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# Comparative rates of transfer of lipid-linked oligosaccharides to endogenous glycoprotein acceptors *in vitro*

**(dolichol phosphate oligosaccharides/glycoprotein processing/asparagine-linked glycopeptides)**

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**ABSTRACT** We have shown previously that particulate preparations of Nil 8 fibroblasts catalyze the synthesis of two oligosaccharide–lipids: one composed of *N*-acetylglucosamine and mannose residues and the other containing, in addition to mannose and *N*-acetylglucosamine, one or two glucose residues. These two oligosaccharide–lipids were purified and added to fresh microsomal preparations. In comparative studies, we find that the glucose-containing lipid-linked oligosaccharide is transferred much more rapidly to endogenous protein acceptors than the glucose-free compound. With materials of comparable specific activities, as much as 41% of the glucose-containing oligosaccharide was transferred to protein as compared to 5% for the glucose-free compound. These results suggest that the attachment of glucose to mannosyl lipid-linked oligosaccharide serves an important role in the transfer of these compounds from lipid carrier to protein acceptor.

Recent studies have shown that dolichol-linked oligosaccharides synthesized by tissue slices (1–3) and generated with microsomal preparations (4, 5) may contain glucose in addition to *N*-acetylglucosamine and mannose. The participation of these glucose-containing oligosaccharide–lipids in the glycosylation of protein, though implied, has not been demonstrated. Evidence for *in vitro* transfer to protein of similar oligosaccharide–lipids that do not contain glucose has been well established (see ref. 6 for a review). The only reports of the transfer of glucose-containing materials from lipid-linked oligosaccharides to glycoprotein acceptors have come from Leloir and coworkers (7, 8). However, in these studies it was unclear whether the glucose-containing oligosaccharide being transferred contained mannose or other sugar constituents in addition to glucose.

In a previous paper from our laboratory, evidence was presented indicating that microsomal preparations from Nil 8 fibroblasts catalyze the transfer of *N*-acetylglucosamine and mannose from their respective nucleotide sugars to a lipid acceptor (4). Upon addition of 0.2 μM UDP-glucose, this oligosaccharide–lipid and also a larger, glucose-containing oligosaccharide–lipid are synthesized. In this paper we report comparative studies concerning the transfer of each of these oligosaccharides from the lipid to protein in an *in vitro* system. We demonstrate that the glucose-containing oligosaccharide is transferred at a faster rate, and to a greater extent, than is the glucose-free oligosaccharide.

## MATERIALS AND METHODS

**Materials.** UDP-[*1*-$^3$H]Glc (4.85 Ci/mmol), GDP-[*U*-$^{14}$C]Man (150 mCi/mmol), and sodium [$^3$H]borohydride (227 mCi/mmol), were purchased from New England Nuclear Corp. Other reagents were obtained as follows: UDP-glucose, GDP-mannose, UDP-*N*-acetylglucosamine from Sigma Chemical Co.; Sephadex LH-20 from Pharmacia Fine Chemicals Inc., and Bio-Gel P-6 from Bio-Rad Laboratories.

**Cell Culture and Membrane Preparation.** Nil 8 cells were maintained and microsomal preparations were obtained as described (4).

**Preparation of Oligosaccharide–Lipids.** The final concentrations of the components in the standard incubation mixture (0.08 ml) were as follows: 20 mM Tris·HCl, pH 7.4, containing 0.15 M NaCl (Tris-buffered saline), 0.4 mM $MnCl_2$, and 20 μM UDP-*N*-acetylglucosamine. In order to generate the respective radiolabeled oligosaccharide–lipids, the following components were added to the incubation mixture: 2 μM GDP-[$^{14}$C]Man ($8 \times 10^4$ cpm) to form [*Man*-$^{14}$C]oligosaccharide–$lipid_{II}$; 2 μM GDP-[$^{14}$C]Man and 1 μM UDP-Glc to form [*Man*-$^{14}$C]oligosaccharide–$lipid_I$; and 0.2 μM UDP-[$^3$H]Glc ($2.7 \times 10^4$ cpm) and 2 μM GDP-Man to form [*Glc*-$^3$H]oligosaccharide–$lipid_I$. Approximately 500 μg of microsomal preparation was added to initiate the reaction. The assay tubes were incubated for 10 min in a shaking water bath set at 37°.

