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Phosphorylated oligosaccharides in lysosomal enzymes: Identification of α-*N*-acetylglucosamine(1)phospho(6)mannose diester groups

(glycoproteins/recognition marker/receptor-mediated uptake)

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ABSTRACT **In human fibroblasts, the recognition of lysosomal enzymes by cell surface receptors is mediated by mannose 6-phosphate residues located on oligosaccharides that can be cleaved by endo-β-*N*-acetylglucosaminidase H. About half of these oligosaccharides, as isolated from β-hexosaminidase and cathepsin D secreted by human skin fibroblasts, are anionic. Most of these are resistant to alkaline phosphatase. The resistance is due to α-*N*-acetylglucosamine residues linked to mannose 6-phosphate by a phosphodiester bond. The major phosphorylated oligosaccharides contain one and two and possibly three phosphate groups blocked by *N*-acetylglucosamine. Besides the blocked phosphate groups these oligosaccharides contain a common inner core consisting of Manα1,6(Manα1,3)Manα1,6(Manα1,3)ManβGlcNAc and either one or two α1,2-linked mannose residues.**

Lysosomal enzymes share an oligosaccharide recognition marker, which plays an important role in the transfer of these enzymes into lysosomes (1). In 1977 Kaplan *et al.* (2) suggested, primarily on the basis of competition experiments, that phosphorylated mannose residues were the principal component of the recognition marker of β-glucuronidase. The same conclusion was reached for other enzymes and in other laboratories (3–5). Later on phosphate groups were found in several lysosomal glycoproteins (6–10) and were localized on carbon 6 of mannose (7–9).

To study the structure of the oligosaccharide bearing the phosphorylated group, we used procedures developed (9, 11) to isolate mannose 6-phosphate residues from lysosomal enzymes. We labeled cultured human fibroblasts in the presence of NH_4Cl, collected the secretions, immunoprecipitated precursors of β-hexosaminidase and of cathepsin D, and isolated the oligosaccharides that were cleaved by endo-β-*N*-acetylglucosaminidase H. We have observed that the phosphate groups are to a large extent buried in a diester with *N*-acetylglucosamine and that the oligosaccharides may contain up to three such phosphodiester residues.

During the preparation of this manuscript Tabas and Kornfeld reported that β-glucuronidase from mouse lymphoma cells contains high-mannose oligosaccharides with phosphate residues blocked by *N*-acetylglucosamine (12).

MATERIALS AND METHODS

Labeling and Immunoprecipitation of Lysosomal Enzymes. Human skin fibroblasts were maintained at 37°C in 5% CO_2 in Eagle's minimal essential medium supplemented with antibiotics, nonessential amino acids, and 10% fetal calf serum (Flow Laboratories, Bonn, Federal Republic of Germany) as described (13). Conditions of labeling cells with [2-^{3}H]mannose and $^{32}P_i$ and immunoprecipitation of β-hexosaminidase and cathepsin D were those described (11) with the following modifications. The labeling medium was prepared without glucose and supplemented with 10 mM NH_4Cl and with 0.5% fetal calf serum that had been incubated at pH 10.4 for 30 min at 37°C to destroy acid hydrolases. NH_4Cl has been included in the medium to increase the yield of secreted precursors of lysosomal enzymes (11). Labeling with [6-^{3}H]glucose and [6-^{3}H]glucosamine was performed as described for [2-^{3}H]mannose. Cultures in 75-cm^2 flasks were incubated for 44 hr with 5 ml of labeling medium supplemented with 0.5 mCi of [2-^{3}H]mannose (22 Ci/mmol), 0.5 mCi of [6-^{3}H]glucose (30 Ci/mmol), 0.5 mCi of [6-^{3}H]glucosamine (19 Ci/mmol), or 0.5 mCi of $^{32}P_i$ (carrier free) (1 Ci = 3.7×10^{10} becquerels).

The immunoprecipitates of β-hexosaminidase and cathepsin D were assayed for impurities by gel electrophoresis in the presence of sodium dodecyl sulfate followed by fluorography (11). More than 95% of the radioactivity detectable by fluorography was located in bands that migrated as the precursor forms of β-hexosaminidase and cathepsin D.

