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Biochemistry

Identification of mannose 6-phosphate in glycoproteins that inhibit the assimilation of β-galactosidase by fibroblasts

(adsorptive endocytosis/recognition marker/lysosomal enzymes)

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ABSTRACT **Bovine testicular β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is rapidly and selectively assimilated by human skin fibroblasts. The assimilation of the enzyme is strongly inhibited by mannose 6-phosphate and by a glycoprotein fraction isolated from bovine testes (glycoprotein inhibitors). These results suggest that β-galactosidase and the glycoprotein inhibitors have a common recognition marker that contains mannose 6-phosphate. The presence of mannose phosphate in the glycoprotein inhibitors was demonstrated by acid hydrolysis of the glycoproteins to liberate mannose phosphate followed by reduction with NaB^3H_4 to give [^{3}H]mannitol phosphate. The ^{3}H-labeled compound was identified by paper electrophoresis and by the release of [^{3}H]mannitol on treatment with phosphatase. The [^{3}H]mannitol phosphate was oxidized with periodate and the resulting phosphorylated fragment, on reduction with NaB^3H_4, yielded [^{3}H]ethylene glycol phosphate, indicating substitution of phosphate on carbon 6 of mannitol. Mannose 6-phosphate was also found in a major carbohydrate-containing fraction of peptides produced from the glycoprotein inhibitors by tryspin digestion. It was estimated that about 2% of the mannose residues were present as mannose 6-phosphate. Phosphorylated oligosaccharides were also identified in hydrolysates of the glycoprotein inhibitors. One, a disaccharide, was identified as α-(mannosyl-6-phosphate)-(1 → 2)-mannose. These observations suggest that the recognition marker of β-galactosidase contains α1,2-linked mannose 6-phosphate; terminal α1,2-linked mannose residues are known to occur in the high-mannose type oligosaccharides present on β-galactosidase.**

Observations that extracellular lysosomal enzymes are assimilated by fibroblasts and serve to correct genetic lysosomal enzyme deficiencies has stimulated interest in the mechanisms by which the enzyme assimilation occurs. It has been proposed that the assimilation (absorptive endocytosis or uptake) of lysosomal enzymes by fibroblasts is initiated by cellular "recognition" of a carbohydrate-containing "marker" bound to the lysosomal enzymes (1). Studies in this laboratory implicated enzyme-bound mannosyl residues in the assimilation of bovine testicular β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) (2). Recently, Kaplan *et al.* (3) suggested that phosphate, probably as mannose 6-phosphate, participated in the assimilation of platelet β-glucuronidase by fibroblasts. This conclusion was based on the strong inhibition of the assimilation of β-glucuronidase by free mannose 6-phosphate and the lack of assimilation of β-glucuronidase when the enzyme was pretreated with alkaline phosphatase. Subsequently, similar experiments implicated mannose phosphate in the assimilation of other lysosomal enzymes by fibroblasts. These enzymes included highly purified α-L-iduronidase (4) and β-galactosidase (5) as well as several other partially purified lysosomal enzymes from platelets (6) and urine (7). These observations strongly suggested that mannose 6-phosphate was a normal constituent of many lysosomal enzymes. However, direct evidence of the occurrence of mannose 6-phosphate in lysosomal enzymes has not been presented. Furthermore, the type of carbohydrate unit of these glycoproteins that may contain mannose 6-phosphate and constitute the "recognition marker" was not apparent.

Elucidation of the structural features that characterize the recognition marker has been restricted by the limited amounts of highly purified enzymes. Fortunately, glycoproteins prepared from urine (4), platelets (6), and bovine testes (2, 5) furnish abundant materials which, based on inhibition studies, bear recognition markers similar to those of lysosomal enzymes.* These glycoprotein inhibitors were found to contain several lysosomal enzymes that may compete for the same cell receptors (4, 5).