The reaction was terminated by the addition of 1 ml of chloroform/methanol (3:2) and 0.1 ml of water, and the suspension was centrifuged at 700 × *g* for 10 min. The upper aqueous phase and lower organic phase were removed, and the interphase was extracted with 3 ml of chloroform/methanol/water (3:2:1), sonicated (Branson Sonic Power, Branson Instruments Inc., power level no. 2), and centrifuged. Again the upper and lower phases were removed and the interphase was pelleted three times in 3 ml of water. The residue was extracted three times with 3 ml of chloroform/methanol/water (1:1:0.3) by the method of Behrens *et al.* (7). The extracted oligosaccharide–lipids were purified on DEAE-cellulose as described (4) and by passage through a column (1 × 40 cm) of Sephadex LH-20 equilibrated in *n*-propanol/water (1:1).

**Mild Acid Hydrolysis.** The lipid samples were dried, redissolved in a mixture of 50 μl of *n*-propanol and 0.1 ml of 20 mM HCl, and were then hydrolyzed for 20 min at 100°.

**Reduction with Sodium [$^3$H]Borohydride.** Samples were subjected to mild acid hydrolysis and extracted by the method of Folch *et al.* (9). The aqueous phases were dried, dissolved in 0.2 ml of 0.1 mM sodium borate buffer, pH 11.4, and reduced by the addition of 0.1 ml of sodium [$^3$H]borohydride in borate buffer. After incubation for 20 hr at 25°, excess borohydride was destroyed by the addition of several drops of 1 M acetic acid. Borate was converted to the acid form by passage through a small column of Dowex AG 50W-X8 ($H^+$ form). The samples were dried, dissolved in methanol, and evaporated to dryness under a stream of nitrogen; this procedure was repeated several times to remove boric acid (as methylborate). An aliquot of each

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Abbreviations: oligosaccharide–$lipid_I$, glucose-containing oligosaccharide–lipid; oligosaccharide–$lipid_{II}$, glucose-free oligosaccharide–lipid.

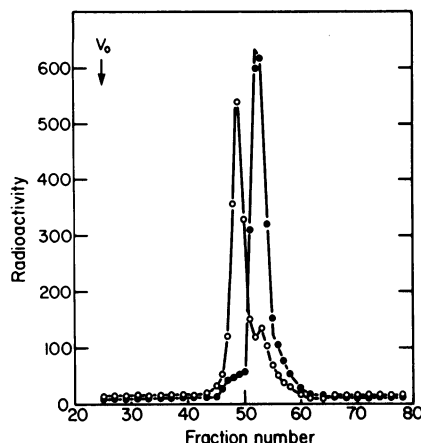


FIG. 1. Bio-Gel P-6 chromatogram of [*Man*-$^{14}$C]oligosaccharides released from lipid by mild acid hydrolysis. [*Man*-$^{14}$C]Oligosaccharide–lipid$_I$ (2500 cpm) and [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$ (3000 cpm) were subjected to mild acid hydrolysis separately, dried, and redissolved in 0.1 M Tris·HCl, pH 8.0/0.02% sodium azide buffer. Each sample was applied to a column of Bio-Gel P-6. Bovine serum albumin was used to determine the void volume, and 1.0-ml fractions were collected. O, [*Man*-$^{14}$C]Oligosaccharide–lipid$_I$; ●, [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$.