Preparation and Hydrolysis of Radioactive Oligosaccharides. The immunoprecipitates from 20 flasks were solubilized in 0.2 ml of 0.125 M Tris·HCl, pH 6.8/1% sodium dodecyl sulfate/10 mM dithiothreitol (11), transferred into dialysis bags, diluted with 0.2 ml of water, and dialyzed for 9 hr against two changes of 50 ml of 20 mM NH_4CHO_2, pH 5.5. The bags were opened and a stock solution of endo-β-*N*-acetylglucosaminidase H (Seikagaku Kogyo, Tokyo, 30 units/mg of protein), 1 unit/ml, dissolved in 10 mM sodium phosphate, pH 6.0 and 0.15 M NaCl was added to give a final concentration of 0.05 unit/ml. Dialysis was continued in vials containing 15 ml of 20 mM NH_4CHO_2, pH 5.5 at 37°C for up to 36 hr. The dialysis solution was lyophilized and desalted on a Bio-Gel P-4 column (Bio-Rad, 1 × 20 cm) in 50 mM acetic acid and lyophilized. Mannose 6-phosphate was the only phosphorylated carbohydrate detectable in acid hydrolysates of the oligosaccharides (for method see ref. 9).

For degradation with alkaline phosphatase from *Escherichia coli* (Sigma, 44 units/mg of protein), lyophilized oligosaccharides were incubated with 0.5–1 unit of enzyme in 20–30 μl of 70 mM Tris·HCl, pH 7.5 for 90–180 min. The products were analyzed by either paper electrophoresis or ion-exchange chromatography on AG 1-X2 (Bio-Rad). For degradation with α-mannosidase from jack bean (Sigma, 20 units/mg of protein) lyophilized oligosaccharides (containing residual Tris buffer) were incubated with 0.15 unit of enzyme in 16 μl of 0.2 M sodium phosphate buffer, pH 6.0, for up to 96 hr under toluene. After 24 and 48 hr the same amount of α-mannosidase was added in 5 μl of 0.15 M NaCl. The products were separated by

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paper chromatography in solvent A (see below) for 15–20 hr. Degradation with neuraminidase from *Vibrio cholerae* (Behringwerke, Marburg, Federal Republic of Germany; 4 units/mg of protein) was performed with 0.001 unit of enzyme in 20 μl of 50 mM sodium acetate buffer, pH 5.5, containing 1 mM $CaCl_2$ and 10 mM sodium phosphate, pH 6.0, for 36 hr at 37°C. Products were analyzed by either electrophoresis at pH 8.0 or ion-exchange chromatography on AG 1-X2. For treatment with α-*N*-acetylglucosaminidase from human urine (14), lyophilized oligosaccharides were incubated with 0.008 unit of enzyme (1.4 units/mg of protein) in 10 μl of 50 mM sodium citrate buffer, pH 4.5, containing 5 mM Na_2HPO_4, for up to 32 hr. Treatment with 0.16 unit of β-hexosaminidase from jack bean (Sigma, 35 units/mg of protein) was performed as described for treatment with α-*N*-acetylglucosaminidase. The products were analyzed by ion-exchange chromatography on AG 1-X2. All enzyme incubations were done at 37°C. One unit of enzyme is defined as that amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate per min.

Mild acid hydrolysis of oligosaccharides was carried out in 0.5 ml of 1 or 3 mM HCl for 30 min at 100°C. After lyophilization, products were separated by high-voltage paper electrophoresis, paper chromatography, or ion-exchange chromatography on AG 1-X2 or else digested with one of the hydrolases.

Analytical Procedures. For ion-exchange chromatography, all steps were carried out at 0–4°C. Oligosaccharides were applied in 0.2 ml of water or of 20 mM Tris·HCl, pH 7.4, on a column of AG 1-X2, Cl^- form, 0.5 × 2 cm. The column was eluted stepwise with 3 ml of water and either 3 ml of 0.3 M HCl (for preparative purposes) or 3 ml of 1 M HCl (for analytical purposes). The eluates were assayed for radioactivity and lyophilized.

Glucosamine was identified by ion-exchange chromatography on a column of AG 50W-X8, H^+ form (Bio-Rad), 0.4 × 43 cm, equilibrated and eluted with 0.3 M HCl (15).