MATERIALS AND METHODS

Diphenylcarbamyl chloride-treated (DCC) trypsin, soybean trypsin inhibitor, jack bean α-mannosidase, and mannose 6-phosphate† were obtained from Sigma. Potato acid phosphatase, glycoaldehyde phosphate diethylacetal, and DL-glyceraldehyde-3-phosphate diethylacetal were obtained from Calbiochem; acetals were hydrolyzed as recommended by the supplier. Concanavalin A (Con A)-Sepharose was obtained from Pharmacia. Sodium boro[^{3}H]hydride [specific activity, 422 mCi/mmol (1 Ci = 3.7×10^{10} becquerels)] was obtained from Amersham. Radioactivity was measured by liquid scintillation spectrometry on squares of Whatman 3 MM paper immersed in a toluene liquid scintillation cocktail. Methyl β-mannopyranoside was prepared from methyl tetra-*O*-acetyl-β-mannopyranoside. The latter compound and synthetic α-mannopyranosyl-(1→ 2)-mannose were kindly provided by I. J. Goldstein (University of Michigan). Mannose 6-sulfate was prepared by direct sulfation of mannose (8). Glucosamine- and *N*-acetylglucosamine-6-phosphate were prepared by published methods (9).

High-voltage electrophoresis was performed on a Gilson model D electrophorator on Whatman 3 MM paper saturated with one of the following buffers: *buffer A*, 1.25 M pyridine/64 mM acetic acid, pH 6.4 (75 V/cm; 30 min); *buffer B*, 1% sodium tetraborate, pH 9.0 (65 V/cm; 25 min); *buffer C*, 1.6 M acetic acid/0.15 M formic acid, pH 2.1 (75 V/cm; 45 min). Organic and inorganic phosphates were estimated by a modification (10) of the procedure of Fisk and Subbarow. Phosphates were detected on electrophoretograms by the procedure of

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Abbreviation: Con A, concanavalin A.

* Bovine testicular glycoprotein inhibitors also inhibit the assimilation of human platelet β-glucuronidase (William S. Sly, personal communication) whereas human urinary glycoprotein inhibitors or highly purified α-iduronidase inhibits the assimilation of bovine β-galactosidase by fibroblasts (Gloria N. Sando, personal communication). These observations support the idea that the three enzymes are assimilated by means of a common system.

† Unless otherwise noted, all sugars are of the D configuration.

Bandurski and Axelrod (11). Protein was estimated by the procedure of Lowry *et al.* (12), sialic acid, by a periodate/resorcinol method (13), and neutral sugars and hexosamines, by gas/liquid chromatography of their glycitol acetate derivatives (14, 15) on a column of 3% OV-275 on Chromosorb W AW (Supelco, Bellefonte, PA) at 215°C.

Treatment of ^{3}H-labeled compounds with acid phosphatase (0.35 unit) or α-mannosidase (0.5 unit) was performed at 37°C for 18 hr in 25-μl incubation mixtures containing 2 μmol of sodium acetate at pH 4.5. After incubation, the mixtures were dried under reduced pressure and reconstituted in an appropriate electrophoresis buffer. Periodate oxidations were performed for 18 hr as described by Spiro (16).

Preparation of Bovine Testicular Glycoprotein Fraction That Inhibits Assimilation of β-Galactosidase. During the preparation of testicular β-galactosidase (17), the galactosidase-free effluent from the first affinity chromatography step was retained for the isolation of glycoprotein inhibitors. The effluent was adjusted to pH 7.5 with 4 M NaOH and dialyzed against two changes (10 vol each) of 0.05 M Tris·HCl/0.15 M NaCl, pH 7.5. Con A-Sepharose (100 ml) was added to the dialyzed solution and the suspension was slowly stirred at 5°C for 6 hr. The suspension was allowed to settle and the glycoprotein–Con A-Sepharose aggregate was washed five times at room temperature by decantation with 500-ml portions of the buffer/salt mixture. The aggregate was packed into a 4-cm-diameter column, an additional liter of the buffer/salt mixture was passed through the column, and the glycoproteins were eluted from the support at room temperature with 0.75 M methyl α-mannoside dissolved in the buffer/salt mixture. Fractions containing protein were combined and dialyzed against two changes (12 liters each) of cold (5°C) saline. Glycoproteins were precipitated from the solution by addition of solid $(NH_4)_2SO_4$ to 75% saturation. After mixing for 30 min, the precipitate was collected by centrifugation and dissolved in 60 ml of 0.05 M Tris·HCl at pH 7.5. The solution was dialyzed against 15 vol of the same buffer and lyophilized. Approximately 1.7 g of glycoprotein inhibitor fraction (32% protein) was obtained from 12 bovine testes.

