Carbohydrate Research, 152 (1986) 183–194
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

LYSOSOMAL-ENZYME TARGETING: THE PHOSPHORYLATION OF SYNTHETIC D-MANNOSYL SACCHARIDES BY UDP-*N*-ACETYL-GLUCOSAMINE:LYSOSOMAL-ENZYME *N*-ACETYLGLUCOSAMINE-PHOSPHOTRANSFERASE FROM RAT-LIVER MICROSOMES AND FIBROBLASTS

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(Received September 4th, 1984; accepted for publication in revised form, November 22nd, 1985)

ABSTRACT

Phosphorylation of the D-mannose residues of lysosomal enzymes is essential for the uptake and intracellular transport of these enzymes to lysosomes. The GlcNAc-P-transferase which is involved in the phosphorylation reaction seems to recognize a signal, probably a protein conformation, common to many lysosomal enzymes. To evaluate the role of the carbohydrate portion of the enzyme in these phosphorylation reactions, the acceptor specificity of GlcNAc-P-transferase from rat-liver microsomes and fibroblasts was examined with the aid of synthetic D-mannosyl disaccharides and derivatives that are closely related to the high-mannose type of oligosaccharides. Four methyl D-mannobiosides were synthesized, and their structures were established by ^{13}C-n.m.r. spectroscopy. Of all the D-mannosyl saccharides tested, α-D-Man-(1→2)-α-D-Man-(1→OMe) was found to be the best acceptor, thereby suggesting that oligosaccharide structure may also have a role to play in recognition by this enzyme.

INTRODUCTION

The phospho-D-mannopyranosyl recognition-marker of lysosomal enzymes that is considered to be responsible for the targeting of newly synthesized, lysosomal enzymes to lysosomes[1,2] is generated in two steps. First, *N*-acetyl-D-glucosamine 1-phosphate is transferred to the 6-hydroxyl group situated on a D-mannose residue of high-mannose type oligosaccharides in lysosomal enzymes. This reaction is catalyzed[3] by a specific UDP-GlcNAc–lysosomal-enzyme *N*-acetylglucosaminephosphotransferase (EC 2.7.8.17; "GlcNAc-P-transferase"). In the next step, the outer *N*-acetyl-D-glucosamine residues are removed[4] by the action of an *N*-acetylglucosamine-1-phosphodiester *N*-acetylglucosaminidase (EC 3.1.4.45).

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Interest in the biochemistry of these two novel enzymes has increased, owing to recent findings that indicated a relationship between the deficiency of these enzymes and certain human genetic-diseases[5,6]

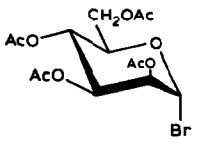
The substrate specificity of GlcNAc-P-transferase had previously been examined with the aid of certain lysosomal enzymes, nonlysosomal glycoproteins, and commercially available methyl α-D-mannopyranoside[2]. However, detailed kinetic, substrate, and product analyses concerning this enzyme have been restricted, because of the limited amounts of high-mannose-type oligosaccharides that are available[4] and because the commercially available methyl α-D-mannopyranoside is not[2] an ideal acceptor for the enzyme. Hence, we have initiated a program to synthesize chemically various D-manno-oligosaccharides sterically related to the high-D-mannose-type units of lysosomal enzymes, and to study the specificity of the enzyme by using those synthetic acceptors with the goal that these studies might establish a synthetic acceptor better than methyl α-D-mannopyranoside for measurement of GlcNAc-P-transferase.

EXPERIMENTAL

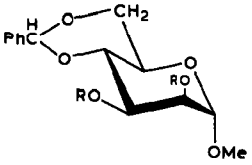
Materials and methods. — UDP-[^{14}C]*N*-acetyl-D-glucosamine was a product of New England Nuclear, Boston, MA. ACS II Scintillation Fluid was purchased from Amersham. Methyl α-D-mannopyranoside was purchased from Sigma Chemical Company.

Melting points were determined with a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured at 25° with a Perkin–Elmer 241 polarimeter. T.l.c. was conducted on plates coated with 0.2-mm layers of silica gel 60G-254 (E. Merck, Darmstadt, F.R.G.); the components were located either by exposure to u.v. light, or by spraying the plates with 10% sulfuric acid in ethanol and heating. Organic solutions were generally dried with anhydrous sodium sulfate. Silica gel used for column chromatography was Baker Analyzed (60–200 mesh). *N,N*-Dimethylformamide was distilled, and stored, over 4Å molecular sieves. Pyridine was distilled, and stored, over potassium hydroxide. Elemental analyses were performed by Robertson Laboratory, Florham Park, NJ, and Galbraith Laboratories, Inc., 2223 Sycamore Drive, P.O. Box 4187 Lonsdale, Knoxville, TN 37921. N.m.r. spectra were recorded at 25° with a Varian XL-100 instrument, ^{1}H-n.m.r. spectra at 100 MHz, and ^{13}C-n.m.r. spectra at 25.2 MHz in the Fourier-transform (F.t.) mode; the positions of the peaks are expressed in p.p.m. from the Me_4Si signal.

