Lipid-Saccharide Intermediates in Glycoprotein Biosynthesis

II. STUDIES ON THE STRUCTURE OF AN OLIGOSACCHARIDE-LIPID FROM THYROID*

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Structural studies have been performed on an oligosaccharide-lipid from thyroid believed to be an intermediate in glycoprotein synthesis. For these investigations the compound was isolated from the gland in unlabeled form as well as differentially radiolabeled in its saccharide, lipid, and phosphate portions by incubation of slices with [14C]- or [3H]glucose, [3H]mevalonic acid and [32P]phosphate, respectively.

The unlabeled oligosaccharide-lipid was obtained in a chloroform/methanol/water (10/10/3) extract in a yield of about 1 nmol/g of thyroid and was purified therefrom by DEAE-cellulose chromatography. The saccharide moiety released from the glycolipid by mild acid hydrolysis was isolated by gel filtration and contained 11 mannose, 1 to 2 glucose, and 2 N-acetylglucosamine residues. The reducing terminal position of the oligosaccharide was occupied by 1 of the glucosamine residues and from these analyses a molecular weight of 2,415 was calculated.

That glucose is an integral part of the molecule was further demonstrated by the finding that during Dowex 50 chromatography it remained as a constituent of the positively charged oligosaccharide produced by deacetylation with alkaline borohydride at 80°.

The phosphorus content of the purified unlabeled oligosaccharide-lipid was determined to be 2 residues per molecule, suggesting the presence of a pyrophosphate bridge between its carbohydrate and lipid portions. Further evidence for such a linkage region was provided by characterization of the products from mild acid and alkaline hydrolysis of the differentially radiolabeled glycolipid. These included dolichyl mono- and pyrophosphate, oligosaccharide phosphate, and free oligosaccharide.

Digestion with α-mannosidase of the radiolabeled glycolipid led to the release of 39% of its mannose while from the free oligosaccharide 53% of this sugar was removed. Acetylation of the [14C]oligosaccharide yielded a mannobiose and mannotriose as well as larger fragments consisting of mannose, glucose, and glucosamine. Smith periodate degradation gave rise to a small core segment (6 glucose residues) made up only of mannose and glucosamine from which half of the mannose residues could be released by α-mannosidase digestion.

From these studies a tentative structure for the carbohydrate moiety of the oligosaccharide-lipid has been proposed. In this formulation an inner core (periodate-resistant) made up of 4 mannose and 2 N-acetylglucosamine residues is attached to the pyrophosphate group by the most internal glucosamine. This core, as well as an additional mannose and 1 to 2 glucose residues, constitutes the α-mannosidase-resistant fragment. More peripherally are found other mannose residues, all in α-linkage. In this structural scheme the glucose is located so as to prevent the enzymatic release of more internally situated α-linked mannose residues.

In a preceding paper it has been shown that thyroid slices synthesize an acidic oligosaccharide-lipid which appears to serve as an intermediate in the attachment of carbohydrate to protein (1). Radiolabeling experiments indicated that the saccharide moiety of this molecule contains mannose, glucose, and glucosamine and is linked through an acid-labile bond to a phosphorylated lipid metabolically derived from mevalonic acid.

It was the purpose of the present investigation to further characterize the thyroid oligosaccharide-lipid in regard to its composition and structure. Analyses on the carbohydrate, phosphate and lipid portions of the molecule have been...
performed after its isolation in unlabeled form by large scale fractionation of calf thyroids. Studies with the radiolabeled compound have yielded information on the structure of its oligosaccharide moiety and the nature of its linkage region to the lipid. For this purpose the products obtained after glycosidase digestion, alkaline borohydride treatment, acetylation, periodate oxidation, and mild acid and alkaline hydrolysis have been isolated and characterized.

**EXPERIMENTAL PROCEDURE**

**Preparation of Radiolabeled Oligosaccharide-Lipid—**Thyroid slices were incubated with [14C]- or [3H]-labeled glucose, [3H]-leucine, or (10/10/3) extraction and chromatography on DEAE-cellulose. In this manner differential radiolabeling of the saccharide, lipid, and phosphate moieties of the glycoprotoxin molecule was achieved. Incubation with the radioactive glucose for 3 h resulted in maximal labeling of the radioactive lipid and was confirmed on a Nuclear Chicago Actigraph III radioscanner. When components labeled with 3H were to be scanned, the windows of this instrument were removed to improve the efficiency.

The following reference compounds were used for paper chromatography and gel filtration: 2-O-α-D-glucopyranosyl-D-glucose prepared from bakers' yeast mannose by acetylation (8); mannitol, mannose, and mannobiose from *Saccharomyces cerevisiae*, generous gift of Dr. Clinton Ballou, University of California at Berkeley; deltolchol purchased from Sigma; and dolichol phosphate synthesized by the procedure of Wedgwood et al. (9).