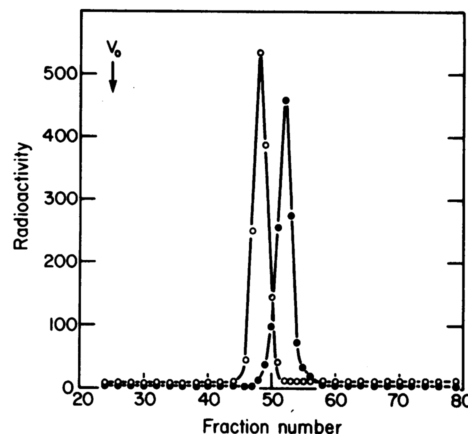


FIG. 2. Bio-Gel P-6 chromatogram of [*Glc*-$^3$H]- and [*Man*-$^{14}$C]oligosaccharides released from lipid by mild acid hydrolysis. [*Glc*-$^3$H]Oligosaccharide–lipid$_I$ (1800 cpm) and [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$ (1500 cpm) were combined, subjected to mild acid hydrolysis, dried, and redissolved in 0.1 M Tris·HCl, pH 8.0/0.02% sodium azide. The sample was applied to a column of Bio-Gel P-6 (200–400 mesh, 1 × 115 cm), equilibrated in the same buffer. Bovine serum albumin was used as the standard to determine the void volume, and 1.0-ml fractions were collected. O, [*Glc*-$^3$H]Oligosaccharide–lipid$_I$; ●, [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$.

sample was then dissolved in 0.1 M Tris, pH 8.0, containing 0.02% sodium azide, applied to Bio-Gel P-6 equilibrated in the same buffer, and eluted as described (4).

**Transfer of Oligosaccharide from Lipid to Protein.** Components of the standard assay mixture were as follows. Labeled oligosaccharide–lipid was added to a tube and dried under a stream of nitrogen; 20 μl of 64 mM $MnCl_2$ and 20 μl of Tris-buffered saline were added and the residue was sonicated followed by the addition of 20 μl of 3% sodium deoxycholate and 100 μl of microsomal protein (3–5 mg). After the appropriate period of time at 25°, the reaction was terminated by the addition of 2 ml of chloroform/methanol (3:2) and 0.2 ml water, sonicated, and centrifuged. The upper and lower phases were removed and the interphase was washed once with 4 ml of water, three times with 4 ml of chloroform/methanol/water (1:1:0.3), and three times with 4 ml of methanol. Between washes, samples were sonicated and centrifuged. The residue was then dissolved in 1 ml of 10% sodium dodecyl sulfate, sonicated, and boiled, and radioactivity was determined.

**Analytical Methods.** Protein was determined by the method of Lowry *et al* (10), with bovine serum albumin as a standard. Radioactivity was measured as described (4).

## RESULTS

**Preparation of the Two Oligosaccharide–Lipids.** Studies in this laboratory have established that microsomal preparations from Nil 8 fibroblasts synthesize two lipid-linked oligosaccharides (4). Incubation of the microsomes with 2 μM GDP-[$^{14}$C]Man and 20 μM UDP-*N*-acetylglucosamine results in the synthesis of one $^{14}$C-labeled oligosaccharide–lipid. Inclusion of 0.20 μM UDP-[$^3$H]Glc in this reaction mixture results in the appearance of a larger, double-labeled oligosaccharide–lipid that contains, in addition to mannose and *N*-acetylglucosamine, one or two glucose residues. Attempts to separate the two oligosaccharide–lipids by DEAE-cellulose, silicic acid chromatography, Sephadex LH-20 gel filtration, and thin-layer chromatography in several solvent systems were unsuccessful.