Methyl 2,3-di-O-benzyl-4,6-O-benzylidene-α-D-mannopyranoside (**3**). — Sodium hydride (8 g) was slowly added to a solution of methyl 4,6-*O*-benzylidene-α-D-mannopyranoside[7] (**2**; 12 g, 42.5 mmol) in dry *N,N*-dimethylformamide at ~0° with constant stirring, followed by dropwise addition of benzyl chloride (20 mL). On completion of the addition, the mixture was allowed to warm gradually to room temperature, and stirred for 20 h. The excess of sodium hydride was decomposed



1



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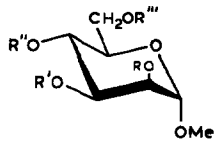
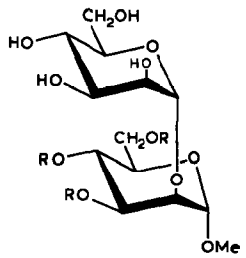
3R = Bzl
Bzl = $PhCH_2$
Ph = C_6H_5



4 R = R′ = R″ = R‴ = H
5 R = H, R′ = R″ = R‴ = Bzl
6 R′ = H, R = R″ = R‴ = Bzl
7 R″ = H, R = R′ = R‴ = Bzl
8 R‴ = H, R = R′ = R″ = Bzl



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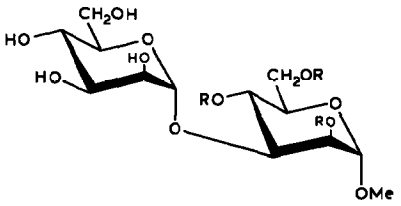
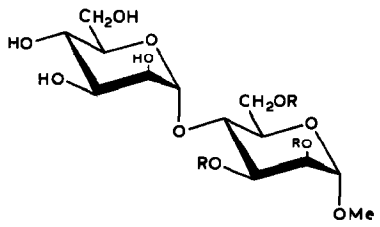
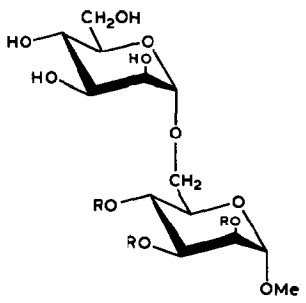
10 R = H



11 R = Bzl
12 R = H



13 R = Bzl
14 R = H



15 R = Bzl
16 R = H

with methanol, the mixture filtered through Celite, and the filtrate evaporated to a brownish syrup which was then purified on a column of silica gel, using 3:2 hexane–chloroform as the eluant, to give compound **3** (11.1 g, 85%); $[\alpha]_D$ +29.6° (*c* 1.1, $CHCl_3$), {lit.[8] $[\alpha]_D$ +26.7° (*c* 2.2, $CHCl_3$)}; ^{1}H-n.m.r. data ($CDCl_3$): δ 7.60–7.20 (m, 15 H, aromatic), 5.62 (s, PhCH), 4.85–3.65 (unresolved signals, 11 H), and 3.24 (s, 3 H, $-CH_3$).

Anal. Calc. for $C_{28}H_{30}O_6$: C, 72.73; H, 6.49. Found: C, 73.00; H, 6.53.

Methyl 2,3,6-tri-O-benzyl-α-D-mannopyranoside (**7**). — To a solution of **3** (11 g, 23.8 mmol) in dry tetrahydrofuran (350 mL) were added 3Å molecular sieves (60

g) and sodium cyanoborohydride (13.5 g), and the mixture was stirred for 0.5 h in an ice bath. Diethyl ether presaturated with hydrogen chloride was then added dropwise until the solution became acidic or the effervescence stopped. After 5 min, the mixture was poured into ice–water, and extracted with dichloromethane. The extracts were combined, successively washed with saturated sodium hydrogencarbonate and water, dried (sodium sulfate), and evaporated, to afford a syrup which was purified by chromatography on a column of silica gel, by using 4:1 (v/v) ethyl acetate–hexane as the eluant to give **7** (8.4 g, 76%) as a syrup; $[\alpha]_D$ +4.5° (*c* 1.68, $CHCl_3$); ^{1}H-n.m.r. data ($CDCl_3$): δ 7.40–7.20 (m, 15 H, aromatic), 4.80 (bs, 1 H, H-1), 4.65–3.6 (complex, 12 H, $PhCH_2$ + ring protons), and 3.27 (s, 3 H, $-CH_3$); ^{13}C-n.m.r. data ($CDCl_3$): δ 99.2 (C-1), 74.1 (C-2), 79.8 (C-3), 67.8 (C-4), 73.6 (C-5), and 70.5 (C-6).