**Paper Chromatography—**Descending paper chromatography was performed on Whatman No. 1 paper in the following solvent systems: pyridine/ethyl acetate/water/acetic acid (6/5/3) in the troughs and pyridine/ethyl acetate/water (11/4/5) in the bottom of the chamber (3) (System A); 1-butanol/ethanol/water (10/10/1) (System B); ethanol/1 M sodium acetate (3/1) (System C). Anisaldehyde reagent (7) was employed for locating polysaccharide components. Labeled compounds were detected by the chromogenic reagent, 5,5'-diaminobenzidine tetrachloride in the second extraction and water with no salt in the third and fourth. The methylated paper was dried in vacuo at room temperature for 2 h and then extracted twice with chloroform/methanol (2/1), using 0.5 ml of original tissue. The paper was impregnated with a linear gradient of ammonium acetate in this system mannose, glucose, glucosamine, and N,N-diacyctylchitobiose standards were treated with 28 nmol of NaBT, (178 μCi/μmol) in 300 μl of 0.125 M sodium borate buffer at pH 9.0 for 16 h at 4°C. The reaction was terminated by the addition of acetic acid to bring the pH to 5 and the mixture was lyophilized. The paper chromatograms were developed at 6°C and contained only the free sugars as R. The paper was divided into neutral sugar and hexosamine fractions by passage through an anion exchange chromatography, employing the Technicon sugar analyzer equipped with a split-stream attachment and the elution scheme previously reported (11). The column effluent was divided so that one-third passed through the analytical system and two-thirds were diverted to a fraction collector to be used for scintillation counting. In this system mannose, glucosamine, and mannosamine were well separated from each other. The glucitol eluted in a position close to standard galactose, while the mannitol eluted between the xylose and glucose standard peaks.

**Isolation of Unlabeled Oligosaccharide-Lipid from Calf Thyroids—**Thyroids obtained from a local slaughterhouse were frozen on dry ice and kept at -20°C until use. The glands (130 g) were sliced and homogenized equipped with a PT-2OST generator (Brinkmann Instruments) at a setting of 7 for two 5-s intervals. The homogenate was centrifuged for 20 min at 10,000 × g in the International centrifuge unit. The supernatant was dialyzed against pH 7.4 buffer containing 5% Triton X-100, 10% glycerol and 20% methanol at 37°C for 16 h and then centrifuged at 20,000 × g for 30 min. The supernatant was dialyzed against 0.15 M NaCl, pH 7.4, containing 4 mM magnesium chloride. The suspension was adjusted to an ice bath with a 20% glycerol solution in a homogenizer equipped with a PT-2OST generator (Brinkmann Instruments) at a setting of 7 for two 5-s intervals. The homogenate was centrifuged at 228,000 × g for 20 min in a Beckman model L5-55 ultracentrifuge, and the pellets were suspended in the Triton buffer to a final volume of 0.6 mg/ml of original tissue. Chloroform (1.8 ml/g) and methanol (1.2 ml/g) were added and the mixture was shaken for 10 min at room temperature. After centrifugation at 500 × g for 15 min at 10°C in the International refrigerated centrifuge unit, the interphase pellet was carefully removed from the material at the interphase. The chloroform/methanol/water (3/2/1) extraction was carried out three and fourth. The interphase pellet was dried in vacuo at room temperature for 2 h and then extracted twice with chloroform/methanol (2/1), using 0.5 ml of original tissue. The paper was impregnated with a linear gradient of ammonium acetate in this system mannose, glucose, glucosamine, and N,N-diacyctylchitobiose standards were treated with 28 nmol of NaBT, (178 μCi/μmol) in 300 μl of 0.125 M sodium borate buffer at pH 9.0 for 16 h at 4°C. The reaction was terminated by the addition of acetic acid to bring the pH to 5 and the mixture was lyophilized. The paper chromatograms were developed at 6°C and contained only the free sugars as R. The paper was divided into neutral sugar and hexosamine fractions by passage through an anion exchange chromatography, employing the Technicon sugar analyzer equipped with a split-stream attachment and the elution scheme previously reported (11). The column effluent was divided so that one-third passed through the analytical system and two-thirds were diverted to a fraction collector to be used for scintillation counting. In this system mannose, glucosamine, and mannosamine were well separated from each other. The glucitol eluted in a position close to standard galactose, while the mannitol eluted between the xylose and glucose standard peaks.

**Filtering of Unlabeled Oligosaccharide-Lipid—**The purified oligosaccharide-lipid was solubilized from the pellet with three portions (0.5 ml/g of tissue) of chloroform/methanol/water (10/10/1, 3). The organic solvents were evaporated in a vacuum rotator at room temperature and the water remaining was removed by lyophilization.

**Purification of Unlabeled Oligosaccharide-Lipid—**Chromatography of unlabeled oligosaccharide-lipid in the chloroform/methanol/water (10/10/3) extract was performed on DEAE-cellulose (DE52, microcrystalline, Whatman No. 1 paper). A larger quantity of the purified oligosaccharide (12 nmol) was prepared from the chloroform/methanol/water (10/10/3) extract of the lipids derived from 210 g of thyroid tissue. Purification was achieved with "C-labeled oligosaccharide-lipid (4 × 10^8 dpm) to make possible the monitoring of the elution of the activity by means of scintillation counting without the necessity of consuming significant amounts of the unlabeled compound in chemical analysis. Tubes containing the oligosaccharide-lipid were pooled, the chloroform/water phase removed by aspiration, and the ammonium acetate was volatilized by repeated lyophilization with water. Sugar analyses performed on the radioactive peak as well as on other pools from the column indicated that the unlabeled oligosaccharide-lipid eluted together with the radioactive compound.

**Determination of Reducing-terminal Residue of Oligosaccharide—**In order to determine the optimal hydrolysis conditions for the release of the carbohydrate portion oligosaccharide-lipid labeled with "C in its sugar components of the amine sugar fraction on the Technicon amino acid analyzer I (130-cm column) using the gradient conventionally employed for amino acids. Again two-thirds of the column effluent was diverted to a fraction collector and the labeled glucosaminolactone detected by scintillation counting.

