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BIOSYNTHESIS, IN CALF PANCREAS MICROSOMES, OF THREE LIPID-LINKED OLIGOSACCHARIDE DIPHOSPHATES FROM A SYNTHETIC DOLICHYL DIPHOSPHATE TETRASACCHARIDE*

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ABSTRACT

Incubation of calf pancreas microsomes with synthetic α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol and GDP-D-[^{14}C]-mannose gave three major lipid-linked oligosaccharide diphosphates. After release of the phospholipid residue by mild acid hydrolysis, the corresponding [^{14}C]oligosaccharides were analyzed by gel-filtration, liquid chromatography, degradation by endo-*N*-acetyl-β-D-glucosaminidases D and H, by jack bean α-D-mannosidase and *Aspergillus oryzae* (1→2)-α-D-mannosidase, acetolysis, and binding to concanavalin A-Sepharose. From the results it could be inferred that the following reaction took place in calf pancreas microsomes: α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol + GDP-D-Man gave GDP + α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol. The next products to be formed were α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol, followed by α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol. The mannose incorporation was enhanced by Triton X-100 and inhibited by Mn^{2+}, and it occurred in the presence of either Mg^{2+} or EDTA. It is likely that the mannose donor was GDP-mannose since, under the conditions used, the formation of dolichyl mannosyl phosphate was negligible and the dolichyl heptasaccharide diphosphate accumulated.

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INTRODUCTION

The biosynthesis of *N*-linked oligosaccharide chains is thought to proceed by a multistep pathway starting by the addition of a 2-acetamido-2-deoxy-D-glucosyl phosphate unit to a dolichyl phosphate carrier[1,2]. A second 2-acetamido-2-deoxy-D-glucose residue is bound to the thus initiated saccharide-*PP*-lipid, and then five residues of mannose are transferred from GDP-D-mannose. These early steps seem highly ordered, since, at each step, only one major intermediate is synthesized[3]. Most of the previous work dealing with this problem, however, employed an *in vitro* system supplemented by a radioactive sugar donor, such as UDP-GlcNAc or GDP-Man[4,5] and, even when attempts were made to purify them[6], the endogenous acceptor(s) was (were) not well defined, leading to confusing results. For instance, beside the major intermediate synthesized at each step, several minor products were described[7], the role of which is totally unknown; perhaps they are used by the cell for further biosynthesis, or they may act as regulatory compounds.

For these reasons, our laboratory has used, for several years, chemically synthesized, exogenous substrates to specify the discrete reactions involved in *N*-linked oligosaccharide-chain biosynthesis[8,9]. Previously, we have shown the formation of α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol starting from synthetic α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol and GDP-D-[^{14}C]mannose as precursors[10]. We describe herein the biosynthesis, in calf pancreas microsomes, of α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol starting from α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol, together with lipid-linked hexa- and hepta-saccharide diphosphates having, respectively, one and two extra mannosyl residues on the α-(1→3) branch of the lipid-linked trimannosyl core.

RESULTS AND DISCUSSION

When the synthetic substrate α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol was incubated with calf pancreas microsomes and GDP-[^{14}C]mannose, 10 to 15% of the radioactivity was extracted by chloroform–methanol and by chloroform–methanol–water. No radioactivity was found in the protein pellet. Analysis by thin-layer chromatography of both extracts showed the presence of three radioactive spots, oligosaccharide-lipids A, B, and C, that migrated more slowly than the starting substrate (Fig. 1). Maximum incorporation of ^{14}C into oligosaccharide-lipids A, B, and C was obtained at pH 6.2. However, the ratio of incorporation varied with the pH; at a higher pH value it was about the same, whereas at a lower pH oligosaccharide-lipid C was favored. The incorporation was not dependent on a divalent cation, as it was similar in the presence of Mg^{2+} or EDTA. However, it decreased with increasing concentration of Mn^{2+} (1 to 10mM). The rates of enzymic synthesis of the respective oligo-

saccharide-lipids varied with the concentration of acceptor substrate (Fig. 2); oligosaccharide-lipid C increased almost linearly as the substrate was increased from 5 to 50 μg, oligosaccharide-lipid B exhibited a similar pattern but the increase was