Anal. Calc. for $C_{28}H_{32}O_6$: C, 72.41; H, 6.90. Found: C, 72.09; H, 6.90.

*Methyl 3,4,6-tri-*O*-benzyl-2-*O*-α-*D*-mannopyranosyl-α-*D*-mannopyranoside* (**9**). — To a mixture of methyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside[9,10] (**5**; 2.32 g, 5.0 mmol) and 4Å molecular sieves (2 g) in dry acetonitrile (25 mL, freshly distilled) were added mercuric bromide (2 g, 5.5 mmol) and mercuric cyanide (1.4 g, 5.5 mmol). Under stirring at room temperature tetra-*O*-acetyl-α-D-mannosyl bromide[11,12] (**1**) (3.6 g, 8.8 mmol) in dry acetonitrile (15 mL) was slowly added, with exclusion of moisture. After stirring for 6 h at room temperature, another batch of **1** (3 g, 7.3 mmol) in dry acetonitrile (10 mL) was added, and the mixture was stirred overnight at room temperature. The suspension was filtered, and the filtrate evaporated to a syrup which was dissolved in chloroform. The solution was washed successively with saturated aqueous potassium chloride, aqueous sodium hydrogencarbonate, water, and aqueous sodium chloride, dried (magnesium sulfate), and evaporated to a syrup which was dissolved in methanol (50 mL). The solution was treated with M sodium methoxide (2.5 mL), and the mixture stirred for 2 h at room temperature. The base was neutralized with Amberlite IR-120 (H^+) resin, the suspension filtered, and the filtrate evaporated to a thick syrup. Final purification was achieved by chromatography on a column of silica gel, using 1:9 methanol–chloroform as the eluant, to give compound **9** as a syrup (1.7 g, 52%); $[\alpha]_D$ +39.8° (*c* 1.28, $CHCl_3$); ^{1}H-n.m.r. data ($CDCl_3$): δ 7.4–7.2 (m, 15 H, aromatic), 5.06 (bs, 1 H, H-1), 5.00–3.6 (complex, 26 H, $-CH_2Ph$ + ring protons), and 3.24 (s, 3 H, $-CH_3$).

Anal. Calc. for $C_{34}H_{42}O_{11} \cdot H_2O$: C, 63.35; H, 6.83. Found: C, 63.65; H, 6.88.

*Methyl 2-*O*-α-*D*-mannopyranosyl-α-*D*-mannopyranoside* (**10**). — Catalytic hydrogenolysis of compound **9** (1 g, 1.6 mmol) was conducted overnight in 95% ethyl alcohol (20 mL) and glacial acetic acid (5 mL), using 10% palladium-on-charcoal (500 mg) under hydrogen at a pressure of 350 kPa. The mixture was filtered through Celite, and the filtrate evaporated to a syrup which was purified by chromatography on a column of silica gel, using 8:2 (v/v) ethyl acetate–ethanol as the eluant, to furnish compound **10** as a syrup (0.53 g, 93%) that crystallized from

aqueous methanol; m.p. 192–194°, $[\alpha]_D$ +64.6° (*c* 0.5, water) {lit.[13] $[\alpha]_D$ +72.0° (*c* 0.49, methanol)}; for ^{13}C-n.m.r. data, see Table I.

*Methyl 2,4,6-tri-*O*-benzyl-3-*O*-α-*D*-mannopyranosyl-α-*D*-mannopyranoside* (**11**). — Methyl 2,4,6-tri-O-*benzyl*-α-D-mannopyranoside[9] (**6**; 2.32 g, 5.0 mmol) in dry acetonitrile (25 mL, freshly distilled) was stirred with 4Å molecular sieves (2 g) at room temperature. To this mixture were sequentially added mercuric bromide (2 g, 5.5 mmol), mercuric cyanide (1.4 g, 5.5 mmol), and, slowly, bromide **1** (3.6 g, 8.8 mmol) in dry acetonitrile (15 mL), with exclusion of moisture. The mixture was stirred for 6 h at room temperature, another batch of bromide **1** (3 g, 7.3 mmol) in dry acetonitrile (10 mL) was added, and the mixture was stirred overnight, filtered, and the filtrate processed as described for compound **9**; compound **11** was isolated as a syrup in 74% yield (2.3 g); $[\alpha]_D$ +49.5° (*c* 0.84, $CHCl_3$); ^{1}H-n.m.r. data ($CDCl_3$): δ 7.40–7.20 (m, 15 H, aromatic), 5.07 (bs, 1 H, H-1), 4.80–3.48 (complex, 26 H, $PhCH_2$- and ring protons), and 3.26 (s, 3 H, $-CH_3$).