A larger quantity of the purified oligosaccharide (12 nmol) was prepared from unlabeled NaBH₄, by treatment with 150 μmol in 1.0 ml of 0.15 M sodium borate buffer at pH 8.0 for 16 h at 4°C. The reduced oligosaccharide was desalted and hydrolyzed in the manner already described.

The glucosamine and glucosaminolactone of the reduced sample were determined on the Technicon NC-2 amino acid analyzer with a phenol program which utilized pH 5 buffers. The sample was applied to the column at this pH and an initial 40-min elution with 0.1 M citrate, pH 5.0, (0.21 M NaCl) was followed by 80 min of 0.1 M citrate, pH 5.1, 0.38 M
The regeneration cycle for this gradient involved 8 min of 0.2 M sodium citrate buffer, pH 4.5. The enzyme was removed by centrifugation and the digested oligosaccharide-lipid was separated from the released monosaccharide by filtration on a column of Bio-Gel P-4 equilibrated with 0.1 M pyridine acetate buffer at pH 5.0.

An aliquot of the oligosaccharide (6 x 10^9 dpm) obtained by the Bio-Gel filtration after a-mannosidase digestion was treated with 0.05 unit of a-mannosidase from melon (gift of Dr. Y.-T. Li, Tulane University) in 0.2 ml of 0.15 M sodium citrate buffer, pH 4.0, at 37°C for 48 h. At the end of the digestion the glycoprotein was separated from any released monosaccharide by passage through a small charcoal-Celite column (10).

Dissociation of Oligosaccharides—Dissociation of the glycosamine residues present in 14C-labeled neutral oligosaccharides was achieved by their treatment with 1 ml of 2 N NaOH containing 2 M NaBH₄ at 80°C for 20 h in capped polypropylene tubes as previously described (11, 14). At the end of the reaction, the sample was diluted with water, neutralized with acetic acid and placed on a 20-ml column of Dowex 50-X2, 200 to 400 mesh (H⁺ form). After an extensive water wash, the reduced deacetylated oligosaccharides were eluted with 120 ml of 1.5 M ammonium hydroxide and the ammonium was removed by lyophilization.

This procedure was applied to the oligosaccharide obtained by Bio-Gel filtration after mild acid hydrolysis of the oligosaccharide-lipid as well as to neutral products obtained from periodate oxidation and acetolysis studies.

The specificity of the deacetylation was in each case confirmed by conversion of the material in the Dowex 50 eluates to neutral compounds through N-acetylation with acetic anhydride by a procedure previously employed (10).

Radioactivity Measurements—Radioactivity was measured either by liquid scintillation counting in a Nuclear Chicago Isocap 300 scintillation counter using Bray’s solution (18) or by the radiochromatography of paper chromatograms in a Nuclear Chicago radioscanner (Actigraph III) equipped with an integrator. All counts were converted to disintegrations per min with appropriate efficiency corrections.

Other Analyses—Phosphorus was determined after digestion with 70% H₂SO₄-acidification of the ascorbic acid method of Chen et al. (20), in which all of the volumes were reduced by one-half to increase the sensitivity of the reaction.

Hydroses for neutral sugars and hexosamines were performed in 1 N HCl for 6 h at 100°C and the hydrosates were passed through coupled columns of Dowex 50 and Dowex 1 as previously described (10). The chromatograms were determined in a Technicon W2 amino acid analyzer utilizing the pH 5.0 program while the neutral sugars were measured on the Technicon sugar analyzer as previously reported (11), except that a Durrum resin (DA-X4) was employed.
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were extracted with chloroform/methanol/water (10/10/3) after thorough extraction with chloroform/methanol/water (3/2/1) and chloroform/methanol (2/1), a lipid fraction was obtained which had as its monosaccharide constituents mannose, glucose, and glucosamine, as well as variable amounts of galactose (Table I). When low levels of galactose-containing lipid were present (Table I) they were effectively removed by the DEAE-cellulose chromatography. Occasionally larger quantities of galactose occurred and the lipid was then repurified prior to the DEAE-cellulose step by recombining it with the lipid-free protein and repeating the chloroform/methanol/water (3/2/1) and chloroform/methanol (2/1) extractions. The oligosaccharide-lipid was then recovered by extraction with chloroform/methanol/water (10/10/3) in a yield of approximately 50% and with only small amounts of galactose.

Chromatography on DEAE-cellulose led to a purification of the oligosaccharide-lipid (Table I), as has already been demonstrated for the labeled material prepared from thyroid slices after incubation with radioactive substrates (1).

Preparation of Unlabeled Oligosaccharide Moiety and Determination of its Reducing-terminal Residue—After mild acid hydrolysis (0.1 N HCl, 20% methanol, 37°, 16 h) of the unlabeled oligosaccharide-lipid and filtration on Bio-Gel P-6, 84% (Table I) of the mannose was recovered in the oligosaccharide peak, which represents a yield similar to that previously obtained with the radiolabeled material (1). The emergence of the unlabeled oligosaccharide in the same peak as the radioactive material suggested that its molecular weight was close to the 2,400 previously determined (1).

After reduction of the Bio-Gel-purified oligosaccharide with NaBT, no radioactivity was detected in the alcohol derivatives of any of its neutral sugar components. On the other hand, when the hexosamine fraction was chromatographed on the amino acid analyzer, a peak of tritium activity emerged in the tubes corresponding to glucosaminitol. From the amount of tritium incorporated it was calculated that 1.0 mol of glucosamine had been reduced for every 2 mol of this sugar originally present in the oligosaccharide.