It was possible, however, to obtain the [*Man*-$^{14}$C]oligosaccharide–lipid$_I$ in almost pure form by increasing the concentration of UDP-Glc in the preparative incubation mixture. As shown in Fig. 1, in the presence of 1 μM UDP-Glc, 85% of the [$^{14}$C]mannose is associated with the larger oligosaccharide–lipid. Furthermore, when UDP-[$^3$H]Glc was substituted for unlabeled UDP-GLc, all of the tritium radioactivity resided in the larger oligosaccharide–lipid (Fig. 2). As expected, in the absence of UDP-Glc only the smaller oligosaccharide–lipid is labeled by [$^{14}$C]mannose (Figs. 1 and 2). In this manner we were able to label each oligosaccharide–lipid separately.*

**Measurement of Oligosaccharide–Lipid Specific Activities.** Since a meaningful comparison of the kinetics of incorporation of each oligosaccharide requires a knowledge of the molar concentration of each added substrate, the exact specific activity of each oligosaccharide–lipid was determined. The free oligosaccharide from [*Man*-$^{14}$C]oligosaccharide–lipid$_I$ (36,000 cpm) and [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$ (36,000 cpm) were treated with sodium [$^3$H]borohydride and the amount of tritium radioactivity corresponding to the single peak of [$^{14}$C]mannose was quantitated. Since sodium [$^3$H]borohydride reacts stoichiometrically with the reducing end of the oligosaccharide generated by mild acid hydrolysis, tritium incorporation provides a measure of the molar concentration of the oligosaccharide. We obtained a specific activity of 22,500 cpm of $^{14}$C/nmol for oligosaccharide–lipid$_I$ and 36,000 cpm of $^{14}$C/nmol for oligosaccharide–lipid$_{II}$. Recovery of $^{14}$C radioactivity throughout the procedure was greater than 85%. Strong acid hydrolysis of the respective oligosaccharides, followed by analysis by paper chromatography, revealed that, as expected, all the $^3$H radioactivity comigrated with glucosaminitol.

**Transfer of Oligosaccharides to Protein.** The addition of equimolar amounts (9.7 nmol) of [*Man*-$^{14}$C]oligosaccharide–lipid$_I$ and [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$ to separate microsomal preparations resulted in a 9-fold greater initial rate of incorporation of the glucose-containing oligosaccharide than the glucose-free oligosaccharide (Fig. 3). Further, 41% of the glucose-containing oligosaccharide moiety was transferred to

* In the presence of other sugar nucleotides (UDP-galactose, UDP-xylose, UDP-glucuronic acid, and TDP-glucose), the distribution of labeled oligosaccharide–lipids is unchanged, as judged by their chromatographic profiles on Bio-Gel P-6.

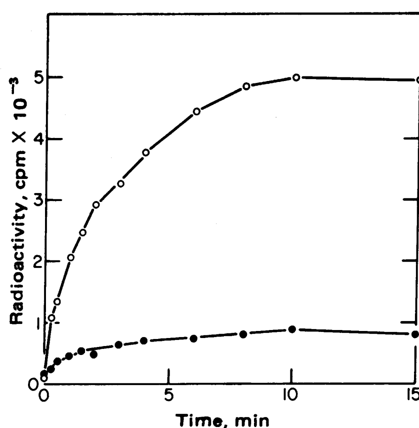


FIG. 3. Kinetics of incorporation of [*Man*-$^{14}$C]oligosaccharides by the endogenous acceptors of Nil cell microsomes. [*Man*-$^{14}$C]Oligosaccharide–lipid$_I$ (220,000 cpm) and [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$ (350,000 cpm) were dried in separate tubes under a stream of nitrogen. To each tube was added 0.4 ml of 64 mM $MnCl_2$ and 0.4 ml of Tris-buffered saline. After sonication, 0.4 ml of 3% deoxycholic acid was added. Each reaction was initiated by the addition of 2.0 ml of microsomal protein (84 mg) and was stirred at 25°. At each time point a 160-$\mu$l aliquot was removed from each tube; the reaction was terminated by addition of 2.0 ml of chloroform/methanol (3:2) and 200 $\mu$l of water. The residue fraction was extracted as described. O, [*Man*-$^{14}$C]Oligosaccharide–lipid$_I$; ● [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$.