Anal. Calc. for $C_{34}H_{42}O_{11}$: C, 65.18; H, 6.71. Found: C, 64.67; H, 7.00.

*Methyl 3-*O*-α-*D*-mannopyranosyl-α-*D*-mannopyranoside* (**12**). — A solution of disaccharide **11** (1 g, 1.6 mmol) in 95% ethyl alcohol (20 mL) and glacial acetic acid (5 mL) was shaken overnight under hydrogen at 350 kPa in the presence of 10% palladium-on-charcoal (500 mg). The mixture was filtered through Celite, and the filtrate evaporated to a syrup which was purified as described for compound **10**, to give compound **12** as an amorphous solid (52.5 mg, 92%); $[\alpha]_D$ +103.3° (*c* 0.62, H_2O) {lit.[14] $[\alpha]_D$ +94.8° (*c* 0.31, water)}; for ^{13}C-n.m.r. data, see Table I.

*Methyl 2,3,6-tri-*O*-benzyl-4-*O*-α-*D*-mannopyranosyl-α-*D*-mannopyranoside* (**13**). — To a solution of **7** (2.32 g, 5.0 mmol) in dry acetonitrile (25 mL, freshly

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS[a] FOR METHYL D-MANNOBIOSIDES IN D_2O

Atom	*Compound*				
	4	**10**	**12**	**14**	**16**
C-1	101.9	100.3	101.7	101.6	100.4
C-2	71.0	79.4	70.5	72.0	70.9
C-3	71.7	71.3[b]	79.2	71.3	71.6
C-4	67.9	67.9	67.1	75.2	67.7[b]
C-5	73.6	73.5	74.3	72.0	71.7[c]
C-6	62.1	62.0	61.8	61.9	66.6
C in OCH_3	56.2	55.8	55.7	55.7	55.9
C-1′		103.2	103.3	102.4	102.0
C-2′		71.2[b]	71.0[b]	71.3	71.6
C-3′		71.3[b]	71.3[b]	71.3	71.9[c]
C-4′		71.0	67.9	67.6	67.5[b]
C-5′		74.3	73.7	74.7	73.9
C-6′		62.1	62.0	62.1	61.9

[a]In p.p.m. downfield from external Me_4Si (no secondary internal standard was used). [b]Indistinguishable; may have to be interchanged. [c]Indistinguishable; may have to be interchanged.

distilled) were added 4Å molecular sieves, mercuric bromide (2 g, 5.5 mmol), and mercuric cyanide (1.4 g, 5.5 mmol) with constant stirring at room temperature. Bromide **1** (3.6 g, 8.8 mmol) in dry acetonitrile (15 mL) was then slowly added, with exclusion of moisture. After stirring for 6 h at room temperature, another batch of bromide **1** (3 g, 7.3 mmol) in dry acetonitrile (10 mL) was added, the mixture was stirred overnight at room temperature and processed, and the disaccharide derivative **13** was isolated as a syrup (2.1 g, 68%) as described for compound **9**; $[\alpha]_D$ +41.8° (*c* 0.92, $CHCl_3$); ^{1}H-n.m.r. data ($CDCl_3$): δ 7.40–7.20 (m, 15 H, aromatic), 5.24 (bs, 1 H, H-1), 4.8–3.6 (complex 26 H, $PhCH_2$ and ring protons), and 3.26 (s, 3 H, $-CH_3$).

Anal. Calc. for $C_{34}H_{42}O_{11}$: C, 65.18; H, 6.71. Found: C, 65.09; H, 6.94.

Methyl 4-O-α-D-mannopyranosyl-α-D-mannopyranoside (**14**). — Debenzylation of compound **13** (1 g, 1.6 mmol) was conducted in ethyl alcohol (20 mL) and glacial acetic acid (5 mL), using 10% palladium-on-charcoal (500 mg) as the catalyst, by shaking overnight under hydrogen at 350 kPa. The mixture was filtered through Celite, and the filtrate evaporated to a syrup. Crystallization from ethyl alcohol furnished compound **14** (0.52 g, 91%); m.p. 192–194° $[\alpha]_D$ −116.7° (*c* 1.05, water) {lit.[15] $[\alpha]_D$ −101.0° (*c* 0.1, water)}; for ^{13}C-n.m.r. data, see Table I.

