The Biosynthesis of the Major Lipid-linked Oligosaccharide of Chinese Hamster Ovary Cells Occurs by the Ordered Addition of Mannose Residues*

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Intact Chinese hamster ovary cells were incubated with [3H]mannose or [3H]galactose to label the mannose and glucose residues, respectively, of the lipid-linked oligosaccharides. The lipid-linked oligosaccharides were then extracted and the oligosaccharide units released by mild acid hydrolysis and separated by descending paper chromatography. A family of oligosaccharides were isolated which contained from 1 to 8 mannose residues linked to a di-N,N'-acetylchitobiose unit. Each oligosaccharide was subjected to α-mannosidase digestion and methylation analysis. The larger oligosaccharides were also subjected to acetolysis. With this information the arrangement of the mannose residues in each of the oligosaccharides could be deduced. We conclude that each oligosaccharide of a given size consists of one predominant isomer and that the addition of mannose residues to the growing lipid-linked oligosaccharide is highly ordered.

In addition to the predominant (glucose)₃-(mannose)(N-acetylglucosamine) lipid-linked oligosaccharide previously characterized (Li et al. (1978) J. Biol. Chem. 253, 7762-7770), the Chinese hamster ovary cells were also found to synthesize a second glucose-containing lipid-linked oligosaccharide composed of 3 residues of glucose (Glc), 5 residues of mannose (Man), and 2 residues of N-acetylglucosamine (GlcNAc). The structure of this oligosaccharide was determined to be: Glc1 → 2Glc1 → 3Glc1 → 3Manα1 → 2Manα1 → 2Manα1 → 3(Manα1 → 6)Man → GlcNAc → GlcNAc.

Recently a second lipid-linked oligosaccharide has been isolated and its structure determined to be that shown in Structure 2 (5).

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\text{Man}_1 \xrightarrow{6} \text{Man}_1 \xrightarrow{3} 4(3)\text{GlcNAc}_1 \xrightarrow{4} \text{GlcNAc}
\]

**Structure 2**

This lipid-linked oligosaccharide, which is present in both vesicular stomatitis virus-infected and uninfected Chinese hamster ovary cells, appears to be a biosynthetic precursor of the major lipid-linked oligosaccharide shown in Structure 1. The finding of only one isomer with the composition Man₅GlcNAc₂ indicated that the biosynthesis of the major lipid-linked oligosaccharide might occur by the ordered addition of mannose and glucose residues. To test this hypothesis, the various lipid-linked oligosaccharide intermediates of Chinese hamster ovary cells were labeled with [3H]mannose, isolated, and then subjected to structural analysis. In each instance the predominant species was a single isomer. These data demonstrate that the addition of mannose residues during the assembly of the lipid-linked oligosaccharide is highly ordered. Based on these findings a scheme for the sequence of mannose addition to the lipid-linked oligosaccharide is proposed.

**EXPERIMENTAL PROCEDURES**

Materials—D-[2-3H]Mannose (2 Ci/mmol) and D-[1-3H]glucosamine (2 Ci/mmol) were from Amersham. D-[G-3H]Galactose (2 Ci/mmol) was from New England Nuclear Corp. Minimal essential medium was obtained from Flow Laboratories, Rockville, Md., and fetal calf serum, glutamine, penicillin, and streptomycin were from Grand Island Biological Co. The 5α70 scintillation mixture was from Research Products International Corp. Elk Grove Village, Ill. Man,GlcitolNAc, Manβ1 → 4GlcNAcβ1 → 4GlcNAc, Manα1 → 2Manα1 → 3Man, and Manα1 → 2Man were prepared as described.

The biosynthesis of the two complex-type oligosaccharide units of the vesicular stomatitis virus G protein is initiated by the en bloc transfer of a high molecular weight oligosaccharide from a lipid carrier to the nascent polypeptide. Following transfer to the G protein, the oligosaccharide is processed extensively to give rise to the completed complex-type oligosaccharide (1–3). The complete structure of the carbohydrate unit of the lipid-linked oligosaccharide is shown in Structure 1 (4).