Descending chromatography was performed on Whatman 3 MM paper for 14–17 hr in solvent A (upper phase of 1-butanol/acetic acid/H_2O, 4:1:5, vol/vol) and solvent B (1-butanol/pyridine/H_2O, 6:4:3, vol/vol).

To determine the size of dephosphorylated oligosaccharides, chromatography was performed on thin-layer silica plates (catalog no. 5626, Merck) in ethanol/1-butanol/acetic acid/pyridine/H_2O, 100:10:3:10:30 (vol/vol) as solvent. High-mannose oligosaccharides isolated from mannosidosis urine were used as standards.

Electrophoresis on Whatman 3 MM paper was performed in (*i*) 30 mM NH_4HCO_3, pH 8.0, for 60–90 min at 50 V/cm, (*ii*) 1.9 M HCO_2H, pH 1.7, for 60 min at 50 V/cm, or (*iii*) 0.08 M pyridine/acetic acid, pH 5.3, for 60 min at 70 V/cm. Sugar standards were located with alkaline $AgNO_3$ (16), radioactivity was quantitated on 1 × 2.5 cm strips, soaked for about 15 min in 1 ml of 0.15 M NaCl and then mixed with 4 ml of scintillation fluid (Instagel, Packard Instruments, Frankfurt, Federal Republic of Germany).

Gel chromatography of oligosaccharides was performed at 4°C on columns of Bio-Gel P-4, 200–400 mesh (1 × 200 cm), in 50 mM acetic acid. The column was calibrated with the following standards: $(\alpha Man)_x$-βMan-*N*-acetyl[1-^{3}H]-glucosaminitol (x = 0–3), bovine serum albumin, *N*-acetylglucosamine, mannose, and ^3H_2O.

Methylation of the oligosaccharides was carried out as described by Hakomori (17). The methylated oligosaccharides were hydrolyzed by 4 M trifluoroacetic acid for 4 hr at 105°C. The hydrolysate was analyzed by thin-layer chromatography (catalog no. 5626, Merck) in solvent E of Li *et al.* (18). A mixture of 2,4-di-, 3,4,6-tri-, and 2,3,4,6-tetramethyl mannose was used as standard and located with 0.2% orcinol in 20% H_2SO_4 (19).

RESULTS

Phosphorylated Oligosaccharides Resistant to Alkaline Phosphatase. The labeled enzymes were isolated from the secretions of cells that had been incubated in the presence of 10 mM NH_4Cl. NH_4Cl increases the fraction of lysosomal enzymes secreted without interfering with the incorporation of ^{32}P into lysosomal enzymes (9).

For preparation of phosphorylated oligosaccharides the immunoprecipitates of β-hexosaminidase and cathepsin D labeled with [2-^{3}H]mannose were digested with endo-β-*N*-acetylglucosaminidase H. The yield of oligosaccharide radioactivity from either enzyme corresponded to half of label present in the immunoprecipitate, and to 0.1% of the [2-^{3}H]mannose added. Of the oligosaccharides released, 48–55% were acidic by the criteria of electrophoresis at pH 1.7 and 8.0 and ion-exchange chromatography. Only a small fraction thereof (6–10%) became neutral upon treatment with neuraminidase, and this probably belonged to the hybrid type. The anionic nonsialylated oligosaccharides were presumed to be phosphorylated. Surprisingly, less than 20% thereof were susceptible to alkaline phosphatase, unless first hydrolyzed with 1 mM HCl. The treatment with the diluted acid increased the electrophoretic mobility of the oligosaccharides at pH 8.0, but not at pH 1.7.

In order to characterize the phosphorylated but phosphatase-resistant oligosaccharides, we purified them by electrophoresis at pH 8.0 followed by chromatography on Bio-Gel P-4 (Fig. 1). None of the fractions recovered was susceptible to phosphatase. Fraction I-1 contained the oligosaccharides susceptible to neuraminidase (60% by radioactivity), which were not studied further. The remaining fractions were rendered phosphatase sensitive by treatment with 1 mM acid. Mild acid hydrolysis also increased the electrophoretic mobility of these fractions (the conversion was not quantitative in all cases) at pH 8.0 but not at pH 1.7, as illustrated for fraction II-2 in Fig. 2. The effect on electrophoretic mobility indicated that mild acid treatment hydrolyzed a phosphodiester linkage. But because no neutral sugar (mannose or L-fucose) had been released, the diester linkage was postulated to be either cyclic or to some unidentified residues.