Methyl 2,3,4-tri-O-benzyl-6-O-α-D-mannopyranosyl-α-D-mannopyranoside (**15**). — To a stirred solution of methyl 2,3,4-tri-*O*-benzyl-α-D-mannopyranoside[16] (**8**; 2.31 g, 5.0 mmol) in dry acetonitrile (25 mL, freshly distilled) containing 4Å molecular sieves (2 g) were added mercuric bromide (2 g, 5.5 mmol) and mercuric cyanide (1.4 g, 5.5 mmol). Bromide **1** (3.6 g, 8.8 mmol) was then added with exclusion of moisture. The mixture was stirred for 6 h at room temperature, more bromide **1** (3 g, 7.3 mmol) was added, and stirring was continued overnight. After processing as described for compound **9**, the disaccharide derivative **15** was obtained as a syrup (2.5 g, 80%); $[\alpha]_D$ +62.2° (*c* 0.74, $CHCl_3$); ^{1}H-n.m.r. data ($CDCl_3$): δ 7.40–7.20 (m, 15 H, aromatic), 5.00 (bs, 1 H, H-1), 5.01–3.45 (complex, 26 H, $PhCH_2$ and ring protons), and 3.22 (s, 3 H, $-CH_3$).

Anal. Calc. for $C_{34}H_{42}O_{11}$: C, 65.18; H, 6.71. Found: C, 64.91; H, 7.00.

Methyl 6-O-α-D-mannopyranosyl-α-D-mannopyranoside (**16**). — A mixture of compound **15** (1 g, 1.6 mmol) and 10% palladium-on-charcoal (500 mg) in 95% ethyl alcohol (20 mL) and glacial acetic acid (5 mL) was shaken overnight under hydrogen at 350 kPa, the mixture filtered through Celite, and the filtrate evaporated to a syrup which was purified as described for compound **10**, to give compound **16** as an amorphous solid (0.52 g, 91%); $[\alpha]_D$ +88.3° (*c* 0.87, H_2O) {lit.[1] $[\alpha]_D$ +90.3° (*c* 0.67, H_2O)}; for ^{13}C-n.m.r. data, see Table I.

The following compounds were prepared as described in the respective references: methyl β-D-mannopyranoside[18], 2-*O*-α-D-mannopyranosyl-D-mannopyranose[19], 3-*O*-α-D-mannopyranosyl-D-mannopyranose[20], 6-*O*-α-D-mannopyranosyl-D-mannopyranose[21], and 2-acetamido-2-deoxy-3-*O*-α-D-mannopyranosyl-D-glucopyranose[22].

Enzyme assay. — Normal fibroblasts (GM 1728) and ML II fibroblasts were

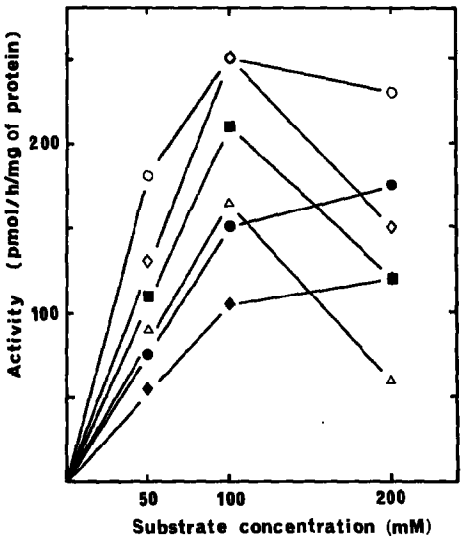


Fig. 1. The effect of the concentration of D-mannosyl saccharides on the GlcNAc-P-transferase activity from normal fibroblasts. Acceptors used included α-D-Man-(1→3)-α-D-Man-(1→OMe) (◆), α-D-Man-(1→4)-α-D-Man-(1→OMe) (■), α-D-Man-(1→6)-α-D-Man-(1→OMe) (●), α-D-Man-(1→2)-D-Man (△), α-D-Man-(1→3)-D-Man (◇), and α-D-Man-(1→6)-D-Man (○). Apparent K_m values were determined for α-D-Man-(1→OMe) and α-D-Man-(1→2)-α-D-Man-(1→OMe); the values are given in "Results and Discussion".

generously provided by Dr. Thomas Shows of the Department of Human Genetics at RPMI, and harvested by scraping with a rubber policeman in 20mM Tris·HCl, pH 7.45, containing 155mM NaCl. Rat-liver microsomes were prepared according to Waheed *et al.*[23] from female rats (200–500 g).