endogenous protein acceptors as compared to 5% of the glucose-free oligosaccharide under these conditions. Since in this experiment the oligosaccharide from oligosaccharide–lipid$_I$ was radiolabeled with [$^{14}$C]mannose, we were unable to determine whether the glucose-containing oligosaccharide was transferred *en bloc* or whether the glucose residue(s) were cleaved during transfer. In order to investigate the latter possibility further, [*Glc*-$^3$H]oligosaccharide–lipid$_I$ and [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$ were prepared and purified separately. The two lipid-linked oligosaccharides were then combined and added to a fresh preparation of microsomes. As shown in Fig. 4, the $^3$H-labeled oligosaccharide was incorporated into protein,

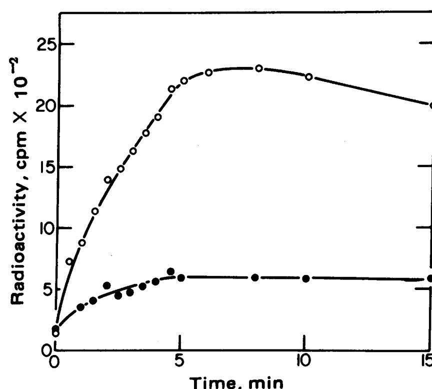


FIG. 4. Kinetics of incorporation of [*Glc*-$^3$H]- and [*Man*-$^{14}$C]-oligosaccharides by the endogenous acceptors of Nil cell microsomes. [*Glc*-$^3$H]Oligosaccharide–lipid$_I$ (200,000 cpm) and [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$ (200,000 cpm) were obtained and dried under a stream of nitrogen. $MnCl_2$ (0.28 ml of 64 mM solution) and 0.28 ml of Tris-buffered saline, pH 7.4, were added and the mixture was sonicated. After the addition of 0.28 ml of 3% deoxycholate, the reaction was initiated by the addition of 1.4 ml of microsomal protein (42 mg) and was stirred at 28°. At the indicated times, 160-$\mu$l aliquots were removed and the reaction was terminated by the addition of 2 ml of chloroform/methanol (3:2) and 200 $\mu$l of water. The residue fraction was extracted as described. O, [*Glc*-$^3$H]Oligosaccharide–lipid$_I$; ●, [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$.

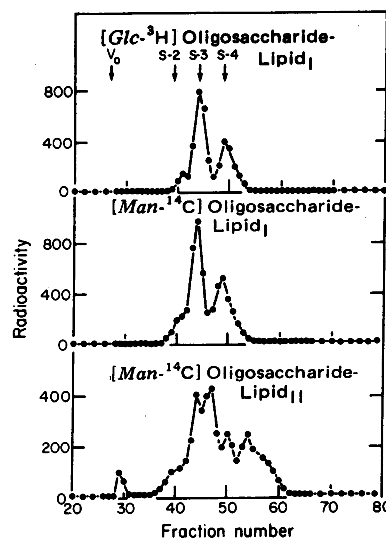


FIG. 5. Bio-Gel P-6 chromatogram of glycopeptides derived by Pronase digestion of labeled microsomal proteins. The nonextractable residue fractions prepared from 10-min incubations with oligosaccharide–lipids were treated with Pronase as described (4). The residue fractions were suspended in 500 $\mu$l of 0.1 M Tris·HCl, pH 8.0/10 mM $CaCl_2$. A 1% solution of predigested Pronase in the same medium (Calbiochem) was added in 200 $\mu$l of aliquots at the following times: 0, 22, 48, and 72 hr. The digestion was carried out at 50°, and the preparations were protected from bacterial contamination by periodic addition of a drop of toluene. After 92 hr the samples were concentrated to 400 $\mu$l under a stream of nitrogen and applied directly to a Bio-Gel P-6 column. The vertical arrows indicate the positions of the exclusion volumes and Sindbis virus glycopeptides S-2, S-3, and S-4. The carbohydrate portions of S-4 and S-3 have molecular weights of 1550 and 1800, respectively. S-2 and S-1 are similar to S-3 except that they contain, respectively, one and two residues of sialic acid.

