The role of glucose-containing dolichyl pyrophosphate-linked oligosaccharides in protein glycosylation by thyroid microsomes is described in an accompanying report (Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1979) J. Biol. Chem. 254, 7668-7674). Glucose was found to be essential for oligosaccharide transfer but a loss of this sugar from the newly synthesized glycoprotein was noted shortly thereafter.

The results of the present investigation demonstrate the occurrence in calf thyroid microsomes of an enzyme which can selectively release glucose from the dolichol-linked oligosaccharide ($K_m = 7 \times 10^{-6} M$) or from the oligosaccharide after transfer to endogenous protein. This glucosidase was less active towards the free oligosaccharide and towards substrate from which the peripheral mannose residues had been removed by a-mannosidase treatment. Enzyme activity was solubilized by Triton X-100, was maximal between pH 6.0 and pH 7.0, and was unaffected by EDTA. Marked stimulation of glucose release was observed in the presence of the anticaotropic ions, SO$_4^-$ and PO$_4^{2-}$. The specificity of the enzyme as well as its neutral pH optima and low $K_m$ value distinguished it from other thyroid glucosidases. A large number of $a$- and $b$-glucosides were ineffective as inhibitors although an $a$-linked glucose tetrasaccharide from nigeran manifested some inhibitory action.

About 85% of the glucose could be released from the oligosaccharide-lipid by the glucosidase without scission of any other bonds and after such treatment the oligosaccharide portion became much more susceptible to a-mannosidase digestion. Since the glucosidic linkages in the oligosaccharide were resistant to cleavage by glucosidases of known anomeric specificities, chromic acid oxidation studies were performed and indicated that these bonds are $a$- in configuration. Information obtained from the action of the thyroid glucosidase as well as periodate oxidation studies suggested that the glucose residues of the oligosaccharide-lipid are attached to the polymannose portion primarily in the form of an $a$-Glc-(1 $\rightarrow$ 3)-Glc disaccharide although some lipid-saccharide molecules apparently contain single glucose residues and a small number have glucose as a component of an $a$-Man-(1 $\rightarrow$ 3)-Glc disaccharide.

Studies with thyroid microsomes under conditions permitting transfer of oligosaccharide from lipid carrier to protein, as well as under conditions where this transfer is inhibited, indicated that the glucose released in this system originated from both the oligosaccharide-lipid donor and the glycoprotein product. This suggests the possibility that thyroid glucosidase, in addition to initiating processing of the oligosaccharide after its transfer to protein, may also control the level of glucose-containing oligosaccharide-lipids available for protein glycosylation.

We have previously reported that thyroid slices, as well as those from a number of other tissues, actively synthesize a dolichyl pyrophosphate-linked oligosaccharide which contains glucose in addition to mannose and $N$-acytlemannosamine residues (2-4). From pulse-chase studies carried out in slices (2) and more recently from investigations with particulate enzyme, described in an accompanying paper (5), it has become evident that the glucose-containing oligosaccharide-lipid serves as an intermediate in glycoprotein biosynthesis by transferring its intact carbohydrate moieties to protein. Indeed, the experiments with the thyroid cell-free system indicate that the glucose is essential for effective transfer reactions to take place and appears to play a specifying role in this glycosylation step (5). These studies, as well as investigations carried out in a number of other laboratories (6-8), now strongly suggest that the glucose-containing oligosaccharide-lipids function as the physiological donors in the en bloc transfer of carbohydrate to protein which represents the initial step in the biosynthesis of the asparagine-linked saccharide units.

Although glucose is still attached to the mannose-$N$-acytetlemannosamine oligosaccharide moiety after transfer from the lipid carrier to nascent protein, this sugar has not been found as a component of asparagine-linked carbohydrate units of mature glycoproteins (9). This paradox prompted us to postulate that glucose may be enzymatically cleaved from the protein-linked oligosaccharide as part of a series of modifying reactions which lead to the typical N-glycosidically-bound saccharide moieties (2).

In this report, we present evidence that thyroid contains a microsomal enzyme which can selectively release glucose from its attachment to the mannose-$N$-acytetlemannosamine oligosaccharide. The properties of this glucosidase are described and suggest that it may indeed carry out the first step in a series of processing reactions of the kind which have been proposed for the vesicular stomatitis virus glycoprotein (8, 10, 11). Since the thyroid glucosidase can act on lipid-linked as well as protein-bound oligosaccharides, it may have an additional physiological function involving the regulation of the amount of appropriate oligosaccharide-lipid donors available for the glycosylation of proteins.
Thyroid Glucosidase Involved in Glycoprotein Processing

EXPERIMENTAL PROCEDURES

Preparation of Enzyme—Fresh calf thyroids were obtained from a local slaughterhouse and brought on ice to the laboratory. After removal of connective tissue, the glands were minced into small pieces and suspended in 5 volumes of ice cold 0.35 M sucrose, 0.1 M Tris/acetate buffer, pH 7.0, containing 25 mM potassium acetate, and 5 mM magnesium acetate. Disruption of the tissue was achieved by two 5-s bursts of a Polytron homogenizer equipped with a PT-20ST generator (Brinkmann Instruments) at a neashot setting of 7. After filtration through a 35-mesh polyethylene sieve, the homogenate was centrifuged successively in a Beckman L-5-65 ultracentrifuge at 2,000 \( \times g \) for 10 min, 15,000 \( \times g \) for 20 min, and 215,000 \( \times g \) for 35 min. The 215,000 \( \times g \) pellet, representing the microsomal fraction, was washed with 1 ml of a 0.35 M sucrose solution containing 2 mM 2-mercaptoethanol by centrifugation at 215,000 \( \times g \) for 35 min and then resuspended in the same buffer with a Dounce homogenizer.