Partial Acid Hydrolysis of Glycoprotein Inhibitors Followed by Reduction with NaB^3H_4. Glycoprotein inhibitor fraction (100 mg) in 1 ml of water was dialyzed at 5°C for 20 hr against 100 ml of water. The resulting suspension (1.2 ml) was transferred to an 18-mm Pyrex tube and an equal volume of a 40% suspension of Dowex 50 X-2, H^+ form, in 0.02 M HCl was added (15). The tube was sealed and heated at 100°C for 15 hr. After cooling, the tube contents were applied to 200 μl of additional Dowex 50 (H^+ form) resin in a small column and the column was washed with 2 ml of water. The effluent was adjusted to approximately pH 8 with 1 M NH_4OH and taken to dryness under reduced pressure in a 10-ml pear flask. NaB^3H_4 (25 mCi, dissolved in 300 μl of 50% dimethylformamide) was added, and reduction was allowed to proceed for 2 hr. One drop of acetic acid was added to decompose remaining $NaBH_4$ and the solution was taken to dryness under reduced pressure five times with the sequential addition of 2 ml of electrophoresis buffer A. Blank reaction mixtures containing albumin (30 mg) with or without added mannose 6-phosphate were processed in the same way.

Standard ^{3}H-labeled glycitols were prepared by direct reduction of the corresponding glycoses (hydrolysis step omitted). Usually, the glycoses (1–2 μmol) were reduced with approximately 5 mCi of NaB^3H_4.

Enrichment of Phosphorylated ^{3}H-Labeled Compounds. The ^{3}H-labeled residue remaining after hydrolysis and reduction was applied to a 0.5 × 3 cm column of Dowex-1 X-8 (200–400 mesh, acetate-form). The column was washed with 15 ml of 0.1 M sodium acetate at pH 7.0 and the phosphorylated compounds were eluted from the column with 0.05 M H_2SO_4; fractions containing radioactivity were adjusted to pH 7.0 with 0.1 M NaOH and concentrated by evaporation under reduced pressure.

Inihibition Studies. Generalized gangliosidosis skin fibroblasts, strain KD, were obtained from the Repository for Mutant Human Cell Strains (Montreal Children's Hospital). Bovine testicular β-galactosidase was prepared and assayed as described (17). Assimilation and inhibition of assimilation of β-galactosidase were tested by the procedure of Hieber *et al.* (2).

A sufficiently wide range of inhibitor concentrations was used to give 8–10 values in the range of 20–80% inhibition of β-galactosidase assimilation. Values for percentage inhibition of β-galactosidase assimilation were plotted against the logarithm of inhibitor concentration, and the concentration required for 50% inhibition was determined from the slope of the line.

RESULTS

A glycoprotein fraction was isolated from bovine testicular extracts by a procedure that included adsorption on Con A-Sepharose and elution with methyl α-mannoside. This fraction (called glycoprotein inhibitors) strongly inhibited the assimilation of β-galactosidase by fibroblasts (2, 5). The carbohydrate composition of the fraction was (per mg of protein): mannose, 0.32 μmol; *N*-acetylglucosamine, 0.17 μmol; galactose, 0.06 μmol; and *N*-acetylneuraminic acid, 0.05 μmol.

Identification of Mannose Phosphate in Partial Hydrolysates of the Glycoprotein Inhibitors. Advantage was taken of the recognized stability of mannose 6-phosphate in acid (18). Preliminary experiments revealed that mannose 6-phosphate was 50% hydrolyzed after 15 hr of Dowex 50-catalyzed hydrolysis; under the same conditions, maximal amounts of free mannose were liberated from the glycoprotein inhibitors.