demonstrating that the transferred oligosaccharide from oligosaccharide–lipid$_I$ retained glucose. Consistent with the results shown in Fig. 3, the [*Glc*-$^3$H]oligosaccharide was transferred at a greater initial rate (at least 4-fold), and to a 4-fold greater extent, than was the glucose-free oligosaccharide. After the period of maximum transfer to endogenous acceptors (10–15 min), approximately 40% of the glucose-containing oligosaccharide–lipid and 70% of the glucose-free oligosaccharide–lipid could be recovered by extraction with chloroform/methanol/water (1:1:0.3). The re-isolated oligosaccharide–lipids were unchanged as judged by Bio-Gel P-6 chromatography of the products of mild acid hydrolysis.

**Characterization of Product of Transfer.** The endogenous radiolabeled acceptor(s) for the oligosaccharide–lipids were characterized as protein by the following methods. The products derived from either [*Glc*-$^3$H]- or [*Man*-$^{14}$C] oligosaccharide–lipid$_I$ were 99% insoluble in trichloroacetic acid after extraction by the method of Folch *et al.* (9), and greater than 95% sensitive to exhaustive Pronase disgestion. In contrast, 92% of the product from [*Man*-$^{14}$C]oligosaccharide–lipid$_{II}$ was insoluble in trichloroacetic acid, and 61% was sensitive to Pronase digestion. Bio-Gel P-6 chromatograms of the Pronase digests of the labeled proteins are shown in Fig. 5. The profiles of radioactivity of the pronase-digested residues generated by transfer from the oligosaccharide–lipids are very similar to corresponding profiles obtained for glycoproteins generated with nucleotide sugars as substrates (4).

## DISCUSSION

As far as we are aware the only previous studies concerning the transfer of glucose-containing and glucose-free oligosaccha-

ride–lipids to protein have been made with the liver microsomal system by Leloir and coworkers (7, 8). Using a glucose-containing oligosaccharide–lipid as the substrate, Parodi *et al.* (8) were able to demonstrate up to 38% transfer of their oligosaccharide to protein at an initial rate of 500 cpm/2 min at 30°. In a later publication, Behrens *et al.* (7) reported transfer of up to 23% of their glucose-free oligosaccharide, at an initial rate of 500 cpm/15 min at 30°. These results may suggest that the glucose-containing oligosaccharide is transferred from lipid to protein preferentially in the liver system. However, since the published experiments were not carried out at the same time or with oligosaccharide–lipid preparations of known specific activity, accurate comparison is impossible.

In our studies the relative rates of incorporation of oligosaccharides of known specific activity were compared by incubating the individually labeled oligosaccharide–lipids either separately or together. When the oligosaccharides were incubated separately, it was clear that the glucose-containing species was transferred almost an order of magnitude more efficiently. When the two oligosaccharides were incubated together, the results suggested possible competition for transfer between the two lipid derivatives. However, the suggestion of competition is only tentative since different reaction conditions were used, e.g., protein concentration and oligosaccharide–lipid concentration. Our results suggest that the major enzyme responsible for transfer of the oligosaccharide from lipid carrier to protein is specific for an oligosaccharide containing glucose, but will also transfer, although to a lesser extent, oligosaccharides that do not contain glucose. It is also possible, however, that more than one enzyme is involved in the transfer reactions and that there are either more acceptor sites for, or a faster rate of reaction of, the glucose-containing oligosaccharide than the glucose-free oligosaccharide.

Lipid-linked intermediates such as those reported here presumably serve as precursors of the asparagine-linked oligosaccharide groups found in glycoproteins. If further studies confirm the importance of glucose-containing structures in the initial transfer of mannosyl oligosaccharide units to protein, it may be that processing occurs before these glycoprotein molecules appear at the cell surface. While several soluble glycoproteins have glucose as a constituent (11–13), mature asparagine-linked oligosaccharides of membrane glycoproteins probably do not (14, 15), strongly suggesting that processing is a selective and important event.

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