Solubilization of enzyme was achieved by gently stirring for 30 min the suspended microsomal membranes with 0.45% Triton X-100 in 0.1 M Tris/acetate, pH 7.0, 2 mM 2-mercaptoethanol buffer at a protein concentration of about 8 mg/ml. The Triton extract was then separated from insoluble material by centrifugation at 78,000 \( \times g \) for 90 min and used for the enzyme studies to be reported unless otherwise specified. All of the above steps were carried out at 2°C.

Preparation of \(^{14}C\)-Labeled Substrates—Oligosaccharide-lipid radiolabeled in its glucose as well as mannose residues was prepared by incubating thyroid slices with \( \alpha \left( ^{1}U - ^{14}C \right) \) glucose, about 270 \( \mu \)Ci/\( \mu \)mol, 0.1 M sodium acetate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol, after which the 2,000 \( \times g \) pellets were washed in chloroform/methanol (10:1.0) and DEAE cellulose chromatography. By varying the amount of \( ^{14}C \) glucose used per gram of slices in the incubations, oligosaccharide-lipids were prepared containing glucose residues with specific activities ranging from 3 \( \times 10^{7} \) to 3 \( \times 10^{8} \) dpm/\( \mu \)mol as determined from sugar analyses after acid hydrolysis. The average ratio of radioactivity of [\(^{14}C\)mannose to [\(^{14}C\)glucose in the oligosaccharide-lipid was 2.7.

The radiolabeled oligosaccharide mixture was prepared by mild acid hydrolysis of the \(^{14}C\)-labeled oligosaccharide-lipid (0.02 n HCl, 20% methanol, for 20 min at 100°C) followed by passage through coupled columns of Dowex 50 X 2 and Dowex 1 X 8.

Digestion with \( \alpha \)-mannosidase of the intact \(^{14}C\)-labeled oligosaccharide-chloroform/methanol (1:1) washes from the protein extractions. For this purpose the \(^{14}C\)-labeled oligosaccharide-lipid was also transferred to endogenous thyroid protein by incubation under the same conditions as previously reported (3) and then acetylated with pyridine/acetic anhydride (1:1) in chloroform/methanol (10:10:3). The protein was removed by centrifugation, washed with this solvent mixture, and the combined supernatant and washes were chromatographed on a column of DEAE-cellulose (DE-52 microgranular) (1.5 \( \times \) 22 cm) in chloroform/methanol/water (10:10:3) mixture. Sulfates were detected by alkaline silver nitrate (16, 17) and labeled compounds localized by radioscanning.

Preparation of Enzymes—For the purpose of product characterization, larger amounts of radiolabeled oligosaccharide-lipid (65,000 to 250,000 dpm \( ^{14}C\)glucose) were incubated for extended periods of time in the presence of tolue. The digests were terminated by the addition of chloroform, methanol, and water to give 3 ml of a chloroform/methanol/water (10:10:3) mixture. The protein was removed by centrifugation, washed with this solvent mixture, and the combined supernatant and wash were chromatographed on a column of DEAE-cellulose (DE-52 microgranular) (1.5 \( \times \) 22 cm) in chloroform/methanol/water (10:10:3). Oligosaccharides released from this purified lipid by mild acid hydrolysis were filtered boiled Bio-Gel P-4 (1.18 \( \times \) 110 cm) in 0.1 M sodium acetate buffer, pH 7.0 (3).

Chromium Trioxide Oxidation—\(^{14}C\)-Labeled oligosaccharide derived from the thyroid oligosaccharide-lipid as well as various disaccharide standards were reduced with NaBH₄ under the conditions previously described (3) and then acetylated with pyridine/acetic anhydride (1:1) in chloroform/methanol. The acetylated oligosaccharides were then oxidized by chromium trioxide in a heating block at 100°C for 60 min (18). After removal of the reagent with a stream of dry nitrogen, the samples was dried with 100 ml of a mixture of equal volumes of chloroform and methanol dissolved in 50% acetic acid at 45°C for 15 min in a Branson ultrasonic bath (19, 20). Upon dilution with 3 ml of H₂O, the reaction mixture was extracted three times with equal volumes of chloroform. The solvent was evaporated from the chloroform extract and the glucosylceramide determined by paper chromatography (21). The acetylation and chromium trioxide oxidation were optimally carried out on 2 \( \mu \)mol of reduced saccharide. Since only about 20 pmol of \(^{14}C\)-labeled oligosaccharide were employed in these

\(^{1} \) All glycosides are in the pyranose form.

