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Structural Studies of Phosphorylated High Mannose-type Oligosaccharides*

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Phosphomannosyl residues on acid hydrolases serve as recognition markers which target these enzymes to lysosomes. We have found that the oligosaccharide units of newly synthesized β-glucuronidase contain phosphate residues in diester linkage between mannose and α-linked *N*-acetylglucosamine residues (Tabas, I., and Kornfeld, S. (1980) *J. Biol. Chem.* 255, 6633-6639). To obtain larger amounts of these molecules for structural studies, total cellular glycopeptides were isolated from [2-^{3}H]mannose-labeled mouse lymphoma cells, and the phosphorylated oligosaccharides were released by endo-β-*N*-acetylglucosaminidase C_{II} and H and isolated by gel filtration and ion exchange chromatography. The fractions were characterized by α-mannosidase digestion before and after removal of the phosphate residues and by acetolysis. We also determined whether the phosphate was present as a phosphomonoester or as a diester.

The phosphorylated oligosaccharides consisted of a family of related molecules, all of which contained a high mannose-type oligosaccharide core. The major class consisted of isomers containing a single phosphate in diester linkage to 1 of 3 mannose residues of the underlying oligosaccharide. The second class contained isomers with two phosphodiester groups located at five different positions of the oligosaccharide. In both of these classes, the phosphodiester group could be converted to a phosphomonoester by pig liver α-*N*-acetylglucosaminidase, indicating that the cover is α-linked *N*-acetylglucosamine. The third class was similar to the second except that the 2 phosphate residues were present as monoester groups. The last class contained molecules with a single phosphomonoester group. These molecules differed from those with single phosphodiester groups in that the core oligosaccharides were smaller. This is consistent with their being more mature species which have undergone partial processing.

These data demonstrate that phosphorylation can occur at 5 separate mannose residues on the high mannose-type oligosaccharides. Individual molecules can have 1, 2, and perhaps even 3 phosphate residues. The majority of the newly synthesized phosphorylated oligosaccharides contain phosphate groups in diester linkage.

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Several lines of evidence indicate that phosphomannosyl residues present on acid hydrolases are essential components of the recognition marker which is necessary for the segregation of these enzymes into lysosomes (1-11). While the biologic importance of these molecules is well established, relatively little is known about the structural details of these phosphorylated oligosaccharides. Several investigators utilizing mature forms of acid hydrolases have shown that the phosphorylated mannose residues are located on high mannose-type oligosaccharide units and that the phosphate is linked to position 6 of the mannose residues (5-8, 11). However the number of phosphorylated mannose residues per oligosaccharide and the precise location of these residues on the oligosaccharide has not been established.

During the course of studies of the intracellular biosynthetic intermediates of the acid hydrolase β-glucuronidase, we isolated a family of phosphorylated oligosaccharides which appeared to contain either 1, 2, or 3 phosphate residues (12). In addition, most of these phosphate residues were present in an unusual phosphodiester linkage between mannose residues of the underlying high mannose-type oligosaccharide and an outer covering residue which was shown to be α-linked *N*-acetylglucosamine in at least some of the cases. Only a small percentage of these newly synthesized phosphorylated oligosaccharides contained exposed phosphate residues. Based on these findings, we proposed that the mechanism by which the mannose residues of lysosomal enzymes are phosphorylated may be the transfer of α-*N*-acetylglucosamine 1-phosphate residues to mannose residues of high mannose-type oligosaccharides. Subsequently, the *N*-acetylglucosamine residues would be removed to expose the phosphate residues and generate the recognition marker necessary for targeting the enzyme to lysosomes.

To elucidate this biosynthetic pathway further, careful structural characterization of this new family of oligosaccharides was necessary. However, the amount of [2-^{3}H]mannose incorporated into β-glucuronidase was not sufficient to perform a detailed analysis of the structure of each intermediate. We reasoned, therefore, that the [2-^{3}H]mannose-labeled cellular material remaining after immunoprecipitation of the β-glucuronidase should contain a much larger amount of similar phosphorylated oligosaccharides, in association with the other lysosomal enzymes and perhaps other glycoproteins that might share this pathway. To isolate this class of molecules, we looked for oligosaccharides which were released from glycopeptides by either endo-β-*N*-acetylglucosaminidase C_{II} or H and which contained a net negative charge. In this paper we report the isolation and characterization of a family of phosphorylated high mannose-type oligosaccharides that are very similar to those found in the biosynthetic intermediates of β-glucuronidase.

EXPERIMENTAL PROCEDURES

Materials—D-[2-^{3}H]Mannose (10 Ci/mmol) and the formula 963 scintillation mixture were from New England Nuclear. Bio-Gel P-6 (200 to 400 mesh) and AG 50W (1-X2) were from Bio-Rad Laboratories. Amberlite MB-3 ion exchange resin was from Mallinckrodt. QAE-Sephadex (Q-25-120) and Sephadex G-25-80 were from Sigma Chemical Co. *p*-Nitrophenyl-α-*N*-acetylglucosaminide was from Calbiochem. The $Man_9GlcNAc$,[1] $Man_8GlcNAc$, $Man_7GlcNAc$, $Man_6GlcNAc$, and $Man_5GlcNAc$ standards were isolated and characterized as previously described (13). Manα1 → 2Mannitol and Manα1 → 3Mannitol were prepared as previously described (14). All other chemicals were of reagent grade.

Enzymes—Pronase was from Calbiochem. *Streptomyces griseus* endo-β-*N*-acetylglucosaminidase H was from Miles. *Clostridium perfringens* endo-β-*N*-acetylglucosaminidase C_{II} was prepared by the method of Ito *et al.* (15). α-Mannosidase from jack bean meal was prepared as described by Li and Li (16). Homogeneous *Escherichia coli* alkaline phosphatase was a gift of Dr. M. Schlesinger, Washington University. Pig liver α-*N*-acetylglucosaminidase was prepared as previously described (17). The enzyme preparation had an activity of 24 milliunits/ml using *p*-nitrophenyl-α-*N*-acetylglucosaminide as substrate.

Radiolabeling of Tissue Culture Cells—The growth of the mouse lymphoma cell line BW5147.3 in suspension culture, the labeling of the cells for 3 h with [2-^{3}H]mannose, and the purification and immunoprecipitation of the β-glucuronidase have been previously described (12). Following this, the two pellets from the heat steps were combined with the supernatant from the immunoprecipitation procedure and these combined fractions, containing all of the cell material with the exception of the β-glucuronidase, served as the starting material for the present study. The material was derived from 5×10^8 cells.

Preparation of Glycopeptides—The above material was brought to 1.0 ml with 0.1 M Tris buffer, pH 8.0, containing 20 mM $CaCl_2$ and 0.2 M glucose 6-phosphate. The glucose 6-phosphate was used to inhibit a phosphatase activity present in the pronase. Pronase (10 mg) was added and the suspension was incubated at 60°C for 15 h. Additional pronase (5 mg) was added at 5 h. The digestion was terminated by boiling for 5 min and the soluble glycopeptide material was applied to a Bio-Gel P-6 (200 to 400 mesh) column (1.5 × 90 cm) using 0.1 M NH_4HCO_3, pH 8.0, as eluting buffer. One-milliliter fractions were collected and monitored for radioactivity.

Endo-β-N-acetylglucosaminidase Digestion of Glycopeptides—The glycopeptides were incubated with 1 milliunit of endo-β-*N*-acetylglucosaminidase C_{II} in 0.05 ml of 0.05 M citrate/phosphate buffer, pH 6.5, or with 1 milliunit of *S. griseus* endo-β-*N*-acetylglucosaminidase H in 0.05 ml of 0.05 M citrate/phosphate buffer, pH 5.5. Incubations were for 18 h at 37°C under a toluene atmosphere. The enzyme-treated glycopeptides were then reapplied to the Bio-Gel P-6 column.

QAE-Sephadex Fractionation of Oligosaccharides—The samples in 2 mM Tris base were applied to columns (0.5 × 2 cm) of QAE-Sephadex previously equilibrated in 2 mM Tris base. The columns were washed with 2 mM Tris base and eluted stepwise with increasing concentrations of NaCl in 2 mM Tris base. A final elution was made with 1.0 M NaCl in 0.1 N HCl. Two-milliliter fractions were collected and monitored for radioactivity.

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.

Enzyme Digestions of Oligosaccharides—All incubations were at 37°C under a toluene atmosphere. The sample was digested in 0.02 ml of pig liver α-*N*-acetylglucosaminidase (24 milliunits/ml) in 0.05 M sodium citrate, pH 4.6, for 48 h. The sample was digested in 0.02 ml of *E. coli* alkaline phosphatase (6 units/ml) in 0.05 M Tris buffer, pH 8.0, for 2 h. The sample was digested in 0.02 ml of jack bean α-mannosidase (50 units/ml) in 0.05 M citrate buffer, pH 4.5 for 1 h unless stated otherwise. Release of free mannose from the [^{3}H]mannose-labeled sample was assayed by subjecting the digest to paper chromatography in Solvent A for 12 h and monitoring the radioactivity co-migrating with authentic mannose.

Paper Chromatography—Descending paper chromatography was performed on Whatman No. 1 paper in Solvent A, ethyl acetate/pyridine/acetic acid/water (5:5:1:3) or Solvent B, 1-butanol/ethanol/water (10:1:2). Strips (1 cm) were cut and soaked in 0.4 ml of water. Four and one-half milliliters of the formula 963 scintillation mixture was added to each vial, and the ^{3}H radioactivity determined.

Acetolysis—The oligosaccharides were reduced with $NaBH_4$ prior to acetolysis. The acetolysis of the phosphorylated oligosaccharide alcohols was performed as previously described (18) with the following modification (19): The acetolysis reaction was terminated by the addition of aqueous 1 M barium acetate sufficient to neutralize the sulfuric acid. The solvents were then evaporated and the acetylated oligosaccharides were extracted three times into 70% methanol. The methanol was removed by evaporation and the products were deacetylated in 0.2 N NaOH for 45 min at room temperature. The reaction was diluted to bring the NaOH concentration to below 5 mM and the sample was applied to a QAE-Sephadex column (0.5 × 2.0 cm) to separate the neutral fragments which passed through the column from the charged fragments which bound and were eluted with 8 ml of 100 mM NaCl in 2 mM Tris base. The eluate was applied to a column (0.5 × 6 cm) of AG 50W (1-X2). The column run through containing the phosphorylated fragments was frozen and lyophilized to remove the HCl.