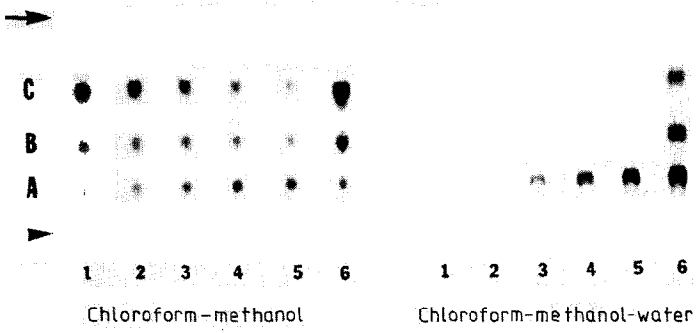

Fig. 1. Thin-layer chromatography–autoradiography of the ^{14}C-labeled lipid-linked oligosaccharides A, B, and C. Time course: (1) 5 min, (2) 10 min, (3) 20 min, (4) 30 min, (5) 60 min, and (6) 30 min (without backwashing). The radioactive products were formed by the incubation of calf pancreas microsomes with an exogenous substrate, α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol, and GDP-[^{14}C]mannose under standard conditions, as described in Experimental. Symbols: ▶, origin; ➔, migration position of synthetic acceptor as revealed by the anisaldehyde spray.

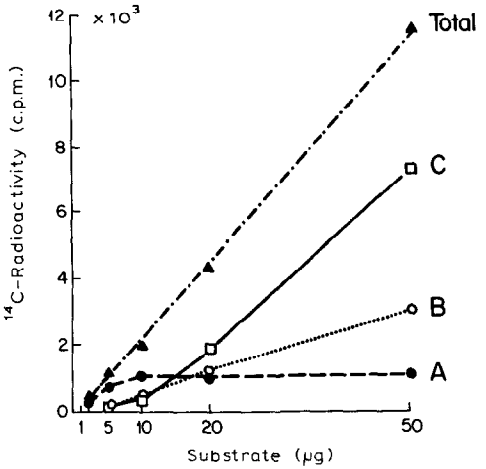

Fig. 2. Enzymic synthesis of lipid-linked oligosaccharides A, B, and C as a function of the synthetic substrate concentation under standard incubation conditions. The extracts of the incubation mixtures were analyzed by t.l.c. as described in the Experimental section. Each point is the sum of the counts for the particular lipid-linked oligosaccharide in the two extracts (chloroform–methanol and chloroform–methanol–water) of an incubation mixture.

less steep, and oligosaccharide-lipid A reached a plateau at a substrate level of 10 μg, suggesting that the synthesis of oligosaccharide-lipid A is a rate-limiting step in

this pathway. With time and in the continuous presence of GDP-[^{14}C]mannose, the pattern of incorporation into the three oligosaccharide-lipids A, B, and C evolved in a different way for each compound (Fig. 3a), suggesting a precursor-product relationship. This was clarified by a pulse-chase experiment, which showed clearly that the radioactivity decreased in oligosaccharide-lipids C and B at very similar rates, and disappeared after 5 min of chase, whereas the radioactivity in oligosaccharide-lipid A increased and reached a plateau, also after a chase of 5 min (Fig. 3b). Preliminary experiments (data not presented) showed that the radioactivity in oligosaccharide-lipid A remained at a constant level for at least 3 hours, indicating that the compound was not being consumed.

As previously pointed out[9], the presence of Triton X-100 enhanced the ^{14}C incorporation into lipid-linked oligosaccharides when the synthetic substrate was added to the reaction medium, and its presence might also be responsible[11,12] for the negligible formation of [^{14}C]Man-*P*-Dol. As the synthesis of Man-*P*-Dol also requires[11] the presence of Mn^{2+}, the use of Mg^{2+} in the present work and the absence of exogenous Dol-*P* in the starting material resulted in the absence of [^{14}C]Man-*P*-Dol. This absence was important, because Man-*P*-Dol is the mannosyl donor for the elaboration from the $Man_5(GlcNAc)_2$- to the $Man_9(GlcNAc)_2$-*PP*-Dol stage[13,14]. Therefore, minimal Man-*P*-Dol formation is consistent with the t.l.c. evidence that the biosynthetic process stopped at the Man_5 stage.