\(^{2} \) The delipidated radiolabeled oligosaccharide-protein from paper (5). The delipidated radiolabeled oligosaccharide was isolated by published procedures for the preparation of labeled glucosylceramide.}

Other Substrates and Sources of Commercial Glucosidases—Enzymes tested for their capacity to release glucosidase from glucosylceramide (Type IV-A, \( \alpha \)-amylase, Sigma), Aspergillus niger (amyloglucosidase, Boehringer Mannheim), and hog pancreas (Type I-a, \( \alpha \)-amylase, Sigma) as well as \( \beta \)-glucosidase from almond emulsion (Mann Research Laboratories). The radiolabeled oligosaccharide mixture was prepared by mild acid hydrolysis of the \(^{14}C\)-labeled oligosaccharide-lipid. The radiolabeled oligosaccharide-lipid was also transferred to endogenous thyroid protein by incubation under the same conditions to yield a mannose-rich radiolabeled oligosaccharide-protein.

\(^{3} \) Preparations were from the \(^{14}C\)-labeled oligosaccharide-protein by pronase digestion followed by Bio-Gel P-4 filtration (5).

\(^{4} \) Other Substrates and Inhibitors—Samples of 1-O-\( \beta \)-glucosylceramide 1 both unlabeled and radiolabeled in its glucose residue (n-\( ^{6}H \)glucose) were kindly made available by Dr. E. Kolodny and S. Raghavan, Eunice Kennedy Shriver Center. [\(^{14}C\)]Maltose was purchased from ICN while [\(^{14}C\)-Glc(1 \( \rightarrow \) 2)-\( \beta \)-Gal(1 \( \rightarrow \) 4)-Hyl] was prepared enzymatically with rat kidney enzyme (12). The following disaccharides were obtained as gifts: nigerose (Dr. I. J. Goldstein, University of Michigan and Dr. J. H. Pazar, Pennsylvania State University); laminaribose (Dr. S. Kirkwood, University of Minnesota (Smith Collection)); kojibiose (Dr. K. Matsuda, Tohoku University); sophorose (Dr. W. Wolfrom, Ohio State University). A tetrasaccharide isolated from nigeran (13) with the structure Glc-(1 \( \rightarrow \) 3)-a-Glc-(1 \( \rightarrow \) 4)-a-Glc(1 \( \rightarrow \) 3)-Glc was kindly provided by Dr. J. H. Nordin (University of Massachusetts at Amherst). All other compounds employed in the glucosidase assays including p-nitrophenyl-\( \alpha \)- and \( \beta \)-glucosides, maltose, cellobiose, isomaltose, gentiobiose, and n-glucal, 1,5-lactone, salicin, and phloridzin were obtained from commercial sources.

Standard Assay of Glucosidase Activity—In the standard assay, the enzyme, approximately 0.35 mg of protein, from the Triton solubilized microsomal pellet (215,000 \( \times g \) was incubated with \(^{14}C\)-labeled oligosaccharide-lipid, containing about 13,000 dpm in its glucosyl residues, in 300 \( \mu l \) of 0.1 M Tris/acetate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol, 0.45% (v/v), Triton X-100, and 0.95 M (NH₄)₂SO₄. The incubations were carried out with shaking at 30°C for 3 h and were terminated by the addition of 6 ml of cold 8% trichloroacetic acid. The supernatant was separated from the precipitated protein by centrifugation and was extracted with cold ether to yield a white powdery residue. The extracted protein was then precipitated with 10% TCA.
studies, it was found necessary to add unlabeled reduced disaccharide (2 μmol of a 1:1 mixture of celllobiitol and maltoito) to the radiolabeled sample to enhance the recovery in the acetylation steps. Control experiments with 14C-labeled and reduced 14C-labeled oligosaccharide indicated that substantial losses (>90%) occurred in this procedure when samples containing less than 200 nmol were employed.

All oxidations were performed along with controls in which the acetylated oligosaccharide was treated with the acetic acid in the absence of CrO3.

Digestion with Mannosidase—Radiolabeled oligosaccharides (1.2 × 106 dpm) obtained by mild acid hydrolysis after treatment of the oligosaccharide-lipid with the thyroid glucosidase were incubated with 2 units of purified jack bean α-mannosidase for 24 h at 37°C under the conditions previously described (3). The reaction was terminated by the addition of cold 8% trichloroacetic acid and after ether extraction and desalting by passage through Dowex 50 (H+ form) and Dowex 1 (formate form), the deproteinized samples were chromatographed on paper in Solvent System B.

Periodate Oxidation—14C-Labeled oligosaccharides (6 × 105 dpm) were oxidized with periodate and reduced with NaBH4, as previously described (3). Sugar analyses were performed after the oxidation and reduction steps.

Assay of Glucosidase—Incubation of the Triton X-100 extract of calf thyroid microsomes with 14C-labeled oligosaccharide-lipid resulted in the selective release of radiolabeled glucose as shown in a typical radioscan of a chromatogram used in the assay (Fig. 1, lower). Since the oligosaccharide-lipid employed as substrate contained almost 3 times as much radiolabel in its mannose residues as in the glucose, the absence of free mannose in this chromatographic assay served as a sensitive index for excluding the presence of even small levels of mannosidase activity. The radiolabeled component present at the origin represents oligosaccharide split from the lipid during the deproteinization step and could also be observed when the substrate was incubated without enzyme (Fig. 1, upper).

