B, and then eluted with the same buffer. After 2 column volumes, the elution was continued with Buffer B containing mannose 6-phosphate (5 or 0.5 mM, as indicated)for an additional two column volumes. 0.5 ml fractions were collected directly into scintillation vials and the ^{14}C and ^{3}H radioactivity determined. For preparative runs, the internal marker was omitted but was chromatographed separately either immediately before or after the oligosaccharides.

Other Procedures - Protein was determined by the method of Lowry, et al. (36). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out by a modification of the method of Laemlii (8,38). β-N-Acetylhexosaminidase activity was assayed as previously described (9), and the activity expressed in units (1 µmole/hr).

RESULTS

Isolation and Fractionation of Anionic Oligosaccharides - P388D_1 cells were labeled, and the cellular and secreted glycoproteins isolated as described under "Experimental Procedures". As shown in Figure 1, the high molecular weight glycoproteins eluting in the void of the Sephadex G-50 column were pooled and digested with endo H, and the released oligosaccharides isolated by rechromatography under the same conditions. Under these conditions, 53% of the label from cellular and 40.2% of the label from the media glycoproteins was released. Retreatment of the unreleased material with endo H did not release any more radioactivity. The released oligosaccharides were desalted and fractionated by QAE-Sephadex gradient elution. As shown in Figure 2, 9.4% of the cellular oligosaccharides and 39% of the those from the media bound to the column and eluted in several different peaks. The various peaks were pooled, taken to dryness, dissolved in 1 ml of Buffer E, and passed over 1 ml columns of Con A-Sepharose equilibrated in the same buffer. After washing with one ml of the same buffer, the eluates were repassed over the columns, in order to ensure maximal binding. The columns were washed with 8 ml of Buffer D, and then eluted with 10 ml of Buffer E containing 100 mM α-methylmannoside at 60°C. In all instances, the majority of the counts bound, and required elution with α-methylmannoside. However, significant amounts of radioactivity passed through the columns in certain fractions (see Table 1). The various fractions were taken to dryness and desalted on columns of G-25 (0.9 x 50 cm) in water. Each fraction was named alphabetically, according to its peak of origin on the QAE-Sephadex gradient. The cellular fractions have the prefix 'c', whereas those from the media have the prefix 'm'; fractions that bound to Con A are labeled II, whereas those that did not are labeled I.

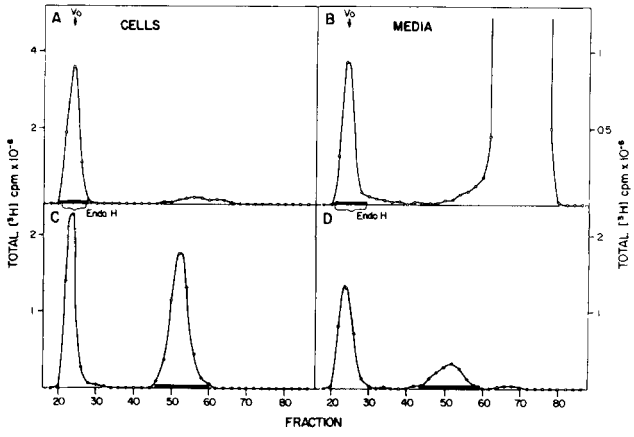


Figure 1 - Sephadex G-50 Chromatography of [2-^{3}H]Mannose-Labeled Oligosaccharides Released from Intact Glycoproteins by Endo H. The labeled material from the cells (Panel A) and the media (Panel B) were prepared and chromatographed on Sephadex G-50 as described under "Experimental Procedures". The material eluting in the void volume was pooled as indicated by the bar, treated with endo H and rechromatographed on the Sephadex G-50 column (Panels C and D). The released oligosaccharides were pooled as indicated in the figure.

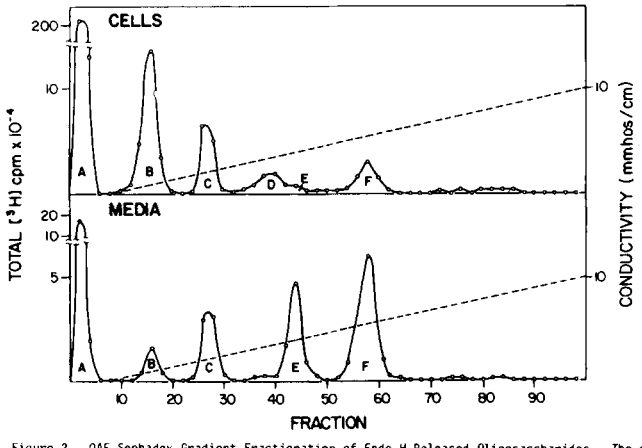


Figure 2 - QAE-Sephadex Gradient Fractionation of Endo H-Released Oligosaccharides. The oligosaccharides from the cells (top) and the media (bottom) were loaded on a QAE-Sephadex column and eluted with a linear gradient as described under "Experimental Procedures". The various peaks were pooled as labeled.