GlcNAc-P-transferase assay was performed according to the procedure described by Waheed *et al.*[24]. Harvested fibroblasts or liver microsomes were homogenized in the minimal volume of 80mM Tris·HCl, pH 7.45, containing 1.2% (w/v) of Triton X-100, 0.25mM 1,4-dithiothreitol, 0.16mM leupeptin, and 3.2mM 2-iodoacetamide, using a Biosonic Sonicator for 90 s. A standard incubation-mixture contained 25 pmol of UDP-[^{14}C]GlcNAc (11 MBq/mol) with 7.5 nmol of cold UDP-GlcNAc, 0.25 μmol of $MgCl_2$, 0.25 μmol of $MnCl_2$, 0.5 μmol of CDP-choline, 0.1 μmol of ADP, the appropriate disaccharide at a final concentration of 100mM (or methyl α-D-mannopyranoside at a final concentration of 200mM), and 30μL of the aforementioned homogenate containing 100–300 μg of protein, to give a final volume of 50 μL. The concentration of the D-mannosyl substrates used was found to be the saturating concentration of the acceptor under the assay conditions (see Fig. 1). The concentrations of α-D-Man-(1→OMe) and α-D-Man-(1→2)-α-D-Man-(1→OMe) used were well above their apparent K_m for the enzyme (see Results and Discussion for details). Control-assay tubes contained either water or methyl α-D-galactopyranoside instead of the disaccharide or methyl α-D-mannopyranoside. The mixture was incubated for 30 min at 37°, and the reaction then

terminated by the addition of 50 μL of 40mM EDTA. Tubes were heated for 5 min in a boiling-water bath, diluted with 1 mL of 2mM Tris base, and centrifuged at 6000*g* for 15 min at 4° in order to pelletize the precipitated protein. The supernatant liquor was applied to a column (0.5 × 1 cm) of QAE-Sephadex (Q-25-120), equilibrated and washed with 2mM Tris. The enzyme product was finally eluted with 30mM NaCl in 2mM Tris. The eluate was lyophilized, the product dissolved in 1 mL of water, and the radiolabelled product counted in a Beckman LS 9000 liquid-scintillation system. The enzyme activity is expressed as pmol of the product formed per h per mg of homogenate protein.

The apparent K_m for some of the substrates was determined by use of a double-reciprocal, Lineweaver–Burk plot.

RESULTS AND DISCUSSION

The readily accessible 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide[11,12] (**1**) has frequently been employed for the synthesis of 1,2-*trans* glycosides, and herein we describe its use for the synthesis of some desired disaccharides. Reaction of methyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside[9,10] (**5**) with bromide **1** in anhydrous acetonitrile in the presence of mercuric cyanide and mercuric bromide afforded a crude mixture of products which, on saponification followed by purification by chromatography on a column of silica gel, gave methyl 3,4,6-tri-*O*-benzyl-2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (**9**) in 52% yield. Treatment of methyl 2,4,6-tri-*O*-benzyl-α-D-mannopyranoside[9] (**6**) with bromide **1** under Helferich-reaction conditions, followed by similar processing, produced methyl 2,4,6-tri-*O*-benzyl-3-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (**11**) in 74% yield.

Reductive ring-opening of methyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-α-D-mannopyranoside (**3**) as described by Garegg *et al.*[25] provided, in 76% yield, methyl 2,3,6-tri-*O*-benzyl-α-D-mannopyranoside (**7**) free from methyl 2,3,4-tri-*O*-benzyl-α-D-mannopyranoside[16] (**8**). Reaction of alcohols **7** and **8** with bromide **1** under similar conditions, followed by saponification of the products and chromatographic separation, gave 4-*O*- and 6-*O*-α-D-mannopyranosyl derivatives **13** and **15**, respectively. Thus, the aglycon hydroxides **5, 6, 7,** and **8** all react satisfactorily with bromide **1** under the conditions of Helferich glycosylation.

Hydrogenolysis of the *O*-deacetylated derivatives **9, 11, 13,** and **15** in the presence of 10% Pd–C catalyst respectively provided the disaccharides **10** (ref. 13), **12** (refs. 14 and 26), **14** (ref. 15), and **16** (ref. 17). The overall yields of **10, 12, 14,** and **16** from **5, 6, 7,** and **8** were 48, 66, 82, and 73%, respectively. The structure of **10, 12, 14,** and **16** were confirmed by ^{13}C-n.m.r. spectroscopy. The chemical shifts and the assignments of ^{13}C-n.m.r. resonances of these four isomeric methyl mannobiosides, shown in Table I, are in close agreement with the values reported[27].

The synthesis of these methyl mannobiosides had already been accomplished by Ogawa and Sasajima[14,15,17] by using 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-manno-

pyranosyl chloride as the glycosylating agent. However, if intermediates having a "persistent" protecting group on the methyl α-D-mannopyranoside unit and a "temporary" protecting group on the α-D-mannopyranosyl residue were available, this would allow subsequent manipulation for the conversion of the D-mannopyranosyl into the corresponding D-talopyranosyl residue or phosphorylated derivatives. Thus, in the present investigation, we used such intermediates as **9** and **11**, which can be employed not only for the synthesis of D-mannobiosides but also for the synthesis of methyl *O*-2-D-talopyranosyl-α-D-mannopyranosides that are likely[28] to serve (*a*) as potential inhibitors of the title enzyme, and (*b*) for the synthesis of D-mannopyranosyl 6′-phosphate derivatives.