Solubilization of Particulate Glucosidase—Incubation of thyroid microsomal particles with various concentrations of Triton X-100 indicated that maximal release of glucose from oligosaccharide-lipid was achieved at a detergent concentration of 0.45% (v/v) (Fig. 2). Treatment of the microsomal fraction with this concentration of Triton brought about 40% of the protein into solution and yielded a supernatant with a specific enzyme activity about 3 times greater than that of the unextracted membranes. This Triton-solubilized fraction was used to study in detail the properties of the microsomal glucosidase. As far as could be ascertained, the glucosidase activity in the Triton supernatant was qualitatively similar to that in the whole microsomal pellet and moreover was free of mannosidase action towards oligosaccharide-lipid, an activity which was occasionally detected after prolonged incubations with the unextracted membranes.

FIG. 1. Radioscans obtained during a standard glucosidase assay (see "Experimental Procedures") after incubation of 14C-labeled oligosaccharide-lipid in the absence (upper scan) and presence (lower scan) of thyroid Triton-solubilized microsomal enzyme (0.37 mg of protein). Chromatography was performed in Solvent System A on Whatman No. 1 paper for 7 days. The positions of migration of standard glucose (Glc) and mannose (Man) on guide strips are shown.

RESULTS

Assay of Glucosidase Activity—Incubation of the Triton extract of calf thyroid microsomes with 14C-labeled oligosaccharide-lipid resulted in the selective release of radiolabeled glucose as shown in a typical radioscan of a chromatogram used in the assay (Fig. 1, lower). Since the oligosaccharide-lipid employed as substrate contained almost 3 times as much radiolabel in its mannose residues as in the glucose, the absence of free mannose in this chromatographic assay served as a sensitive index for excluding the presence of even small levels of mannosidase activity. The radiolabeled component present at the origin represents oligosaccharide split from the lipid during the deproteinization step and could also be observed when the substrate was incubated without enzyme (Fig. 1, upper).

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Thyroid Glucosidase Involved in Glycoprotein Processing

**Fig. 2 (left).** Effect of Triton X-100 concentration on the release of glucose from $^{14}$C-labeled oligosaccharide-lipid by thyroid microsomal enzyme (215,000 x g pellet). Incubations were performed under standard conditions with thyroid microsomes (1.1 mg of protein) and substrate containing 13,600 dpm (50 pmol) of $[^{14}]$Cglucose at various Triton X-100 (v/v) concentrations.

**Fig. 3 (center).** Effect of time on the release of glucose from $^{14}$C-labeled oligosaccharide-lipid by thyroid Triton-solubilized microsomal enzyme. Incubations were performed under standard conditions with 0.37 mg of enzyme protein and substrate containing 15,600 dpm (480 pmol) of $[^{14}]$Cglucose.

**Fig. 4 (right).** Effect of enzyme concentration on release of glucose from $^{14}$C-labeled oligosaccharide-lipid. Incubations were performed under standard conditions with varying amounts of thyroid Triton-solubilized microsomal enzyme and substrate containing 13,600 dpm (480 pmol) of $[^{14}]$Cglucose.

**Fig. 5 (left).** Effect of pH upon thyroid glucosidase activity. Incubations were carried out at the indicated pH values under standard conditions with 0.42 mg of Triton-solubilized microsomal enzyme and $^{14}$C-labeled oligosaccharide-lipid containing 12,200 dpm (46 pmol) of $[^{14}]$Cglucose. The buffers employed were as follows: △, acetate; ○, phosphate; ■, Pipes; ●, Tris. The solid line (---) and the dashed line (-- -- --) represent different experiments.

**Fig. 6 (right).** Effect of ammonium sulfate concentration on thyroid glucosidase activity. Incubations were carried out under standard conditions with 0.37 mg of Triton-solubilized microsomal enzyme and $^{14}$C-labeled oligosaccharide-lipid containing 12,200 dpm (46 pmol) of $[^{14}]$Cglucose at various concentrations of (NH$_4$)$_2$SO$_4$.

The enzyme also acted on the oligosaccharide after transfer to protein (Table I). Glycopeptides prepared from this oligosaccharide-protein demonstrated reduced activity, and protein glycosylated with an α-mannosidase-treated oligosaccharide-lipid was an even less effective substrate for the glucosidase.

**Comparison with Other Thyroid Glucosidase Activities—**The rather unusual specificities of the glucosidase, as well as the low $K_m$ value determined for the oligosaccharide-lipid, suggested that the enzyme was distinct from various other known glucosidases. Indeed, a comparison of the $K_m$ values of various glucosidase activities in the Triton extract of thyroid microsomes indicated that the value for the oligosaccharide-lipid was about 2 to 3 orders of magnitude lower than that for a variety of other substrates (Table III). Moreover, the ratio of glucosidase activity at neutral to acidic pH was considerably higher for the lipid-bound oligosaccharide than for the other compounds examined. The ratio of glucosidase activity at pH 7.0 to pH 4.2 when the oligosaccharide-protein was used as substrate was similar to that for the oligosaccharide-lipid (10.2 compared to 11.8).