Characterization of the Anionic Species Present in Each Fraction - Aliquot of each fraction were treated with either mild acid, neuraminidase, alkaline phosphatase, or combinations of these treatments and then analyzed for the number of negative charges by batch elution from small columns of QAE-Sephadex. The elution of an untreated aliquot of each fraction was followed simultaneously. As expected, many of the fractions contained mixtures of different anionic species. Some examples are shown in Figures 3 and 4. The panels on the left of Figure 3 show the analysis of fraction cBII, which originated from peak B of the QAE-Sephadex gradient of the cellular material and bound to Con A. With no treatment (3A), all of the molecules had one net negative charge (eluting at 20 mM NaCl). After mild acid treatment, (Panel 3B) 61% of the radioactivity increased to two net negative charges (eluting at 70 mM), while 36% became neutral. Upon neuraminidase treatment, (Panel 3C) a similar 36% became neutral. Upon combined treatment with mild acid and alkaline phosphatase all of the radioactivity became neutral (3D). Thus, the fraction consisted of a mixture of oligosaccharides with one sialic acid (converted to neutral by mild acid or neuraminidase) and others with one phosphodiester (converted to a phosphomonoester by mild acid, and subsequently neutralized by alkaline phosphatase). Panels E, F, and G show the behavior of fraction cBI (that originally ran through Con A) upon no treatment, mild acid treatment, and neuraminidase treatment, respectively. All of the negative charge in these molecules can be accounted for by sialic acid.

Panels A, B, C, and D of Figure 4 show the analysis of fraction cDII after no treatment, mild acid treatment, neuraminidase treatment, and alkaline phosphatase treatment, respectively. This fraction carries two net negative charges per oligosaccharide, both of which are resistant to alkaline phosphatase. Mild acid and neuraminidase cause complete neutralization of 4-7% of the molecules, suggesting that these molecules carry two sialic acid residues. 70-80% of the molecules increase in negative charge upon mild acid treatment to a total of four net negative charges (eluting at 140 mM NaCl), suggesting that these molecules originally carried two phosphodiesters. The remainder of the oligosaccharides show a decrease of one negative charge upon neuraminidase treatment. However, upon mild acid treatment, they show no apparent change in charge. This is because although one negative charge (due to the sialic acid) is lost, another negative charge is gained because the other group, a phosphodiester, is converted to a phosphomonoester by the acid treatment. Thus, this fraction consists of a mixture of three different species. Panels E, F, G, and H show the similar treatment of fraction mEII. More than 95% of this fraction behaves as a single homogenous anionic species. It originally has three net negative charges (eluting with 100 mM NaCl) and loses one charge with either mild acid or neuraminidase treatment (Panels F and G). Alkaline phosphatase causes loss of two negative charges (Panel H) and combined treatment with neuraminidase and alkaline phosphatase causes complete loss of negative charge (data not shown). This fraction, therefore, contains molecules that have one sialic acid and one phosphomonoester on the same oligosaccharide.

Similar analysis of all the other fractions was carried out. The results are summarized numerically in Table 1. It can be seen that while several fractions were heterogenous, all of the fractions that ran through Con A (those labeled I) contained sialic acids as the negative charge. Thus, all of the phosphodiester and phosphomonoester-containing oligosaccharides bound to Con A-Sepharose under the conditions used.

In order to further characterize some of the species in the subfractions, aliquots were treated with neuraminidase and the various subfractions collected by batch elution on QAE-Sephadex (see Table 2). Each subfraction was then desalted on BioGel P2 and further analyzed by mild acid and/or alkaline phosphatase treatment. As shown in Table 2, it was possible to isolate relatively pure subfractions and to conclusively demonstrate that their remaining anionic charge was due to either phosphomonoesters or phosphodiesters.

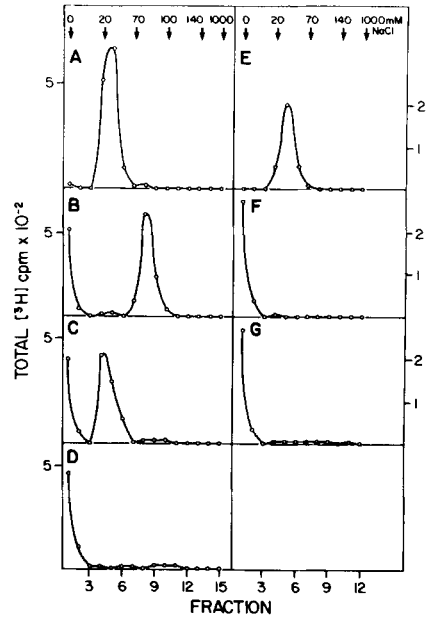


Figure 3 - QAE-Sephadex Batch Elution for Analysis of Anionic Species. Fractions cBII (Panels A, B, C, and D) and cBI (Panels E, F, and G) were applied to QAE-Sephadex after no treatment (Panels A and E), mild acid treatment (Panels B and F), neuraminidase treatment (Panels C and G) or mild acid plus alkaline phosphatase treatment (Panel D), and then eluted batchwise with increasing concentrations of NaCl as shown.

