Glycoprotein Biosynthesis in Plants

DEMONSTRATION OF LIPOID-LINKED OLIGOSACCHARIDES OF MANNOSE AND N-ACETYLGLUCOSAMINE*

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Previous studies from this laboratory have shown that particulate preparations from maturing cotton fibers catalyze the transfer of mannose from GDP-[14C]mannose into mannosylphosphorylpolysoprenol (Forsee, W. T., and Elbein, A. D. (1973) J. Biol. Chem. 248, 2858–2867). In this report, we show that these particulate preparations also catalyze the incorporation of mannose from GDP-[14C]mannose into lipid-linked oligosaccharides and into glycoprotein. The oligosaccharide-lipids were treated with dilute acid to liberate the water-soluble oligosaccharides and these oligosaccharides could then be separated into seven or eight distinct radioactive peaks by paper chromatography in isobutyric acid/NH₃OH/H₂O (57/4/39). The smallest of the oligosaccharides appears to be a trisaccharide with the structure Man & GlcNAc-GlcNAc. Thus the oligosaccharides attached to the lipids apparently range in size from those having 3 glucose units to those having approximately 8 to 10 glucose units. The radioactivity in the smaller-sized oligosaccharide-lipids could be chased into the larger oligosaccharide-lipids by a second incubation in the presence of unlabeled GDP-mannose. The sugar at the reducing ends of the oligosaccharides was identified as GlcNAc while some mannose (20 to 30%) was present in α linkages at the nonreducing ends. The [14C]mannose-labeled glycoprotein was isolated by treatment of the insoluble residue with sodium dodecyl sulfate, or water-soluble [14C]mannose-labeled glycopeptides were obtained by Pronase digestion of the insoluble material. GlcNAc was present in the glycopeptides and was shown to be involved in the linkage of at least some of the oligosaccharide chains to the protein.

When p-hydroxymercuribenzoate (p-HMB) was added to incubations containing GDP-[14C]mannose and enzyme, it inhibited the formation of the oligosaccharide-lipids but allowed the particulate preparations to accumulate large amounts of [14C]mannosylphosphorylpolysoprenol. However, when this inhibition was reversed by the addition of dithiothreitol, radioactivity rapidly disappeared from the mannosylphosphorylpolysoprenol with the concomitant appearance of radioactivity in the [14C]mannose-labeled oligosaccharide lipids. Nearly all of this radioactivity was in the largest oligosaccharides, suggesting that the mannosylphosphorylpolysoprenol is an intermediate in the synthesis of at least the more distal mannose residues of the oligosaccharides.

Particulate fractions of cotton fibers also catalyze the incorporation of GlcNAc from UDP-[3H]GlcNAc into lipids and into glycoprotein. In the absence of GDP-mannose, essentially all of the GlcNAc is incorporated into a lipid which is soluble in CHCl₃/CH₃OH (2/1) and which was characterized as an N,N'-diacetylchitobiosyl-(pyro)-phosphoryl-lipid. However, when GDP-mannose is added to incubation mixtures containing UDP-[3H]GlcNAc, very little chitobiosyl-lipid is formed and most of the radioactivity is found in lipid-linked oligosaccharides. The addition of GDP-mannose to particulate preparations containing [3H]GlcNAc-labeled chitobiosyl-lipid results in the rapid disappearance of radioactivity in the chitobiosyl-lipid with the concomitant appearance of radioactivity in the oligosaccharide-lipids. The [3H]GlcNAc-labeled oligosaccharides isolated from the oligosaccharide-lipids elute from Sephadex G-25 in the same area as the [14C]mannose-labeled oligosaccharides and both of these oligosaccharides give the same series of radioactive peaks on paper chromatography. The GlcNAc-labeled oligosaccharides also contain GlcNAc at the reducing end and some mannose in α linkages at the nonreducing end. [3H]GlcNAc-labeled glycopeptides were also isolated from the insoluble residue by Pronase digestion.

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GDP-[β-3H]mannose and glucose from UDP-[α-3H]glucose into CHCl<sub>3</sub>/CH<sub>3</sub>OH-soluble products which were identified as mannosyl- and glucosylphosphorylpolysaccharides (1, 2). Similar types of lipids have also been reported in other plant systems (3–6), as well as in microbial and mammalian systems (see review by Lennarz and Secher, Ref. 7, for detailed coverage).