Mild acid hydrolysis released three oligosacharides (A, B, and C), which

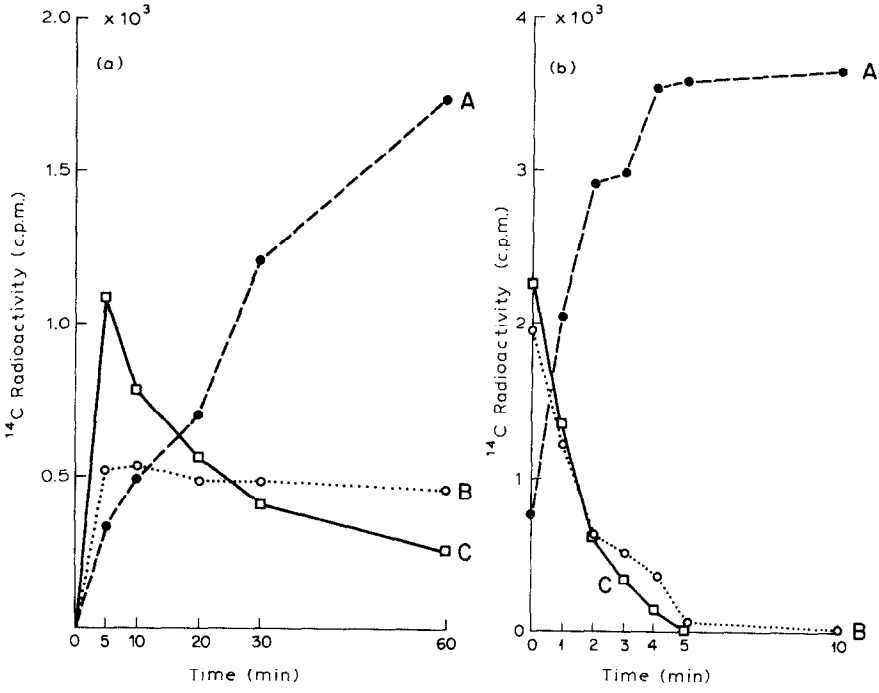

Fig. 3. Time course of the enzymic synthesis of lipid-linked oligosaccharides A, B, and C: (a) in the continuous presence of GDP-[^{14}C]mannose; (b) after a 5-min pulse with GDP-[^{14}]mannose, and then a 10-min chase with cold GDP-mannose (see Experimental). The data were obtained in the same way as those plotted in Fig. 2.

were purified by gel filtration over Bio-Gel P-4. These products cochromatographed (t.l.c.) with standard $Man_5(GlcNAc)_2$, $Man_4(GlcNAc)_2$, and $Man_3(GlcNAc)_2$, respectively. Analysis by l.c. gave the same results, based on comparison with standard oligosaccharides from loco-sheep urine[15]. The three oligosaccharides were resistant to the action of endo-*N*-acetyl-β-D-glucosaminidase H (data not shown), and only oligosaccharide C was sensitive to endo-*N*-acetyl-β-D-glucosaminidase D, which removed the reducing 2-acetamido-2-deoxy-D-glucose residue. Thus, oligosaccharide C is a pentasaccharide having two 2-acetamido-2-deoxy-D-glucose residues and, hence, three mannose residues. From the known specificity[16] of endo-*N*-acetyl-β-D-glucosaminidase D, a branched structure (**1**) is likely for oligosaccharide C. The resistance of oligosaccharides A and B to endo-*N*-acetyl-β-D-glucosaminidases D and H suggested that both have a substituted α-D-mannopyranosyl-(1→3) residue and lack the α-D-Man*p*-(1→3)-α-D-Man*p*-(1→6)-D-Man structure required for endo-*N*-acetyl-β-D-glucosaminidase H activity[17].