After 15-hr hydrolysis of the glycoprotein inhibitors, the neutral and anionic compounds were reduced with NaB^3H_4 to convert liberated sugar phosphate to the corresponding [^{3}H]-glycitol phosphate. When an aliquot of the reduced hydrolysate was subjected to paper electrophoresis (Fig. 1*a*), high background radioactivity obscured the area where authentic [^{3}H]-mannitol 6-phosphate migrated. The remainder of the reduced hydrolysate was subjected to an ion-exchange procedure for the enrichment of phosphorylated compounds. The enriched fraction contained a ^{3}H-labeled compound that corresponded in electrophoretic migration to authentic [^{3}H]mannitol 6-phosphate (Fig. 1*b*). When an aliquot of the enriched fraction was treated with acid phosphatase, radioactivity that corresponded to [^{3}H]mannitol 6-phosphate was not found and neutral ^{3}H-labeled compounds appeared (Fig. 1*c*). Two other areas, A and B in Fig. 1*b*, also appeared to contain phosphorylated compounds, possibly oligosaccharides (see below).

To confirm the identity of the compound that comigrated with [^{3}H]mannitol 6-phosphate, paper electrophoresis was performed on a preparative scale; the compounds that corresponded in migration to mannitol 6-phosphate were eluted from the paper and subjected to paper electrophoresis in buffer B (Fig. 2*a*); the major radioactivity again corresponded in migration to authentic [^{3}H]mannitol 6-phosphate. When an aliquot of the ^{3}H-labeled glycitol phosphate was treated with acid phosphatase, a neutral ^{3}H-labeled compound was obtained that corresponded in migration to [^{3}H]mannitol on electrophoresis in buffer B (Fig. 2*b*). The possibility that the charge on the unknown compound resulted from the presence of a sulfate or carboxyl group was unlikely because at pH 2.1 there are wide differences in the migration of the unknown compound relative

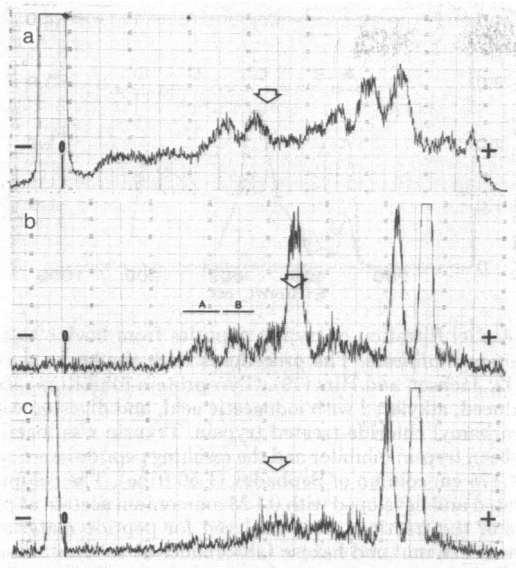

FIG. 1. Paper electrophoresis of a hydrolysate of glycoprotein inhibitors after reduction with NaB^3H_4. (*a*) Scan of radiochromatogram of aliquot of the crude mixture after reduction. (*b*) The same reaction mixture after enrichment of phosphorylated compounds. (*c*) An aliquot of the enriched fraction treated with acid phosphatase. Electrophoresis was performed in buffer A. The arrows mark the migration of authentic [^{3}H]mannitol 6-phosphate run on an adjacent channel of the paper.

to mannitol 6-sulfate and L-gulonic acid (Fig. 2*c*). Although buffer systems A, B, and C do not clearly distinguish [^{3}H]-mannitol 6-phosphate from *N*-acetyl [^{3}H]glucosaminitol 6-phosphate, after removal of phosphate, [^{3}H]mannitol could be readily distinguished from *N*-acetyl[^{3}H]glucosaminitol in buffer B (Fig. 2*b*) as well as from [^{3}H]glucosaminitol, [^{3}H]galactitol, and [^{3}H]glucitol.

It was estimated that 1.5×10^6 cpm of [^{3}H]mannitol phosphate (approximately 0.12 μmol) was obtained from 100 mg of glycoprotein inhibitors.

Substitution of Phosphate at Carbon 6 of [^{3}H]Mannitol. Although the properties of [^{3}H]mannitol phosphate from the glycoproteins were similar to the properties of authentic [^{3}H]mannitol 6-phosphate, they did not allow definitive proof that phosphate was present at carbon 6. The position of substitution was established by periodate oxidation of [^{3}H]mannitol phosphate followed by reduction of the phosphorylated fragment with NaB^3H_4. If substitution were on carbon 6 of mannitol, [^{3}H]ethylene glycol phosphate would be expected; [^{3}H]glycerol 2-phosphate should arise if phosphate were substituted on carbons 2 through 5.