High Voltage Paper Electrophoresis—Manα1 → 2mannitol and Manα1 → 3mannitol were separated by high voltage paper electrophoresis according to the method of Verma *et al.* (20).

Reduction of Oligosaccharides—The oligosaccharides were reduced with $NaBH_4$ as previously described (18).

RESULTS

Isolation of High Mannose-type Oligosaccharides from [2-^{3}H]Mannose-labeled Mouse Lymphoma Cells—Our previous studies with purified β-glucuronidase demonstrated that the phosphorylated oligosaccharides exhibited several properties which could be used to retrieve them from a mixture of total cellular glycopeptide material. They behaved as unusually large molecules when subjected to gel filtration on Bio-Gel P-6 at pH 8.0 (either in the form of a glycopeptide or an oligosaccharide), were cleaved from the peptide with either endo-β-*N*-acetylglucosaminidase C_{II} or H and bound to QAE-Sephadex due to their net negative charge. The fractionation scheme which we used in this study took advantage of these properties. The [2-^{3}H]mannose-labeled cellular material was treated with pronase to generate glycopeptides and then subjected to gel filtration on a Bio-Gel P-6 column. As shown in Fig. 1, a family of glycopeptides of different sizes were obtained and these were pooled as shown in the figure.

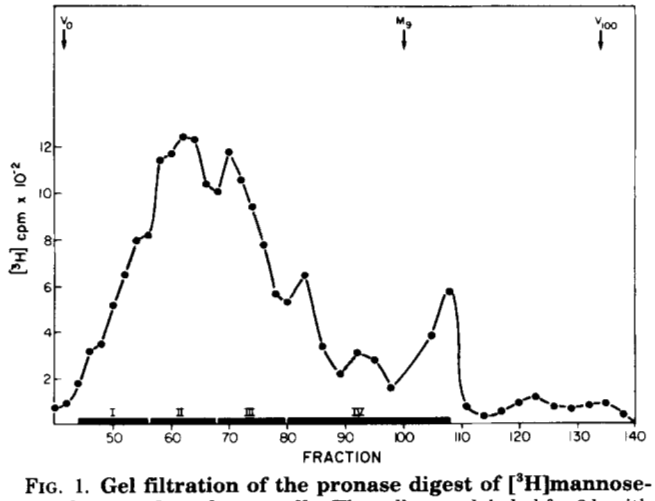


FIG. 1. **Gel filtration of the pronase digest of [^{3}H]mannose-labeled mouse lymphoma cells.** The cells were labeled for 3 h with [2-^{3}H]mannose, and the whole cell material was prepared and digested with pronase as described under "Experimental Procedures." The digest was applied to a Bio-Gel P-6 column and 1-ml fractions were collected. Aliquots (5 μl) were monitored for ^{3}H radioactivity. V_0 was determined with bovine serum albumin and V_{100} with galactose. M_9 indicates the elution position of the standard $Man_9GlcNAc$. The *Fractions I, II, III,* and *IV* were pooled as indicated.

[1] The abbreviations used are: GlcNAc, *N*-acetylglucosamine; GlcitolNAc, *N*-acetylglucosaminitol; Man, mannose.

The material in Pools I, II, and III were treated separately with endo-β-*N*-acetylglucosaminidase C_{II} which cleaves the *N,N'*-diacetylchitobiose unit of most high mannose-type oligosaccharides (15) and the samples were then resubjected to gel filtration on the Bio-Gel P-6 column. The profiles obtained with the Pool I and II material are shown in Fig. 2, *Panels A* and *C*. In each case some of the released material eluted in the position of typical high mannose-type oligosaccharides (Peaks IE and IIE). In addition, several other peaks of released material (IB, IC, ID, IIC, and IID) eluted significantly earlier, denoting either a greater size and/or net negative charge relative to the standard $Man_9GlcNAc$ oligosaccharide.

The radioactive material that eluted in the position of the original glycopeptides (IA and IIA + B) was pooled, digested with endo-β-*N*-acetylglucosaminidase H and subjected once again to gel filtration on the Bio-Gel P-6 column, as shown in Fig. 2, *Panels B* and *D*. In both cases, additional oligosaccharides were released and this material, labeled IAc, IAd, IIAc, and IIAd was pooled as noted in the figure. The fractions labeled IAb and IIAb were pooled individually and further characterized. Fractionation of the material in Peaks IAa and IIAa will be described later.

When Pool III was digested with endo-β-*N*-acetylglucosaminidase C_{II} and then analyzed by gel filtration, virtually all of the oligosaccharide material was released and eluted in the position of the standard $Man_9GlcNAc$ oligosaccharide. In addition, all of this oligosaccharide material behaved as neutral species when passed through a column of QAE-Sephadex and therefore was not characterized further.

The Released Oligosaccharides of Pools I and II are Similar—Pools I and II from the gel filtration of the original pronase digest were separated on the basis of their elution position on Bio-Gel P-6 (see Fig. 1). As illustrated in Fig. 2, both gave rise to similar fractions following endo-β-*N*-acetylglucosaminidase digestion. Further studies showed that the corresponding fractions from each of the two pools (*e.g.* IAc and IIAc, etc.) were virtually identical in all respects. Thus, the original difference in elution from the Bio-Gel P-6 column was most likely due to differences in the size and/or charge of the peptide portion of the glycopeptides. Therefore, to conserve space, only the data obtained with the Pool II material will be presented in the sections that follow.

Fractionation of the Pool II Oligosaccharides on QAE-Sephadex—To determine if the endo-β-*N*-acetylglucosaminidase-released oligosaccharides of Pool II contained molecules with a net negative charge, the various fractions were desalted on Sephadex G-25 columns, brought up in 2 mM Tris base, and fractionated on columns of QAE-Sephadex. Material which bound to the column was eluted with stepwise increments of NaCl in 2 mM Tris base. The results are shown in Fig. 3. Almost all of the radioactive material in Fractions IIC, IIAb, and IIAc bound to the QAE-Sephadex and could be eluted with NaCl. Fraction IIC gave rise to two peaks of radioactivity, eluting at 10 mM (IIC_{10}) and 50 mM (IIC_{50}) NaCl concentrations. Most of the radioactivity in Fractions IIAb and IIAc eluted in single peaks ($IIAb_{50}$ and $IIAc_{10}$, respectively). Each of these fractions, therefore, contained oligosaccharides with a net negative charge. In contrast, most of the radioactive material in Fractions IID, IIE, and IIAd passed through the QAE-Sephadex, indicating that these fractions (termed IID_0, IIE_0, and $IIAd_0$) did not contain negatively charged oligosaccharides. All of the fractions were pooled as indicated in the figure and desalted on Sephadex G-25 columns.

Most of the Negatively Charged Oligosaccharides Contain Phosphate in Phosphodiester Linkage—To determine whether the net negative charge of Fractions $IIAc_{10}$, IIC_{10}, IIC_{50}, and $IIAb_{50}$ was due to covalently bound phosphomonoester residues, aliquots of the fractions were treated with *E. coli* alkaline phospatase and reapplied to QAE-Sephadex columns. As shown in Fig. 4, the elution position of oligosaccharides $IIAc_{10}$, IIC_{10}, and $IIAb_{50}$ was unchanged, demonstrating that alkaline phosphatase did not remove the net negative charge from these samples. In contrast, 70% of the IIC_{50} fraction now passed through the column, indicating the presence of an exposed phosphate residue in the majority of oligosaccharides of this fraction.

Since our previous experiments had demonstrated that the newly synthesized oligosaccharides of β-glucuronidase contained phosphate residues in phosphodiester linkage (12), we reasoned that oligosaccharides $IIAc_{10}$, IIC_{10}, $IIAb_{50}$, and a portion of IIC_{50} probably contained similar linkages to account for their resistance to alkaline phosphatase. We therefore subjected aliquots of the negatively charged oligosaccharides to mild acid treatment (pH 2.1, 100°C, 30 min) to cleave the postulated phosphodiester bonds and then re-examined the behavior of the oligosaccharides on QAE-Sephadex as well as their susceptibility to alkaline phosphatase. As shown in Fig. 5, oligosaccharides $IIAc_{10}$, IIC_{10}, and $IIAb_{50}$ showed an increase in net negative charge as evidenced by the increased NaCl

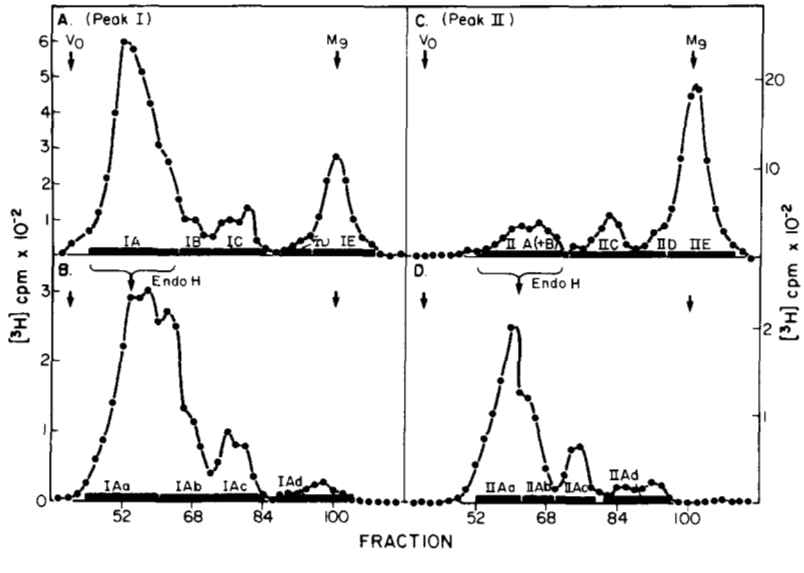


FIG. 2. **Gel filtration of endo-β-*N*-acetylglucosaminidase digests of [2-^{3}H]mannose-labeled glycopeptides.** *Peaks I* and *II* (see Fig. 1) were digested with endo-β-*N*-acetylglucosaminidase C_{II}, and applied to Bio-Gel P-6 columns. Fractions of 1 ml were collected and aliquots (10 μl) were monitored for radioactivity. *Peaks IA, IB, IC, ID,* and *IE* (*Panel A*) and *Peaks IIA(+B), IIC, IID,* and *IIE* (*Panel C*) were pooled as indicated. Fractions IA and IIA(+B), which eluted in the same position as the original glycopeptides, were then digested with endo-β-*N*-acetylglucosaminidase H and reapplied to the Bio-Gel P-6 columns. *Fractions IAa, IAb, IAc,* and *IAd* (*Panel B*) and *IIAa, IIAb, IIAc,* and *IIAd* (*Panel D*) were pooled as indicated. M_9 indicates the elution position of $Man_9GlcNAc$.