When the hexosamine fraction from the oligosaccharide reduced with unlabeled NaBH₄ was analyzed directly on the Technicon amino acid analyzer a ratio of glucosaminitol to glucosamine of 1.2 was found (Table I), which indicated that glucosamine is in the reducing terminal position and that each oligosaccharide contains a second more internal glucosamine as well. On the basis of 2 N-acetylglucosamine residues per molecule the calculated molecular weight of the purified oligosaccharide from the oligosaccharide-lipid is 2,415, which is in close agreement with the molecular weight estimated by the gel filtration studies (1). The nonstoichiometric glucose-oligosaccharide ratio suggests that some microheterogeneity occurs in this molecule. If the assumption is made that all of the mannose in the chloroform/methanol/water (10/10/3) extract is found in the oligosaccharide-lipid it can be calculated that there is 0.90 nmol of this glycolipid per g of thyroid.

Nature of Oligosaccharide-Lipid Linkage Region—In the preceding study it was demonstrated that thyroid slices incubated with [³²P]phosphate incorporated radioactivity into a component that co-chromatographed on DEAE-cellulose with oligosaccharide-lipid labeled either in its saccharide or lipid portions (1).

When phosphorus determinations were performed on the DEAE-cellulose-purified oligosaccharide-lipid, nearly 2 residues per carbohydrate unit were found (Table I), which would be consistent with the acidic behavior of this glycolipid.

When DEAE-purified oligosaccharide-lipid labeled in its phosphate (from [³²P]P) and its lipid (from [³H]mevalonic acid) moieties was treated under several conditions of acid and alkaline different distributions of radioactivity and various products were observed in the upper and lower phases after chloroform/methanol/water (3/2/1) extraction of the hydrolysates (Table II). Although each one of the treatments led to the recovery of all of the H in the lower phase, the ³²P was almost completely recovered in the lower phase only after the 0.1 N HCl, 20% methanol hydrolysis at 37°, while it was more evenly distributed between the upper and lower phases subsequent to the stronger acid (0.25 N HCl, 50% propanol, 90°) or alkaline (0.1 N NaOH, 20% methanol, 90°) treatments. The 90° acid and alkaline hydrolysates yielded a single labeled component in the lower phase which migrated to the position of standard dolichyl monophosphate (Rf = 0.65) upon paper chromatography in Solvent System D (Fig. 1). In contrast the milder 37° acid treatment produced a component with approximately

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield* (mol/mmol oligosaccharide)</th>
<th>Composition (nmol/mmol oligosaccharide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform/methanol/water (10/10/3) extract</td>
<td>10.1</td>
<td>Mannose: 7.0, Glucose: 1.4, Glucosamine: 2.0, Galactose: 0.9, Glucosaminitol: 0.9, Phosphorus: 1.9</td>
</tr>
<tr>
<td>Purified oligosaccharide (DEAE-cellulose peak)</td>
<td>5.6</td>
<td>Mannose: 8.0, Glucose: 1.4, Glucosamine: 2.0, Galactose: 0.9, Glucosaminitol: 0.9, Phosphorus: 1.9</td>
</tr>
<tr>
<td>Purified oligosaccharide (Bio-Gel P-6 peak)</td>
<td>4.7</td>
<td>Mannose: 10.9, Glucose: 1.5, Glucosamine: 2.0, Galactose: 0.9, Glucosaminitol: 0.9, Phosphorus: 1.9</td>
</tr>
</tbody>
</table>

*Expressed per g of original thyroid used for the isolation.
*Phosphorus was only determined on the purified oligosaccharide-lipid.
*Expressed on the basis of 2 glucosamine residues per molecule of oligosaccharide.

Occasional preparations with higher galactose contents were repurified by a second series of extractions prior to DEAE-cellulose chromatography (see text).
TABLE II

Effect of acid and alkaline hydrolysis on oligosaccharide-lipid radiolabeled in the phosphate (\(^{32}P\)) and lipid (\(^{3}H\)) moieties

<table>
<thead>
<tr>
<th>Conditions of hydrolysis</th>
<th>Distribution of radioactivity</th>
<th>Identification of products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upper phase</td>
<td>Lower phase</td>
</tr>
<tr>
<td>0.1 mol HCl, 20% methanol, 37(^\circ)C, 16 h</td>
<td>12</td>
<td>88</td>
</tr>
<tr>
<td>0.25 mol HCl, 50% propanol, 90(^\circ)C, 3 h</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>0.1 mol NaOH, 20% methanol, 90(^\circ)C, 3 h</td>
<td>69</td>
<td>31</td>
</tr>
</tbody>
</table>

\(^a\) Identified by paper chromatography.

\(^b\) Refers to distribution of radioactivity between upper and lower phase of chloroform/methanol/water (3/2/1) extraction; each sample contained oligosaccharide-lipid labeled with 11,250 dpm of \(^{32}P\) and 42,460 dpm of \(^{3}H\) derived from \([8^\text{H}]\text{mevalonic acid.}\)

\(^c\) Only a trace of phosphate was evident chromatographically; studies with oligosaccharide-lipid labeled with \(^{14}C\) in the saccharide moiety have shown that the carbohydrate is released primarily (85\%) as the free oligosaccharide into the upper phase under these conditions of hydrolysis (1).

\(^d\) Studies with oligosaccharide-lipid labeled with \(^{14}C\) in the saccharide moiety indicated that carbohydrate is released into the upper phase as a neutral component.

twice the radioactivity which moved more slowly on chromatography (\(R_c = 0.59\)) and was presumed to be dolichyl pyrophosphate (Fig. 1).