Mannitol phosphate (3.7×10^5 cpm) obtained from the glycoprotein inhibitors was oxidized with periodate for 18 hr, and excess periodate was converted to iodate by the addition of ethylene glycol (16). Authentic [^{3}H]mannitol 6-phosphate (2×10^5 cpm) was added to the reaction mixture as an internal standard and the mixture was subjected to ion exchange chromatography as described for enrichment of phosphorylated compounds. The now unlabeled glycose phosphate fragment was located by its simultaneous elution with [^{3}H]mannitol 6-phosphate. After neutralization and concentration, the fraction containing [^{3}H]mannitol 6-phosphate and unlabeled glycose phosphate was reduced with 5 mCi of NaB^3H_4 and subjected to electrophoresis in buffer A (Fig. 3*a*). A ^{3}H-labeled compound was found that migrated at the same rate as authentic [^{3}H]-

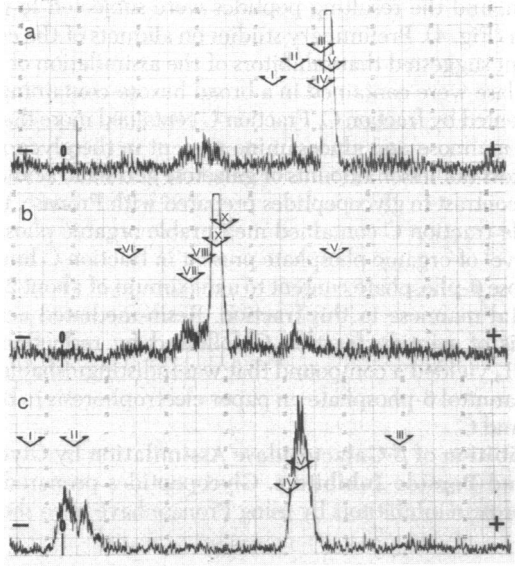

FIG. 2. Identification of [^{3}H]mannitol phosphate from the glycoprotein inhibitors. (*a*) Scan of radiochromatogram of [^{3}H]glycitol phosphate from the glycoprotein inhibitors. The compound migrating with [^{3}H]mannitol 6-phosphate was eluted from an electrophoretogram as described in Fig. 1*b* and then subjected to paper electrophoresis in buffer B. (*b*) Paper electrophoresis in buffer B of the [^{3}H]glycitol phosphate treated with acid phosphatase. (*c*) Paper electrophoresis of the [^{3}H]glycitol phosphate in buffer C. The arrows indicate the migration of authentic standards run on adjacent channels: I, [^{3}H]glucosaminitol 6-phosphate; II, L-[^{3}H]gluonic acid; III, [^{3}H]mannitol 6-sulfate; IV, *N*-acetyl[^{3}H]glucosaminitol 6-phosphate; V, [^{3}H]mannitol 6-phosphate; VI, [^{3}H]glucosaminitol; VII, *N*-acetyl[^{3}H]glucosaminitol; VIII, [^{3}H]glucitol; IX, [^{3}H]mannitol; and X, [^{3}H]galactitol.