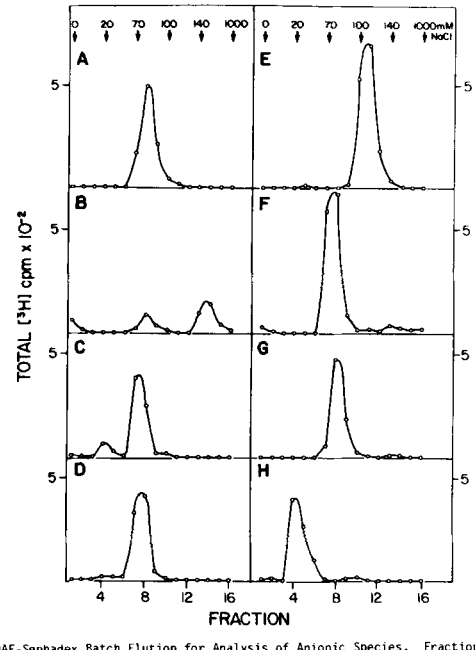


Figure 4 - QAE-Sephadex Batch Elution for Analysis of Anionic Species. Fractions cDII (Panels A, B, C, and D) and mEII (Panels E, F, G, and H) were applied to QAE-Sephadex columns after no treatment (Panels A and E), mild acid treatment (Panels B and F) neuraminidase treatment (Panels C and G) or alkaline phosphatase treatment (Panels D and H), and eluted stepwise with increasing concentrations of NaCl as shown.

In this manner, it was possible to account for virtually all of the various anionic species present in the endo H-released oligosaccharides from both the cells and medium, as being various combinations of phosphodiesters, phosphomonoesters, or sialic acids. A complete catalogue of all the species found, with their original source, behavior on Con A, and the actual total cpm isolated in each species from the cells and media is given in Table 3 (see main body of the manuscript). In addition, there was a small amount of radioactivity with a negative charge greater than that of 2 phosphomonoesters (see Figure 2, the region beyond peak F). Although this material bound to Con A, there was an inadequate number of counts to allow an adequate characterization of the species present.

Structural Studies of Oligosaccharides with one Sialic Acid Residue - Oligosaccharides with one sialic acid were present in several different fractions (see Table 3). Since they were all released by endo H we reasoned that they were probably hybrid-type structures (39,40). As expected for such structures, jack bean α-mannosidase released a portion of the radioactivity as free mannose from each of the fractions, but the amount varied from 37-56% for the various fractions (data not shown). In addition, some of the fractions originally bound to Con A, whereas others did not, again suggesting variability in the number and/or the locations of accessible mannose residues. We therefore carried out a detailed structural study of these oligosaccharides. In order to conserve space, only the studies performed on one major fraction (cBII) will be described, and the others will then be contrasted with this.

Table 1

Analysis of Anionic Species in Endo H Released Oligos

Fraction Name	Treatment	% of Total CPM Eluting at (mM NaCl) 0	20	70	100	140	1M
cBII	None	0.5	99	0.5	0	0	0
	H^+	36	2.5	61	0.5	0	0
	N	36	63	1	0	0	0
	H+ and AP	99	1	0	0	0	0
mBII	None	1	98	1	0	0	0
	H^+	57	1	40	1	0	0
	N	58	41	1	0	0	0
cBI	None	0	100	0	0	0	0
	H^+	97	1	1	1	0	0
	N	99	1	0	0	0	0
mBI	None	1	99	0	0	0	0
	H^+	98	1	1	0	0	0
	N	99	0	1	0	0	0
cCII	None	1	0	99	0	0	0
	AP	99	0	1	0	0	0
mCII	None	1	0	99	0	0	0
	AP	99	0	1	0	0	0
cDII	None	0	0	98	2	0	0
	H^+	7	0	14	79	0	0
	N	3	10	85	2	0	0
	AP	0	3	97	0	0	0
cDI	None	2	0	94	4	0	0
	H^+	90	0	5	5	0	0
	N	88	0	8	4	0	0
cEII*	None	1	0	0	99	0	0
	H^+	28	0	60	0	13	0
	N	29	8	53	10	0	0
mEII	None	0	0	2	98	0	0
	H^+	2	0	95	0	3	0
	N	1	0	96	3	0	0
	AP	0	99	1	0	0	0
	N and AP	99	0	0	0	0	0
cFII	None	0	2	3	1	95	0
	AP	95	2	2	2	0	0
mFII	None	0	0	2	2	96	0
	AP	97	0	2	1	0	0

Aliquots of the various fractions were subjected to different treatments as shown, and then applied to columns of QAE-Sephadex and eluted stepwise as described. (Some examples are shown graphically in Figures 3 and 4). (N = neuraminidase, H^+ = mild acid, AP = alkaline phosphatase).