Paper chromatography was performed in Solvent System C for 16 h on the aqueous phases obtained after the various hydrolyses and indicated that after treatment with 0.1 mol HCl at 37\(^\circ\)C there was little radioactivity located either at the origin or in the free phosphate position (migration = 19 cm). The stronger acid hydrolysis, however, produced a substantial phosphate peak, while the treatment with 0.1 mol NaOH yielded both free phosphate and a component which stayed close to the origin. The latter appears to be the oligosaccharide phosphate, as after similar alkaline treatment of oligosaccharide-lipid labeled with \(^{14}C\) in the saccharide moiety most of the radioactivity was recovered in a negatively charged compound (retained on Dowex 1) that stayed close to the origin upon chromatography in Solvent System C. Furthermore, treatment of this \(^{14}C\)-labeled component with alkaline phosphatase converted it into a neutral molecule.

The results of these experiments indicate that mild acid treatment cleaves the glycosylphosphate linkage with the formation of oligosaccharide and dolichyl pyrophosphate, while somewhat stronger acid also splits the pyrophosphate bond and yields, in addition to the oligosaccharide, free phosphate and dolichyl monophosphate. In contrast, alkaline hydrolysis appears to cleave the pyrophosphate bond first leading to the formation of oligosaccharide phosphate and dolichyl monophosphate with the additional formation of free phosphate which may arise from further splitting of both of these primary products.

**Mannosidase Digestions**—When oligosaccharide-lipid labeled with \(^{14}C\) in the mannose and glucose components of the carbohydrate portion was digested with \(\alpha\)-mannosidase in the presence of 1.25% Triton, 29% of the radioactivity (representing 39\% of the mannose) was released in 6 h at 37\(^\circ\)C. Without Triton in the incubation only 11\% of the radioactivity was removed. Digestion of the water-soluble oligosaccharide derived from the oligosaccharide-lipid led to the release of 40\% of the radioactivity (representing 53\% of the mannose residues) in the same period.

Chromatography of the \(\alpha\)-mannosidase digest of \(^{14}C\)-labeled oligosaccharide-lipid on DEAE-cellulose permitted resolution of the modified glycolipid from the released mannose (Fig. 2).
The latter emerged from the column with the solvent wash while the mannose-depleted oligosaccharide-lipid eluted with the buffer gradient at a position slightly ahead of the native glycolipid (Fig. 2), indicating that affinity for the DEAE-cellulose is enhanced by the presence of a larger oligosaccharide unit.

Filtration on Bio-Gel P-4 of the α-mannosidase digest of 14C-labeled oligosaccharide yielded a symmetrical oligosaccharide peak which was separated from the free mannose (Fig. 3). The reduced size of the digested oligosaccharide was apparent from its retarded emergence from the column when compared to the undigested compound (Fig. 3). Analysis of the neutral sugars in the α-mannosidase-digested oligosaccharide obtained by the Bio-Gel filtration indicated that it was significantly enriched in regard to its glucose content, as would be expected if this sugar was an integral constituent of the saccharide moiety to which the mannose also belongs (Fig. 4). Attempts to remove the remaining mannose by treatment with β-mannosidase were not successful.

Preparation and Fractionation of Reduced, Deacetylated Oligosaccharide-Lipid—Treatment of the 14C-labeled neutral oligosaccharide with alkaline borohydride reagent at 80°C for the purpose of deacetylating the glucosamine residues led to its conversion to reduced, positively charged material which could be adsorbed on Dowex 50-X2. Chromatography of this reduced, deacetylated oligosaccharide on a long column of this exchanger at pH 5.0 resulted in the resolution of one major (peak A) and one minor (peak B) component which emerged at salt concentrations of 175 mM and 195 mM, respectively (Fig. 5). Extending the length of the alkaline borohydride treatment did not change the elution pattern and the radioactivity in peaks A and B remained in a ratio of about 4.5:1.

Analyses of these two peaks indicated that they contained both mannose and glucose as their neutral sugar constituents. While the ratio of [14C]mannose to [14C]glucose in peak A was similar to that found in the untreated oligosaccharide (3.3), this ratio in peak B was twice as great (6.7). Since the molar ratios of the sugar components shown in Table I suggest that some oligosaccharides have 2 glucose residues whereas others have only 1, peak A may represent the former and peak B the latter type of molecule.

N-Acetylation completely converted both peaks A and B to neutral components indicating that retention on Dowex 50 had indeed been the result of a deacetylation reaction.

Periodate Oxidation of Oligosaccharide—When 14C-labeled oligosaccharide was treated with sodium periodate for 24 h at 4°C, approximately 45% of the glucose and 60% of the mannose were destroyed. Filtration on Bio-Gel P-4 of the oligosaccharide...
after a Smith periodate degradation resulted in its separation into two groups of different sized radiolabeled components (Fig. 6). Peak a yielded on acid hydrolysis labeled mannose but no glucose as its neutral sugar constituents. Furthermore, the additional presence of N-acetylglucosamine was demonstrated by its complete adsorption on Dowex 50-X2 after alkaline borohydride treatment at 80°C and its reversion to a neutral oligosaccharide upon N-acetylation. The approximate molecular weight of the material in peak a, based on its position of elution from the Bio-Gel P-4 column in comparison with standard mannose oligosaccharides (Fig. 6) was 1,080 which is equivalent to an oligosaccharide with 4 mannose and 2 N-acetylglucosamine residues. Digestion of this fragment with \(\alpha\)-mannosidase led to the release of half (52%) of the \(^{14}C\)mannose residues.