In the present study we show that particulate fractions of cotton fibers also catalyze the incorporation of mannose from GDP-[β-3H]mannose and GlcNAc from UDP-[α-3H]GlcNAc into a series of lipid-linked oligosaccharides which appear to have GlcNAc at the reducing terminus and some α-linked mannose at the nonreducing ends. The oligosaccharide-lipids appear to be formed by the sequential addition of mannose to the GlcNAc-disaccharide-lipid. Mannose and GlcNAc are also incorporated into the oligosaccharide chains of glycoproteins by the cotton enzyme. Previous studies from the laboratories of Lennarz (8, 9) and Heath (10, 11) have shown that in mammalian systems mannosylphosphorylpolysaccharide serves as the precursor for the formation of a (Man)₆-GlcNAc-lipid and this oligosaccharide-lipid is then the precursor for the oligosaccharide chain of the glycoprotein. Thus the plant systems appear to resemble, in many respects, results obtained with animal systems. However, the present study appears to be one of the few reports showing the formation of a whole series of oligosaccharide-lipids. A series of oligosaccharide-lipids has also been observed in aorta tissue incubated with GDP-[β-3H]mannose (12). This cotton system may therefore be useful for studying the sequential formation of individual oligosaccharide-lipids. A preliminary report of some aspects of this work has been presented (13).

**MATERIALS AND METHODS**

**Chemicals—**All solvents were analytical reagent grade unless otherwise indicated. GDP-[β-3H]mannose (278 μCi/μmol), UDP-[α-3H]GlcNAc (5 to 15 Ci/μmol), UDP-[α-3H]GlcNAc (45 to 55 μCi/μmol), and NaBH₄ (4.7 mCi/mg) were purchased from New England Nuclear Co. GDP-mannose, UDP-GlcNAc, and DEAE-cellulose were obtained from Sigma Chemical Co. Sephadex G-25 and G-50 were from Pharmacia Co., Pronase was from Calbiochem, and Silica Gel G plates were from Brinkmann Instruments Co. α-Mannosidase was prepared from jack bean meal (14) and β-mannosidase was purified from Aspergillus fumigatus.'

**Analytical Methods—**Radioactivity was measured in a Packard liquid scintillation spectrometer using Bray's cocktail for aqueous samples and toluene scintillator for lipid samples, or for paper strips. Paper chromatograms were scanned with a Packard radiotchromatogram scanner.

**Chromatographic Procedures—**Oligosaccharides and monosaccharides were chromatographed on Whatman No. 3MM paper in the following solvent systems: Solvent A, CHCl₃/CH₃OH/H₂O (57/4/39); Solvent B, 1-propanol/ethyl acetate/H₂O (57/4/39); Solvent C, 1-butanol/pyridine/0.1 N HCl (5/3/2); Solvent D, ethyl acetate/acetic acid/formic acid/H₂O (18/3/1/4). Sugars were visualized with alkaline silver nitrate (15) or with periodate-permanganate spray (16).

**Enzymatic Treatments of Oligosaccharides and Glycopeptide—**A series of lipid-linked oligosaccharides—Assays for the incorporation of mannose or GlcNAc were prepared by incubating 0.2 ml of enzyme with the appropriate sugar nucleotide (usually 50,000 cpm of [β-3H] or [α-3H]) and 10 mM MgCl₂ all in a final volume of 0.25 ml. Incubations were done at 37°C for varying periods of time. In some cases, as indicated in the text, incubations were run with labeled GDP-mannose and unlabeled UDP-GlcNAc or vice versa. The reactions were stopped by the addition of 2 ml of CHCl₃, 2 ml of CH₃OH, and 1.7 ml of H₂O. The reactions were mixed vigorously and the phases were separated by centrifugation. The lower organic phase, which contained the mannosylphosphorylpolysaccharide and the chitobiose-lipid, was removed and saved. The upper aqueous phase was extracted once with 2 ml of CHCl₃/CH₃OH/H₂O (1/1/0.3). After standing for 15 min at room temperature, the pellet was removed by centrifugation and the 1/1/0.3 solution was dried in scintillation vials for the determination of radioactivity. The remaining pellet was washed two or three times with water and then plated on 1-cm pieces of Whatman No. 3MM paper. The papers were dried and placed in scintillation vials for the determination of their radioactive content.

**Chemical Methods for Analysis of Oligosaccharides and Glycopeptides—**Mild acid hydrolysis of the oligosaccharide-lipids was done in 0.1 N HCl in 50% 1-propanol at 100°C for 10 min. Samples were neutralized with NaHCO₃ and lipid was removed by extraction with CHCl₃/CH₃OH. The aqueous phase was removed and dried and the oligosaccharides were isolated and purified by gel filtration and paper chromatography. Strong acid hydrolysis of oligosaccharides and glycopeptides was done in 4 N HCl at 100°C for 2 to 4 hours. After hydrolysis, HCl was removed by evaporation. Sugars were separated and identified by ion exchange chromatography and paper chromatography.