**Inhibitors—**Several oligosaccharides and glycosides were examined as possible inhibitors of glucosidase activity. For this purpose, these compounds were incubated with $^{14}$C-labeled oligosaccharide-lipid (2.5 μM) for 3 h at molar ratios of inhibitor to substrate ranging from 1:1 to 100:1. Under these conditions, the following compounds showed less than 10% inhibition.

**Table I**

| Reaction mixture | Enzyme activity | Relative activity |
|------------------|-----------------|------------------|
| Control incubation$^a$ | 8,860 | 100 |
| Omit enzyme | 0 | 0 |
| Heat 4 min at 80°C | 0 | 0 |
| Add 0.05 M EDTA | 9,300 | 105 |
| Add 0.02 M Mn$^{2+}$ | 9,210 | 104 |
| Add 0.01 M glucose | 9,130 | 103 |
| Incubations without (NH$_4$)$_2$SO$_4$ | 1,200 | 14 |
| No addition | 8,260 | 93 |
| Add 1.5 M potassium phosphate, pH 7.0 (0.92 M HPO$_4^{2-}$) | 594 | 7 |
| Add 1.0 M Tris acetate, pH 7.0 | 0 | 0 |
| Add 2.0 M sodium perchlorate | 1,430 | 16 |

$^a$ Control incubation was carried out under the standard assay condition (see "Experimental Procedures") with 0.39 mg of Triton-solubilized microsomal enzyme and $^{14}$C-labeled oligosaccharide-lipid containing 13,000 dpm (52 pmol) of $[^{14}]$Cglucose.
inhibition of $[^{14}C]$glucose release from the oligosaccharide-lipid: kojibiose, sophorose, nigerose, laminaribiose, maltose, cellobiose, isomaltose, gentiobiose, 1-O-$\beta$-D-glucosylceramide, salicin, phlorizin, p-glucono-1,5-lactone, p-gluc, and p-nitrophenyl $\alpha$- and $\beta$-D-glucosides. However, a tetrasaccharide from nigeran ($\alpha$-Glc-(1 $\rightarrow$ 3)-$\alpha$-Glc-(1 $\rightarrow$ 4)-$\alpha$-Glc-(1 $\rightarrow$ 3)-Glc) did exert a modest inhibitory activity on the release of glucose from the radiolabeled oligosaccharide-lipid: the percentage inhibition at various concentrations of the tetrasaccharide were as follows: 16% at 12.5 $\mu$M; 24% at 25 $\mu$M; 41% at 250 $\mu$M; and 57% at 1.25 mM.

Characterization of the Products of Glucosidase Treatment—Under the conditions of the glucosidase assay, no transfer of oligosaccharide to endogenous protein took place and all the radioactivity was recovered in the chloroform/methanol/water (10:10:3) extract. Upon DEAE-cellulose chromatography of the $^{14}C$-labeled oligosaccharide-lipid after extended digestion with the thyroid enzyme, it emerged from the column primarily in a position ($R$) of that observed for the co-chromatographed tritium-labeled oligosaccharide-lipid (Fig. 8). However, a small amount of material (C) eluted in the position of the undigested compound. Peak A, which emerged from the column before start of the gradient, con-

![Fig. 7. Effect of oligosaccharide-lipid concentration (expressed on the basis of glucose content) on thyroid glucosidase activity. Standard conditions of incubation were employed with varying concentrations of oligosaccharide-lipid-containing $[^{14}C]$glucose at a specific activity of 3.2 $\times$ $10^6$ dpm/nmole and 0.32 mg of Triton-solubilized microsomal enzyme. The inset shows the Line- weaver-Burk plot of the data.](http://www.jbc.org/)
tained the sugars released by the enzyme, and paper chromatography of the material in that peak revealed that it contained only free \( ^{14}C \) glucose (Fig. 9A). Moreover, paper chromatography of the neutral sugars from an acid hydrolysate of the major oligosaccharide-lipid fraction (Fig. 8, Peak B) indicated that mannose alone was now present (Fig. 9B). In contrast, chromatography of a hydrolysate of the minor component (Fig. 8, Peak C) showed that it still contained some glucose in addition to the mannose (Fig. 9C).

The oligosaccharide obtained by mild acid hydrolysis of the DEAE-cellulose-purified, glucosidase-treated oligosaccharide-lipid yielded a single peak on Bio-Gel P-4 filtration which emerged from the column slightly later than the native oligosaccharide (Fig. 10). The oligosaccharide derived from the glucosidase-treated lipid-saccharide was found to be more susceptible to \( \alpha \)-mannosidase digestion than the oligosaccharide obtained from the undigested compound (Fig. 11). While \( \alpha \)-mannosidase, as we have previously reported (3), released only a limited amount of mannose from the native oligosaccharide leaving a large residual polymer (Fig. 11, upper scan) the action of this enzyme on glucosidase-treated material resulted in the appearance of most of the radioactivity as free mannose or as smaller oligosaccharides which migrated away from the origin on paper chromatography (Fig. 11, lower scan). Treatment with \( \alpha \)-mannosidase of oligosaccharides obtained after partial glucosidase digestion indicated that the amount of mannose released was directly related to the extent of prior glucose removal.