The lower molecular weight fraction (peak b) obtained after the Smith periodate oxidation was separated on paper chromatography into glycerol and one major slower component with an \(R_{Man}\) of 0.80 (Fig. 7). Analysis of this latter material after acid hydrolysis indicated that it consisted of radiolabeled glucose, mannose, and glycerol. Since its migration was more rapid than that of mannobiose (all \(\alpha\)-linked isomers were tested (14)) this component may represent a mixture of mannosylglycerol and glucosylglycerol.

No erythritol was found in peak b either by direct paper chromatography or by chromatography after acid hydrolysis, indicating that neither glucose nor mannose are singly substituted at C-4. Treatment of the material in this peak with alkaline borohydride at 80°C did not result in any conversion to positively charged molecules which could be retained on Dowex 50, indicating that the oligosaccharides of this fraction, unlike those of peak a, were devoid of N-acetylglucosamine.

After the periodate oxidation 4.9% of the radioactivity in the original oligosaccharide was recovered as an anionic component presumed to be formic acid, indicating that approximately 29% of the neutral sugars of this polymer are either in terminal nonreducing positions or solely substituted by 1,6 bonds.

**Acetolysis of Oligosaccharide—**Further information on the distribution of sugars within the oligosaccharide was obtained from a study of fragments produced by the technique of acetolysis (16). Treatment of the purified \(^{14}C\)-labeled oligosaccharide in this manner led to the formation of neutral components which were resolved into two peaks (I and II) by filtration on Bio-Gel P-4 which accounted for all of the radioactivity placed on the column (Fig. 8). Paper chromatography of the lower molecular weight fraction (peak II) in Solvent System A indicated the presence of a small amount of free mannose as well as two slower components which migrated like oligosaccharides (Fig. 9). Oligosaccharide A yielded only \(^{14}C\)mannose upon acid hydrolysis and was believed to be mannotriose on the basis of its chromatographic migration \((R_{Man} = 0.49)\), as well as from the finding that after reduction with sodium borohydride and subsequent hydrolysis, mannose and mannitol in ratio of 2:1 to 1 were identified by chromatography in Solvent System B. Oligosaccharide B was also

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**Fig. 5.** Chromatography on Dowex 50-X2 of deacetylated oligosaccharide (2N NaOH, 2 m NaBH₄, 80°C, 20 h) labeled with \(^{14}C\) in its mannose and glucose constituents. The sample (1.2 x 10⁶ dpm) was applied to a column (1.0 x 82 cm) in 0.02 M pyridine acetate buffer, pH 5.0. After a wash with this buffer a linear gradient of pyridine acetate, pH 5.0, was started at tube 11 as described under "Experimental Procedure." Lettered areas (A and B) designate tubes which were pooled for further study. The recovery of radioactivity from the column was 90%.

**Fig. 6.** Gel filtration of products obtained after a Smith periodate degradation of the \(^{14}C\)-labeled oligosaccharide. After the mild acid hydrolysis steps the sample (3.6 x 10⁶ dpm) was neutralized and placed on a Bio-Gel P-4 column (1.8 x 112 cm). Elution was performed with 0.1 M pyridine acetate, pH 5.0, at a flow rate of 12 ml/h. The positions of elution from the column of standard mannose (M), mannobiose (M₂), mannotriose (M₃), mannotetraose (M₄), and mannohexaose (M₅) are indicated. V₀ refers to the void volume of the column. Lettered areas (a and b) designate tubes which were pooled for further study.

**Fig. 7.** Radioscan of paper chromatographic separation of components found in peak b from Bio-Gel P-4 column (Fig. 6) after a Smith periodate degradation of the \(^{14}C\)-labeled oligosaccharide. Chromatography was performed in Solvent System A on Whatman No. 1 paper for 20 h. The areas designated by bars were eluted for further study. The positions of migration of standard glycerol (Glyc), erythritol (Eryth) mannose, and \(\beta\)-D-\(\alpha\)-D-mannosylmannose (Man-Man) on guide strips are indicated.
Fig. 8. Filtration on Bio-Gel P-4 of the products obtained by acetolysis of the \(^{14}\)C-labeled oligosaccharide. The sample \((2.6 \times 10^9\) dpm) after removal of O-acetyl groups was placed on the column \((1.8 \times 112\) cm) in 0.1 M pyridine acetate, pH 5.0. Elution was carried out with the same buffer at a flow rate of 12 ml/h. The position of elution of standard compounds and the void volume are indicated with the same abbreviations as in Fig. 6. Roman numerals designate the tubes which were pooled for further study.

Fig. 9. Radioscan of paper chromatogram showing separation of components found in peak II from Bio-Gel P-4 column (Fig. 8) after acetolysis of the \(^{14}\)C-labeled oligosaccharide. Chromatography was performed in Solvent System A for 20 h on Whatman No. 1 paper. The areas designated by the capital letters were eluted for further study. The positions of migration of standard mannose, mannobiose \((\text{Man}_2)\), mannotriose \((\text{Man}_3)\), and mannotetraose \((\text{Man}_4)\) are indicated.

Fig. 10. Comparison of radioscan of chromatographic separation of neutral sugar components obtained after acid hydrolysis of the untreated \(^{14}\)C-labeled oligosaccharide and its acetolysis products. Scan A, untreated oligosaccharide; scan B, Bio-Gel P-4, Peak I (Fig. 8); scan C, Bio-Gel P-4 peak II (Fig. 8). Chromatography was performed in Solvent System B for 120 h. The positions of migration of standard galactose, glucose, and mannose on guide strips are shown.