The three oligosaccharides A, B, and C bound to concanavalin A-Sepharose, thus confirming a branched structure for oligosaccharide C, as the other possible $Man_3(GlcNAc)_2$ isomers that could result from the addition of an α-D-mannopyranosyl group to the α-D-Man*p*-(1→6) branch would not bind to concanavalin A-Sepharose[18]. The binding of oligosaccharides A and B to concanavalin A-Sepharose reduced the number of possible structures for these two oligosaccharides.

Jack bean α-D-mannosidase treatment of oligosaccharides A, B, or C gave free [^{14}C]mannose, as monitored by l.c. or Bio-Gel P-2 filtration, indicating that the D-[^{14}C]mannopyranosyl groups were added as the α anomer. Oligosaccharide C was insensitive to (1→2)-α-D-mannosidase treatment, only 1% of the [^{14}C]mannose being released (Bio-Gel P-2 filtration). In contrast, oligosaccharides B and A were partially degraded by the (1→2)-α-D-mannosidase, which liberated [^{14}C]mannose representing 55 and 75%, respectively, of their total radioactivity (as compared with expected values of 50 and 66%). Further treatment of the first radioactive peak from the Bio-Gel P-2 column [product of the digestion of oligosaccharide A or B with (1→2)-α-D-mannosidase] with endo-*N*-acetyl-β-D-glucosaminidase D gave the same result as the treatment of oligosaccharide C with this enzyme, *i.e.*, a shift in the l.c. position of the solute from that of $Man_3(GlcNAc)_2$ to that of $Man_3GlcNAc$.

On acetolysis of the three borohydride-reduced oligosaccharides A, B and C, only one mannose residue was released in each case, as shown by l.c. analysis (see Fig. 4). Owing to the undesired cleavage between the 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucitol residues[19], there were small proportions of the corresponding products having only the penultimate 2-acetamido-2-deoxy-D-glucose residue (as the glucitol). The early-eluted radioactively-labeled peaks were interpreted as random products of degradation; they represent less than one third of the total recovered radioactivity. As α-D-(1→6)-linked residues are preferentially cleaved by acetolysis, it is clear that, in each product, the α-(1→6)-

α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc
3
↑
1
α-D-Man*p*

1

α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc
3
↑
1
α-D-Man*p*-(1→2)-α-D-Man*p*

2

α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Glc*p*N
3
↑
1
α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*

3

linked D-mannose residue was not substituted. Thus, the addition of D-[^{14}C]-mannose residues during the incubation could have taken place only by first forming, then extending, the α-D-(1→3)-linked branch of the β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc core. This conclusion is in agreement with the results of the digestion with specific (1→2)-α-D-mannosidase. In one set of experiments, the main product of acetolysis of oligosaccharides B and A was isolated by l.c. and investigated for binding to concanavalin A-Sepharose. For oligosaccharides B and A 94 and 96%, respectively, of the radioactivity bound to the column and was eluted by methyl α-D-mannopyranoside, showing that the products of acetolysis had the structures required for Con-A binding[18].

The aforementioned results showed unambiguously the structures of oligosaccharides A, B, and C. Oligosaccharide C has structure **1** since it was sensitive to endo-*N*-acetyl-β-D-glucosaminidase D and not H, but insensitive to (1→2)-α-D-mannosidase. It bound to concanavalin A–Sepharose, its acetolysis only released the nonradioactive (1→6)-linked α-D-mannopyranosyl group, and jack bean α-D-mannosidase released all the α-D-mannosyl residues, including the radioactively-labeled ones.