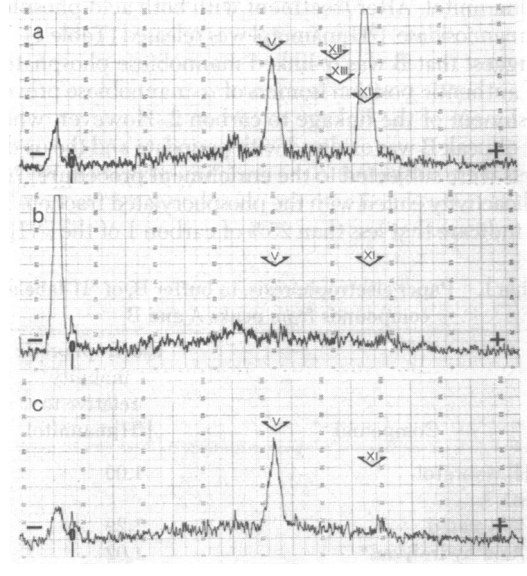

FIG. 3. Paper electrophoresis in buffer A of the phosphorylated fragment obtained by periodate oxidation of [^{3}H]mannitol phosphate. After periodate treatment the fragment was reduced with NaB^3H_4 (see text for details). (*a*) Scan of radiochromatogram of the phosphorylated fragment arising from [^{3}H]mannitol phosphate from the glycoprotein inhibitors. Authentic [^{3}H]mannitol 6-phosphate was added after periodate treatment as an internal standard. (*b*) The same sample as in *a* but treated with acid phosphatase. (*c*) A periodate-treated extract of electrophoresis paper equivalent to that from which [^{3}H]mannitol phosphate was eluted; the extract was processed in the same manner as described for [^{3}H]mannitol phosphate. The arrows mark the migration of authentic compounds on adjacent channels: V, [^{3}H]mannitol 6-phosphate; XI, [^{3}H]ethylene glycol phosphate; XII, [^{3}H]glycerol 3-phosphate; XIII, glycerol 2-phosphate.

ethylene glycol phosphate; no radioactive peak was found that corresponded to the migration of glycerol 2-phosphate. The presence of phosphate on the ^{3}H-labeled compound was confirmed by treatment with acid phosphatase (Fig. 3*b*). These results indicated that phosphate was present at carbon 6 of [^{3}H]mannitol phosphate. A blank obtained by treatment of an eluant of electrophoresis paper equivalent to that from which the [^{3}H]mannitol phosphate was eluted did not produce detectable [^{3}H]ethylene glycol phosphate (Fig. 3*c*), indicating the phosphorylated fragment did not arise from materials derived from the electrophoresis paper.

Partial Identification of Phosphorylated Oligosaccharides Present in Hydrolysates of the Glycoprotein Inhibitors. An effort was made to identify two radioactive compounds (A and B in Fig. 1*b*) that migrated on paper electrophoretograms more slowly than did [^{3}H]mannitol 6-phosphate. The migration of both A and B was altered after treatment with phosphatase (Fig. 1*c*). In experiments in which Dowex-50-mediated hydrolysis was conducted for only 7 hr, compounds A and B comprised a much larger fraction of the phosphorylated compounds. The increased yield of these compounds after short hydrolysis times suggested that A and B represented incompletely hydrolyzed fragments containing mannose 6-phosphate residues. Compounds A and B were eluted from preparative electrophoretograms that were run in the same fashion as those described in Fig. 1*b* and were analyzed as shown in Table 1. Hydrolysis of A and B with 2 M HCl for 4 hr gave [^{3}H]mannitol but no [^{3}H]mannitol 6-phosphate. Because less than 25% of the authentic [^{3}H]mannitol 6-phosphate was hydrolyzed under the same conditions, it may be assumed that the [^{3}H]mannitol moieties of A and B are not phosphorylated.

After treatment with acid phosphatase, B gave a compound that comigrated on electrophoresis with α-mannosyl-(1 → 2)-[^{3}H]mannitol. After treatment with both acid phosphatase and α-mannosidase, [^{3}H]mannitol was released (Table 1). These data suggest that B was α-linked mannobiose phosphate, but lack of authentic position isomers of α-mannobiose prevented establishment of the linkage to carbon 2. However, when an aliquot of peak B was oxidized with periodate and the oxidation products were subjected to the enrichment procedure, 76% of the radioactivity eluted with the phosphorylated fraction. These results indicate that less than 25% of carbon 1 of the [^{3}H]mannitol moiety was converted to [^{3}H]formaldehyde, a result that would be anticipated only if carbon 2 of [^{3}H]mannitol were substituted. We therefore tentatively assigned to peak B the structure α-(mannosyl-6-phosphate)-(1 → 2)-[^{3}H]mannitol.

Table 1. Paper electrophoresis, in buffer B, of ^{3}H-labeled compounds from peaks A and B.