(*) The original fraction cEII was thought to be contaminated with molecules containing only 2 negative charges, because of crossover from the much larger adjacent peak D (see upper Panel of Figure 2, peaks D and E.) The material was therefore preparatively fractionated batchwise on QAE-Sephadex in a manner similar to that shown above. As expected, some of the radioactivity eluted at 70 mM NaCl. The majority of the radioactivity eluted at 100 mM NaCl. This latter fraction was pooled and desalted, and is hereafter called cEII.

Table 2

Demonstration of Subfractions

Starting Fraction	Subfraction	Treatment	% of Total CPM Eluting at (mM NaCl) 0	20	70	100	140	1000
cDII	---	N	3	10	<u>85</u>	2	0	0
	$cDIIN_{20}$	H^+	8	<u>0</u>	92	0	0	0
		H^+ and AP	86	<u>4</u>	10	0	0	0
	$cDIIN_{70}$	H^+	2	0	<u>3</u>	0	95	0
		H^+ and AP	95	0	<u>4</u>	0	1	0
cEII	---	N	29	8	53	<u>10</u>	0	0
	$cEIIN_{20}$	H^+	9	<u>1</u>	90	0	0	0
		H^+ and AP	82	<u>1</u>	17	0	0	0
	$cEIIN_{70}$	H^+	2	1	<u>97</u>	0	0	0
		H^+ and AP	96	0	<u>4</u>	0	0	0
	$cEIIN_{100}$	H^+	2	0	0	<u>3</u>	95	0
		H^+ and AP	95	0	0	<u>3</u>	2	0

Aliquots of fractions cDII and cEII were treated with neuraminidase, boiled, diluted with 2 mM Tris base, applied to columns of QAE-Sephadex, and batch eluted with increasing concentrations as shown. Each subfraction thus generated was desalted on Sephadex G-25, subjected to other treatments and then reapplied to the columns as before. (N = neuraminidase; H^+= mild acid; AP = alkaline phosphatase) The underscored numbers indicate the original elution position of each fraction i.e., where they would have eluted without any further treatment.

Fraction cBII consisted of a mixture of molecules with one phosphodiester or one sialic acid. (see Table 1). Two different methods were used to separate these subfractions. Treatment with neuraminidase, followed by application to QAE-Sephadex resulted in a neutral fraction that had lost its sialic acid residue (hereafter called fraction $cBIIN_0$). Alternatively, treatment with the purified α-GlcNAc-1-phosphodiester glycosidase resulted in cleavage of the phosphodiesters, with a consequent increase in negative charge. The material that continued to elute with 20 mM NaCl after this treatment consisted of the intact sialylated molecules (hereafter called fraction $cBIIP_{20}$).

Fraction $cBIIN_0$ lost 50% of its radioactivity as free mannose upon treatment with jack bean α-mannosidase. HPLC analysis of this fraction revealed an unexpected degree of heterogeneity (Figure 5, Panel A). Several peaks, of size ranging from $Mannose_5GlcNAc_1$ to $Mannose_7GlcNAc_1$ were seen. Treatment with β-galactosidase before the HPLC analysis caused an almost complete overall shift of the peaks, of about one sugar residue (Figure 5, Panel B). This suggested that most, if not all, of these molecules had one terminal galactose residue after removal of the sialic acid. Treatment with β-galactosidase and β-hexosaminidase resulted in a further decrease in size (Panel C), to the degree expected if one additional GlcNAc residue had been removed (31). However, the heterogeneity of the peaks persisted. Combined treatment with both the above enzymes and α-mannosidase caused release of the majority of the radioactivity as free mannose (which was reduced to mannitol prior to the HPLC analysis) (Panel D). The remaining radioactivity migrated as two distinct peaks, instead of the single peak expected of Manβ1-4GlcitolNAc. This suggested that one possible cause of the observed heterogeneity might be some unexplained substitution of this disaccharide.