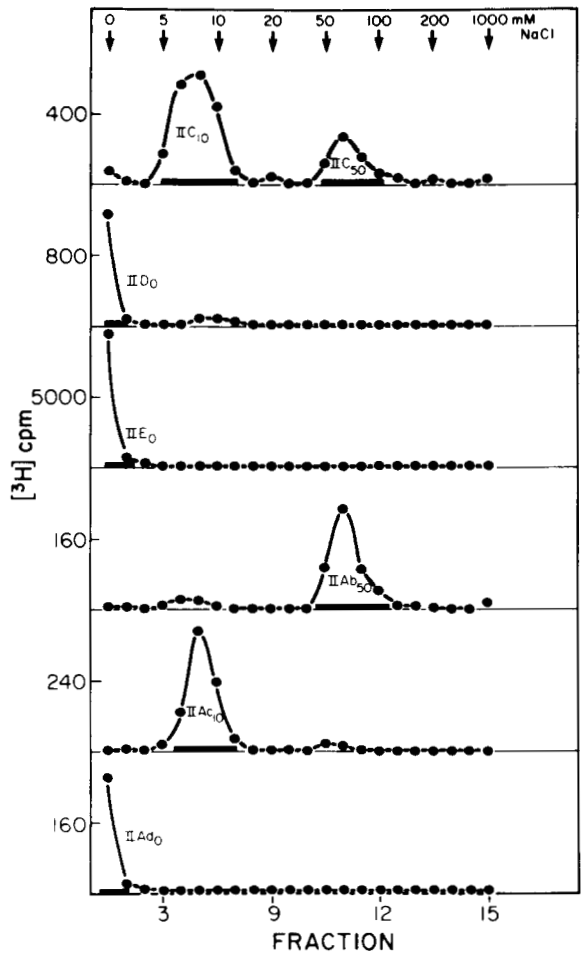


FIG. 3. **QAE-Sephadex fractionation of endo-β-*N*-acetylglucosaminidase C_{II} and H released oligosaccharides from Peak II.** Fractions IIC, IID, IIE, IIAb, IIAc, and IIAd (see Fig. 2) in 2 ml of 2 mM Tris base were applied to columns (2 × 0.5 cm) of QAE-Sephadex. The columns were washed with two more 2-ml fractions of 2 mM Tris base and then eluted stepwise with 5 to 1000 mM NaCl in 2 mM Tris base, as indicated by the *arrows*. Aliquots (10 μl) of the 2-ml fractions were monitored for radioactivity. Fractions IIC_{10}, IIC_{50}, IID_0, IIE_0, $IIAb_{50}$, $IIAc_{10}$, and $IIAd_0$ were pooled as indicated by the *bars*.

concentrations required to elute them from the QAE-Sephadex column. Approximately 70% of Fraction IIC_{50} showed no change with acid treatment, consistent with the per cent of this fraction which was susceptible to the original alkaline phosphatase treatment. However, a portion of the IIC_{50} fraction (25%) did show an increase in net negative charge ($IIC_{50 \rightarrow 200}$), accounting for the original material in the IIC_{50} fraction which was resistant to alkaline phosphatase.

All of the oligosaccharides that showed an increase in net negative charge ($IIAc_{10 \rightarrow 50}$, $IIC_{10 \rightarrow 50}$, $IIC_{50 \rightarrow 200}$, and $IIAb_{50 \rightarrow 200}$) became completely neutral when treated with alkaline phosphatase, as demonstrated by the loss of binding to QAE-Sephadex (data not shown). Fraction $IIC_{50 \rightarrow 50}$, which showed no change in net negative charge, remained susceptible to alkaline phosphatase just as it had been prior to the acid treatment.

These data demonstrate that all of the major negatively charged oligosaccharides contain covalently bound phosphate residues. However, only Fraction $IIC_{50 \rightarrow 50}$ contained phosphomonoester residues in the native state. The other molecules contain phosphate residues with an acid-labile "blocking" moiety, most likely in a phosphodiester linkage.

To define further the acid lability of the presumed phos-

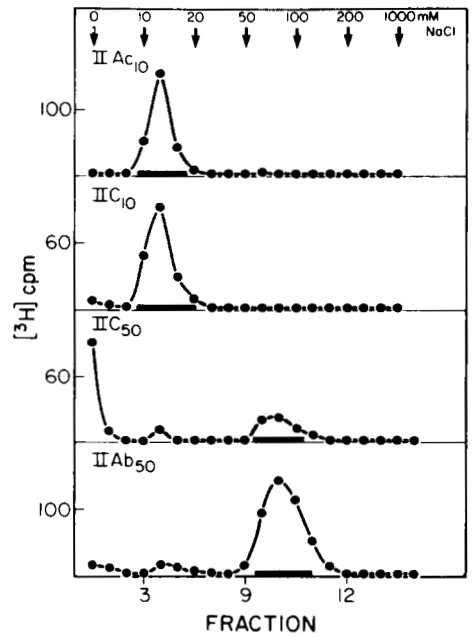


FIG. 4. **Alkaline phosphatase digestion of negatively charged oligosaccharides from Peak II.** Aliquots of $IIAc_{10}$, IIC_{10}, IIC_{50}, and $IIAb_{50}$ were treated with *E. coli* alkaline phosphatase. The reaction mixtures were diluted to 2 ml with 2 mM Tris base (final salt concentration 3.6 mM) and applied to columns (0.5 × 2 cm) of QAE-Sephadex. The columns were washed with two more 2-ml fractions of 2 mM Tris base, and then eluted stepwise with 10 to 1000 mM NaCl in 2 mM Tris base, as indicated by the *arrows*. Fractions of 2 ml were collected and 200-μl aliquots were monitored for radioactivity. The *bars* indicate the elution position of the untreated oligosaccharides.

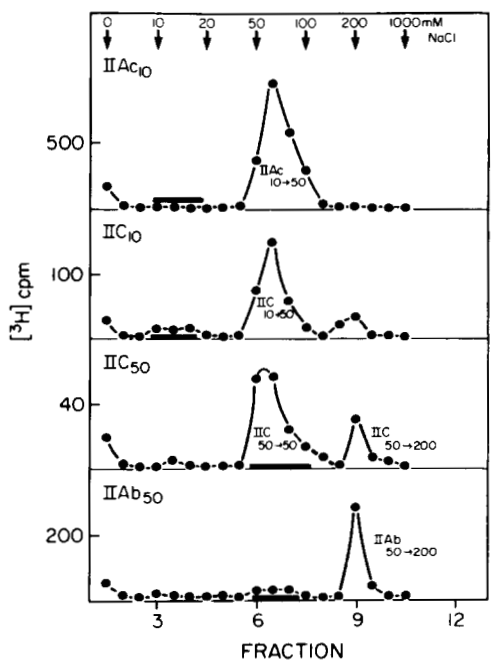


FIG. 5. **Effects of mild acid treatment on the negatively charged oligosaccharides from Peak II.** Aliquots of $IIAc_{10}$, IIC_{10}, IIC_{50}, and $IIAb_{50}$ were brought to pH 2.1 with HCl and incubated at 100°C for 30 min. The samples were then frozen, lyophilized to remove the HCl, brought up in 2 ml of 2 mM Tris base, and applied to columns (2 × 0.5 cm) of QAE-Sephadex. After washing with two more 2-ml fractions of 2 mM Tris base, the columns were eluted stepwise with 10 to 1000 mM NaCl in 2 mM Tris base, as indicated by the *arrows*. Fractions of 2 ml were collected and 200-μl aliquots were monitored for radioactivity. Peaks $IIAc_{10 \rightarrow 50}$, $IIC_{10 \rightarrow 50}$, $IIC_{50 \rightarrow 50}$, $IIC_{50 \rightarrow 200}$, and $IIAb_{50 \rightarrow 200}$ were pooled and desalted. The black bands indicate the elution position of the untreated oligosaccharides.