Oligosaccharides were reduced in 20 mM Na₂CO₃ in the presence of excess NaBH₄ for 24 to 30 hours at room temperature. Following reduction, the mixtures were acidified with acetic acid and passed through Dowex 50 H⁺ to remove Na⁺. The wash from this column was concentrated to dryness and the residue was dissolved reportedly in CH₃OH and dried to remove borate. The tritiated oligosaccharides were purified on Sephadex G-25 before being subjected to strong acid hydrolysis.

In order to determine the nature of the carbohydrate–peptide linkage and the sugar involved in this linkage, the glycopeptides subjected to strong alkaline digestion in the presence of NaBH₄ (19, 20). The glycopeptide mixture was treated with 1 N NaOH containing 1 mM NaBH₄ for 6 hours at 100°C. The sample was then neutralized with glacial acetic acid and concentrated to dryness. The sample was suspended in CH₃OH and concentrated to dryness several times to remove borate. The oligosaccharides were partially purified on Sephadex G-25 before being subjected to strong acid hydrolysis.

**Enzymatic Treatments of Oligosaccharides and Glycopeptide—**Oligosaccharides to be treated with α- or β-mannosidase were placed in 0.05 M acetate buffer, pH 5.0, and enzyme (0.1 to 1 mg) was added. After incubation, trichloroacetic acid was added to a final concentration of 10% to precipitate the protein which was then centrifuged. The supernatant liquid was extracted repeatedly with ether to remove trichloroacetic acid before being analyzed by paper chromatography.

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Proteolytic digestion of the glycoprotein was done using Pronase. The washed pellet was suspended in 2 ml of 0.02 M Tris buffer, pH 7.5, containing 0.01 M CaCl₂, and 2 mg of Pronase were added. After 24 hours the insoluble residue was isolated by centrifugation and the supernatant liquid was removed and saved. The pellet was resuspended in Tris buffer containing CaCl₂, and treated with another sample of Pronase as indicated above. The supernatant liquid, containing the glycopeptides, was heated for 10 min at 100° to destroy the Pronase and was then lyophilized. The glycopeptides were purified by gel filtration on Sephadex G-50 and by paper chromatography and paper electrophoresis.

RESULTS

Incorporation of Mannose into Lipid-linked Sugars and into Glycoprotein

Fig. 1 shows that the particulate enzyme from cotton fibers catalyzes the incorporation of mannose from GDP-[³⁴C]mannose into three different products. During the first 5 min there was a rapid incorporation of radioactivity into a product which was soluble in CHCl₃/CH₃OH (2/1). The amount of radioactivity in this product leveled off after 10 min and then slowly declined during long incubations (not shown in this figure). This product was characterized previously as a mannosylphosphorylpolyisoprenol (1, 2). Radioactivity was also incorporated at a somewhat slower rate into material which was not extracted by CHCl₃/CH₃OH or by water, but which was extracted with CHCl₃/CH₃OH/H₂O (1/1/0.3). Incorporation of radioactivity into this product was linear for about 5 to 8 min and then proceeded at a lower rate over the next 10 or 15 min. This material has been characterized partially as a series of lipid-linked oligosaccharides (see below). The third product formed in these reactions is insoluble in organic solvents and in water. This material is a mixture of polysaccharide and glycoprotein. Approximately 10% of this water-insoluble radioactivity could be solubilized by treatment with sodium deoxycholate or by digestion with Pronase. Some characterization of the glycoprotein is given below.

Isolation and Partial Characterization of [³⁴C]Mannose-labeled Oligosaccharide-lipids

In order to isolate sufficient amounts of [³⁴C]mannose-labeled oligosaccharide lipids for characterization, incubation mixtures were scaled up about 30 times to contain 1.5 x 10⁶ cpm of GDP-[³⁴C]mannose and 6 ml of enzyme. Following an incubation of 20 min, this mixture yielded about 180,000 cpm of mannosyl-oligosaccharide-lipid and about 250,000 cpm of water-insoluble material.

Chromatographic Properties—When the intact mannosyl-oligosaccharide-lipids (i.e. the 1/1/0.3 soluble material) were streaked on Whatman No. 3MM paper and chromatographed in Solvent A, most of the radioactivity migrated in a broad area near the solvent front (Rₛ = 0.6 to 0.9). On the other hand, when the lipids were chromatographed in water, most of the radioactivity remained at the origin. However, if the oligosaccharide-lipid was first heated at 100° in 0.01 N HCl for 15 min and then streaked on paper and chromatographed in H₂O, the radioactivity now migrated at the solvent front. These chromatographic properties are suggestive of acid-labile lipid-linked oligosaccharides. In addition the mannosyl-oligosaccharide-lipids bound to DEAE-cellulose and could be eluted with a solvent of CHCl₃/CH₃OH/H₂O (1/1/0.3) containing 0.1 M ammonium formate. However, since it was difficult to remove the ammonium formate from the lipids, this method was not used for routine purification. Instead, the lipids were hydrolyzed in 0.01 N HCl at 100° for 15 min in 50% propanol and the water-soluble oligosaccharides were purified by paper chromatography.