By alkaline borohydride treatment at \(80^\circ\) it was possible to show that two types of oligosaccharides were present in peak I. Approximately half (51%) of the \(^{14}\)C-labeled material in this peak was adsorbed on Dowex 50-X2 after this treatment, while the other half remained neutral and was not retained by this ion exchanger. The positive charge on the component retained by Dowex 50 was presumed to have resulted from the deacetylation of glucosamine and could be eliminated by subsequent \(N\)-acytlylation. Both the neutral and cationic fractions obtained after the alkaline borohydride treatment contained glucose and mannose as their neutral sugar components, although in somewhat different ratios. The higher molecular weight material obtained after acetolysis (peak I) was found to be resistant to the action of the jack bean \(\alpha\)-mannosidase.

**DISCUSSION**

In the preceding paper evidence was presented for the formation by thyroid slices of an oligosaccharide-lipid, believed to be an intermediate in glycoprotein biosynthesis, which could be radiolabeled in its oligosaccharide portion by incubation with glucose, mannose, or glucosamine and in its lipid moiety with mevalonic acid (1). Because \(^{32}\)P could also be incorporated into the molecule during incubation with \(^{32}\)P-phosphate it was felt likely that the linkage between the carbohydrate and lipid involved a phosphate ester bond.

In the present study further structural information on the oligosaccharide-lipid has been obtained from investigations carried out both on the radiolabeled material from thyroid slices and on the unlabeled glycolipid directly isolated from calf thyroid particles in a yield of approximately 1 nmol/g of the gland.
Analyses carried out on the carbohydrate moiety liberated by mild acid hydrolysis from the chromatographically purified unlabeled oligosaccharide-lipid indicated that it consisted of 11 mannose, 1 to 2 glucose, and 2 N-acetylglucosamine residues with a calculated molecular weight of 2,415 which is in close agreement to the size (2,400) estimated for the radioactive oligosaccharide by gel filtration studies (1). One of the 2 glucosamine residues of the acid liberated oligosaccharide was found to be in the reducing-terminal position and therefore likely to be involved in linking the saccharide moiety to the remainder of the molecule.

The finding of close to 2 phosphorus residues per molecule of the unlabeled oligosaccharide-lipid indicated that a pyrophosphate bridge was involved in connecting the carbohydrate and lipid portions of the molecule. The occurrence of such a pyrophosphate group was supported by the characterization of the products of partial acid and alkaline hydrolysis of the differentially radiolabeled oligosaccharide-lipid. Upon treatment with 0.1 N NaOH at 90°C the lipid was primarily liberated as the polyisoprenyl monophosphate, while the saccharide appeared as the oligosaccharide phosphate; acid hydrolysis (0.25 N HCl) at that temperature again yielded the lipid as the monophosphate derivative, but the oligosaccharide was obtained in its free form in addition to inorganic phosphate. Treatment with acid at 37°C however liberated no inorganic phosphate but resulted in the formation of the free oligosaccharide and a component which appeared to be polyisoprenyl pyrophosphate.

Studies carried out on the lipid moiety of the oligosaccharide-lipid indicated that it is a polyisoprenol and most probably dolichol. The chromatographic migration of the phosphorylated lipid released after alkaline or acid hydrolysis at 90°C was identical to that of chemically synthesized dolichyl monophosphate. Moreover, when the compound released by such treatment from the unlabeled oligosaccharide-lipid and purified by thin layer chromatography in chloroform/methanol/water (60/25/4) was incubated with thyroid particle enzyme it served as an excellent acceptor for the transfer of [14C]mannose from GDP-mannose to form [14C]mannolipid and also stimulated the transfer of mannose from the nucleotide to methyl α-D-mannopyranoside to form the methvmannobioside. It has previously been shown that the latter reaction is quite specific in that it could not be carried out by fucaprenyl phosphate (21). The ready labeling of the lipid moiety by radioactive mevalonic acid is further consistent with its identity as a polyisoprenol. The occurrence of endogenous dolichyl phosphate has previously been demonstrated by the formation of dolichyl mannosyl phosphate by thyroid particulate enzymes in the presence of GDP-mannose (21). Furthermore, dolichol has been implicated as the lipid moiety in the polyisoprenol-bound saccharides from a number of other tissues (22).

The 2 glucosamine residues of the carbohydrate moiety are believed to occur in the N-acetyl form, since the oligosaccharide released by mild acid hydrolysis is neutral. It could however be converted to a positively charged molecule by treatment with alkaline borohydride at a temperature which brings about deacetylation of N-acetylglucosamine. The deacetylated oligosaccharide was chromatographed on Dowex 50-X2 by virtue of its positive charges in order to assess the extent of its microheterogeneity. This form of chromatography has previously been used to uncover small variations in saccharide composition in glycopeptides of the thyroglobulin unit A (17). Since 83% of the radiolabeled oligosaccharide appeared in a discrete major peak from the Dowex 50 column the existence of a fairly uniform carbohydrate unit may be inferred. The mannose:glucose ratio of the minor peak suggested that it contains an oligosaccharide that differs from the major component in having 1 rather than 2 glucose residues.

Some information on the distribution of sugars within the oligosaccharide has been obtained from a study of partially degraded forms of this molecule produced by digestion with α-mannosidase, as well as by acetolysis and Smith periodate degradation. A scheme depicting some of the structural features of the carbohydrate moiety of the oligosaccharide-lipid deduced from these studies is presented in Fig. 11.