The presence of a cyclic mannose 4,6-phosphate was excluded by the finding of only glycerol [^{32}P]phosphate after periodate oxidation and $NaBH_4$ reduction of ^{32}P-labeled oligosaccharides (Fig. 3). This result is incompatible with a 4,6 or 3,6 cyclic ester (a cyclic mannose 2,6-phosphate appeared unlikely from steric considerations) and led us to search for some other residue linked to the phosphate.

Identification of the Group Linked to Mannose via a Phosphodiester Linkage. A mixture of oligosaccharides was prepared from either β-hexosaminidase or cathepsin D isolated from the secretions of cells that had been labeled in the presence of [6-^{3}H]glucose. The efficiency of labeling was poor (about 1/30th of that obtained with [2-^{3}H]mannose) and precluded extensive purification. Mild acid hydrolysis of the mixed oligosaccharides liberated 4–5% of the radioactivity as material migrating in solvent B in the position of *N*-acetylglucosamine, xylose, and fucose.

Identification of the group liberated by mild acid hydrolysis was made possible by use of oligosaccharides obtained from enzymes labeled with [6-^{3}H]glucosamine. Labeling with this sugar was a third as efficient as with [2-^{3}H]mannose. More than 85% of radioactivity in the oligosaccharides was present in

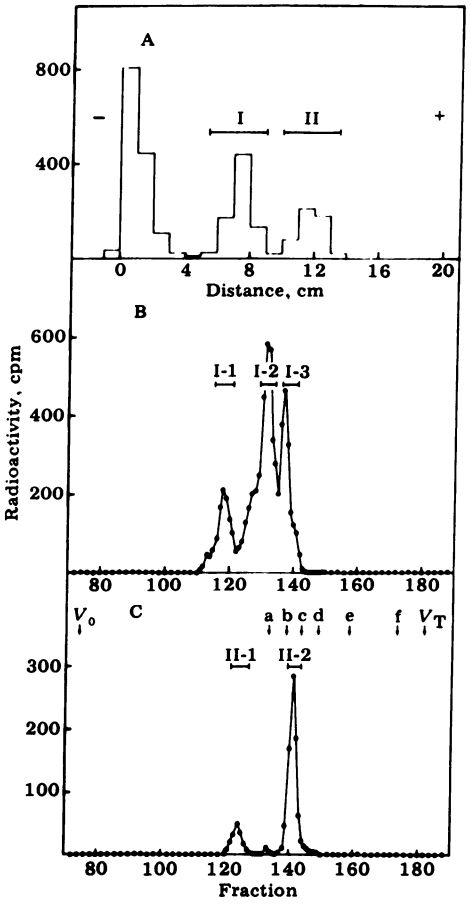


FIG. 1. Separation of oligosaccharides from β-hexosaminidase. The [2-^{3}H]mannose-labeled oligosaccharides released by endo-β-*N*-acetylglucosaminidase H were digested with alkaline phosphatase and subjected to electrophoresis at pH 8.0 (*A*) for 70 min. The acidic oligosaccharides were pooled as indicated and pool I (*B*) and pool II (*C*) were separated on a Bio-Gel P-4 column. The column was calibrated with bovine serum albumin (V_0), ^3H_2O (V_T), and the following sugars: a–d were oligosaccharides of the composition (αMan)$_x$βMan-*N*-acetyl[1-^{3}H]glucosaminitol with *x* ranging from 3 to 0, *N*-acetylglucosamine (e), and mannose (f). The oligosaccharide fractions I-1, I-2, I-3, II-1, and II-2 were pooled as indicated for further characterization.

glucosamine, as shown by ion-exchange chromatography after strong acid hydrolysis. All of the radioactivity liberated by mild acid hydrolysis behaved as *N*-acetylglucosamine upon paper chromatography in solvent B and was deacetylated to glucosamine by strong acid hydrolysis as illustrated for fraction II-2 in Fig. 4.