The oligosaccharides, obtained by mild acid hydrolysis of the [³⁴C]mannose-labeled oligosaccharide-lipids, were chromatographed on paper in Solvent A. When this chromatogram was cut into strips and counted in the liquid scintillation counter, the results shown in Fig. 2 were obtained. The oligosaccharide mixture was separated into seven or eight distinct peaks in this solvent. In this particular experiment, radioactivity in each peak is low since these results were from a small scale incubation. However, the same profile of oligosaccharides was observed consistently from experiment to experiment and is also seen in large scale incubation mixtures (see Fig. 3). The actual amount of radioactivity in a given oligosaccharide did vary, depending on the length of the incubation, amount of enzyme, and so on. In general, with increasing time of incubation there appeared to be a shift in the radioactivity from the smaller-sized (i.e. faster migrating) oligosaccharides to the larger oligosaccharides. Unfortunately, since the particulate enzyme undoubtedly contains various sized, endogenous, oligosaccharide-lipids which can serve as acceptors of mannose to the smaller oligosaccharides.

Fig. 1. Time course of incorporation of mannose from GDP-[³⁴C]mannose into various products by the particulate enzyme from cotton fibers. Enzyme assays and extraction procedures were done as described in the text. The various products were characterized as mannosylphosphorylpolyisoprenol (man-p-poly) (soluble in 2/1 CHCl₃/CH₃OH); [³⁴C]mannose-labeled oligosaccharide-lipids (oligo-lipids) (soluble in 1/1/0.3 CHCl₃/CH₃OH/H₂O); and water-insoluble products (combination of glycoprotein and polysaccharide).

The radioactive peak which migrated near the mannone area...
of the papers (Figs. 2 and 3) was eluted and rechromatographed in Solvent D. In this solvent, a GlcNAc-disaccharide (see Fig. 9) had an $R_{\text{GlcNAc}}$ of 0.72 while mannose had an $R_{\text{GlcNAc}}$ of 0.74 and glucosamine an $R_{\text{GlcNAc}}$ of 0.21. The radioactive material gave a major peak with an $R_{\text{GlcNAc}}$ of 0.4 as well as a smaller peak at the origin. This mobility of 0.4 was suggestive of a trisaccharide while the peak at the origin was thought to be a tetra- or pentasaccharide. When the “trisaccharide” was treated with $\beta$-mannosidase essentially all of the radioactivity was released as mannose. However, $\alpha$-mannosidase had no effect on this material. The “trisaccharide” was also isolated from experiments with UDP-$[^{3}H]$GlcNAc (see below for details of these studies). When this $[^{3}H]$-labeled compound was treated with $\beta$-mannosidase, the radioactivity now chromatographed in the disaccharide area ($R_{\text{GlcNAc}} = 0.75$). The “tetrasaccharide” peak was also treated with $\alpha$- and $\beta$-mannosidase. In this case, $\beta$-mannosidase had no effect whereas $\alpha$-mannosidase released about 30% of the original radioactivity in two new peaks. One of these migrated with mannose whereas the other migrated in the trisaccharide area of the paper. Thus, the oligosaccharides seen in Figs. 2 and 3 appear to vary in size from 3 sugar residues probably to 8 or 10 sugar residues.

Chemical Properties—Suggestive evidence for the presence of phosphate in the $[^{14}C]$mannose-labeled oligosaccharide-lipids was based on the fact that they bound to DEAE-cellulose and could be eluted with ammonium formate. The intact oligosaccharide-lipids were also treated with 0.1 N NaOH in 50% propanol at 100°C for 15 min (11). This procedure resulted in the release of a negatively charged radioactive material which was water-soluble and which bound to Dowex 1-Cr-\(\text{I}^{-}\). The radioactivity was eluted from this column with 1 M KCl, desalted on Sephadex G-10, and treated with alkaline phosphatase. This treatment gave rise to neutral $[^{14}C]$-oligosaccharides. Thus, the oligosaccharides appear to be attached to the lipid by a phosphoryl or pyrophosphoryl linkage. Strong acid hydrolysis of the oligosaccharides liberated all of the radioactivity as mannose (see Fig. 5).