Man1 → 2Manα1

Man1 → 2Manα1

Glc1 → 2Glc1 → 3Glc1 → 3Manα1 → 2Manα1 → 2Manα1

**Structure 1**

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cells were labeled for 10 to 15 min with either [2-^H]mannose or [3-^H]galactose as described under “Experimental Procedure.” The released oligosaccharides from the CHCl;/CHrOH (2:l) extract, the hydrolyzed sample was then passed through a column of Amberlite MB-3 mixed bed resin. Because of the large amount of lipid in the CHCl;/CHrOH (2:l) extract, the hydrolyzed sample was first partitioned between 5 ml of CHCl;/CHrOH (9:1) and 9 ml of H2O. The H2O layer, containing the released oligosaccharides, was then passed through a column of Amberlite MB-3. The oligosaccharide were then separated by descending paper chromatography. Under these labeling conditions, [2-^H]mannose is incorporated exclusively into mannose while 90 to 95% of the label derived from [G-^H]galactose is incorporated into galactose (4).

RESULTS

Isolation of Oligosaccharides—Chinese hamster ovary cells were labeled for 10 to 15 min with either [2-^H]mannose or [3-^H]galactose as described under “Experimental Procedures.” The lipid-linked oligosaccharides were then extracted, subjected to mild acid hydrolysis, and the released oligosaccharides were separated by descending paper chromatography in Solvent A (Fig. 1). A series of ten [^H]mannose-labeled oligosaccharide fractions, in addition to the major lipid-linked oligosaccharide were detected as shown in Fig. 1. Each fraction was eluted as indicated, and purified by repeated descending paper chromatography in Solvent A, or Solvent B, or both. Oligosaccharide Fraction V has been characterized previously as Man2GlcNAc2 (5). Based on their relative mobility on paper chromatography oligosaccharide Fractions I through IV, isolated from the chloroform/methanol (2:1) extract, were estimated to contain 3 through 6 glycosic units, respectively (Fig. 1A). Similarly, oligosaccharide Fractions IV through IX, isolated from the chloroform/methanol/water (1:1:0.3) extract, were estimated to contain 6 through 11 glycosic units, respectively (Fig. 1B). In the experiment shown in Fig. 1 there was too little radioactivity in the Peak VII region (Man3GlcNAc2) to analyze further. However in a second similar experiment we were able to isolate enough of the Peak VII species to characterize. The overlapping oligosaccharides in Fractions VIIIa and VIIIb were clearly separated after chromatography for 6 days in Solvent B. The faster migrating material (VIIIa) proved to contain glucose in addition to mannose (see below). Region IX turned out to be a mixture of oligosaccharides. We were not able to obtain enough Man3GlcNAc2 for complete characterization. There was also

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**Synthesis of Lipid-linked Oligosaccharide**

The radioactivity and the isolation of lipid-linked oligosaccharides from Chinese hamster ovary cells has been previously described (2, 4). Briefly, confluent monolayer cultures grown in 150-mm diameter tissue culture Petri dishes were incubated for 10 to 15 min with 1 mCi of [2-^H]mannose or [3-^H]galactose in 4 ml of Dulbecco’s phosphate-buffered saline (0.9% NaCl solution) (9). The lipid-linked oligosaccharides were then isolated from the cells. The CHCl;/CHrOH and the CHCl;/CHrOH/H2O fractions were each subjected to mild acid hydrolysis. The released oligosaccharides from the CHCl;/CHrOH/H2O fraction were passed through a column (0.5 x 3.0 cm) of Amberlite MB-3 mixed bed resin. Because of the large amount of lipid in the CHCl;/CHrOH (2:l) extract, the hydrolyzed sample was first partitioned between 5 ml of CHCl;/CHrOH (9:1) and 9 ml of H2O. The H2O layer, containing the released oligosaccharides, was then passed through a column of Amberlite MB-3. The oligosaccharide were then separated by descending paper chromatography. Under these labeling conditions, [2-^H]mannose is incorporated exclusively into mannose while 90 to 95% of the label derived from [G-^H]galactose is incorporated into galactose (4).

**Chromatographic Techniques—**Descending paper chromatography was performed on Whatman No. 1 paper in the following solvent systems: Solvent A, ethyl acetate/pyridine/acetic acid/water (5:5:1:3); Solvent B, l-butanol/pyridine/water (4:3:4). Labeled compounds were localized by radioscanning of the chromatogram with a TMC Vanguard Instrument Corp. model 885 dual channel autoscanner. Alternatively, 1-cm strips cut from the chromatogram were soaked in 0.3 ml of water in scintillation vials and counted after the addition of ammonium hydroxide (50:200:1.0:1.35) to separate methylated glucose species, or benzene/acetone/water/ammonium hydroxide (50:200:1.0:1.35) to separate methylated glucose species. The standards were located by spraying the plate with 5% concentrated sulfuric acid in ethanol and heating at 100°C. Labeled compounds were located by scraping 0.5-cm segments of the silica gel from the plate into 1 ml of water. 4 ml of 3a70 and counting in a scintillation counter.