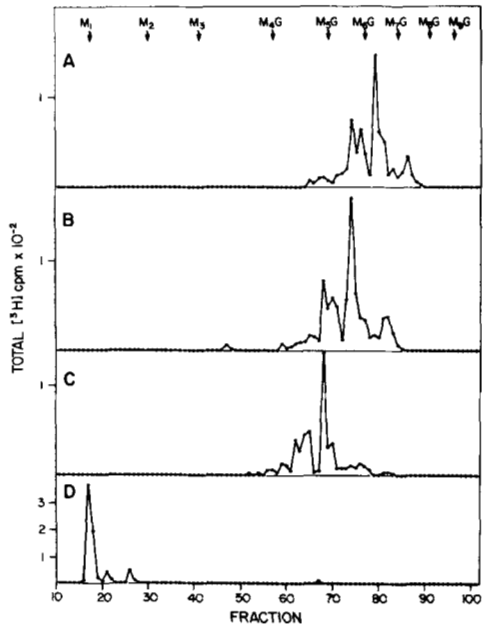


Figure 5 - HPLC Analysis of Oligosaccharide Size. Fraction cBII was treated with neuraminidase, and the oligosaccharides that became neutral were isolated. These neutral oligosaccharides were subjected to no treatment (Panel A), digestion with β-galactosidase (Panel B), digestion with β-galactosidase and β-hexosaminidase (Panel C), or digestion with β-galactosidase, β-hexosaminidase and α-mannosidase (Panel D). Following the treatments, the oligosaccharides were reduced with $NaBH_4$, desalted on Amberlite MB-3, and fractioned by HPLC as described under "Experimental Procedures". The standards are M_1, Mannitol; M_2, Mannobiitol; M_3, Mannotriitol; M_4G, $Mannose_4GlcitolNAc$; M_5G, $Mannose_5GlcitolNAc$; M_6G, $Man_6GlcitolNAc$; M_7G, $Man_7GlcitolNAc$; M_8G, $Man_8GlcitolNAc$; M_9G, $Man_9GlcitolNAc$.

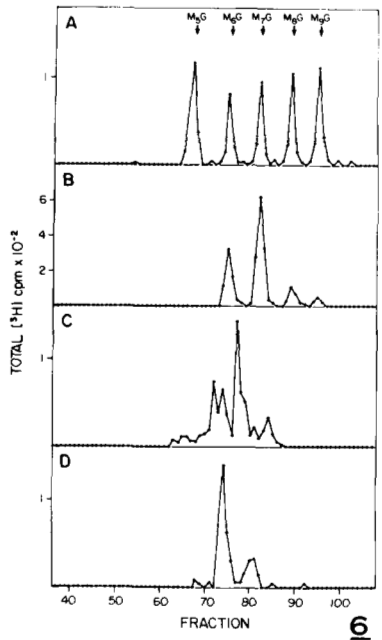
We next considered other possible structural features that could explain such heterogeneity. One possibility was that some of the oligosaccharides were substituted with fucose residues. To rule out this possibility, an aliquot was hydrolyzed in 2N HCl for 6 hours at 100°C, and the resulting monosaccharides fractionated by paper chromatography in Solvent B for 14 hours. Greater than 99.5% of the counts migrated as free mannose, and none as free fucose (data not shown). Another possibility was the presence of repeating Gal→GlcNAc units of varying sizes. However the overall size of these oligosaccharides was somewhat smaller than might be expected if such substitutions were present. Furthermore, endo-β-galactosidase treatment had no effect on the HPLC profile of these oligosaccharides (data not shown). The next possibility we considered was that some of the molecules might carry an additional GlcNAc residue, either as a "bisecting" residue on the β-linked mannose, or as an additional branch on the mannose linked α1-3 to the β-linked mannose. To study this possibility, we performed a methylation analysis of each of the peaks seen on HPLC. A preparative run similar to Panel A of Figure 5 was carried out except that smaller (0.3 ml) fractions were collected. This allowed the separation of at least 8 distinct peaks, each of which was separately subjected to methylation. All the fractions contained a residue of 2,4 di-methylmannose that arises from the β-linked mannose, which would not be present if a "bisecting" GlcNAc residue was present. None of the peaks gave rise to 3,6 dimethylmannose that would have been generated if branching was present on the α1-3 linked side of the molecule. The methylation analysis suggested that the major peaks had 4 to 5 mannose residues, although 3 or 6 residues could be present in the smaller or larger oligosaccharides, respectively. Furthermore, we noted that adjacent peaks could have an identical methylation profile. Thus, the unknown factor causing the heterogeneity on HPLC analysis had no detectable effect on the methylation profile. Similar heterogeneity was seen upon HPLC analysis of several of the other fractions that carried one sialic acid residue (data not shown).