The fractions corresponding to I-1 through I-3 as well as II-1 and II-2 prepared from either β-hexosaminidase or cathepsin D oligosaccharides labeled with [6-^{3}H]glucosamine were analyzed for the ratio of acid-labile to acid-resistant *N*-acetylglucosamine (the latter is assumed to be located at the reducing terminal of the oligosaccharides). The hydrolysates were lyophilized and subjected to paper electrophoresis at pH 8.0, to paper chromatography in solvent B, and to ion-exchange chromatography on AG 1-X2. The ratio of free *N*-acetylglucosamine (derived from the phosphodiester group) to oligosaccharide-bound N-acetylglucosamine (from the reducing end) varied from 0.2 to 3.0 (Table 1).

To distinguish between the two possible anomeric configurations of the *N*-acetylglucosamine 1-phosphate residues, the oligosaccharides were treated with either α-*N*-acetylglucosaminidase or β-hexosaminidase. Only α-*N*-acetylglucosaminidase liberated *N*-acetylglucosamine (up to 90% of the acid-labile *N*-acetylglucosamine residues in fraction I-2).

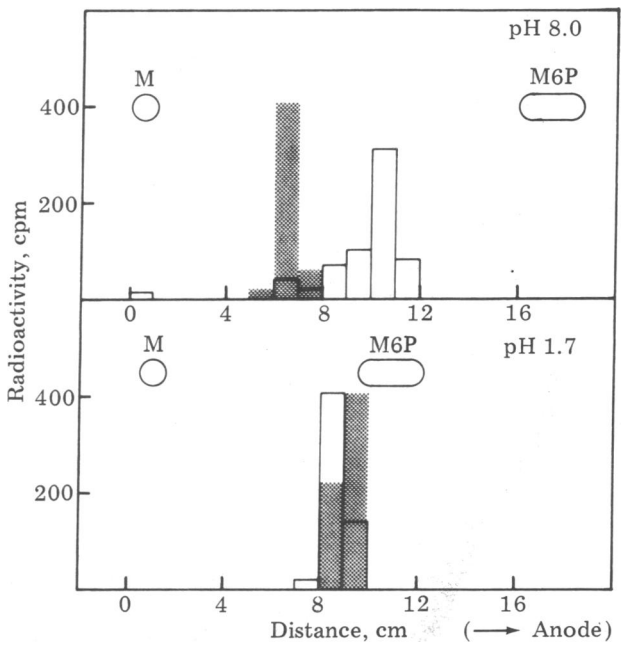


FIG. 2. Effect of mild acid hydrolysis on electrophoretic mobility of oligosaccharide fraction II-2. High-voltage electrophoresis was performed at pH 8.0 or at pH 1.7 for the oligosaccharide fraction II-2, either untreated (shaded bars) or hydrolyzed with 1 mM HCl (open bars). Mannose (M) and mannose 6-phosphate (M6P) served as standards.

Structural Analysis of Oligosaccharides Resistant to Phosphatase. The presence of phosphate groups leads to a re-