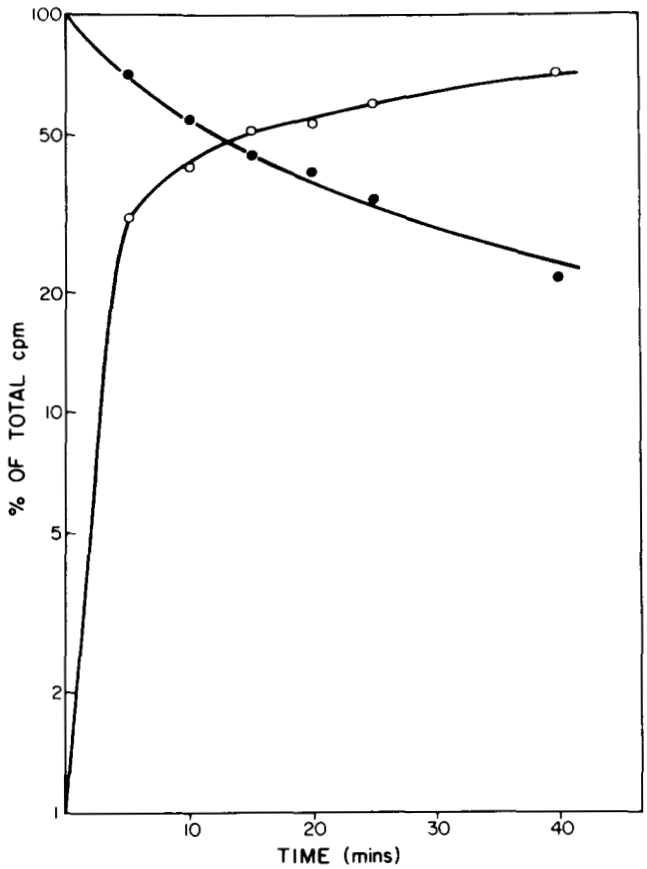


FIG. 6. **Rate of "uncovering" of the phosphate residues in oligosaccharide IIC_{10} by mild acid treatment.** An aliquot of Fraction IIC_{10} in 30 μl of H_2O was added to 570 μl of 0.01 N HCl equilibrated to 80°C on a heating block. The final pH of a similarly prepared blank was 2.15. Aliquots (100 μl) were removed at 5½, 10, 20, and 25 min, and immediately frozen on a dry ice-acetone bath. The samples were then lyophilized, brought up in 1 ml of 2 mM Tris base, and applied to columns (0.5 × 2 cm) of QAE-Sephadex. The columns were washed with five more 1-ml fractions of 2 mM Tris base, and then eluted with six 1-ml fractions of 10 mM NaCl in 2 mM Tris base and six 1-ml fractions of 50 mM NaCl in 2 mM Tris base. The total radioactivity eluted at 10 mM (●) and 50 mM (○) NaCl was determined and expressed as a percentage of the total counts per min for each time point.

phodiester bond, we studied the rate of increase in net negative charge of oligosaccharide Fraction IIC_{10} under various conditions of hydrolysis. As shown in Fig. 6, hydrolysis at pH 2.15 at 80°C caused a rapid conversion of IIC_{10} to $IIC_{10\rightarrow 50}$, as expected for a phosphodiester bond (21). However, the kinetics of this reaction indicate that the molecules in this fraction are cleaved with at least two half-lives. The explanation for this curve is not clear. Since Pool $IIC_{10\rightarrow 50}$ consists of at least three different isomers with phosphate residues in different positions on the underlying oligosaccharide backbone (see below), one possibility is that the differences in acid lability relate to the presence of these isomers.

The Phosphate Covering Moiety is Released by α-N-Acetylglucosaminidase—We next sought to determine the nature of the molecule which was present in the phosphodiester linkage and released by mild acid treatment. In our earlier study of β-glucuronidase, *N*-acetylglucosamine in α linkage was the only covering moiety which we could identify (12). We therefore tested the ability of pig liver α-*N*-acetylglucosaminidase to remove the covering group from the various oligosaccharide fractions. This enzyme is capable of releasing *N*-acetylglucosamine from UDP-GlcNAc and thus can cleave α-linked *N*-acetylglucosamine residues which are present in a phosphodiester linkage. The results of treating Fraction IIC_{10} with this enzyme are shown in Table I. The enzyme caused changes identical with those produced by mild acid treatment, *i.e.* an increase in binding to QAE-Sephadex and a conversion to alkaline phosphatase susceptibility. The effects of the enzyme were abolished by prior boiling or by the addition of 5 mM *N*-acetylglucosamine, an inhibitor of the enzyme. Similar results were obtained with the other fractions which contained covered phosphate residues ($IIAc_{10}$, $IIAb_{50}$, $IIAb_{10}$, and IAb_{50}). Molecules that contained only exposed phosphomonoester residues were unchanged by this treatment (IC_{50}, IAc_{50}). These data indicate that most, if not all, of the oligosaccharides which have covered phosphate residues contain α-*N*-acetylglucosamine in phosphodiester linkage with the underlying oligosaccharide.

TABLE I

Treatment of Fraction IIC_{10} with pig liver α-N-acetylglucosaminidase

Aliquots of Fraction IIC_{10} (1000 cpm) were treated with 20 μl of pig liver α-*N*-acetylglucosaminidase (24 milliunits/ml) in 50 mM citrate, pH 4.5, at 37°C for 48 h. Five millimolar mannose was added to all incubations to inhibit a trace of α-mannosidase activity. The reaction mixtures were diluted to 1 ml with 2 mM Tris base, and applied to columns of QAE-Sephadex (0.5 × 1 cm). The columns were then washed with three more 1-ml fractions of 2 mM Tris base, and eluted stepwise with increasing concentrations of NaCl in 2 mM Tris base. The distribution of elution of the radioactivity is expressed as a percentage of the total counts per min in each case.

Treatment	NaCl required for elution from QAE-Sephadex		
	<1 mM	10–20 mM	>50 mM
	%	%	%
1. None	2	95	3
2. pH 2.1, 100°C 30 min	8	4	88
3. α-*N*-Acetylglucosaminidase	3	17	80
4. α-*N*-Acetylglucosaminidase (boiled enzyme)	6	90	4
5. α-*N*-Acetylglucosaminidase + 5 mM GlcNAc	8	81	11
6. Alkaline phosphatase alone	2	97	1
7. α-*N*-Acetylglucosaminidase + alkaline phosphatase	82	15	3

TABLE II

Recovery of radioactivity in the various fractions and subfractions

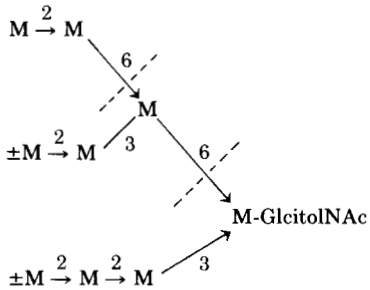
Fractions I to IV were derived from the original Bio-Gel P-6 column of the pronase digest of the 3-h labeled mouse lymphoma cells (see Fig. 1).

Fraction	Subfraction	Total radioactivity	Phosphate residues	
			No.	Type
		cpm		
I		885,000		
	$IAb_{50\rightarrow 200}$	35,000	2	Diester
	$IB_{50\rightarrow 50}$	7,000		
	$IB_{50\rightarrow 200}$	11,000		
	$IAc_{10\rightarrow 50}$	36,000	1	Diester
	$IAc_{50\rightarrow 50}$	29,000	1	Monoester
	$IC_{10\rightarrow 50}$	35,000	1	Diester
	$IC_{50\rightarrow 50}$	23,000	1	Monoester
II		1,975,000		
	$IIAb_{50\rightarrow 200}$	40,000	2	Diester
	$IIAc_{10\rightarrow 50}$	31,000	1	Diester
	$IIC_{10\rightarrow 50}$	162,000	1	Diester
	$IIC_{50\rightarrow 50}$	56,000	1	Monoester
	$IIC_{50\rightarrow 200}$	11,000	2	Diester
III		2,382,000	0	
IV		1,109,000		

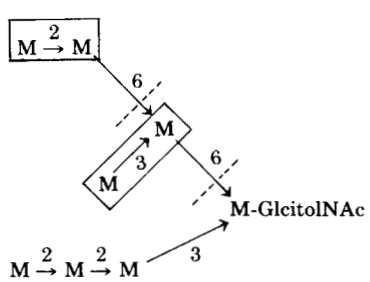
Our subsequent studies were performed to obtain more detailed structural information about individual fractions. Fractions $IIAc_{10}$, IIC_{10}, IIC_{50}, and $IIAb_{50}$ as well as their corresponding fractions from Peak I were subjected to mild acid hydrolysis followed by QAE-Sephadex chromatography to obtain more highly purified samples and to separate the molecules with one uncovered phosphate from these with two covered phosphates. The recovery of radioactivity in each fraction as well as the total starting radioactivity is listed in Table II. Altogether, the phosphorylated oligosaccharides recovered in these fractions comprised 7.5% of the total glycopeptide radioactivity. Studies with Fractions $IIAc_{10 \rightarrow 50}$, $IIC_{10 \rightarrow 50}$, $IIC_{50 \rightarrow 50}$, $IIAb_{50 \rightarrow 200}$, and $IIC_{50 \rightarrow 200}$ will be described in the following sections.

Characterization of Molecules with one Phosphodiester Group: Structure of $IIAc_{10 \rightarrow 50}$—Treatment with jack bean α-mannosidase released 50% of the radioactivity as free mannose with the residual radioactivity remaining at the origin of a paper chromatogram (Fig. 7, *Panel A*). Following removal of the phosphate residue with alkaline phosphatase, α-mannosidase released 88% of the radioactivity as free mannose while the remaining radioactivity migrated with the Manβ1 → 4GlcNAc standard (Fig. 7, *Panel B*). When an aliquot of the dephosphorylated $IIAc_{10 \rightarrow 50}$ was subjected to paper chromatography in Solvent A for 5 days, three radioactive peaks were obtained which co-migrated with $Man_9GlcNAc$ (47%) $Man_8GlcNAc$ (36%), and $Man_7GlcNAc$ (17%) (Fig. 8, *Panel A*). These data, taken together with the endo-β-*N*-acetylglucosaminidase susceptibility of the molecule, indicate that the core structures underlying the phosphate residues are typical high mannose-type molecules. In addition, the finding that the phosphorylated species are partially protected from α-mannosidase digestion indicates that the phosphate group is located at or near the nonreducing termini of these molecules.