**Glycosidase Digestions—**All incubations were at 37°C under a 5% CO2 atmosphere. The sample was digested in 0.02 ml of jack bean a-mannosidase (50 units/ml) in 0.05 M citrate buffer, pH 4.5, for 16 h. The sample was digested in 0.025 ml of hen oviduct /-mannosidase (0.166 unit/ml) in 0.05 M citrate phosphate, pH 5.0, for 12 h. The sample was digested in 0.03 ml of endo-^-N-acetylglucosaminidase CII (20 milliunits/ml) in 0.05 M citrate phosphate. pH 6.5, for 18 h. The sample was digested with 1 milliunit of endo-^-N-acetylglucosaminidase D in 0.06 ml of citrate phosphate, pH 6.0, for 24 h.

**Structural Analysis—**Methylation analysis, borohydride reduction, and acetylation were performed as previously described (4).

**FIG. 1. Paper chromatograms of radiolabeled oligosaccharides from the chloroform/methanol (2:1) and chloroform/methanol/water (1:1:0.3) extracts of Chinese hamster ovary cells.** The chromatograms were developed in ethyl acetate/pyridine/acetic acid/water (5:5:1:3) or for every 19 h (A) or for four days (B, C, and D). Oligosaccharides were eluted as indicated by the brackets. A, [^H]mannose-labeled chloroform/methanol (2:1) extract; B and C, [^H]mannose-labeled chloroform/methanol/water (1:1:0.3) extract. The arrows indicate the positions of the standards. 1, Man2GlcNAc2, 2, Man3GlcNAc2; 2, 4GlcNAc; 3, (Man3)GlcNAc2; 4, Glc-Man3GlcNAc2.
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FIG. 2. Thin layer chromatogram of methylated species from [3H]mannose-labeled oligosaccharides I to IV. A, I (Man$_3$GlcNAc$_2$); B, II (Man$_3$GlcNAc$_2$); C, III$_{endo}$D (Man$_3$GlcNAc$_2$); D, IV (Man$_4$GlcNAc$_2$). The arrows indicate the positions of the standards: 1, 2,3,4,6-tetra-Me-Man; 2, 2,4,6-tri-Me-Man; 3, 2,3,6-tri-Me-Man; 4, 2,3,4-tri-Me-Man; 5, 3,4,6-tri-Me-Man; 6, 2,4-di-Me-Man; 7, 2,3-di-Me-Man.

a small amount of material which migrated slightly slower than the Glc$_3$Man$_3$GlcNAc$_2$ standard.

The chloroform/methanol/water (1:1:0.3) fraction from [3H]galactose-labeled cells contained a major labeled oligosaccharide corresponding to Glc$_3$Man$_3$GlcNAc$_2$ plus a second oligosaccharide which migrated in the region of Man$_3$GlcNAc$_2$ (Fig. 1D). The latter oligosaccharide (A in Fig. 1D) migrated as a single peak in Solvent B. The small amount of oligosaccharide material which migrated slower than Glc$_3$Man$_3$GlcNAc$_2$ (C in Fig. 1D) was not further characterized. The chromatogram of the chloroform/methanol (2:1) fraction of these cells contained only one peak which migrated with free glucose and was presumably derived from glucose-P-dolichol (data not shown).

When similar experiments were performed with [3H]glucosamine, very little radioactive material was obtained from the chloroform/methanol (2:1) fraction although peaks migrating with free N-acetylglucosamine and Man$_3$GlcNAc$_2$ were detected. Similarly, the chloroform/methanol/water (1:1:0.3) fraction contained low levels of radioactivity corresponding to the major [3H]mannose-labeled compounds (data not shown). Since our studies were designed to determine the sequence of mannose addition to the growing lipid-linked oligosaccharide, the labeling studies with [3H]glucosamine were not pursued. Based on previous studies, we assumed that in each case the reducing end of the oligosaccharide chain released from the lipid contained a di-N$\beta$-acetylchitobiose unit (4, 5). The finding that endo-$\beta$-N-acetylglucosaminidase C$_4$ and D were active against several of the oligosaccharides provides further evidence to support this assumption.