Oligosaccharides B and A shared common structural properties, except that

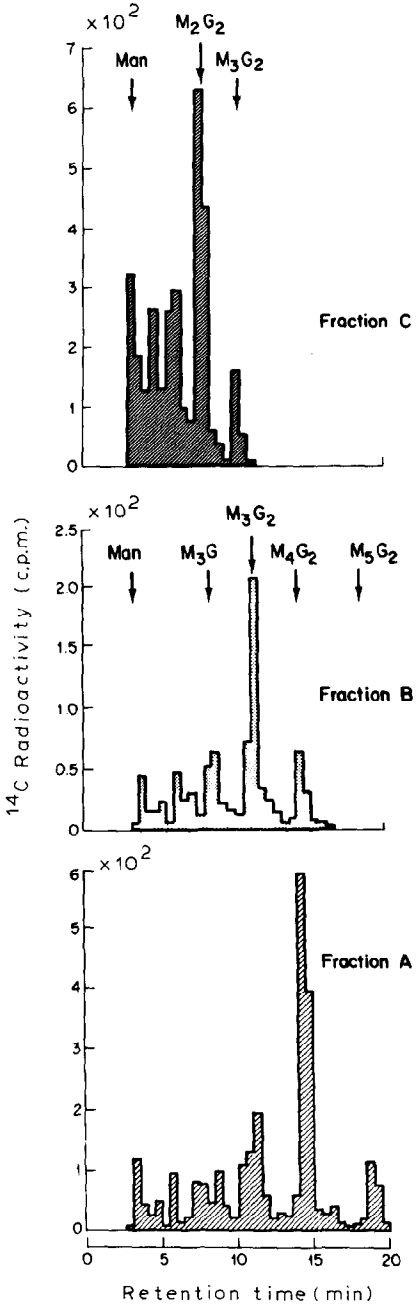

Fig. 4. Liquid chromatography of the acetolysis products from ^{14}C-labeled oligosaccharides C, B, and A, as described in the Experimental section. The elution positions of oligosaccharide standards are indicated by abbreviated formulas in which M is D-mannose, and G is *N*-acetyl-D-glucosamine; the standards are common for products A and B.

oligosaccharide B behaved as a $Man_4(GlcNAc)_2$ and oligosaccharide A as a $Man_5(GlcNAc)_2$ compound. Both bound to concanavalin A, both were resistant to endo-*N*-acetyl-β-D-glucosaminidases D and H, and both were sensitive to jack bean α-D-mannosidase, which released all the radioactively-labeled mannosyl residues. (1→2)-α-D-Mannosidase removed one D-mannosyl residue from oligosaccharide B and two from A. Acetolysis reduced the size of oligosaccharides B and A by one nonradioactive mannosyl residue each, confirming that the α-D-(1→6) branch was not substituted during the incubation. Taken together, these results suggested structures **2** and **3**, respectively, for oligosaccharides B and A.

It is apparent that (1→3)-α-D-mannosyltransferase is able to catalyze the donation, by GDP-mannose, of a D-mannopyranosyl group to α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol. This enzyme is probably different from the (1→6)-α-D-mannosyltransferase characterized by Sasak *et al.*[10], as in that case the optimum pH as well as the cation requirement were different: Mg^{2+}, Mn^{2+}, and Ca^{2+} were equally active, and EDTA was strongly inhibitory. On the contrary, the present (1→3)-α-D-mannosyltransferase is likely to be the same as the one described by Herscovics *et al.*[9], as the cation effect was the same: inhibition by Mn^{2+} and insensitivity to EDTA. This suggests that the (1→3)-α-D-mannosyltransferase from calf pancreas can accomodate either β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol or α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol as acceptors. In other words, this enzyme does not have enough specificity to distinguish whether or not the acceptor contains an α-D-Man*p*-(1→6) residue. But it must be pointed out that the Man*p*-(1→6)-containing intermediate has never been characterized in calf pancreas microsomes or in other tissues, such as bovine lactating-gland microsomes[4] and Chinese hamster ovary cells[3]. Perhaps this intermediate was not actually looked for or identified, because it may have resulted from a minor biosynthetic pathway. Furthermore, from the pulse-chase experiment, it seems that the transformation $Man_2(GlcNAc)_2$-*PP*-Dol to $Man_4(GlcNAc)_2$-*PP*-Dol was very fast, as the maximum incorporation of radioactivity into oligosaccharides C and B occurred within 5 min after the start of incubation. Finally, most of the previous research on *N*-glycan-chain biosynthesis used endogenous acceptors which were not well-defined and were present in very low amounts[5,12,14].