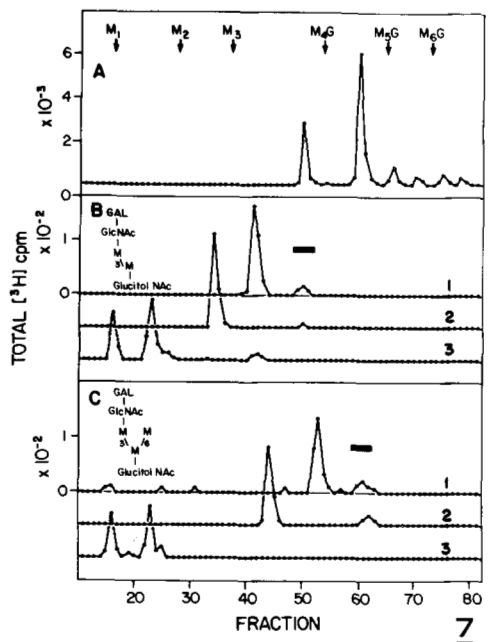
To rule out the possibility that this heterogeneity arose from some chemical artifact during the preparation of oligosaccharides for HPLC, we also studied similarly treated high-mannose oligosaccharide standards. As shown in Figure 6, Panel A, no such heterogeneity was seen in these standards. To rule out the possibility that an artifact may have arisen from some step during the purification of the oligosaccharides, we also studied molecules which had one phosphomonoester after removal of the phosphate. (Fractions cCII and mCII of the same experiment - see Table 1). The profile obtained with fraction cCII after removal of the phosphate and reduction is shown in Panel B of Figure 6. In keeping with previous findings, these molecules contained primarily $Man_6GlcNAc_1$ and $Man_7GlcNAc_1$ (19), and showed no such heterogeneity of peaks. We next discovered that the heterogeneity could be markedly decreased if the removal of the sialic acid was accomplished by mild acid, rather than by neuraminidase treatment. Panel C of Figure 6 is identical to Panel A of Figure 5, and shows the profile following neuraminidase treatment. Panel D shows the effect of mild acid treatment prior to the reduction step. Note that the heterogeneity is markedly diminished. However, the peaks still do not exactly co-migrate with the high-mannose standards in Panel A. This is expected because the additional N-acetylglucosamine residue in each oligosaccharide contributes less than one mannose equivalent in this HPLC system (31).

Thus, while we still cannot conclusively rule out an artifact, it appears likely that some of these sialylated hybrid-type molecules have an acid-labile substitution on the core Manβ1-4GlcNAc that alters their behavior on HPLC.

The above studies suggested that the molecules contain the common sequence S.A.→Gal→GlcNAc→Manα1→3Manβ1→4GlcNAc. In order to prove this, we sought to isolate the sialic acid-containing fragment. Fraction $cBIIP_{20}$, which contained the intact molecules with their sialic acid residues, was digested with β-galactosidase, β-hexosaminidase, and α-mannosidase, and the negatively charged fragment was re-isolated on QAE-Sephadex. The fragment was subjected to mild acid treatment to remove both the sialic acid residue and the unexplained acid-labile substitution. The neutralized fragments were reduced and analyzed on HPLC. Two major peaks were seen (see Figure 7, Panel A). Both peaks, when subjected to sequential digestion with β-galactosidase, β-hexosaminidase and α-mannosidase, showed an orderly decrease in size as expected, with eventual digestion to mannose and Manβ1→4GlcitolNAc (see Figure 7, Panel B and C). The first peak originally migrated in the position expected of the predicted fragment that would have been protected by the sialic acid residue e.g., Gal→GlcNAc→Manα1→3-Manβ1→4GlcitolNAc. We suspected that the second peak represented an incomplete digestion product, with a surviving terminal mannose linked α1-6 to the β-linked mannose (see inset to Figure 7, Panel C). To prove this we analyzed the second peak by methylation analysis. This yielded 2,4-dimethylmannose, 3,4,6-trimethylmannose, and 2,3,4,6-tetramethylmannose in a ratio of 1.0:1.25:1.25, which is compatible with the predicted structure. The incomplete digestion of the terminal α1→6 linked mannose could be the result of steric hindrance from the undigested α1→3 linked branch, or the presence of the unexplained substitution in the core.

These data show that the sialylated molecules present in fraction cBII have an overall structure that is similar to previously reported hybrids (40), except for the presence of the sialic acid, and the absence of the "bisecting" GlcNAc residue.