Compound	Electrophoretic mobility relative to [^{3}H]mannitol
[^{3}H]Mannitol	1.00
Peak A	
Untreated	1.29
Acid hydrolyzed*	1.02
Peak B	
Untreated	1.53
Acid hydrolyzed*	1.01
Treated with phosphatase	0.74
Treated with phosphatase and α-mannosidase	1.00
[^{3}H]Mannitol 6-phosphate	
Untreated	1.80
Acid hydrolyzed*	1.78 (1.00)†
α-Mannosyl-(1→2)-[^{3}H]mannitol	0.74

* Acid hydrolysis was performed in 2 M HCl at 100°C for 4 hr.
† Acid hydrolysis of [^{3}H]mannitol 6-phosphate gave 24% [^{3}H]mannitol and 76% [^{3}H]mannitol 6-phosphate.

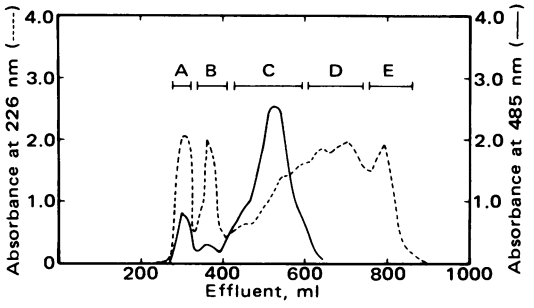

FIG. 4. Gel filtration of tryptic peptides from bovine testicular glycoprotein inhibitors. The procedures were similar to those described by Jackson and Hirs (19). Glycoprotein inhibitors (800 mg) were reduced, alkylated with iodoacetic acid, and digested with diphenylcarbamyl chloride-treated trypsin. Trypsin was inactivated with soybean trypsin inhibitor and the resulting peptides were applied to a 3 × 175 cm column of Sephadex G-50 (fine). The column was equilibrated and developed with 0.1 M ammonium acetate at pH 7.0. Aliquots of the fractions were analyzed for peptide material (absorbance at 226 nm) and hexose (absorbance at 485 nm). Fractions were pooled as indicated by the bars and repeatedly lyophilized to remove ammonium acetate.

When peak A was oxidized with periodate and subjected to the enrichment procedure, only 23% of the radioactivity eluted with the phosphorylated fraction. These results suggest that the major portion of the [^{3}H]mannitol moieties of A may be substituted at other than carbon 2.

Digestion of Glycoprotein Inhibitors with Trypsin. Glycoprotein inhibitors were reduced, alkylated, and digested with trypsin, and the resulting peptides were subjected to gel filtration (Fig. 4). Preliminary studies on aliquots of the column effluent suggested that inhibitors of the assimilation of β-galactosidase were contained in a broad hexose-containing peak represented by fraction C. Fraction C contained more than 75% of the mannose and glucosamine present in the glycoprotein inhibitors but lesser amounts of galactose and sialic acid (Table 2). In contrast to glycopeptides prepared with Pronase, tryptic peptide fraction C contained measurable organic phosphate. The level of organic phosphate present in fraction C limits the mannose 6-phosphate content to a maximum of about 2.5% of the total mannose in this fraction. Resin-mediated acid hydrolysis of peptide fraction C followed by reduction with NaB^3H_4 yielded a compound that was indistinguishable from [^{3}H]mannitol 6-phosphate on paper electrophoresis in buffers A, B, and C.

Inhibition of β-Galactosidase Assimilation by Glycoprotein and Peptide Inhibitors. Glycopeptides prepared from glycoprotein inhibititors by using Pronase have been shown to

Table 2. Analyses of tryptic peptides prepared from the glycoprotein inhibitors

Analysis	Sephadex G-50 fractions of tryptic peptides				
	A	B	C	D	E
Protein, mg	13.4	7.6	49.2	70.4	9.6
Carbohydrate, μmol					
Mannose	1.8	2.5	29.1	1.3	<0.5
Glucosamine	1.7	3.0	19.3	1.2	<0.5
Galactose	1.0	1.3	2.4	<0.5	<0.5
N-Acetylneuraminic acid	0.2	0.4	1.3	0.1	<0.1
Phosphate (organic), μmol	0.1	0.0	0.7	—	—