Structure of I (Man$_3$GlcNAc$_2$)—When the [3H]mannose-labeled oligosaccharide I was methylated followed by hydrolysis and separation of the methylated mannose derivatives by thin layer chromatography only 2,3,4,6-tetra-Me-Man was detected, indicating that there is only 1 residue of mannose which is located at the nonreducing terminus (Fig. 2A). The mannose residue was resistant to jack bean $\alpha$-mannosidase but was released by incubation with hen oviduct $\beta$-mannosidase (Fig. 3, A and B). Based on these data and the fact that the oligosaccharide migrated with authentic Man$_3$GlcNAc$_2$ $\beta_1 \rightarrow$ 4GlcNAc$_2$$\beta_1 \rightarrow$ 4GlcNAc when subjected to paper chromatography (Fig. 3A), its probable structure is: Man$_3$GlcNAc$_2$ $\rightarrow$ GlcNAc.$^1$

Structure of II (Man$_3$GlcNAc$_2$)—Methylation of oligosaccharide II gave rise to 2,3,4,6-tetra-Me-Man and 2,4,6-tri-Me-Man.

$^1$The presence of radioactivity at the origin of the thin layer chromatogram represents a variable and, in our hands, unpredictable finding (compare the various runs in Figs. 2, 6, and 8). Fortunately, the presence or absence of radioactivity at the origin does not seem to influence the ratios of the various methylated species significantly.
Man in the ratio of 1.0:0.8 (Fig. 2D). No 2,3,4,6-di-Me-Man was detected. α-Mannosidase treatment resulted in the release of 49% of the label as free mannose and the residual oligosaccharide now migrated with Manβ1 → 4GlcNAcβ1 → 4GlcNAc (Fig. 3C). These results suggest that the structures of oligosaccharide II is: Manα1 → 3(Manα1 + 6)Manp → GlcNAc + GlcNAc.

Structure of III (Man,GlcNAc) — α-Mannosidase digestion of oligosaccharide III released 70% of the radioactivity as free mannose and the residual 30% of the radioactivity now migrated with Manβ1 → 4GlcNAcβ1 → 4GlcNAc (Fig. 3D). Treatment of oligosaccharide III with endo-β-N-acetylglucosaminidase D converted it to an oligosaccharide (IIIendO) which migrated with authentic Man,GlcNAc when subjected to paper chromatography in Solvent A (Fig. 4). Methylation that the structure of oligosaccharide III is: Manα1 + 3(Manα1 + 6)Manp → GlcNAc + GlcNAc.

Structure of IV (Man,GlcNAc) — Methylation of oligosaccharide IV gave rise to 2,3,4,6-tetra-Me-Man, 3,4,6-tri-Me-Man, and 2,4-di-Me-Man in the ratio of 2.0:1.0:0.9 (Fig. 2D). α-Mannosidase treatment released 83% of the radioactivity as free mannose and the residual oligosaccharide migrated with the Manβ1 → 4GlcNAcβ1 → 4GlcNAc standard (Fig. 3E). Acetylation of the Man,GlcNAc2 molecule resulted in the release of free mannose and the formation of a major fragment which migrated on paper chromatography as if it were 1 residue smaller than the Man,GlcNAc2 standard (Fig. 5A). Since acetylation preferentially cleaves Manα1 → 6Man linkages (10), the structure of oligosaccharide IV is most likely: Manα1 → 2Manα1, 3(Manα1 + 6)Manβ → GlcNAc → GlcNAc.

Structure of V (Man,GlcNAc) — The complete structural analysis of oligosaccharide V is described in our previous publication (5) and is shown in Fig. 10.

Structure of VI (Man,GlcNAc) — Methylation of oligosaccharide VI gave rise to 2,3,4,6-tetra-Me-Man, 3,4,6-tri-Me-Man, and 2,4-di-Me-Man in the ratio of 2.3:1.7:1.0:1.0 (Fig. 6A). α-Mannosidase released 84% of the radioactivity as free mannose and the residual oligosaccharide migrated with the Manβ1 → 4GlcNAcβ1 → 4GlcNAc standard (Fig. 3F). Since 4Me-Man migrated with Manβ1 → 4GlcNAcβ1 → 4GlcNAc (Fig. 3C), the most likely structure of oligosaccharide VII is: Manα1 → 2Manα1, 3(Manα1 + 6)Manβ → GlcNAc → GlcNAc.