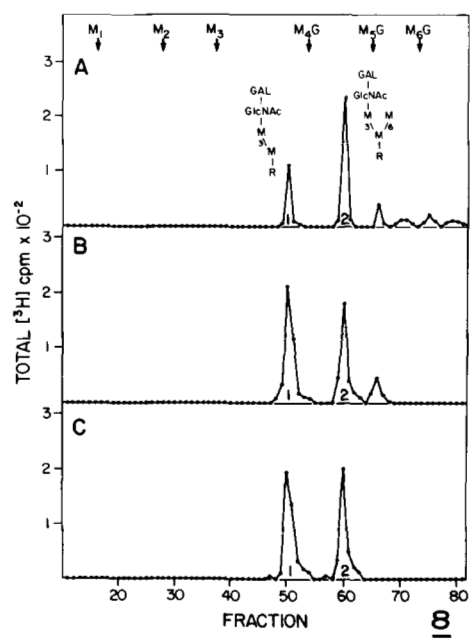


Figure 6 - HPLC Fractionation of Oligosaccharides. Standard high mannose oligosaccharides (Panel A), fraction cCII neutralized with alkaline phosphatase (Panel B), the neutral fraction generated from cBII by treatment with neuraminidase (Panel C) and the neutral fraction generated from cBII by treatment with mild acid (Panel D) were reduced with $NaBH_4$, desalted and fractionated on HPLC as described under "Experimental Procedures". The standards are M_5G, $Mannose_5GlcitolNAc$; M_6G, $Man_6GlcitolNAc$; M_7G, $Man_7GlcitolNAc$, M_8G, $Man_8GlcitolNAc$; M_9G, $Man_9GlcitolNAc$.

Figure 7 - HPLC Fractionation of Oligosaccharides. Panel A: Fraction cBII was treated with the αGlcNAc 1-phosphodiester glycosidase, and refractionated on QAE-Sephadex to separate the oligosaccharides with phosphomonoesters (2 negative charges) from those with sialic acid (1 negative charge) which were unaffected by the treatment. The latter fraction was desalted and treated with a mixture of β-galactosidase, β-hexosaminidase and α-mannosidase. The digest was then passed over QAE-Sephadex to isolate the surviving charged fragment. This fragment was desalted on BioGel P2, then treated with mild acid, reduced with $NaBH_4$, desalted on Amberlite MB-3, fractionated on HPLC, and an aliquot monitored for radioactivity. The two major peaks shown in Panel A were analyzed further as shown in Panels B (first peak) and C (second peak). Separate aliquots were subjected to treatment with (1) β-galactosidase (2) β-galactosidase and β-hexosaminidase or (3) β-galactosidase, β-hexosaminidase and α-mannosidase. In the case of (3) it was necessary to repeat the reduction with $NaBH_4$ to reduce the [2-^{3}H]mannose released. In all cases, the digests were desalted prior to HPLC analysis. The structures shown are based on this and other information (see text). The standards are as described in the legend to Figure 5.

Figure 8 - HPLC Fractionation of Oligosaccharides. Panel A is identical to Panel A of Figure 7. Panel B and C show the oligosaccharides remaining after the identical treatment (see legend to Figure 7) of fractions cBI and alkaline phosphatase digested mEII respectively. The proposed structures of peaks 1 and 2 in Panel A are shown. Note that peaks 1 and 2 coincide in each case with those in Panel A. The standards are as described in the legend to Figure 5.

When similar experiments were carried out on other fractions with one sialic acid, the identical two fragments were recovered (for example, see Figure 8, Panel B). Again, the only detectable causes of heterogeneity were the number of mannose residues and the presence of the unexplained acid labile substitution.

Structure of Oligosaccharides with more than one Sialic Acid Residue - We also identified molecules which carried two or three negative charges that could be removed by neuraminidase treatment alone (fractions $cDIIN_0$, and $cEIIN_0$ respectively, - see Table 2). We reasoned that the presence of two or more sialic acids most likely resulted from branching on the chain containing the mannose linked α1-3 to the β-linked mannose, since this would increase the number of galactose residues available for sialylation. However, HPLC analysis of the neutralized core fragments did not show the expected increase in overall size, although the heterogeneity of peaks was once again seen (data not shown). Furthermore, methylation analysis of the whole mixtures showed no evidence for a 3,6 or 3,4-dimethylmannose replacing a residue of 3,4,6-trimethylmannose, thus making branching on the α1-3 linked side very unlikely. The limited amounts of these fractions prevented us from performing further studies to localize the sialic acid residues. Presumably, as in the case of the monosialylated molecules, one of the sialic acids substitutes a galactose residue. Since other previously known linkages of the sialic acids include the polysialosyl linkage (41) and linkage to the galactose-substituted N-acetylglucosamine residue (42), we speculate that similar linkages might explain the observed findings.