Concerning the (1→2)-α-D-mannosyl transfer from GDP-mannose to $Man_3(GlcNAc)_2$-*PP*-Dol to give the hexa-, and then the hepta-saccharide-*PP*-Dol, the results agree with the scheme proposed by Chapman *et al.*[3]. Furthermore, it seems that, at each of the steps involved in this study, only a single oligosaccharide isomer could be clearly identified, confirming also that this pathway is highly ordered[3], at least in its early steps.

EXPERIMENTAL

Materials. — The dolichyl tetrasaccharide diphosphate α-D-Man*p*-(1→6)-β-

D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*PP*-Dol was synthesized from dolichyl phosphate and the peracetyl derivative of the tetrasaccharide phosphate α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*NAc-*P*, which had been synthesized from a tetrasaccharide isolated from bovine mannosidosis urine[15]. The coupling of the "activated" Dol-*P* and peracetylated tetrasaccharide phosphate was performed as described previously[20]. The GDP-[^{14}C]mannose (10.4 GBq/mmol) and sodium [^{3}H]borohydride (19.5 GBq/mmol) were obtained from New England Nuclear (Billerica, MA). Endo-*N*-acetyl-β-D-glucosaminidases D and H were purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and from Miles (Naperville, IL), respectively. Jack bean α-D-mannosidase was purchased from Boehringer Mannheim (Indianapolis, IN), concanavalin A-Sepharose from Pharmacia Fine Chemicals (Piscataway, NJ), and precoated plates of Silica gel G (0.25 mm thick) from E. Merck (Darmstadt, W. Germany). All reagent chemicals were from Sigma or Fisher. The oligosaccharide standards, $Man_2(GlcNAc)_2$ to $Man_5(GlcNAc)_2$, and $Man_2GlcNAc$ to $Man_5GlcNAc$, were isolated from the urine of swainsonine-poisoned sheep[21]. The cation- and anion-exchange resins used were AG 50W-X8 and Ag 1-X8, respectively, from Bio-Rad Laboratories (Richmond, CA).

Chromatographic methods. — High pressure liquid chromatography (l.c.) (at ~7 MPa) was performed with an model 5020 instrument (Varian Associates, Palo Alto, CA), equipped with a microcomputer-based, interactive CRT–keyboard control for solvent and flow programming. Oligosaccharides were eluted isocratically from a reversible Amino-Spherisorb-5 μm column ("Hi-chrom", Regis Chemical Co., Morton Grove, IL) by 7:3 (v/v) acetonitrile–15mM potassium phosphate buffer (pH 5.2) flowing at 2 mL/min. Peaks were detected by absorbance at 195 nm with a variable wavelength u.v. detector (Erma Optical Co. Japan). When needed, the oligosaccharides were reduced by treatment with sodium borohydride overnight at room temperature[22]. When radioactive samples were analyzed, 30-s fractions were collected and their radioactivity counted.

Bio-Gel P-4 (minus 400 mesh) and Bio-Gel P-2 (200–400 mesh) (Bio-Rad, Richmond, CA) were used in 0.1M pyridinium acetate (pH 5) and water, respectively. Concanavalin A-Sepharose affinity chromatography was carried out as follows. The gel (100 μL) was introduced into a Pasteur pipette and washed with "Con A-buffer" (10mM $MnCl_2$, 10mM $CaCl_2$, and 10mM $MgCl_2$ in water). After the sample was applied, the column was eluted with the same buffer (1.2 mL), and then with 100mM methyl α-D-mannopyranoside (1.2 mL) in the same buffer. The radioactivity was counted on aliquots (120 μL) added to 3 mL of Hydrofluor scintillation cocktail (National Diagnostics, Highland Park, NJ).

Chloroform–methanol and chloroform–methanol–water extracts were applied to silica gel plates, which were developed three times in the solvent system 60:35:6 (v/v) chloroform–methanol–water. The radioactive products were detected by a 3 day contact with Kodak X-Omat AR film. When necessary, the radioactive spots were scraped out and eluted with 10:10:3 (v/v) chloroform–

methanol–water. To locate the standard oligosaccharides, plates were sprayed with the anisaldehyde reagent[23] after autoradiography. The various radioactive products were isolated by preparative t.l.c. under the same conditions.