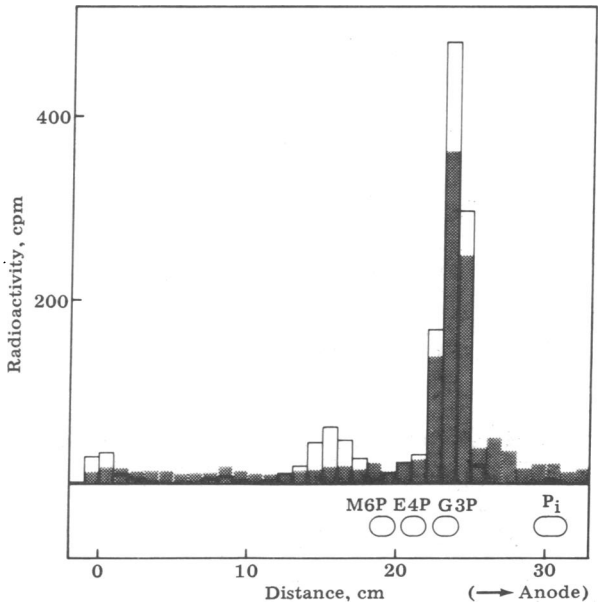


FIG. 3. Paper electrophoresis of ^{32}P-labeled oligosaccharides from β-hexosaminidase after periodate oxidation. Phosphatase-resistant ^{32}P-labeled oligosaccharides were treated with 7.5 mM sodium periodate for 10 hr in the presence of 60 nmol mannose 6-phosphate as carrier. Residual periodate was destroyed with ethylene glycol. The sample was reduced with 10 mM $NaBH_4$ at pH 8.5 and hydrolyzed with 0.2 M HCl for 90 min at 80°C. Electrophoretic separation was performed at pH 5.3 with [^{3}H]glycerol 3-phosphate as internal standard, which is indicated by the shaded bars. Open bars indicate ^{32}P. Mannose 6-phosphate (M6P), erythritol 4-phosphate (E4P), glycerol 3-phosphate (G3P), and $^{32}P_i$ served as standards.

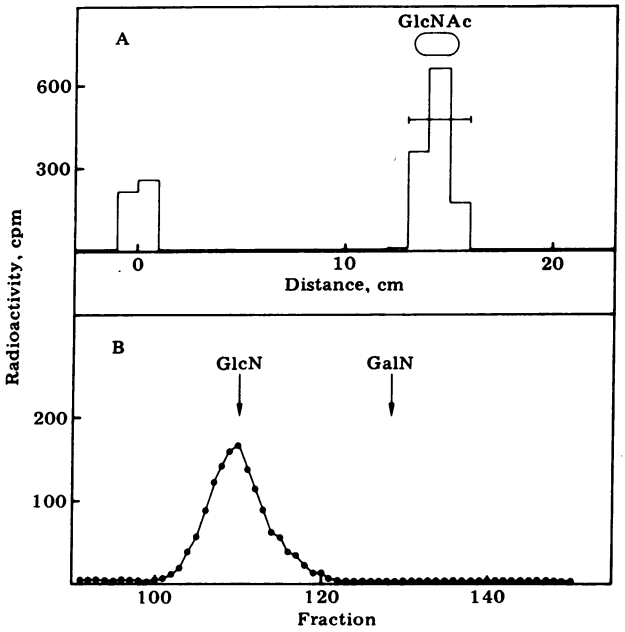


FIG. 4. Liberation of *N*-acetylglucosamine from fraction II-2 by mild acid hydrolysis. Fraction II-2 isolated from [^{3}H]glucosamine-labeled oligosaccharides of β-hexosaminidase was treated with 3 mM HCl for 30 min at 100°C, lyophilized, and subjected to paper chromatography in solvent A. The material migrating as the *N*-acetylglucosamine (GlcNAc) standard was eluted, hydrolyzed with 6 M HCl for 4 hr at 110°C, and applied to a AG 50W-X8 column in 0.3 M HCl. Glucosamine and galactosamine were added as internal standards. Their elution positions are marked by arrows (GlcN, GalN).

tardation of oligosaccharides on Bio-Gel P-4 under the acidic conditions used by us. Therefore the oligosaccharide fractions were rechromatographed on Bio-Gel P-4 after hydrolysis with 3 mM HCl and treatment with alkaline phosphatase. The apparent molecular weights of the dephosphorylated oligosaccharides varied between 1150 and 2800 (Table 1). Some fractions contained minor amounts of oligosaccharides with apparent molecular weights lower than the major species. The dephosphorylated forms of fractions I-2 and II-2, which accounted for more than half of the acidic oligosaccharides resistant to alkaline phosphatase (see Table 1), had almost identical molecular weights. In thin-layer chromatography their dephosphorylated forms migrated as did a $(Man)_7GlcNAc$ standard, whereas fraction I-3 migrated as did a $(Man)_6GlcNAc$ standard.

Table 1. Characteristics of acidic oligosaccharides resistant to alkaline phosphatase

Source	Pool	Distribution,* % Exp. A	Distribution,* % Exp. B	Apparent molecular weight† Before dephosphorylation	Apparent molecular weight† After dephosphorylation	Phosphodiester GlcNAc/reducing GlcNAc
β-Hexosaminidase	I-1	11	11	2180	2700‡	0.2‡
	I-2	23	23	1020	1570	1.3
	I-3	15	18	740	1150 (440)	1.4
	II-1	4	9	1540	2650 (1290)	0.4
	II-2	27	32	560	1520 (1090)	2.9
Cathepsin D	I-1	16		2140	2800	0.5‡
	I-2	29		970		1.7
	II-1	9		1540		0.4
	II-2	17		560	1480	3.0
	III-1	16		430		

* Distribution of radioactivity in the pools is given as percent of radioactivity in oligosaccharides resistant to phosphatase applied to the column.