Structure of VII (Man,GlcNAc) — Methylation of oligosaccharide VII gave rise to 2,3,4,6-tetra-Me-Man and 2,4,6-tri-Me-Man in the ratio of 1.00:7. No 3,4,6-tri Me-Man was detected. Based on these data, the most likely structure for oligosaccharide VI is: Manα1 → 2Manα1 → 2Manα1 → 3(Manα1 → 3Manα1 → 6)Manβ → GlcNAc → GlcNAc (see Fig. 10).

and the other which migrated as a disaccharide. The minor peaks are probably the result of a small degree of overdegradation. Methylation of the disaccharide gave rise to 2,3,4,5-tetra-Me-Man and 2,4,5-di-Me-Man in the ratio of 1.00:7. No 3,4,5-tri Me-Man was detected. Based on these data, the most likely structure for oligosaccharide VII is: Manα1 → 2Manα1 → 2Manα1 → 3Manα1 → 6)Manβ → GlcNAc → GlcNAc (see Fig. 10).

Structure of VIII (Man,GlcNAc) — Methylation of oligosaccharide VIIIb gave rise to 2,3,4,6-tetra Me-Man, 3,4,6-tri Me-Man, 2,4,6-tri-Me-Man, and 2,4-di-Me-Man in the ratio of 2.1:2.8:0.9:1.2 (Fig. 6B). α-Mannosidase digestion released 85% of the radioactivity as free mannose and the residual oligosaccharide migrated with the Manβ1 → 4GlcNAcβ1 → 4GlcNAc (Fig. 7B). Acetylation of oligosaccharide VIII following endo-β-N-acetylglucosaminidase C1 digestion and reduction produced two major fragments, one of which migrated with Man,GlcitolNAc while the other migrated as a trisaccharide (Fig. 5C). The most likely structure of oligosaccharide VII is: Manα1 → 2Manα1 → 2Manα1 → 3Manα1 → 2Manα1 → 3Manα1 → 6)Manβ → GlcNAc → GlcNAc (see Fig. 10).

![Fig. 4. Descending paper chromatogram of the endo-β-N-acetylglucosaminidase D digestion products of oligosaccharide III. The chromatogram was developed for 48 h in ethyl acetate/pyridine/acetic acid/water (5:5:1:3). The arrows point to 1, intact oligosaccharide III; and 2, a Man,GlcNAc standard.](http://www.jbc.org/Downloaded)
Structure of VIIIb (Man₃GlcNAc₂)—Methylation of oligosaccharide VIIIb gave rise to 2,3,4,6-tetra-Me-Man, 3,4,6-tri-Me-Man, and 2,4-di-Me-Man in the ratio of 3.4:2.9:1.7 (Fig. 6C). Digestion with α-mannosidase released 88% of the radioactivity as free mannose while the residual oligosaccharide migrated with Man₁ → 4GlcNAcβ → GlcNAc (Fig. 7C). Acetylation of endo-β-N-acetylglucosaminidase CII-treated and NaBH₄-reduced oligosaccharide VIIIb resulted in major fragments which co-migrated with Man₃GlcitolNAc, Man₄, and free mannose (Fig. 5D). The most likely structure of the oligosaccharide is: Man₁ → 2Man₁ → 3(Man₁ → 6)Man₁ → 6(Ma₁ → 2Man₁ → 2Man₁ → 3)Man₁ → GlcNAc → GlcNAc (see Fig. 10).

Structure of VIIIa (Glc₂Man₃GlcNAc₂)—The [¹⁴C]glucose-labeled Peak A oligosaccharide (Fig. 1D) migrated slightly faster than Man₃GlcNAc and identically with the [¹⁴C]mannose-labeled Peak VIIIa compound when the two were chromatographed together. The following studies, performed on the [¹⁴C]glucose- and [¹⁴C]mannose-labeled fractions indicated that this compound had the composition Glc₂Man₃GlcNAc₂. Methylation of the [¹⁴C]mannose-labeled oligosaccharide gave rise to 2,3,4,6-tetra-Me-Man, 3,4,6-tri-Me-Man, and 2,4-di-Me-Man in the ratio of 0.8:1.9:0.8:1.0 (Fig. 8A). Methylation of the [¹⁴C]glucose-containing species gave rise to 2,3,4,6-tetra-Me-Glc, 2,4,6-tri-Me-Glc, and 3,4,6-tri-Me-Glc in the ratio of 1:1:1.0 (Fig. 8C). These data indicate that the oligosaccharide contains 5 residues of mannose and 3 residues of glucose. α-Mannosidase digestion of [¹⁴C]mannose-labeled material released 20% of the radioactivity (or 1 residue) as free mannose (Fig. 9A). Methylation of the residual