inhibit the assimilation of β-galactosidase by fibroblasts (5). The presently described tryptic peptide fraction C exhibited inhibitor activity approximately 5 times higher than that of glycopeptides prepared with Pronase. (Pronase glycopeptides at 0.48 μmol of mannose per ml produced 50% inhibition whereas only 0.097 μmol of mannose of the tryptic glycopeptides per ml was required.) The inhibitory activity of the tryptic peptides was only slightly less than that found for free mannose 6-phosphate (0.15 μmol/ml). However, when the mannose 6-phosphate content of the tryptic glycopeptides was used as a basis for comparison, peptide fraction C was 32 times more inhibitory than free mannose 6-phosphate. These results indicate that structural features other than the presence of mannose 6-phosphate may contribute to the effectiveness of the assimilation inhibitors. It is perhaps significant that synthetic α-mannopyranosyl-(1 → 2)-mannose has an inhibitory activity more than 17 times that of methyl α- or β-mannopyranoside (5); we have suggested that α1,2-linked mannosyl residues occur at the terminus of carbohydrate residues of β-galactosidase and contribute to the recognition marker of this enzyme (5).

DISCUSSION

Mannose 6-phosphate has long been recognized as a constituent of yeast mannans (20) and phosphomannans (21). The initial indication that mannose phosphate may be a constituent of lysosomal enzymes was in the report by Kaplan *et al.* (3) who demonstrated that mannose 6-phosphate was a potent inhibitor of the assimilation of platelet β-glucuronidase by fibroblasts. The assimilation of the same lysosomal enzymes was also markedly decreased by treating the enzymes with alkaline phosphatase, suggesting that the enzymes contained phosphate residues. Recently, phosphorylated oligosaccharides have been obtained from a preparation of α-*N*-acetylglucosaminidase (22). In the present study, mannose 6-phosphate was identified as a constituent of bovine glycoproteins that inhibited the assimilation of bovine testicular β-galactosidase by fibroblasts. The compound was identified in partial acid hydrolysates as [^{3}H]mannitol 6-phosphate after reduction with NaB^{3}H$_4$.‡ About 2% of the total mannose was phosphorylated.

It is notable that glycopeptides obtained by the action of Pronase on bovine testicular β-galactosidase exhibited no evidence for the presence of mannose 6-phosphate (5), an observation that may be due to the action of phosphatases in the Pronase preparation. However, analysis of the glycopeptides produced by the action of Pronase afforded an insight into the carbohydrate structures of β-galactosidase. Chemical and enzymatic analysis indicated the glycopeptides had the general structure

$$\alpha\text{Man} \xrightarrow{1,2} (\alpha\text{Man})_{3-4} \rightarrow \beta\text{Man} \rightarrow \beta\text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \left\{\begin{matrix}\\ \text{Asn.}\\ \\ \end{matrix}\right.$$

In the present report, partial identification of phosphorylated oligosaccharides obtained from glycoprotein inhibitors tentatively associates mannose 6-phosphate with α(1,2)-linked mannosyl residues.

Evidence is presented in this report that the recognition marker encompasses a structure larger than a single residue of mannose 6-phosphate. Glycopeptide-bound mannose 6-phosphate has a substantially higher inhibitory activity than does free mannose 6-phosphate. These results suggest that structures associated with the mannose 6-phosphate may contribute to the recognition marker. Kaplan *et al.* (24) came to a similar conclusion to explain the stronger inhibition of β-glucuronidase assimilation by phosphomannan oligosaccharides. We have previously reported that the disaccharide α-mannopyranosyl-(1 → 2)-mannose is a relatively strong inhibitor of β-galactosidase assimilation, and conceivably its occurrence in the oligosaccharide chains of the glycoprotein inhibitors may contribute to their inhibitory action. Alternatively, the greater inhibitory activity of larger polymers may result from a multiplicity of binding sites within the molecule (4, 24) or from contributions of the peptide portions of the molecules. Evidence has been reported that *N,N*-diacetylchitobiose, reported to link mannose to the polypeptide chain of β-galactosidase, does not inhibit β-galactosidase assimilation (2). Glucosamine residues of β-galactosidase may therefore play a relatively minor role in recognition phenomena.

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‡ During preparation of this manuscript, experiments similar to those described here demonstrated mannose 6-phosphate in highly purified bovine testicular β-galactosidase (23). Details of these experiments will be presented elsewhere.

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