**Incubation with Nonthyroid Glucosidases**—In an attempt to determine by enzymatic means the anomeric configuration of the glucosidic linkages in the lipid-bound oligosaccharide, incubations were carried out with glucosidases of known \( \alpha \)- or \( \beta \)-specificities. The \( ^{14}C \)-labeled oligosaccharide obtained from the lipid-oligosaccharide by mild acid hydrolysis as well as its \( \alpha \)-mannosidase-digested derivative were incubated with these enzymes for various periods of time (up to 48 h) at appropriate pH values. All of the enzymes tested were completely ineffective in releasing \( ^{14}C \) glucose from these oligosaccharides. The glucosidases tested and the pH of the incubation were as follows:

| Enzyme | pH |
|--------|----|
| \( \alpha \)-glucosidase | 5.0 |
| \( \beta \)-glucosidase | 5.0 |
| \( \alpha \)-mannosidase | 5.0 |

**Fig. 9.** Paper chromatography of sugar components present in Peaks A, B, and C from DEAE-cellulose column (Fig. 8). The material in Peak A was chromatographed directly while that in Peaks B and C was hydrolyzed with acid and passed through coupled Dowex 50 and Dowex 1 columns before chromatography. Paper strips were scanned for radioactivity after chromatography was performed in Solvent System A for 5 days. The positions of migration of standard glucose (Glc) and mannose (Man) are indicated.

**Fig. 10.** Filtration on Bio-Gel P-4 of \( ^{14}C \)-labeled oligosaccharide released by mild acid hydrolysis from glucosidase-digested and DEAE-cellulose-purified oligosaccharide-lipid similar to Peak B (Fig. 8). The sample (8.3 \times 10^3 dpm) was applied to the column (1.8 \times 110 cm) in 0.1 M pyridine acetate buffer, pH 5.0, and elution was carried out with this buffer at a flow rate of 12 ml/h. Recovery of radioactivity from the column was complete. The elution of native oligosaccharide (- - -) was determined by co-chromatography with \( ^{1}H \)-labeled oligosaccharide obtained by mild acid hydrolysis of an undigested \( ^{1}H \)-labeled oligosaccharide-lipid. The void volume of the column (V0) and the position of elution of mannose (Man) are indicated by arrows.

**Fig. 11.** Paper chromatography of products from \( \alpha \)-mannosidase digests of native (upper scan) and glucosidase-treated (lower scan) \( ^{14}C \)-labeled oligosaccharides. The mannosidase digestion was carried out as described under “Experimental Procedures” on equimolar amounts of oligosaccharides prepared by mild acid hydrolysis from untreated and thyroid-glucosidase-digested, DEAE-purified, \( ^{14}C \)-labeled oligosaccharides, respectively. The deproteinized and desalted samples were chromatographed on Whatman No. 1 paper in Solvent System B for 24 h and then scanned for radioactivity. The position of migration of standard mannose (Man) is shown.
follows: almond ß-glucosidase (pH 4.5); yeast α-glucosidases (pH 4.5 and 6.8); rice α-glucosidase (pH 6.8); fungal α-glucosidases from A. niger (pH 5.0) and A. oryzae (pH 4.5); and hog pancreatic α-amylase (pH 7.0).

Chromium Trioxide Oxidation—Since the anomeric configuration of the glucose residues in the lipid-linked oligosaccharide could not be determined by the use of glucosidases of established specificity, a CrO₃ oxidation procedure was employed to distinguish between α- and β-glycosidic bonds (Table IV). The oxidation, when carried out on standard reduced acetylated disaccharides (25), resulted in the expected high recovery of α-linked glucose as in maltose and melibiose but in substantial destruction of the β-linked sugar as in celllobiose and lactose (Table IV).

Chromium-trioxide treatment of the reduced, acetylated oligosaccharide from the thyroid-lipid intermediate resulted in a 75% yield with a ratio of [14C]glucose to [14C]mannose actually slightly higher than in the unoxidized control (Table IV). Since all of the mannose residues of the oligosaccharide except the most internal one are believed to be α in anomeric configuration (4, 8, 26), the glucose-to-mannose ratios observed indicate that the glucose in at least the recovered 75% of the oligosaccharide-lipid molecules are also in α-glycosidic linkages. The presence of one β-linked mannose which would be destroyed by the CrO₃ oxidation probably accounts for the slight increase in the ratio of glucose-to-mannose radioactivity compared to the unoxidized control. The incomplete recovery of the CrO₃-treated oligosaccharide may be related to the difficulty of applying this procedure to picomole amounts of radiolabeled saccharides (see “Experimental Procedures”).