Fig. 6. Thin layer chromatograms of methylated species from [¹⁴C]mannose-labeled oligosaccharides VI, VII, and VIIIb. A, VI (ManGlcNAc); B, VII (ManGlcNAc); C, VIIIb (ManGlcNAc). The arrows point to the standard methylated compounds, which are the same as those shown in Fig. 2.

Fig. 7. Descending paper chromatograms of α-mannosidase digestion products from: A, VI (ManGlcNAc); B, VII (ManGlcNAc); C, VIIIb (ManGlcNAc). The standards are: 1, Man₁ → 4GlcNAcβ → 4GlcNAc; 2, mannose.

Fig. 8. Thin layer chromatograms of methylated [¹⁴C]mannose- or [¹⁴C]glucose-labeled species from oligosaccharide VIIIa (Glc₂Man₃GlcNAc₂). A, methylated [¹⁴C]mannose-labeled species from intact VIIIa; B, methylated [¹⁴C]mannose species from the α-mannosidase-digested fragment; C, methylated [¹⁴C]glucose-labeled species from intact VIIIa. The arrows indicate the position of the standard Compounds 1 to 7 are the same as indicated in Fig. 2. Standards 8 to 12 are: 8, 3,4,6-tri-Me-Glc; 9, 2,3,4-tri-Me-Glc; 10, 2,4,6-tri-Me-Glc; 11, 2,3,6-tri-Me-Glc; 12, 2,3,4,6-tetra-Me-Glc.
**DISCUSSION**

The purpose of this study was to determine whether the synthesis of the major lipid-linked oligosaccharide of intact Chinese hamster ovary cells proceeds by the ordered or random addition of mannose residues. If ordered addition of the residues occurred, then each precursor oligosaccharide of a given size should consist of a single isomer whereas random mannose addition would lead to the formation of multiple isomers of the oligosaccharides. By incubating the cells with large amounts of \[^{3}H\]mannose (1 mCi/4 ml) we were able to isolate a series of lipid-linked oligosaccharides ranging in size from Man\(_{3}\)GlcNAc\(_{2}\) to Man\(_{9}\)GlcNAc\(_{5}\). The oligosaccharides were subjected to structural analysis which demonstrated that each species consists of one predominant isomer. In our previous study on the Man\(_{9}\)GlcNAc\(_{2}\) species, we performed a pulse-chase experiment with \[^{14}C\]glucosamine which demonstrated that the lipid-linked Man\(_{9}\)GlcNAc\(_{2}\), Man\(_{9}\)GlcNAc\(_{2}\), and Man\(_{9}\)GlcNAc\(_{2}\) species could be chased into the large Glc-Man\(_{9}\)GlcNAc\(_{2}\) species, indicating that these species are in fact precursors of the major lipid-linked oligosaccharide. Unfortunately there was not enough label in the other species to follow their fate. If we make the reasonable assumption that these oligosaccharides are also biosynthetic precursors of the final lipid-linked Glc-Man\(_{9}\)GlcNAc\(_{2}\) molecule which is transferred to nascent proteins, then we can propose a scheme for the synthesis of this molecule, as shown in Fig. 10. This scheme indicates that the addition of mannose residues to the growing lipid-linked oligosaccharide is highly ordered. Several aspects of the scheme deserve special comment. The Man\(_{9}\)GlcNAc\(_{2}\) species has a structure identical with the core portion of many complex-type oligosaccharides (11). Yet complex-type oligosaccharides are not formed by the direct transfer of the Man\(_{9}\)GlcNAc\(_{2}\) species to protein, but rather by the transfer of the Glc-Man\(_{9}\)GlcNAc\(_{2}\) species which is then processed extensively (1–3). The Man\(_{9}\) and Man\(_{9}\) species which are involved in the biosynthesis of the major lipid-linked oligosaccharide represent different isomers than the Man\(_{9}\) and Man\(_{9}\) species which are generated by processing of the Glc-Man\(_{9}\)GlcNAc\(_{2}\) molecule after it has been transferred to the protein acceptor. This provides further evidence that these molecules are not transferred directly to protein but rather represent true intermediates in the biosynthesis of the final lipid-linked oligosaccharide.