**Determination of Reducing and Nonreducing Ends of Oligosaccharides**—In order to determine the sugar at the reducing ends of the oligosaccharides, the $[^{14}C]$mannose-labeled oligosaccharides were purified on Sephadex G-25 (see Fig. 12). The broad peak of radioactivity was pooled, concentrated to dryness, and suspended in dilute Na$_2$CO$_3$ at pH 10. The oligosaccharides were then reduced with NaBH$_4$. Excess borohydride was destroyed with acetic acid and the mixture was purified on Sephadex G-25 as shown in Fig. 4. Two peaks of $[^{3}H]$ were observed on this column but the first peak ran...
slightly ahead of the $^{14}$C peak. Since the oligosaccharide mixture is composed of a series of oligosaccharides, it seemed possible that the discrepancy in the $^3$H and $^{14}$C peaks was due to a much greater uptake of $^3$H by the larger-sized oligosaccharides. That this was apparently the case was shown by the fact that when the $[^{3}H]$ oligosaccharides were chromatographed on paper in Solvent A, most of the $^3$H was associated with the larger-sized oligosaccharides. Most likely this uptake of $^3$H reflects the amount of each of these endogenous oligosaccharides that are present in the particulate enzyme and suggests that the larger-sized oligosaccharides are present in much higher concentration. The $[^{3}H, ^{14}C]$ mannose-labeled oligosaccharides from the G-25 column were hydrolyzed in 4 N HCl at 100° for 4 hours. The hydrolysate was concentrated to dryness and HCl was removed by the repeated addition and evaporation of water. The sample was then streaked on paper and chromatographed in Solvent C as shown in Fig. 5. All of the $^{14}C$ was found in the mannose area of the paper whereas a peak of $^3$H corresponded to glucosaminitol. The $^3$H at the origins of these papers has not been identified further but apparently it results from a radioactive contaminant in the NaBSH$_2$O$_4$ since it has also been observed by others (21). The $^3$H peak corresponding to glucosaminitol was eluted and passed through Dowex 50-H$^+$. All of the radioactivity bound to the column was eluted with 1 N HCl. The tritium in the IICl eluate also chromatographed with glucosaminitol in Solvent D. Further, on paper electrophoresis in borate buffer, a procedure which separates glucosaminitol from galactosaminitol (21), the radioactive sugar migrated with authentic glucosaminitol. These data indicate that the major sugar at the reducing ends of the oligosaccharides is GlcNAc. However, at this point, we cannot be certain that GlcNAc is at the reducing terminus of each of the oligosaccharides.

Fig. 5. Paper chromatographic identification of the sugars released from the $[^{3}H, ^{14}C]$mannose-labeled oligosaccharide by strong acid hydrolysis. The oligosaccharide peak from Sephadex G-25 was hydrolyzed in 4 N HCl as described in the text. After removal of HCl, the hydrolysate was streaked on Whatman No. 3MM papers and run in Solvent C. Papers were cut into 1-cm strips and counted in the liquid scintillation counter. Standards are glucosaminitol (GlcNol), mannitol (MANol), arabinol (ARAol), xylitol (XYLol), and mannose (MAN).

$\beta$-mannosidase, $\alpha$- and $\beta$-glucosidase, and $\beta$-galactosidase. The $\beta$-mannosidase was a preparation from Aspergillus fumigatus which was free of $\alpha$-mannosidase, $\alpha$- and $\beta$-glucosidase, and $\alpha$- and $\beta$-galactosidase. Following treatment, the reactions were stopped either by heating or by the addition of 10% trichloroacetic acid, and the insoluble material was removed by centrifugation. Trichloroacetic acid was removed by extraction with ether. The supernatant liquid was then chromatographed in Solvent C and papers were scanned to locate the radioactive mannose. Approximately 20 to 25% of the radioactivity was released from the oligosaccharides by treatment with $\alpha$-mannosidase, but not by treatment with $\beta$-mannosidase. Approximately the same amount of radioactivity (25 to 30%) was released when the $\alpha$- and $\beta$-mannosidase were used together. Thus the nonreducing ends of these oligosaccharides contain at least some mannose in $\alpha$ linkages.

**Effect of p-Hydroxymercuribenzoate on Formation of Lipid-linked Intermediates**

As shown in Fig. 6, the addition of increasing amounts of p-hydroxymercuribenzoate to incubations of GDP-$[^{14}C]$mannose and enzyme results in an increase in the amount of $[^{14}C]$mannosylphosphorylpolyisoprenol and a decrease in the amount of $[^{14}C]$mannose-labeled oligosaccharide-lipids. At 4 mM p-HMB$^*$ there was essentially no oligosaccharide-lipid formed. This inhibition by p-HMB could be overcome by the addition of excess dithiothreitol. This amount of dithiothreitol when added alone neither stimulated nor inhibited the incorporation of mannose into the various lipid products.