Structures of Molecules with Sialic Acids and Phosphate Groups - As shown in Tables 1, 2, and 3 we were able to identify molecules carrying various combinations of sialic acids and phosphomonoesters or phosphodiesters on the same oligosaccharide. However, only one fraction (mEII) had sufficient material to allow a complete structural analysis. This fraction which contained a major portion of the anionic oligosaccharides in the media, (see Figure 2) consisted primarily (95%) of molecules that had one sialic acid and one phosphomonoester. We first approached the structural analysis by removal of both the negative charges with the appropriate enzyme treatments. When the resulting neutral core was analyzed on HPLC, many peaks were seen in the size range $Mannose_6GlcNAc_1$ to $Mannose_8GlcNAc_1$ (data not shown). We next removed the phosphate group only, and subjected the molecule to digestion with β-galactosidase, β-hexosaminidase, and α-mannosidase. 56% of the radioactivity was released as free mannose. Thus, the underlying oligosaccharides were somewhat larger than those on molecules with one sialic acid alone, and had greater numbers of accessible mannose residues. The negatively charged fragment resulting from the mixed glycosidase digestion was then treated with mild acid, and analyzed on HPLC. As shown in Figure 8, Panel C, the material now consisted exclusively of two fragments that co-migrated exactly with those obtained by similar treatments of other sialylated hybrids (Panels A and B). Thus, it is reasonable to conclude that these molecules also contain the common sequence S.A.→Gal→GlcNAc→Manα1→3Manβ1→4GlcNAc, and that they sometimes carry the acid-labile group that causes altered behavior on HPLC.

To identify the location(s) of the phosphomonoester group on these oligosaccharides, we removed the sialic acid residue, and then subjected the phosphorylated oligosaccharide to acetolysis, which selectively cleaves α1-6 linkages. The acetolysis fragments were fractionated on QAE-Sephadex, and the negatively charged fragments dephosphorylated and analyzed. As shown in Figure 9, Panel A, all of the charged fragments co-migrated with mannobiose after removal of the phosphate residue. As shown in the inset to Panel B of Figure 9, mannobiose fragments can arise from two different branches of the oligosaccharide after cleavage of the α1-6 linkages. To differentiate between these two possibilities, we reduced the mannobiose fragments, and subjected them to high voltage paper electrophoresis, which separates reduced mannobioses linked α1-2 from those linked α1-3. As shown in Panel B of Figure 9, all of the reduced mannobiose fragments co-migrated with Manα1-2 mannitol. Thus, the phosphate residue could only be present on one of two positions on the oligosaccharide, which are indicated on the inset to Panel B of Figure 9.

Thus, these molecules carry the sialic acid residue in the same location as the non-phosphorylated hybrids do, but the phosphate residue can only be present on two of the three possible positions.

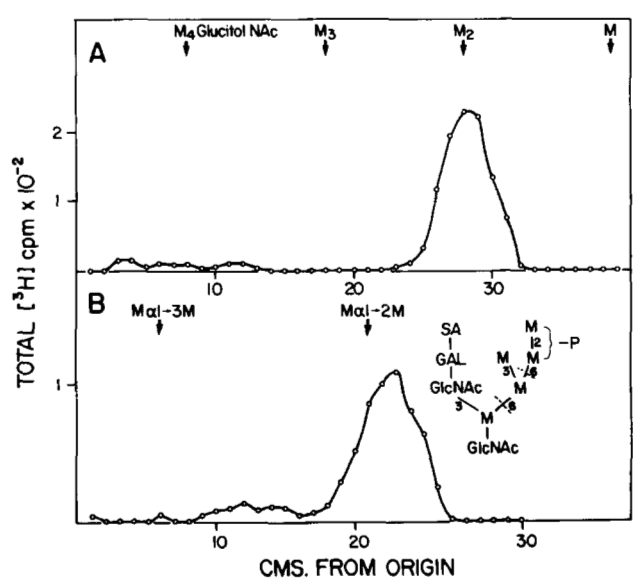


Figure 9 - Characterization of Negatively Charged Acetolysis Fragments. Neuraminidase treated fraction mEII was subjected to acetolysis, and the negatively charged fragments isolated on QAE-Sephadex. Panel A - The fragments were characterized by paper chromatography in Solvent A for 14 hours after removal of the phosphate with alkaline phosphatase. The standards are M, Mannose; M_2, Mannobiose; M_3, Mannotriose, and $M_4GlucitolNAc$, $Man_4GlucitolNAc$. Panel B - The mannobiose fragments were reduced and fractionated by high voltage paper electrophoresis as described in "Experimental Procedures". The standards are Mα1→3M:Mannoseα1→3-Mannitol; Mα1→2M:Mannoseα1→2Mannitol. The structural diagram in the inset show the acetolysis cleavage pattern (dotted lines) and the possible positions of the phosphomonoester (P) based upon this analysis.

The spectrum of anionic oligosaccharides released by endo-beta-N-acetylglucosaminidase H from glycoproteins. Structural studies and interactions with the phosphomannosyl receptor.

A Varki and S Kornfeld

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