To localize the phosphate residue further, the fraction was subjected to acetolysis, a procedure which cleaves Manα1 → 6Man linkages preferentially. Assuming the typical high mannose structure, the acetolysis procedure would be expected to produce the following fragmentation pattern:



Following fragmentation the sample was applied to a QAE-Sephadex column to separate the neutral fragments which passed through the column (82% of total radioactivity) from the negatively charged fragments which bound to the column (18% of radioactivity) and were eluted with 100 mM NaCl. Aliquots of the charged fragments were treated with alkaline phosphatase and then subjected to paper chromatography in Solvent A along with an aliquot of the neutral fraction. As shown in Fig. 9, *Panel A*, mannobiose was the only fragment that had carried a negative charge. The neutral fragments (*Panel B*) consisted of the remaining products expected from acetolysis of this mixture of $Man_{7-9}GlcitolNAc$, namely $Man_4GlcitolNAc$, $Man_3GlcitolNAc$, mannotriose, mannobiose, and a small amount of free mannose. These findings demonstrate that the phosphate residue must be located in those portions of the oligosaccharide that could give rise to mannobiose upon acetolysis, namely the following:



To differentiate between these two possibilities, we utilized a high voltage paper electrophoresis system which separates

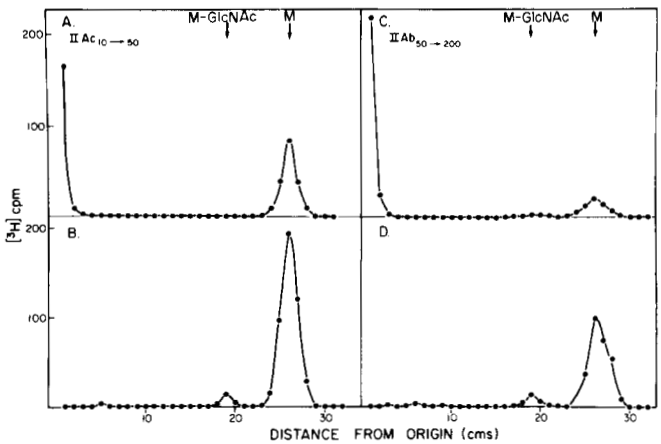


FIG. 7. **Jack bean α-mannosidase digestion of phosphorylated and dephosphorylated oligosaccharides.** Aliquots of $IIAc_{10 \rightarrow 50}$ and $IIAb_{50 \rightarrow 200}$ were digested with jack bean α-mannosidase either before (*Panels A* and *C*) or following (*Panels B* and *D*) treatment with *E. coli* alkaline phosphatase. The entire reaction mixture was spotted on Whatman No. 1 paper and developed in Solvent A for 12 h. The chromatogram was cut into 1-cm strips and the radioactivity was determined. The markers are: *M-GlcNAc*, Manβ1 → 4GlcNAc; *M*, mannose.

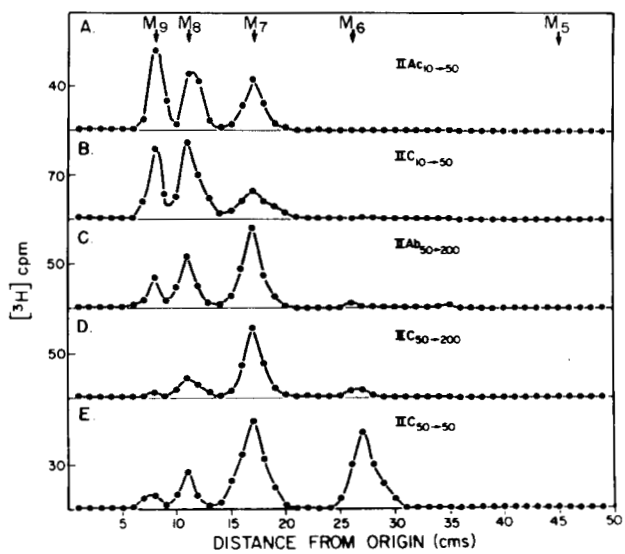


FIG. 8. **Paper chromatography of oligosaccharides from Peak II.** Aliquots of oligosaccharides $IIAc_{10 \rightarrow 50}$, $IIC_{10 \rightarrow 50}$, $IIAb_{50 \rightarrow 200}$, $IIC_{50 \rightarrow 200}$, $IIC_{50 \rightarrow 50}$ were treated with alkaline phosphatase and applied to QAE-Sephadex. The neutral runthrough fractions were desalted and subjected to paper chromatography in Solvent A for 5 days. The chromatograms were cut into 1-cm strips and the radioactivity determined. The standard markers are M_9, $Man_9GlcNAc$; M_8, $Man_8GlcNAc$; M_7, $Man_7GlcNAc$; M_6, $Man_6GlcNAc$; M_5, $Man_5GlcNAc$.

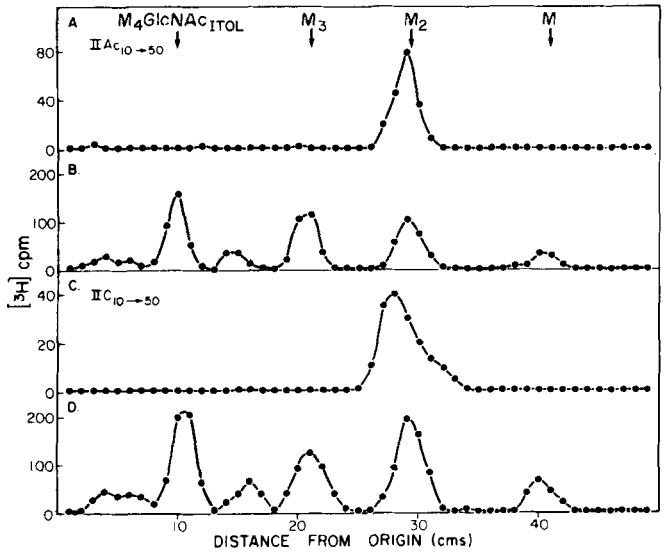


FIG. 9. **Paper chromatography of the acetolysis fragments of Fractions $IIAc_{10\to50}$ and $IIC_{10\to50}$.** Aliquots of $IIAc_{10\to50}$ and $IIC_{10\to50}$ were subjected to acetolysis and the products were fractionated into negatively charged and neutral species as described under "Experimental Procedures." Aliquots of the negatively charged species were treated with alkaline phosphatase, desalted on Amberlite MB-3, and spotted on Whatman No. 1 paper (*Panels A* and *C*). The neutral fragments were desalted directly on Amberlite MB-3 and aliquots were spotted on the paper (*Panels B* and *D*). The chromatograms were developed in Solvent A for 18 h. One-centimeter strips were cut and the radioactivity was determined. The standards are: $M_4GlcNAc_{ITOL}$, Man_4N-acetylglucosaminitol; M_3, Manα1 → 2Manα1 → 3Man; M_2, Manα1 → 2Man; *M*, free mannose.

Manα1 → 2Mannitol from Manα1 → 3Mannitol (20). The mannobiose fragments from both the neutral and the negatively charged fractions were reduced with $NaBH_4$ and the phosphate residue was removed from the latter fraction with alkaline phosphatase. Fig. 10 demonstrates that both Manα1 → 2Mannitol and Manα1 → 3Mannitol were present in both fractions, but in approximately reciprocal amounts. Thus, 80% of the negatively charged mannobiose was Manα1 → 2Man, whereas most of the neutral mannobiose was Manα1 → 3Man. The neutral Manα1 → 3Man presumably was derived from the original $Man_8GlcNAc_1$ and $Man_7GlcNAc_1$ molecules.

The finding that the phosphorylated mannobiose elutes from QAE-Sephadex with 100 mM NaCl indicates that the molecule contains a single phosphate residue since mannose 6-phosphate also elutes from the column with the same NaCl concentration. We presume that the phosphate on the negatively charged Manα1 → 3Man is located on the nonreducing mannose since the reducing mannose is substituted in position 6 in the original oligosaccharide and the phosphate has been shown to be linked to position 6 of mannose in several lysosomal enzymes (5–8, 11). However, in the case of the Manα1 → 2Man fragment, the phosphate could be on either mannose residue. We used two approaches to localize the phosphate within this fragment further. The first approach took advantage of the fact that jack bean α-mannosidase cannot cleave phosphorylated, nonreducing mannose residues. Therefore, we treated the mixture of negatively charged mannobiose fragments with α-mannosidase which would release mannose from Manα1 → 2Man-P but not from P-Manα1 → 2Man or from P-Manα1 → 3Man. Following an overnight incubation, the reaction mixture was boiled to inactivate the α-mannosidase and then alkaline phosphatase was added to generate neutral species. Finally, the sample was subjected to paper chromatography in Solvent A to separate mannobiose from free mannose. We recovered 30% of the radioactivity as free mannose and the rest as mannobiose. Assuming that all of the free mannose was derived from Manα1 → 2Man-P, 30% of the original negatively charged mannobiose would be Manα1 → 2Man-P, 50% would be P-Manα1 → 2Man and 20% would be P-Manα1 → 3Man.

The second approach was to reduce the negatively charged mannobiose fragments with $NaBH_4$ and then subject them to acid hydrolysis under conditions (1 N HCl, 100°C, 4 h) which are sufficient to cleave the glycosidic bonds but not the mannose 6-phosphate linkage (5–8, 11). The resulting monosaccharides were then separated into neutral and negatively charged species by chromatography on QAE-Sephadex. The negatively charged species were eluted from the column, treated with alkaline phosphatase, and then both fractions were subjected to descending paper chromatography in Solvent B for 3 days to separate [^{3}H]mannose from [^{3}H]mannitol. P-Manα1 → 2Man and P-Manα1 → 3Man should give rise to mannose-phosphate and mannitol whereas Manα1 → 2Man-P should give rise to free mannose and mannitol-phosphate. Following paper chromatography the neutral fraction contained 37% mannose and 63% mannitol, whereas the negatively charged fraction gave rise to 84% mannose and 16% mannitol. Therefore, these data are consistent with the results obtained with the α-mannosidase digestion of the negatively charged mannobiose fragments and suggest that phosphate residues are present on either of the 2 mannose residues of the Manα1 → 2Man fragment.