The kinetics of formation of the various lipids in the presence of 4 mM p-HMB was studied as shown in Fig. 7. During the first 8 min of incubation there was a rapid synthesis of mannosylphosphorylpolyisoprenol but little $[^{14}C]$mannose-labeled oligosaccharide-lipid was formed. At 8 min, 10 mM dithiothreitol

$^*$The abbreviation used is: p-HMB, p-hydroxymercuribenzoate (sodium salt).
and 10^{-4} \text{ M} \text{ unlabeled GDP-mannose were added and incubations were continued for an additional 22 min. This amount of unlabeled GDP-mannose was sufficient to dilute the labeled GDP-[\text{\textsuperscript{14}C}]mannose to where it was ineffective as a mannosyl donor. Thus, one of the controls shown at the right-hand side of this figure shows that virtually no radioactivity is incorporated into any lipids when 10^{-4} \text{ M} \text{ GDP-mannose is added to incubations containing GDP-[\text{\textsuperscript{14}C}]mannose. The results show that as soon as p-HMB inhibition is reversed by dithiothreitol there is a rapid appearance of [\text{\textsuperscript{14}C}]mannose-labeled oligosaccharide-lipid along with a substantial disappearance of mannosylphosphorylpolyisoprenol. Just as in the case of the GDP-mannose stimulation of GlcNAc incorporation (Fig. 10), the decrease in radioactivity in the mannosylphosphorylpolyisoprenol is greater than the appearance in the oligosaccharide-lipid. It seems probable that the explanation for this discrepancy is that the oligosaccharide-lipids are not completely stable and that they break down during the incubation. Thus stoichiometry is not possible at this time. However, since GDP-mannose could not be serving as a mannosyl donor for the oligosaccharide-lipids, it seems likely from this experiment that the mannosylphosphorylpolyisoprenol is the direct precursor for at least some of the mannosyl residues in the oligosaccharide-lipids.

Incorporation of GlcNAc into Lipid-linked Disaccharide

As shown in Fig. 8, in the absence of GDP-mannose, radioactivity from UDP-[\text{\textsuperscript{3}H}]GlcNAc is rapidly incorporated into lipids which are soluble in CHCl$_3$/CH$_2$OH (2/1), but little radioactivity is found in the CHCl$_3$/CH$_2$OH/H$_2$O (1/1/0.3)-soluble products. The best divalent cation for this incorporation was Mg$^{2+}$ while Ca$^{2+}$ was about one-half as effective. The CHCl$_3$/CH$_2$OH-soluble material was isolated from large scale incubations of enzyme and UDP-[\text{\textsuperscript{3}H}]GlcNAc. When this material was applied to DEAE-cellulose more than 95% of the radioactivity was retained and could be eluted with 0.1 M ammonium formate. Table I compares the chromatographic mobility of the GlcNAc-lipid to that of mannosylphosphorylpolyisoprenol in three different solvent systems. The GlcNAc-lipid had a fairly rapid migration in the acidic solvent but moved very little in either the neutral or basic solvent system. These data along with the chromatographic properties on DEAE-cellulose suggest that this lipid probably contains either a phosphoryl or pyrophosphoryl linkage.

The [\text{\textsuperscript{3}H}]GlcNAc-labeled lipid was susceptible to mild acid hydrolysis (0.01 N HCl, 100°, 10 min). This treatment released a water-soluble, neutral compound which, as shown in Fig. 9A, migrated much slower than GlcNAc on paper chromatograms in Solvent D. This migration was suggestive of a disaccharide. N,N'-Diacetylchitobiose, isolated from hydrolysates of chitin.

![Graph](http://www.jbc.org)
FIG. 8. Incorporation of \([^{3}H]\)GlcNAc from UDP-\([^{3}H]\)GlcNAc into various lipid products. Reaction mixtures were as described in the text and contained UDP-\([^{3}H]\)GlcNAc, enzyme, and Mg\(^{2+}\). At the times indicated, the reactions were terminated and the 2/1 CHCl\(_3\)/CH\(_2\)OH (GlcNAc-disaccharide-lipid) and the 1/1/0.3 CHCl\(_3\)/CH\(_2\)OH/H\(_2\)O (GlcNAc-oligosaccharide-lipids) products were isolated and their radioactive content was determined.