The phosphorylation of these D-mannosyl disaccharides by GlcNAc-P-transferase from liver microsomes and from fibroblasts, compared to that of methyl α-D-mannopyranoside, is shown in Table II. Enzyme obtained from these two different sources displayed similar activity towards the acceptors tested. Based upon these observations, we assume that both sources contain the same enzyme, having similar characteristics. Also, when fibroblasts from ML II disease, which lack GlcNAc-P-transferase[24], were used as an enzyme source, all of the disaccharides tested showed the same, or less, ability to serve as acceptor of UDP-[^{14}C]-GlcNAc as that exhibited by the controls (results not shown), which clearly indicated that it is only GlcNAc-phosphate, not GlcNAc, that has been transferred to the D-mannosyl saccharides. Most of the controls and ML II fibroblasts had <10% of normal GlcNAc-P-transferase activity. Moreover, it is unlikely that the β-GlcNAc-transferase would transfer GlcNAc from UDP-GlcNAc to these disaccharides, as a terminal α-D-Man-(1→3)-β-D-Man-(1→ sequence is the minimum requirement for β-GlcNAc-transferase activity[29].

Of all the disaccharides tested in the present studies, methyl 2-*O*-α-D-manno-

TABLE II

GlcNAc-P-TRANSFERASE ACTIVITIES[a] IN LIVER MICROSOMES AND NORMAL FIBROBLASTS, USING A VARIETY OF SYNTHETIC D-MANNOSYL SACCHARIDES

Acceptor	*GlcNAc-P-transferase activity (picomol/h/mg of protein) (mean ±s.d.)*	
	Liver	*Normal fibroblasts*
α-D-Man-(1→OMe)	162 ±57	165 ±34
α-D-Man-(1→2)-α-D-Man-(1→OMe)	361 ±43	327 ±53
α-D-Man-(1→3)-α-D-Man-(1→OMe)	71 ±16	69 ±19
α-D-Man-(1→4)-α-D-Man-(1→OMe)	101 ±18	124 ±49
α-D-Man-(1→6)-α-D-Man-(1→OMe)	165 ±13	158 ±49
α-D-Man-(1→2)-D-Man	173 ±69	152 ±47
α-D-Man-(1→3)-D-Man	92 ±42	59 ±13
α-D-Man-(1→6)-D-Man	193 ±23	191 ±22
α-D-Man-(1→3)-D-GlcNAc	44 ±11	4 ±20
β-D-Man-(1→OMe)	49 ±16	58 ±23

[a]Results are the mean ±s.d. of 9 sets of experiments in duplicate.

pyranosyl-α-D-mannopyranoside was found to be the best acceptor for phosphorylation. In fact, the apparent K_m for this disaccharide was about one-fifth that of methyl α-D-mannopyranoside. The apparent K_m values for methyl α-D-mannopyranoside and α-D-Man-(1→2)-α-D-Man-(1→OMe) were 111 and 20mM, respectively, when fibroblast homogenate was used as an enzyme source. The apparent K_m values for UDP-GlcNAc were 40 and 74μM, respectively, when normal fibroblast and liver microsomes were used as enzyme sources and methyl 2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside as the acceptor. The rate of formation of product under the assay conditions was found to be constant over the incubation period of 60 min (see Fig. 2), and proportional to the amount of liver microsomes added, up to 300 μg of protein (see Fig. 3).

Although α-D-Man-(1→2)-Man is a more potent inhibitor of enzyme pinocytosis than are D-mannose and methyl α- and β-D-mannopyranoside[30], this disaccharide is a poorer acceptor for GlcNAc-P-transferase than is α-D-Man-(1→2)-α-D-Man-(1→OMe). Probably, the strict α configuration of the D-mannose residue is a requirement for the enzyme phosphorylation. This was confirmed by the observation that methyl β-D-mannopyranoside is a poor acceptor for this enzyme (see Table I).

The disaccharide α-D-Man-(1→6)-α-D-Man-(1→OMe) behaves similarly to methyl α-D-mannopyranoside as an acceptor, whereas α-D-Man-(1→6)-D-Man provided a slightly higher activity. On the other hand, the α-D-(1→3)-linked D-mannosyl disaccharide derivatives α-D-Man-(1→3)-α-D-Man-(1→OMe), α-D-Man-(1→3)-