We conclude that Fraction $IIAc_{10\to50}$ consists of a mixture of high mannose oligosaccharides that are isomers containing a single phosphate residue per molecule which is positioned

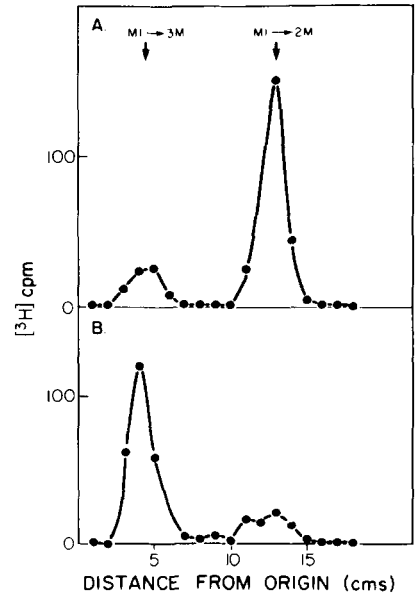


FIG. 10. **Paper electrophoresis for the separation of Manα1 → 3Mannitol from Manα1 → 2Mannitol.** Fraction $IIAc_{10\to50}$ was subjected to acetolysis and the neutral and negatively charged fragments were separated. The negatively charged fragments, which consisted entirely of mannobiose (see *Panel A*, Fig. 9), were treated with alkaline phosphatase to generate neutral species. The neutral fragments from the acetolysis were desalted and subjected to paper chromatography in Solvent A for 18 h. The mannobiose fragments (see Fig. 9, *Panel B*) were eluted from the paper. Both mannobiose fractions were reduced with $NaBH_4$, desalted, and subjected to paper electrophoresis in 0.1 M sodium molybdate, pH 5, at 20 V/cm for 90 min on Whatman No. 3 paper. The paper was cut into 1-cm strips and the radioactivity determined. *Panel A* shows the mannobiose derived from the negatively charged fraction and *Panel B* shows the mannobiose from the neutral fraction. The standards are: *M1 → 3M*, Manα1 → 3Mannitol; *M1 → 2M*, Manα1 → 2Mannitol.

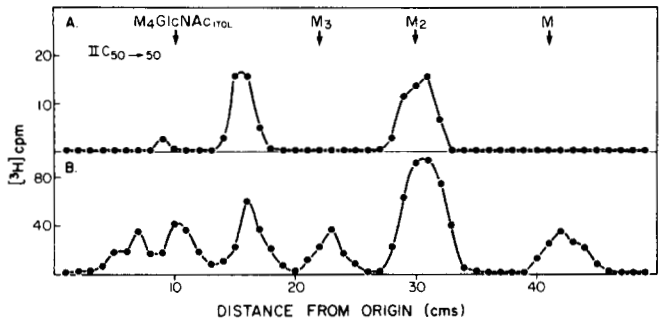


FIG. 11. **Paper chromatography of the acetolysis fragments of Fraction $IIC_{50\rightarrow 50}$.** An aliquot of Fraction $IIC_{50\rightarrow 50}$ was subjected to acetolysis and the fragments were fractionated into neutral and negatively charged species and prepared for paper chromatography as in Fig. 9. *Panel A* shows the negatively charged fragments after removal of the phosphate; *Panel B* shows the neutral fragments. The standards are described in Fig. 9.

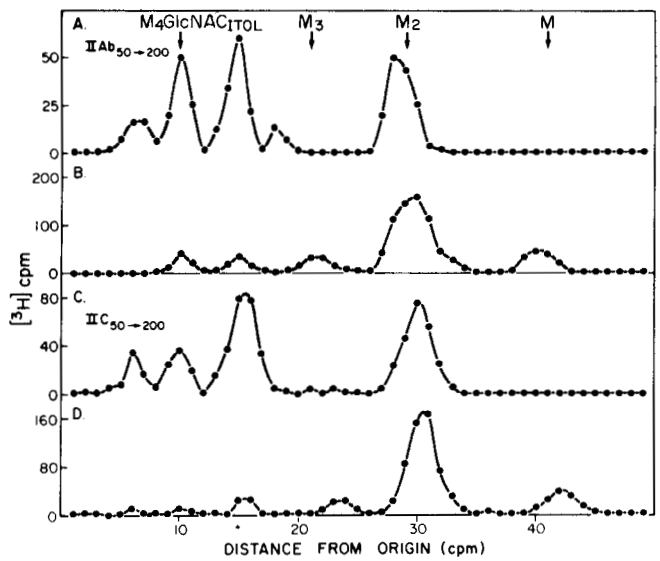


FIG. 12. **Paper chromatography of the acetolysis fragments of Fractions $IIAb_{50\rightarrow 200}$ and $IIC_{50\rightarrow 200}$.** Aliquots of Fractions $IIAb_{50\rightarrow 200}$ and $IIC_{50\rightarrow 200}$ were subjected to acetolysis and the fragments were fractionated into neutral and negatively charged species and prepared for paper chromatography as described in Fig. 9. *Panels A* and *C* show negatively charged framents after removal of the phosphate residues. *Panels B* and *D* show the neutral fragments. The standards are described in Fig. 9.

on 1 of 3 mannose residues. The isomers are shown in Fig. 16 and consist of approximately 80% of Structures A and B and 20% of Structure C. Since the fraction is derived from $IIAc_{10}$ which has only covered phosphates, the phosphate residue is shown in diester linkage with an outer *N*-acetylglucosamine residue.

Structure of $IIC_{10\rightarrow 50}$—Treatment with α-mannosidase released 54% of the radioactivity as free mannose. Following removal of the phosphate residue, α-mannosidase released most of the radioactivity as free mannose and the remaining radioactivity migrated with the Manβ1 → 4GlcNAc standard. Paper chromatography of dephosphorylated $IIC_{10\rightarrow 50}$ in Solvent A revealed $Man_9GlcNAc$ (41%), $Man_8GlcNAc$ (44%), and $Man_7GlcNAc$ (15%) (Fig. 8, *Panel B*). Acetolysis of the fraction gave rise to the same pattern obtained with $IIAc_{10\rightarrow 50}$ (Fig. 9, *Panels C* and *D*). When the mannobiose fragments were subjected to paper electrophoresis, the charged fraction gave rise to 78% Manα1 → 2Mannitol and 22% Manα1 → 3Mannitol whereas the neutral fraction gave rise to 33% Manα1 → 2Mannitol and 67% Manα1 → 3Mannitol. Thus, Fraction $IIC_{10\rightarrow 50}$ consists of a mixture of isomers which are extremely similar, if not identical, to those present in Fraction $IIAc_{10\rightarrow 50}$ (see Fig. 16, *Structures A, B,* and *C*).

Characterization of Molecules with One Phosphomonoester Group: Structure of $IIC_{50\rightarrow 50}$—Treatment with α-mannosidase released 63% of the radioactivity as free mannose. Following removal of the phosphate residue, α-mannosidase released 84% of the radioactivity as free mannose and the remaining radioactivity migrated with the Manβ1 → 4GlcNAc standard. Paper chromatography of dephosphorylated $IIC_{50\rightarrow 50}$ in Solvent A revealed $Man_9GlcNAc$ (5%), $Man_8GlcNAc$ (18%), $Man_7GlcNAc$ (42%), and $Man_6GlcNAc$ (35%) (Fig. 8, *Panel E*).

The acetolysis fragmentation pattern of $IIC_{50\rightarrow 50}$ is shown in Fig. 11. Seventeen per cent of the radioactivity was recovered in the negatively charged fragments, consistent with there being only 1 phosphate residue per molecule. However, in addition to the mannobiose fragments seen with $IIAc_{10\rightarrow 50}$ and $IIC_{10\rightarrow 50}$, a larger fragment migrating in the position expected for $Man_3GlcNAcitol$ was also detected. Treatment of this fragment with α-mannosidase prior to the removal of the phosphate group did not change its migration indicating that the phosphate residue is located on the nonreducing terminus. The proposed structure for this isomer is shown in Fig. 16, *Structure L*.

There was not enough of the negatively charged mannobiose fragments to analyze them further. However, paper electrophoresis of the neutral mannobiose fragments revealed 47% Manα1 → 2Mannitol and 53% Manα1 → 3Mannitol. It seems likely, therefore, that the negatively charged mannobiose fragments were also a mixture of the 1 → 2 and the 1 → 3 species and that Fraction $IIC_{50\rightarrow 50}$ contains isomers with the structures shown in Fig. 16, *Structures J* and *K*. The phosphates are shown uncovered in Structures J, K, and L since the phosphates in these molecules were susceptible to alkaline phosphatase prior to acid treatment.

Characterization of Molecules with Two Phosphodiester Groups: Structure of $IIAb_{50\rightarrow 200}$—Treatment with α-mannosidase released only 22% of the radioactivity as free mannose

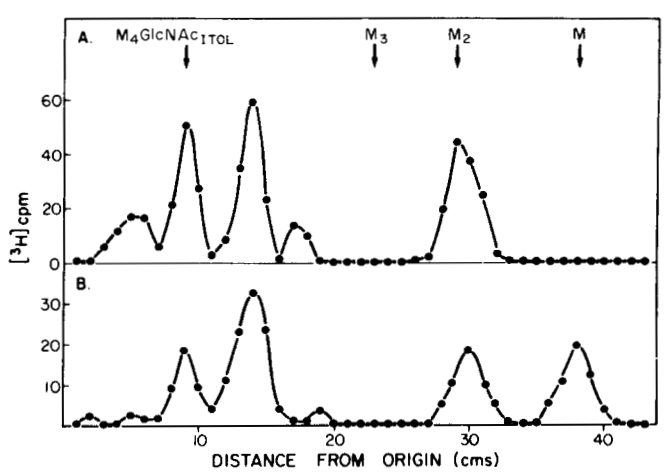


FIG. 13. **Jack bean α-mannosidase digestion of the negatively charged acetolysis fragments of $IIAb_{50\rightarrow 200}$.** An aliquot of the negatively charged acetolysis fragments of $IIAb_{50\rightarrow 200}$ was subjected to digestion with jack bean α-mannosidase for 18 h. The enzyme was then inactivated by boiling for 5 min and the pH of the reaction mixture adjusted to 8.0. The sample was treated with alkaline phosphatase for 2 h at 37°C, desalted on Amberlite MB-3, and subjected to paper chromatography in Solvent A for 18 h (*Panel B*). For comparison, *Panel A* shows a similar paper chromatogram of the negatively charged acetolysis fragments following alkaline phosphatase treatment only. The standards are described in Fig. 9.