† Molecular weights were estimated on a Bio-Gel P-4 column in 50 mM acetic acid calibrated with oligosaccharide standards exposing *N*-acetyl[1-^{3}H]glucosaminitol at the reducing terminal. Under these conditions phosphorylated oligosaccharides are retarded. Values in parentheses refer to minor components.

‡ In pool I-1 60% of the oligosaccharides were sialylated. The molecular weight and the *N*-acetylglucosamine ratio refer to the phosphorylated oligosaccharides in pool I-1.

The relative amounts of mannose residues susceptible to α-mannosidase were less than 1% in fractions II-1 and II-2 and 44% and 34% in fractions I-2 and I-3, respectively. Digestion of dephosphorylated oligosaccharides with an α-mannosidase free of β-mannosidase activity allowed an estimation of the ratio of α- to β-linked mannose residues. These ratios were 6.1:1 and 6.2:1 in fractions I-2 and II-2, respectively, which agree with the molecular weights determined by thin-layer chromatography. Methylation analysis of fractions I-2 and II-2 yielded 2,4-di-, 3,4,6-tri-, and 2,3,4,6-tetramethylmannose in a ratio of 1.8:2:3 and 1.6:2:2.9, respectively (Fig. 5).

Partial mild acid hydrolysis (4.5 instead of 30 min) and high-voltage electrophoresis at pH 8 of the products were performed to study the number of phosphodiester groups. After treatment of fraction I-2 the radioactivity at the position of the untreated material was diminished by about 60% and the corresponding amount of radioactivity was found at the position of the single product, which is formed upon hydrolysis under standard conditions and migrates about the same distance as untreated fraction II-2. In partial hydrolysates of fraction II-2 two products were present besides the starting material. One migrated like the final product obtained under the standard conditions of hydrolysis (cf. Fig. 2) and lost all its ionizable groups when treated with phosphatase. The other was found slightly ahead of the middistance between the unhydrolyzed and the hydrolyzed fraction II-2. Upon incubation with phosphatase it was converted to a material migrating like fraction I-2.

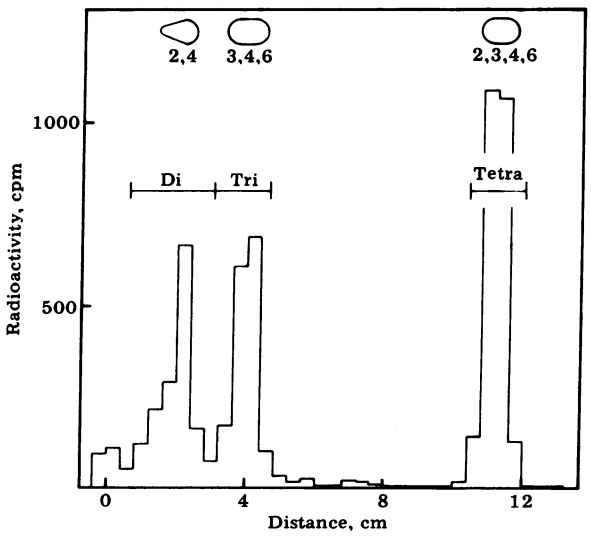


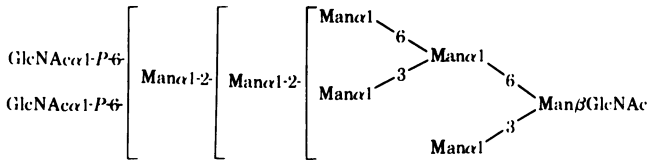
FIG. 5. Thin-layer chromatography of partially methylated [^{3}H]mannose derivatives of oligosaccharide II-2. The relative amounts of di-, tri-, and tetramethylmannose were calculated from the radioactivity in the areas indicated by horizontal bars. The standards were 2,4-di-, 3,4,6-tri-, and 2,3,4,6-tetramethyl mannose.