Acetolysis. — This procedure was performed according to Tai *et al.*[24], except that the acetolyzate was kept for 16 h at 30°, or for 9.5 h at 35° (ref. 25), and a $NaBH_4$ reduction was performed before and after acetolysis. The products were identified by l.c. analysis.

Glycosidase treatments. — Digestion with endo-*N*-acetyl-β-D-glucosaminidase D was performed as previously described[10]. Digestion with endo-*N*-acetyl-β-D-glucosaminidase H was performed as described by Sasak *et al.*[26], except that the buffer was 50mM Tris maleate (pH 5.8), and 2 milliunits of enzyme were added. Digestion with jack bean α-D-mannosidase was performed as described by Sasak *et al.*[26], except that 2.5 units of enzyme were added at zero time only. Digestion products were characterized by l.c. or Bio-Gel P-2 chromatography. Oligosaccharides A, B, and C were treated with a crude (1→2)-α-D-mannosidase from *Aspergillus oryzae* (kindly provided by I. K. Vijay). This enzyme was prepared by extraction and precipitation by ammonium sulfate and acetone according to Yamamoto *et al.*[27], and was not further purified. The crude enzyme was shown to contain almost no other mannosidase activity, and the incubation was done in presence of swainsonine to inhibit any residual (1→3)- or (1→6)-α-D-mannosidase activity. Therefore a typical incubation was performed as follows: Oligosaccharide A, B, or C (30,000 or 50,000 c.p.m.) was incubated with the enzyme preparation (5 μL, 0.7 mg of protein) in 0.1M sodium acetate (45 μL, pH 5.3) in the presence of swainsonine (0.15 μg) for 30 min at 37°. The sample was boiled for 3 min and, after centrifugation, the supernatant was applied to a Bio-Gel P-2 column (30 × 1 cm) in water; fractions (350 μL) were collected and the radioactivity was counted on 25-μL aliquots. The fractions comprising the first radioactive peaks from oligosaccharides A and B were in each case pooled and then treated by endo-*N*-acetyl-β-D-glucosaminidase D as described above.

Incubation conditions. — The standard incubation mixture contained synthetic dolichyl tetrasaccharide diphosphate (5 μg as measured by anthrone assay), 5mM $MgCl_2$, 0.5% Triton X-100 in 40mM Tris maleate buffer (pH 6.2), and calf pancreas microsomes (2 mg of protein) prepared as previously described[8]. GDP-[^{14}C]mannose (185 kBq) was added to start the reaction, which proceeded for 30 min at 37°. The final volume was 500 μL. The reaction was stopped, products were extracted by the addition of 2:1 (v/v) chloroform–methanol (2.5 mL), and the labeled products further extracted with 2:1 (v/v) chloroform–methanol, and then 10:10:3 (v/v) chloroform–methanol–water (3 times each). The extracts were dried separately under N_2, redissolved in a known volume of 2:1 (v/v) chloroform–methanol or 10:10:3 (v/v) chloroform–methanol–water, and aliquots were counted for radioactivity and analyzed by t.l.c.

In further experiments this same protocol was used, with variation in the

metal salt, the pH value (5–8), the amount of substrate (1–50 μg), and the time of incubation (5–60 min).

A pulse-chase experiment was performed as follows. The incubation mixture was scaled up ten times, with maintenance of the proportions of every constituent. The pulse was 5 min long and the chase was performed by adding cold GDP-mannose (100 nmol, 100 times the amount of GDP-[^{14}C]mannose). Aliquots (500 μL) were taken out at 0, 1, 2, 3, 4, 5, and 10 min, discharged into 2:1 (v/v) chloroform–methanol (2.5 mL), and processed as just described. The distribution of the radioactive lipid-linked oligosaccharides was monitored by t.l.c. and autoradiography as described above, then the spots were scraped out and their radioactivity counted.

Oligosaccharide release. — The oligosaccharide residues were released from the lipid intermediates by mild acid treatment (20mM HCl, 30 min, 100°) followed by an extraction with 2:1 (v/v) chloroform–methanol. The aqueous phase was made neutral with 20mM NaOH.

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