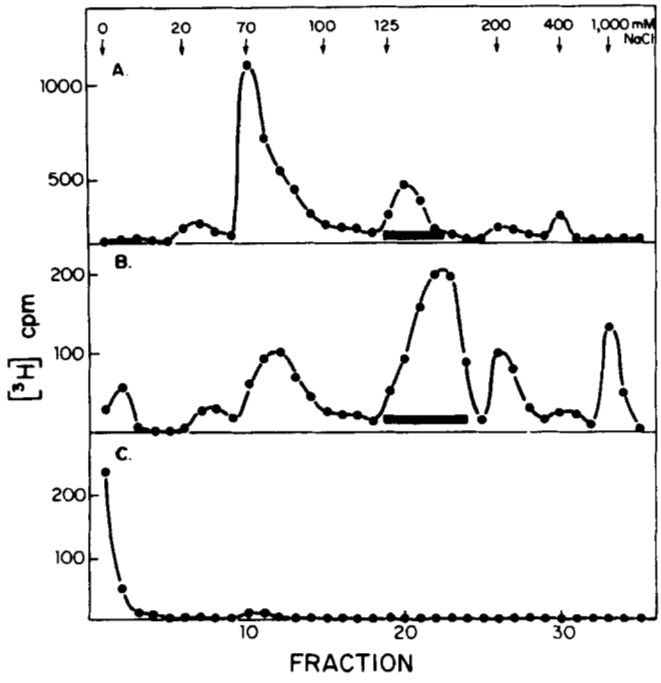


FIG. 14. **Isolation of Fraction $IA_{125 \rightarrow 125}$.** Fraction IA_a (see Fig. 2) in 2 mM Tris base was applied to a column (2 × 0.5 cm) of QAE-Sephadex. After washing with 2 mM Tris base, the column was eluted stepwise with 20 to 1000 mM NaCl in 2 mM Tris base as shown in *Panel A*. The material eluting with 125 mM NaCl was desalted, brought to pH 2.1 with HCl, and incubated at 100°C for 30 min. The sample was then frozen, lyophilized, suspended in 2 mM Tris base, and applied to a fresh column of QAE-Sephadex which was eluted with the same increment of NaCl (*Panel B*). The material which still eluted with 125 mM NaCl was pooled as noted and called $IA_{125 \rightarrow 125}$. The fractions which eluted with 200 mM, 400 mM, and 1000 mM NaCl were also pooled for further studies. *Panel C* shows the behavior of an aliquot of $IA_{125 \rightarrow 125}$ on QAE-Sephadex following treatment with alkaline phosphatase.

(Fig. 7, *Panel C*), consistent with two of the three branches of the underlying high mannose oligosaccharide being resistant to the action of the glycosidase. Following removal of the phosphate residues, α-mannosidase released 88% of the radioactivity as free mannose and the remaining radioactivity migrated with the Manβ1 → 4GlcNAc standard (Fig. 7, *Panel D*). Paper chromatography of dephosphorylated $IIAb_{50 \rightarrow 200}$ in Solvent A revealed $Man_9GlcNAc$ (20%), $Man_8GlcNAc$ (31%), and $Man_7GlcNAc$ (49%) (Fig. 8, *Panel C*).

The results of acetolysis of $IIAb_{50 \rightarrow 200}$ are shown in Fig. 12, *Panels A* and *B*. The negatively charged fragments, comprising 55% of the total radioactivity, consisted predominantly of $Man_4GlcitolNAc$, $Man_3GlcitolNAc$, and mannobiose. The finding that each of these fragments was eluted from QAE-Sephadex with 100 mM NaCl indicated that each contains a single phosphate residue. The major neutral fragment was mannobiose, but there were also small amounts of $Man_4GlcitolNAc$, $Man_3GlcitolNAc$, mannotriose, and mannose. Paper electrophoresis of the mannobiose fragments revealed that the charged species contained 84% Manα1 → 2Man and 16% Manα1 → 3Man whereas the neutral species consisted of 20% Manα1 → 2Man and 80% Manl → 3Man. To investigate the position of the phosphate residue in the larger acetolysis fragments further, an aliquot of the negatively charged fraction was digested with α-mannosidase. The α-mannosidase was then inactivated by boiling and the material was treated with alkaline phosphatase and subjected to paper chromatography. As shown in Fig. 13, α-mannosidase treatment caused a small decrease in the amount of the $Man_4GlcitolNAc$ fragment relative to the $Man_3GlcitolNAc$ fragment (compare the treated sample in *Panel B* to the original sample in *Panel A*), but the ratio of these two fragments was approximately 1:2 after the enzyme treatment. Thus, it appears that both the terminal and the penultimate mannose residues may be phosphorylated although the latter is the predominant site.

The fact that 200 mM NaCl is required to elute the negatively charged oligosaccharides in Fraction $IIAb_{50 \rightarrow 200}$ from QAE-Sephadex indicates that all the molecules contain more than 1 phosphate residue. The finding that 80% of the total radioactivity in the large acetolysis fragments (Man_4·GlcitolNAc and $Man_3GlcitolNAc$) was recovered in negatively charged species indicates that most of the oligosaccharides contain 1 phosphate residue on this portion of the molecule. The second phosphate must be located on a mannose residue which gives rise to mannobiose upon acetolysis. Since 84% of the negatively charged mannobiose is Manα1 → 2Man, this is the predominant site for the second phosphate. Therefore, the major species in $IIAb_{50 \rightarrow 200}$ is depicted in *Structure D* of Fig. 16, with *Structure E* being a minor species. In addition, since some $Man_4GlcitolNAc$ and $Man_3GlcitolNAc$ were present in the neutral fraction of the acetolysate, there must be a small percentage of the molecules with the structure depicted in *F* of Fig. 16.

Structure of $IIC_{50 \rightarrow 200}$—This fraction proved to be very similar to $IIAb_{50 \rightarrow 200}$. Jack bean α-mannosidase initially released only 32% of the radioactivity as free mannose whereas after alkaline phosphatase treatment, it released 86% of the radioactivity as mannose and the remainder migrated as Manβ1 → 4GlcNAc. Paper chromatography of dephosphorylated $IIC_{50 \rightarrow 200}$ in Solvent A revealed predominantly $Man_7GlcNAc$ (71%) with small amounts of $Man_9GlcNAc$ (3%), $Man_8GlcNAc$ (18%), and $Man_6GlcNAc$ (8%) (Fig. 8, *Panel D*).

The acetolysis fragmentation pattern is shown in Fig. 12, *Panels C* and *D*. The negatively charged fragments, comprising 45% of the radioactivity, contained predominantly $Man_3GlcitolNAc$ and mannobiose with a lesser amount of $Man_4GlcitolNAc$ and a small amount of underdegraded material. The major neutral species was mannobiose. There was not enough material to characterize the fragments further.

Based on these data, Fraction $IIC_{50 \rightarrow 200}$ is felt to contain structures similar to those present in $IIAb_{50 \rightarrow 200}$, *i.e.* Structures D, E, and F of Fig. 16.

Characterization of Molecules with Two Phosphomonoes-

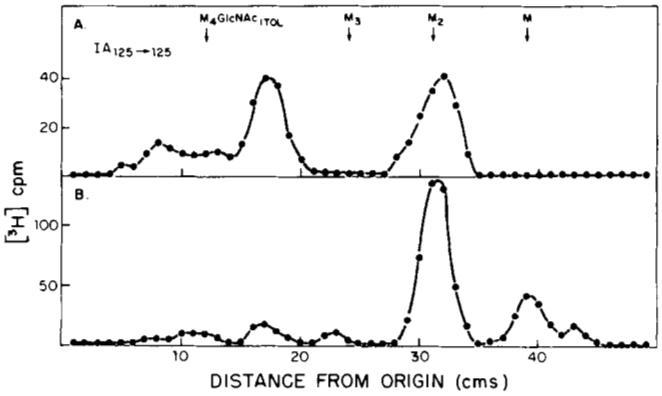


FIG. 15. **Paper chromatography of the acetolysis fragments of Fraction $IA_{125 \rightarrow 125}$.** An aliquot of Fraction $IA_{125 \rightarrow 125}$ was subjected to acetolysis and the fragments were separated into neutral and negatively charged species and prepared for paper chromatography as in Fig. 9. *Panel A* shows the negatively charged fragments after removal of the phosphate; *Panel B* shows the neutral fragments. The standards are described in Fig. 9.

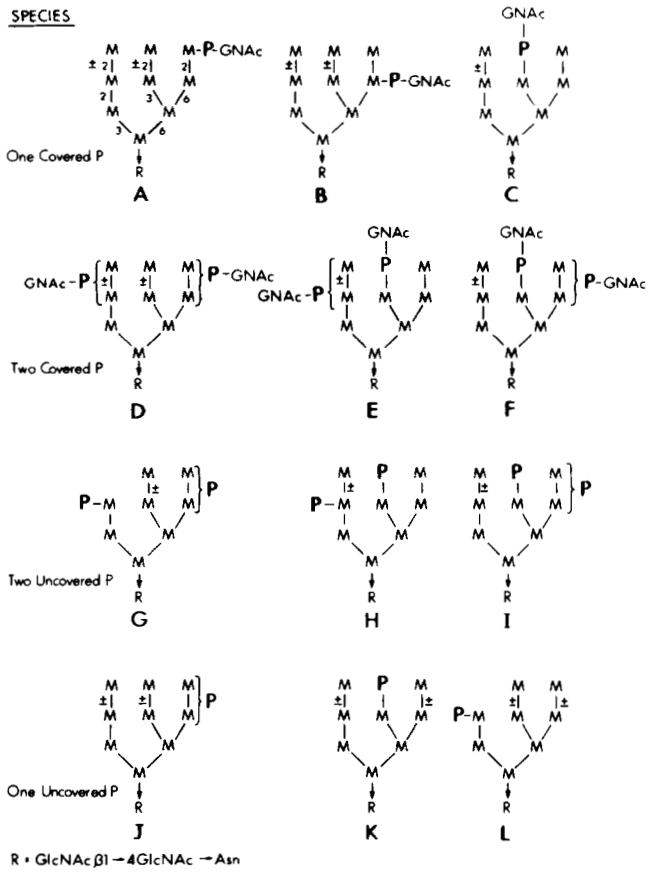


FIG. 16. **Proposed structures of the various phosphorylated oligosaccharides.** *Structures A, B,* and *C* are present in Fractions $IIAc_{10\rightarrow 50}$ and $IIC_{10\rightarrow 50}$; *Structures D, E,* and *F* are present in Fractions $IIAb_{50\rightarrow 200}$ and $IIC_{50\rightarrow 200}$; *Structures G, H,* and *I* are present in Fraction $IA_{125\rightarrow 125}$; and *Structures J, K,* L are present in Fraction $IIC_{50\rightarrow 50}$. *GNAc* refers to *N*-acetylglucosamine.

ter Groups: Structure of $IA_{125\rightarrow 125}$—During the course of these studies we noted that conversion of oligosaccharides from their diester form to the monoester form was associated with an earlier elution from Bio-Gel P-6, *i.e.* the molecules behaved as larger species even though the covering *N*-acetylglucosamine was removed. We therefore reasoned that molecules containing two or more phosphomonoester groups might migrate in the original IA_a fraction even after they had been released from the peptide by endo-β-*N*-acetylglucosaminidase treatment. To determine whether Fraction IA_a contained such species, the fraction was applied to QAE-Sephadex and eluted with stepwise increments in NaCl. As shown in Fig. 14, *Panel A*, almost all the radioactive molecules bound to the column and were eluted in a series of steps. The material eluting with 125 mM NaCl was treated with mild acid, desalted, and refractionated on QAE-Sephadex as shown in Fig. 14, *Panel B*. The material which still eluted with 125 mM NaCl was selected as the best candidate for having had two exposed phosphates initially and was therefore subjected to further analysis.[2] The fraction was called $IA_{125\rightarrow 125}$.