The Man\(_{9}\)GlcNAc\(_{2}\) species is of particular interest, since it serves as a branch point, being converted primarily to Glc-Man\(_{9}\)GlcNAc\(_{2}\) but also to Glc-Man\(_{9}\)GlcNAc\(_{2}\). This latter species has been shown to be transferred to nascent protein in uninfected Chinese hamster ovary cells under certain experimental conditions. These results are consistent with the proposal of Turco et al. that glucose residues are required as a signal for oligosaccharide transfer to protein (13). An identical lipid-linked Glc-Man\(_{9}\)GlcNAc\(_{2}\) species has recently been found in a mutant clone of mouse lymphoma cells which has a block in the conversion of lipid-linked Man\(_{9}\)GlcNAc\(_{2}\) to Man\(_{9}\)GlcNAc\(_{2}\) (14, 15). The Glc-Man\(_{9}\)GlcNAc\(_{2}\) species was also shown to be transferred to protein in the mutant lymphoma cell line and to be processed to form typical complex-type oligosaccharides (14).

Several limitations inherent in the techniques used in these studies should be pointed out. The acetolysis procedure results in some degree of under- and over-degradation, especially in oligosaccharides that contain a di-N,N'-acetylchitobiose unit (5). This makes it difficult to evaluate the significance of the minor fragments that are detected.

The methylation procedure may be complicated by the presence of variable amounts of radioactivity at or near the origin and the possibility of some variation in the recoveries of the different methylated species. To control for this potential problem, compounds of known structure were included in each methylation run and these gave the expected products in the correct ratios. In addition, the assumption is made that all of the mannose residues are uniformly labeled during the period of incubation with the \[^{3}H\]mannose. The larger the oligosaccharide, the greater the possibility for some degree of nonuniform labeling. In spite of these limitations, the data clearly demonstrate that each of the oligosaccharides which was examined consists of one predominant isomer. For the reasons just mentioned, we cannot, however, exclude the possibility that small amounts of other isomers of the various oligosaccharides also exist.

Hercovics et al. (16) have previously investigated the carbohydrate composition of lipid-linked oligosaccharides isolated from calf pancreas microsomes. These investigators demonstrated the presence of a heterogeneous mixture of oligosaccharides containing N-acetylglucosamine, mannose, and glucose in varying proportions ranging from 2:5:1 to 2:10:3; however, further structural studies were not performed. Chambers et al. have studied the transfer of mannose to lipid-

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A. Chapman and S. Kornfeld, unpublished observations.

2 The arrangement of the 3 glucose residues into the sequence Glc1 → 2Glc1 → 3Glc → rather than Glc1 → 3Glc1 → 2Glc → was chosen because the former is the sequence present in the major lipid-linked oligosaccharide (4). The data in the present paper do not allow us to distinguish between these two possible sequences.
linked oligosaccharides in vitro using particulate enzyme preparations from pig aorta (17). Using either GDP-mannose or mannosylphosphoryldolichol as the mannose donor to endogenous lipid-linked oligosaccharides, they obtained a heptasaccharide and larger oligosaccharides as the predominant products. Acetolysis of the heptasaccharide released most of the radioactivity as free mannose, suggesting that the residue transferred in vitro was linked \( \alpha 1 \to 6 \). This is difficult to understand in view of our findings suggesting that the hexasaccharide Man\(_5\)GlcNAcz is converted to Man\(_5\)GlcNAcz by the formation of an \( \alpha 1 \to 2 \) linkage. However, their finding that acetolysis of the octasaccharide released most of the radioactivity as a mannobiose is consistent with our structures for the Man\(_5\)GlcNAcz and Man\(_5\)GlcNAcz species.

Although Glc\(_3\)Man\(_6\)GlcNAcz and Man\(_5\)GlcNAcz are consistently the two most abundant lipid-linked species found in Chinese hamster ovary cells, significant variations in the relative proportions of the various species have been observed in different experiments. In addition, other cell types may have different proportions of the various species. Consequently, it is difficult to draw any conclusions concerning regulation of the biosynthetic pathway.

In conclusion, we have proposed a sequence of mannose addition to form the lipid-linked oligosaccharide as it occurs in vivo. This information should be helpful in dissecting out the enzymes involved in this pathway.

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