DISCUSSION

Structural studies of lysosomal enzymes are hampered by heterogeneities that may arise during the exposure of the hydrolases to each other within the lysosomes and by relatively low content of the enzymes in the cells or their secretions. To circumvent these limitations the present study focused on the oligosaccharide components in lysosomal enzymes that are secreted by fibroblasts cultured in presence of NH_4Cl. Although this compound did not affect the uptake properties of β-hexosaminidase (data not shown) as secreted under the conditions used, the physiological significance of the relative content and the structural features of oligosaccharides as observed in this study must be considered with caution.

This study focused on phosphorylated oligosaccharides cleavable with endo-β-*N*-acetylglucosaminidase H and resistant to phosphatase. These oligosaccharides correspond to about one fourth of mannose label that can be found in immunoprecipitates of either β-hexosaminidase or cathepsin D. In both enzymes the oligosaccharides concerned can be separated by charge and size into five major components corresponding to 4–32% of mannose label in the fraction of oligosaccharides resistant to phosphatase. Four of these components are found in both enzymes studied.

The analyses concerning the structure of the oligosaccharide II-2 suggest the following formula:



Inner core

The formula of the inner core has been constructed in analogy to the structure of high-mannose oligosaccharides from human sources determined by others (20), because our methylation analysis data are ambiguous concerning the position of the second branching point. Oligosaccharide II-2 corresponds then to one or a mixture of several isomers depending on the positions of the α1,2-linked mannose and of *N*-acetylglucosamine 1-phosphate residues. At present there is some ambiguity as to the number of the phosphodiester groups. Resistance to α-mannosidase and number of *N*-acetylglucosamine residues released during the mild acid hydrolysis indirectly indicated three, whereas the data on the effect of partial hydrolysis on charge and on sensitivity to phosphatase were much in favor of only two phosphodiester groups in oligosaccharide II-2. Oligosaccharides I-2 and I-3 appear to have a structure very similar to that of oligosaccharide II-2. They contain seven and six mannose residues, respectively, and most probably only one phosphodiester group. Like oligosaccharides from β-hexosaminidase and cathepsin D synthesized by human skin fibroblasts, those isolated from mouse lymphoma cells (12) may contain up to three α-*N*-acetylglucosamine 1-phosphate groups on three-branched high-mannose oligosaccharides (A. Varki and S. Kornfeld, personal communication).

The finding of *N*-acetylglucosamine linked to mannose 6-phosphate residues via phosphodiester bonds raises several questions concerning the synthesis, degradation, and functions of these oligosaccharides. The synthesis of phosphodiester linkages in yeast mannan is accomplished by transfer of mannose 1-phosphate from GDP-mannose to the C6 hydroxyl of a mannose within the nascent mannan (21, 22). In an analogy, in the biosynthesis of lysosomal enzymes a derivative of *N*-acetylglucosamine such as UDP-*N*-acetylglucosamine or *N*-acetylglucosamine(1)diphosphodolichol may serve as donor of both the phosphate and the sugar esterified to it via its anomeric oxygen. If the suggested precursor–product relationship between the diester and the monoester is true, a nonlysosomal hydrolytic reaction can be anticipated, in which mannose 6-phosphate residues are uncovered. A microsomal enzyme uncovering the phosphate in the diester-containing oligosaccharides has been found in our laboratory (unpublished), as well as in the laboratory of Kornfeld (23).

Because the recognition markers of lysosomal enzymes are sensitive to alkaline phosphatase (2–5), it is unlikely that the observed mannose 6-phosphate residues substituted with *N*-acetylglucosamine mediate binding of lysosomal enzymes to cell surface receptors. Competition studies on binding of β-galactosidase receptors purified from bovine liver indicate rather directly that the recognition system for lysosomal enzymes involves free mannose 6-phosphate residues rather than those involved in a diester linkage (G. Sahagian and G. W. Jourdian, personal communication). The synthesis of the recognition marker in cryptic form may serve to separate the biosynthesis and recognition events. The hydrolysis may determine the site of recognition as well as the relative rate of secretion and retention of lysosomal enzymes.

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