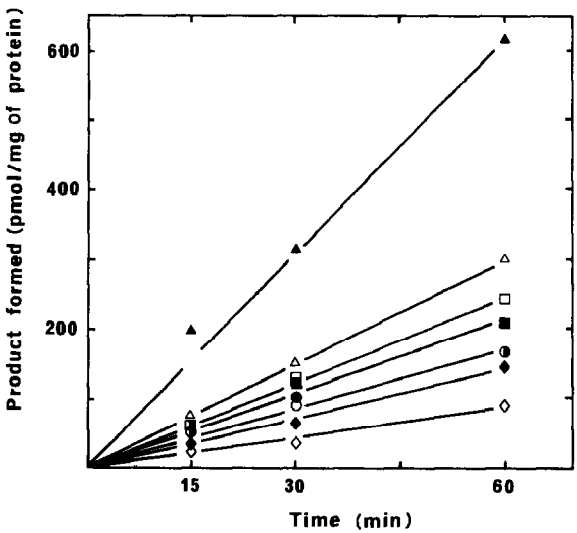


Fig. 2. The effect of incubation time on GlcNAc-P-transferase activity from liver microsomes. The assays used saturating concentration of the D-mannosyl saccharides. Acceptors used included α-D-Man-(1→OMe) (□), α-D-Man-(1→2)-α-D-Man-(1→OMe) (▲), α-D-Man-(1→3)-α-D-Man-(1→OMe) (◆), α-D-Man-(1→4)-α-D-Man-(1→OMe) (■), α-D-Man-(1→6)-α-D-Man-(1→OMe) (●), α-D-Man-(1→2)-D-Man (△), α-D-Man-(1→3)-D-Man (◇), and α-D-Man-(1→6)-D-Man (○).

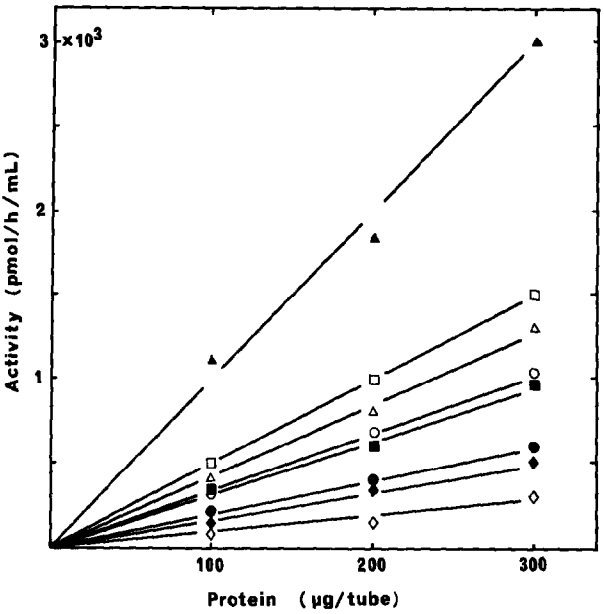


Fig. 3. Amount of protein *versus* activity of GlcNAc-P-transferase from liver microsomes. The assays used saturating concentration of the D-mannosyl saccharides. Acceptors used included α-D-Man-(1→OMe) (□), α-D-Man-(1→2)-α-D-Man-(1→OMe) (▲), α-D-Man-(1→3)-α-D-Man-(1→OMe) (◆), α-D-Man-(1→4)-α-D-Man-(1→OMe) (■), α-D-Man-(1→6)-α-D-Man-(1→OMe) (●), α-D-Man-(1→2)-D-Man (△), α-D-Man-(1→3)-D-Man (◇), and α-D-Man-(1→6)-D-Man (○).

D-Man, and α-D-Man-(1→3)-D-GlcNAc were found to be poor acceptors, particularly when compared to α-D-Man-(1→2)-α-D-Man-(1→OMe). Structural analyses of the phosphorylated oligosaccharides on various newly synthesized lysosomal enzymes have been performed. Interestingly, the existence of α-D-Man-(1→2)-D-Man disaccharide units having phosphoric ester (mono or di) groups on the outer chains of the carbohydrate moiety of these lysosomal enzymes has been tentatively established[31]. These structural studies seem to suggest the preference of these disaccharide units for phosphorylation by GlcNAc-P-transferase.

Our present studies clearly show that α-D-Man-(1→2)-α-D-Man-(1→OMe) is the acceptor preferred over other D-mannobiose disaccharide derivatives; this strongly supports the contention of Waheed *et al.*[32] that GlcNAc-P-transferase recognizes in lysosomal enzymes the carbohydrate moiety, in addition to the protein moiety reported earlier[33].

ACKNOWLEDGMENTS

We thank Drs. Richard DiCioccio, Thomas O. Mueller, and S. A. Abbas for valuable discussions with them, Conrad Piskorz and Dr. Shin Yazawa for their help in the preparation of the manuscript, and Mrs. Marie Vallina for kindly typing the manuscript. This investigation was supported by PHS Grant Number GM 31425 awarded by the National Institute of General Medical Sciences, DHHS.

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