**TABLE I**

<p>| Thin layer chromatographic mobilities of GlcNAc-labeled disaccharide-lipid |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Solvent systems are described in the text. The mannosylphosphoryl-polyisoprenol was isolated from incubations of GDP-[(^{14}C)]mannose with enzyme. |</p>
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<th>Compounds</th>
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<tr>
<td>Mannosylphosphoryl</td>
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<td>polyisoprenol</td>
<td>0.08</td>
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<td>GlcNAc-disaccharide-lipid</td>
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run in the same area of the papers (not shown). The radioactive peak from Fig. 9A was eluted and hydrolyzed in 4 N HCl at 100\(^\circ\) for 4 hours. This treatment resulted in the formation of a \(^1\)H-labeled sugar which bound to Dowex 50-H\(^+\) and after elution from this column with HCl, it had the same mobility as glucosamine in Solvent C (Fig. 9B), as well as in Solvent D. The radioactive material migrating like a disaccharide was also reduced with NaBH\(_4\), and then hydrolyzed in 4 N HCl at 100\(^\circ\) for 4 hours. The hydrolysate was then spotted on paper and analyzed electrophoretically in ammonium molybdate buffer. This method has been reported to separate glucosamine from glucosaminitol (22). As shown in Fig. 9C, two radioactive peaks were observed which corresponded to these two standards. The \(^[3]H\)glucosamine, which represents the nonreducing end of the molecule, contained about 4 times more radioactivity than the \([^{3}H]\)glucosaminitol. These data indicate that GlcNAc from UDP-\([^{3}H]\)GlcNAc is incorporated into an \(N,N'\)-diacetylchitobiosyl-(pyro)-phosphoryl-lipid.

Formation of \([^{3}H]\)GlcNAc-labeled Oligosaccharide-Lipids

As shown in Fig. 8, in the absence of GDP-mannose very little \([^{3}H]\)GlcNAc is incorporated into the oligosaccharide-lipids (i.e. products soluble in CHCl\(_3\)/CH\(_2\)OH/H\(_2\)O (1/1/0.3)). However, as shown in Fig. 10, when various amounts of GDP-mannose were added to incubation mixtures containing UDP-\([^{3}H]\)GlcNAc and enzyme, there was a marked stimulation in the incorporation of \([^{3}H]\)GlcNAc into the oligosaccharide-lipids with a decrease of radioactivity in \([^{3}H]\)GlcNAc-labeled disaccharide-lipid. In this experiment, the decrease in radioactivity in the disaccharide-lipid was not stoichiometric with the increase in the oligosaccharide-lipids, probably because of breakdown of the oligosaccharide-lipids during the incubation. Similar findings were previously reported by Lucas et al. (9). In fact, when the aqueous phase from these incubations was examined, a number of radioactive compounds which migrated like oligosaccharides were observed.

In order to demonstrate further the effect of unlabeled GDP-mannose on the incorporation of \([^{3}H]\)GlcNAc into the oligosaccharide-lipids, the particulate enzyme was incubated for 10 min with UDP-\([^{3}H]\)GlcNAc in order to label the GlcNAc-disaccharide-lipid. Following this incubation, the particles were isolated by centrifugation at 40,000 \(\times\) g and the supernatant liquid was discarded. This centrifugation presumably removes unreacted UDP-\([^{3}H]\)GlcNAc. The particles were resuspended in buffer containing Mg\(^{2+}\) and NaF, and 10\(^{-4}\) M
unlabeled GDP-mannose was added. Aliquots of the incubation mixture were removed with time and the radioactivity in the GlcNAc-disaccharide-lipid and in the GlcNAc-oligosaccharide-lipids was determined. Fig. 11 shows that in the presence of GDP-mannose, there was a fairly rapid transfer of radioactivity from the GlcNAc-disaccharide-lipid to the GlcNAc-oligosaccharide-lipids. In this particular experiment, 50% of the radioactivity that disappeared from the CHCl₃/CH₂OH (2/1)-soluble products could be accounted for in the CHCl₃/CH₂OH/H₂O (1/1/0.3)-soluble materials. Although this conversion was still not stoichiometric probably due to breakdown, the presence of NaF apparently inhibited breakdown to some extent. This experiment shows that the GlcNAc-disaccharide-lipid is acting as an acceptor of mannose and that the addition of mannose units to this lipid results in formation of the oligosaccharide-lipids.