Periodate Oxidation Studies—Although the extensive release of glucose from the oligosaccharide-lipid indicated that this sugar is primarily located in positions external to the mannose residues, the resistance of about 15% of the glucose to enzymatic release suggested that some glucose may be located in a more internal position. To explore this possibility, periodate oxidation was performed on the oligosaccharide

| Saccharide         | Chromium trioxide | Recovery | Ratio \([\text{[14C]}\text{Glc}/\text{[14C]}\text{Man}]\) |
|--------------------|-------------------|----------|--------------------------------------------------|
| Oligosaccharide    | +                 | 75       | 0.39                                             |
| Oligosaccharide    | -                 | 92       | 0.35                                             |
| Maltose            | +                 | 95       |                                                  |
| Maltose            | -                 | 98       |                                                  |
| Celllobiose        | +                 | 18       |                                                  |
| Celllobiose        | -                 | 93       |                                                  |
| Lactose            | +                 | 8        |                                                  |
| Lactose            | -                 | 93       |                                                  |
| Melibiose          | +                 | 96       |                                                  |
| Melibiose          | -                 | 94       |                                                  |

All compounds were reduced with sodium borohydride and acetylated prior to oxidation.

The conditions for acetylation and chromium trioxide oxidation are described under “Experimental Procedures.”

It has become apparent in recent years that glucose-containing dolichyl pyrophosphate-linked oligosaccharides are

![Fig. 12](http://www.jbc.org/)

**FIG. 12.** Time course of glucose release during incubation of thyroid microsomes (215,000 x g pellet) with 14C-labeled oligosaccharide-lipid under conditions in which transfer of oligosaccharide to endogenous protein occurs (Mn⁺⁺, 0.01 M) and under conditions in which this transfer is inhibited (EDTA, 0.05 M). Incubations were performed at 25°C with freshly prepared microsomal membranes (1.8 mg of protein) and 14C-labeled oligosaccharide-lipid (33,000 dpm total radioactivity; 5,800 dpm glucose radioactivity) in 100 μl 0.1 M Tris acetate, pH 7.0, buffer containing 25 mM potassium acetate and 2 mM 2-mercaptoethanol in the presence of manganese (Mn⁺⁺) or EDTA. The amount of 14C-glucose liberated was determined by paper chromatography of the incubations after desalting and removal of protein, while the incorporation of radioactivity into glycoprotein was measured by counting of the hydrolyzed, delipidated protein pellet (see “Experimental Procedures”). No free mannose was released during the course of these incubations before and after α-mannosidase digestion and these studies indicated that while 53% of the glucose was destroyed in the native oligosaccharide, 66% was susceptible to oxidation in the mannosidase-treated derivative. Although this difference is small, it is consistent with the substitution of about 13% of the glucose residues with peripheral mannose.

**Relationship of Glucose Release to Oligosaccharide Transfer to Protein by Thyroid Microsomes**—Since the specificity studies indicated that the thyroid glucosidase was capable of releasing glucose from the oligosaccharide-lipid as well as from glycoproteins derived from it, an attempt was made to differentiate between the two activities by incubating intact microsomal membranes with radiolabeled oligosaccharide-lipid under conditions where transfer of oligosaccharide to protein takes place as well as under conditions where this transfer is inhibited (Fig. 12). As reported in the accompanying paper (5), a rapid transfer of oligosaccharide from lipid carrier to endogenous protein takes place in the presence of manganese but this reaction is completely abolished when EDTA is added. While the glucose released in the presence of the cation can originate from both glycoprotein and oligosaccharide-lipid, the glucose liberated during incubation with EDTA would arise from the oligosaccharide-lipid alone. It is evident from the data presented in Fig. 12 that in this microsomal system glucose appears to originate from both potential substrates in comparable amounts as this sugar is released in the absence of glycoprotein synthesis, and its liberation is approximately doubled when transfer of oligosaccharide to protein occurs.

**DISCUSSION**

It has become apparent in recent years that glucose-containing dolichyl pyrophosphate-linked oligosaccharides are...
widely distributed in eukaryotic cells (27) and participate in the biosynthesis of N-glycosidically-linked carbohydrate units of glycoproteins by transferring their saccharide moiety en bloc to nascent polypeptide chains. Furthermore, the studies described from our laboratory (5, 28) as well as those reported by Turco et al. (6) suggest that glucose plays an important specifying role in this transfer of carbohydrate from lipid carrier to protein. Since glucose, however, has not been observed in asparagine-linked saccharide units (9), the enzymatic removal of this sugar must constitute a necessary step in any processing reactions leading to the mature carbohydrate moieties.

The microsomal glucosidase described in the present study would appear to be well suited to serve as a processing enzyme. The rather unusual substrate specificity of this enzyme, its low $K_m$ value, and its neutral pH optimum distinguish it from other glucosidases and are consistent with it carrying out such a physiological role. Indeed, the preference of this glucosidase for an oligosaccharide with an intact polymannose portion suggests that it is the first in a series of processing enzymes.

Since the thyroid glucosidase has been shown to release glucose from the oligosaccharide linked to lipid as well as from the oligosaccharide attached to protein, an additional function relating to the lipid-saccharide intermediate might be ascribed to this enzyme. As suggested in the schemes shown in Fig. 13, the glucosidase, in addition to initiating the processing of the oligosaccharide after its transfer to protein, may act on the oligosaccharide-lipid donor itself to regulate its availability for protein glycosylation. Since we have shown (5) that glucose acts as a specifier necessary for the transfer reaction to protein, the glucosidase could control the pool of effective oligosaccharide-lipid donors and thereby influence the rate of attachment of carbohydrate to protein in N-glycosidic linkage. While the physiological importance of such glucosidase action on oligosaccharide-lipid remains to be further clarified, the function of the thyroid glucosidase as a processing enzyme is in agreement with recent studies on glycoprotein synthesis in virus-infected cells (8, 10, 11) and hen oviculum (30).