Approximately half of the mannose residues appear to be peripherally located, linked by α-glycosidic bonds, since they are readily removed by digestion with α-mannosidase. Evidence for terminal chains made up of α-linked mannose residues was also provided by acetolysis, which yielded both a mannohexose and a mannotriose sensitive to α-mannosidase action. These chains appear to be linked to a core region which contains the remainder of the mannose as well as all of the glucose and N-acetylg glucosamine residues. Fragments containing segments of this internal portion were isolated after periodate degradation, after α-mannosidase digestion, and after acetolysis. A periodate resistant region was obtained which contained 6 glucose residues consisting only of mannose and N-acetylg glucosamine, while mannosidase digestion left a somewhat larger core segment which contained an additional mannose and all of the glucose residues (Fig. 11). Acetolysis appeared to cleave the core portion remaining after removal of the mannohexose and mannotriose side chains nonspecifically into two overlapping fragments of about 7 glucose residues each. One of these segments contained the glucosamine and represented the most internal region, while the other contained solely mannose and glucose.

While about 50% of the mannose could be removed from the periodate-resistant mannosyl-N-acetylg glucosamine fragment through the action of α-mannosidase the glucose-containing oligosaccharides obtained from acetolysis were resistant to the action of this enzyme. These findings suggest that the glucose residues are located in positions which interfere with the more complete degradation of the native oligosaccharide by the action of α-mannosidase.

The acetolysis and periodate oxidation studies indicated that the glycosidic linkages between the sugars of the oligosaccharide moiety are diverse and probably involve some branching. On the basis of the known susceptibility of different glycosidic linkages to acetolysis (16) it would appear that the mannohexose and mannotriose which are obtained in high yield after such treatment of the oligosaccharide, are linked to the core portion of this molecule by 1,6 bonds (Fig. 11). Because about 60% of the mannose residues were destroyed by periodate oxidation with the formation of glycerol but no erythritol it is likely that they are in terminal nonreducing positions or substituted by 1,2 and/or 1,6 bonds. On the basis of 14C formic acid formation it appears that about one-quarter of the neutral sugar residues are indeed in terminal positions or singly substituted by 1,6 linkages.

The finding of what appeared to be mannosylglycerol and glucosylglycerol among the fragments formed by the Smith periodate degradation suggests that 1 of the mannose and 1 of

*M. J. Sprou and K. G. Sprou, unpublished observations.*
the glucose residues are in positions resistant to this oxidation, due either to C-3 substitution or branching, and are moreover linked themselves to periodate-sensitive saccharide components (Fig. 11).

In the preceding paper (1) it was pointed out that glucose was present as a constant constituent of the \(^{14}C\)-labeled oligosaccharide-lipid from thyroid slices through various purification steps. In the present study it has been possible to provide additional evidence that glucose, which is a rather unexpected constituent of a mannose-N-acetylglucosamine unit, is indeed an integral part of the oligosaccharide. Glucose was found to remain with the mannose and glucosamine upon chromatography on Dowex 50 of the deacetylated oligosaccharide. Moreover, after removal of peripheral mannose with \(\alpha\)-mannosidase, the shortened oligosaccharide contained all of the glucose along with the \(N\)-acetylglucosamine and the remaining mannose residues. Acetolysis treatment has further indicated that the glucose is associated with mannose and \(N\)-acytigelucosamine in a fragment representing the core region.

While it has been established that 1 of the \(N\)-acytigelucosamine residues is involved in the linkage to the phosphate bridge on the basis of its reduction in the free oligosaccharide, the position of the 2nd \(N\)-acytigelucosamine residue has not yet been determined. By analogy with the linkage region of the asparagine-linked carbohydrate units of glycoproteins (23) and the oligosaccharide-lipid formed by glycerol enzymes (24), one may surmise that it is located next to the innermost \(N\)-acytigelucosamine as part of an \(N\)-acytigelucosamine unit.

Although Leloir and his collaborators (2) originally implicated glucose as a constituent of oligosaccharide-lipids synthesized by liver, more recent studies with enzymes from myeloma (25) and oviduct (26) have shown that these cell-free systems form radiolabeled lipid-bound saccharide units with only mannose and \(N\)-acytigelucosamine as their sugar constituents. These simpler oligosaccharides also appear to differ from the lipid-linked oligosaccharide formed by thyroid slices in that they are of smaller size. In contrast to the 11 mannose residues found in the compound from thyroid, only 5 to 7 residues of this sugar have been reported to occur in the oligosaccharides formed by the oviduct and myeloma enzymes, although no analyses on the unlabeled oligosaccharide-lipids from these latter tissues have been carried out, in contrast to thyroid. It appears however that all of the lipid-bound oligosaccharides have in common the occurrence of 2 \(N\)-acytigelucosamine residues (22).

Because recent studies in our laboratory have indicated that particulate enzyme preparations of thyroid also synthesize small lipid-linked oligosaccharides from GDP-mannose, it seems likely that the cell-free systems, unlike slices, are unable to complete the carbohydrate unit bound to lipid which accumulates physiologically. Perhaps some key enzymatic step, such as the addition of glucose, cannot be adequately carried out after disruption of the cell, thereby preventing formation of the appropriate acceptor for further mannosylation to take place.

Thyroid does not appear to be unique in the ability of its slices to form the larger glucose-containing lipid-bound oligosaccharide units, and in a subsequent paper a comparison is made of the oligosaccharide-lipids produced by slice incubations from a number of tissues (27).

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