Treatment of $IA_{125\rightarrow 125}$ with alkaline phosphatase caused a total conversion to neutral species, demonstrating that the negative charge was due to exposed phosphate residues (Fig. 14, *Panel C*). Jack bean α-mannosidase treatment of the native molecules released 26% of the radioactivity as free mannose, but following alkaline phosphatase treatment the enzyme released 88% of the radioactivity as free mannose and the remaining material migrated with the Manβ1 → 4GlcNAc standard. Paper chromatography of the dephosphorylated molecules revealed 79% $Man_7GlcNAc$ and 21% $Man_8GlcNAc$.

The acetolysis fragmentation pattern is shown in Fig. 15. As in the case of $IIAb_{50\rightarrow 200}$ and $IIC_{50\rightarrow 200}$, the negatively charged species contained predominantly $Man_3GlcitolNAc$ and mannobiose while the major neutral species was mannobiose. High voltage paper electrophoresis of the neutral fraction revealed that it contained 44% Manα1 → 2Man and 56% Manα1 → 3Man. There was not enough of the negatively charged fragments to characterize them further.

Based on these data, the most likely structures for the oligosaccharides present in $IA_{125\rightarrow 125}$ are shown in Fig. 16, *Structures G, H,* and *I*, with *Structure G* being the predominant species. These species are extremely similar to those found in $IIAb_{50\rightarrow 200}$ with the exception that the phosphates are in monoester linkage.

DISCUSSION

The present study was prompted by the demonstration that the intracellular biosynthetic intermediates of the acid hydrolase β-glucuronidase contain phosphorylated oligosaccharides in which the phosphate residues are present in diester linkage between mannose residues of the underlying high mannose-type oligosaccharide and outer α-linked *N*-acetylglucosamine residues (12). In order to better understand this biosynthetic pathway it was necessary to determine the structure of these molecules. However, the amount of [2-^{3}H]mannose which could be incorporated into the β-glucuronidase oligosaccharides was not sufficient to allow the required detailed structural studies. We reasoned that if the other lysosomal enzymes shared this biosynthetic pathway, it should be possible to isolate adequate amounts of similar material from the whole cell glycopeptides. This, in fact, proved to be the case. About 7.5% of the [2-^{3}H]mannose incorporated into glycoproteins during a 3-h labeling period was recovered in phosphorylated oligosaccharides. These phosphorylated oligosacharides were released from the underlying peptides by successive treatments with endo-β-*N*-acetylglucosaminidase C_{II} and H. We chose this sequence to determine whether there were molecules which were susceptible to endo-β-*N*-acetylglucosaminidase H, but not to endo-β-*N*-acetylglucosaminidase C_{II}, since endo-β-*N*-acetylglucosaminidase H has a broader specificity (22). In fact, the molecules released with endo-β-*N*-acetylglucosaminidase H turned out to be identical with those released with endo-β-*N*-acetylglucosaminidase C_{II} and, therefore, a longer digestion with endo-β-*N*-acetylglucosaminidase C_{II} probably would have released all the molecules that endo-β-*N*-acetylglucosaminidase H released in the second digest.

In all respects, these phosphorylated oligosaccharides were very similar to those found in β-glucuronidase, making it reasonable to speculate that they were derived from biosynthetic intermediates of many lysosomal enzymes. In other experiments, we have found that human diploid fibroblasts also synthesize this class of molecules. In contrast, fibroblasts from a patient with I-cell disease, a disorder characterized by the failure to phosphorylate acid hydrolases, failed to synthesize these molecules. This observation provides further support for our argument that the whole cell phosphorylated oligosaccharides are derived from acid hydrolases. However, we cannot rule out the possibility that other glycoproteins may share this pathway.

The phosphorylated oligosaccharides proved to be a family of molecules which shared a number of structural features.

[2] In our initial procedure, molecules with two exposed phosphates were eluted from QAE-Sephadex with 200 mM NaCl after failing to be eluted with 100 mM NaCl. Subsequent studies showed that, in fact, 125 mM NaCl was sufficient to elute these molecules, and therefore, the elution scheme was changed in this experiment.

They all contained an underlying high mannose-type unit although the actual number of mannose residues per molecule varied from nine to six. The most likely explanation for this is that some of the oligosaccharides had been processed by the α-mannosidase which is specific for α1,2-linked mannose residues (23). The presence of the phosphate residues presumably would act to prevent more extensive processing since these residues were shown to block the action of jack bean α-mannosidase. While all the molecules contained phosphate residues, they differed in the number, the location, and the nature of the phosphate groups. Approximately 75% of the molecules contained phosphate residues in diester linkage, while 25% contained phosphomonoester groups. In all the species tested, the phosphodiester group could be converted to a phosphomonoester by mild acid hydrolysis or by treatment with pig liver α-*N*-acetylglucosaminidase. The latter finding indicates that the "cover" in most, if not all, of these species is α-linked *N*-acetylglucosamine. The majority of molecules contained a single phosphate residue while about 20% contained two phosphates. In addition, the preparation probably contained small amounts of oligosaccharides with 3 phosphate residues. One likely candidate was Fraction $IA_{125 \rightarrow 200}$ (Fig. 14, *Panel B*) which was converted to a neutral species by alkaline phosphatase but which was unfortunately lost before further studies could be performed. This fraction represented about 1% of the total phosphorylated oligosaccharides. It must be emphasized that the current study only deals with cells labeled for a single time point (3 h), and therefore, the relative amounts of the different species might be significantly different at other time points.

Of the 9 mannose residues in the underlying oligosaccharide, five were shown to be phosphorylated although the distribution of phosphorylation was not random and three of the five sites were preferred. As summarized in Fig. 17, all of the mannose residues which were phosphorylated were located either at nonreducing termini or at positions penultimate to these residues. The mannoses most commonly phosphorylated were *residues i* and *h*. While either of these mannose residues could be phosphorylated, in any individual oligosaccharide either one or the other, but not both, was actually phosphorylated. The next most common site of phosphorylation was *residue d*. Interestingly, in all the molecules in which mannose *residue f* was phosphorylated, *residue g* was missing. Either *residue g* must be removed before *residue f* can be phosphorylated or else *residue g* is removed by the processing α-mannosidase shortly after the phosphorylation of *residue f*. The β-linked mannose (*residue a*), as well as *residues b, c,* and *g* were never found to be phosphorylated.

In our previous paper, we suggested that phosphorylation of the oligosaccharides occurs by the transfer of α-*N*-acetylglucosamine 1-phosphate residues to mannose residues, and that subsequently, the *N*-acetylglucosamine is removed to expose the phosphate residue (12). The present data are consistent with this proposal in that the molecules containing one phosphodiester linkage have core oligosaccharides which are significantly larger than the cores of the molecules with phosphomonoester groups (predominantly $Man_9GlcNAc$ and $Man_8GlcNAc$ *versus* $Man_7GlcNAc$ and $Man_6GlcNAc$). Since the molecules with one phosphodiester linkage have the least processing of their mannose residues, they probably represent the most newly synthesized species. We are currently performing pulse-chase experiments in an attempt to obtain more definitive evidence about the sequence in which the various species are formed.

Although we have concentrated our efforts on characterizing the phosphorylated oligosaccharides, we have also identified a number of mannose-containing negatively charged oligosaccharides which are resistant to alkaline phosphatase both before and after mild acid treatment. One example of this type of oligosaccharide is $IA_{125 \rightarrow 1000}$ (Fig. 14, *Panel B*) which had a large increase in net negative charge following mild acid treatment but which was resistant to alkaline phosphatase. Currently, we are trying to identify the moiety responsible for the negative charge of these species.

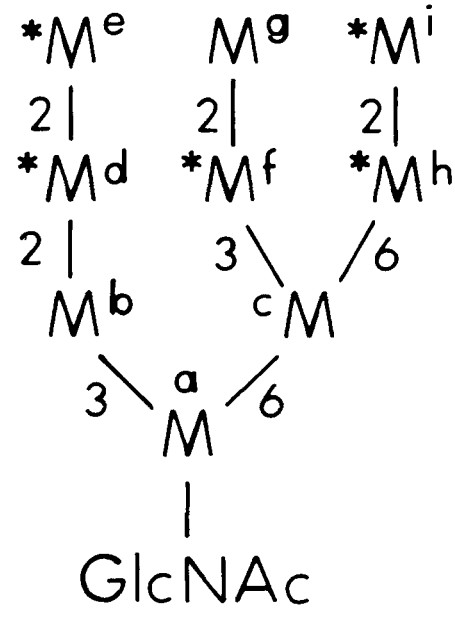


FIG. 17. **Location of the phosphate residues on the underlying oligosaccharide.** The figure shows the complete, unprocessed high mannose oligosaccharide with 9 mannose residues. It is assumed that the branching pattern and the linkages are the same as in other high mannose-type oligosaccharides (14). Each mannose residue (M) has been assigned a letter so that the individual residues can be referred to in the text. The residues marked with the * are phosphorylated in one or more of the phosphorylated oligosaccharide species.

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