The occurrence of glucose in the oligosaccharide-lipid as a specifier for protein glycosylation, followed by its removal shortly after this event, represents an interesting example of the importance of molecular determinants in biological processes and in some ways may be analogous to the role of signal peptides in the interaction of nascent proteins with membranes of the endoplasmic reticulum (31), since these terminal sequences are also excised from the protein after their function is completed.

Some of the properties of the thyroid glucosidase probably relate to its function as an integral component of cellular membranes. The intact oligosaccharide-lipid was found to be a substantially better substrate than the lipid-free oligosaccharide suggesting that the hydrophobic dolichol moiety which is probably physiologically sequestered within the membrane may interact with an enzyme site. The facilitating effect of lipophilic substituents on substrates for a membrane-derived enzyme has previously been observed in a study of thyroid mannosyltransferases (32). The importance of hydrophobic interactions is also suggested by the considerable stimulation of glucosidase activity observed in the presence of antichaperone ions such as $SO_4^{2-}$ and $HPO_4^{2-}$ which would be expected to effect a partial reconstitution of the disassembled membranes (24).

The observation that $\alpha$- and $\beta$-glucosidases from various sources were ineffective in releasing glucose from either the untreated or $\alpha$-mannosidase-pretreated oligosaccharides provided further evidence that the thyroid glucosidase is a specialized enzyme reserved to remove glucose from its rather unusual attachment to a polymannose moiety. This resistance of the glucose to various glucosidases of known specificities precluded making an assignment of the anomeric configuration of the glucosidic linkages on an enzymatic basis. The chromic acid oxidation studies, however, provided evidence that the glucose residues in the oligosaccharide lipid are primarily present in $\alpha$-glycosidic linkage. This would be consistent with our finding that an $\alpha$-linked tetrasaccharide from nigeran (13) with the sequence, $\alpha$-Glc-(1 $\rightarrow$ 3)-$\alpha$-Glc-(1 $\rightarrow$ 4)-Glc-(1 $\rightarrow$ 3)-Glc, inhibited glucose release from the oligosaccharide-lipid by the thyroid enzyme. The failure of the $\alpha$-(1 $\rightarrow$ 3)-linked disaccharide (nigerose) or, for that matter, all other $\alpha$-linked glucose-containing disaccharides to inhibit glucose release suggests that the enzyme may have a large binding site which requires several glycosidically-linked sugar residues.

The present investigation has expanded our understanding of the structure of the thyroid oligosaccharide-lipid beyond that previously reported (3). The fact that about 85% of the

![Fig. 13. Schematic presentation of the physiological roles which may be ascribed to the thyroid microsomal glucosidase(s). One function may be to initiate processing of the oligosaccharide after its transfer to protein to yield the mature, high mannose (Unit A) and complex (Unit B) types of carbohydrate units such as occur in thyroglobulin (29). In addition, glucosidase action on the oligosaccharide-lipid may serve to regulate the amount of appropriate donor available for protein glycosylation.](http://www.jbc.org/)
glucose could be liberated by the thyroid enzyme without scission of any other bonds indicated that the glucose is primarily located in positions external to the polymannose portion of the molecule. Furthermore, the periodate resistance of about 50% of the glucose observed in our previous report as well as in the present study suggest that this sugar occurs primarily in the form of an α-Glc-(1→3)-Glc disaccharide attached to mannose on one of the three branches of the oligosaccharride. This disposition of the glucose residues is compatible with the structure recently proposed by Li et al. (23) for the oligosaccharide-lipid involved in vesicular stomatitis virus G glycoprotein biosynthesis. However, our data indicates that a small percentage of the thyroid oligosaccharide-lipid molecules contain glucose residues internal to mannose since they are resistant to periodate oxidation until the mannose is removed, and this would also account for the lack of complete release of the glucose by the thyroid enzyme. It would therefore appear from the data of the current study as well as the information previously reported (3) that the 1.5 mol of glucose which occur per mole of the thyroid oligosaccharide-lipid occur α-glycosidically linked to the polymannose portion of the molecule in the following forms: two-thirds as the α-Man-(1→3)-Glc disaccharide, one-fifth as the monosaccharide, and the remainder (approximately 13%) as a component of an α-Man-(1→3)-Glc disaccharide.

While our previous findings suggested that the presence of peripherally located glucose protects a portion of the polymannose segment of the molecule from α-mannosidase degradation (3), this could be directly demonstrated in the present studies with the aid of the thyroid glucosidase, as susceptibility of the oligosaccharide in the α-mannosidase increased with progressive removal of the glucose. Furthermore, since the thyroid glucosidase is active on the intact oligosaccharide-lipid, it has proven to be a valuable tool in evaluating the function of glucose residues in the glycosylation process as reported in the accompanying paper (5).

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