Partial Characterization of [³H]GlcNAc-labeled Oligosaccharide-Lipids

In order to obtain sufficient amounts of [³H]GlcNAc-labeled oligosaccharide-lipids for characterization, large scale reaction mixtures were prepared which contained UDP-[³H]GlcNAc, GDP-mannose, and enzyme. In some cases, mixtures were first preincubated with UDP-[³H]GlcNAc before the addition of GDP-mannose. In other cases, incubation mixtures contained both radioactive sugar nucleotides, i.e. UDP-[³H]GlcNAc and GDP-[¹⁴C]mannose. The oligosaccharide-lipids formed in these incubations were isolated as described above.

Chromatographic Properties—The [³H]GlcNAc-labeled oligosaccharide-lipids showed the same chromatographic properties as described above for the [¹⁴C]mannose-labeled oligosaccharide-lipids. That is, on paper chromatograms run in water most of the radioactivity remained at the origin whereas in Solvent A it migrated near the solvent front. The GlcNAc-oligosaccharide-lipids were also susceptible to mild acid hydrolysis. This treatment released water-soluble [³H]GlcNAc-labeled oligosaccharides, which as shown in Fig. 12 emerged from Sephadex G-25 in a rather broad peak between the standards blue dextran and stachyose. The [¹⁴C]mannose-labeled oligosaccharides also emerged from the Sephadex G-25
FIG. 12. Sephadex G-25 chromatography of the [14C]mannose-labeled oligosaccharides, the [3H]GlcNAc-labeled oligosaccharides, and the [14C]mannose-labeled glycopeptides. The labeled oligosaccharides and glycopeptides were isolated as described in the text and each was placed on the same Sephadex G-25 column (1.5 x 80 cm) and run in H2O. Fractions (4 ml) were collected and analyzed for radioactivity. BD, blue dextran; S, stachyose; G, glucose.

column in the same area suggesting that they were of similar size. Also shown in this figure for comparison is the migration of [14C]mannose-labeled glycopeptides obtained from the insoluble material by Pronase digestion.

The [3H]GlcNAc-labeled oligosaccharides also gave a number of radioactive peaks when subjected to paper chromatography in Solvent A. Fig. 13 compares the profile shown by the oligosaccharides labeled with [14C]mannose (Tracing A) compared to those labeled with [3H]GlcNAc (Tracing B). In this experiment two incubations were prepared: Incubation A contained UDP-GlcNAc, enzyme, and Mg2+ whereas Incubation B contained UDP-[3H]GlcNAc, enzyme, and Mg2+. Following a 10-min incubation, GDP-mannose was added to each tube. That is, Incubation A received GDP-[14C]mannose while incubation B received 2 x 10–4 M unlabeled GDP-mannose. After 5 min of incubation, 2 x 10–4 M GDP-mannose was added to Tube A. After another 5 min, both incubations were terminated and the oligosaccharides were isolated. Both incubations gave an identical series of oligosaccharides suggesting that [14C]mannose and [3H]GlcNAc were in the same oligosaccharides. Apparently the specific oligosaccharides which accumulate depend on the length of incubation, the concentration of GDP-mannose and perhaps UDP-GlcNAc, and probably on the amounts and sizes of the endogenous oligosaccharide-lipids present in the enzyme preparation.

Determination of Reducing and Nonreducing Ends of [3H]GlcNAc-labeled Oligosaccharides—The GlcNAc-oligosaccharides obtained from the Sephadex G-25 were reduced with NaBH4 and then subjected to complete acid hydrolysis. The hydrolysate was concentrated to dryness to remove HCl and then subjected to paper electrophoresis in ammonium molybdate buffer. As was shown for the disaccharide-lipid (Fig. 9), two radioactive peaks were observed on these papers, one of which corresponded to glucosaminitol and the other to glucosamine. In this case also, the glucosamine contained approximately 4 times as much 3H as the glucosaminitol indicating that these enzyme extracts must contain some endogenous GlcNAc-lipid. These data indicate that at least some of the [3H]GlcNAc in the GlcNAc-oligosaccharides is positioned at the reducing terminus.

The three major GlcNAc-oligosaccharides seen in Fig. 13 were treated with α- and β-mannosidase and each reaction mixture was chromatographed in Solvent D to determine the effect of these enzymes on chromatographic mobility. For each of the three oligosaccharides, it was observed that treatment with α-mannosidase, but not with β-mannosidase, resulted in the formation of a faster migrating [3H]oligosaccharide (or [3H]oligosaccharides). This faster migrating material which apparently arises as a result of the removal of mannose residues amounted to about 15 to 25% of the original radioactivity. Thus, the GlcNAc-oligosaccharides apparently have some terminal mannose residues linked in α linkages. In some experiments, both 3H and 14C (from UDP-[3H]GlcNAc and GDP-[14C]mannose) were incorporated into the oligosaccharides. Both of these isotopes remained together on gel filtration and paper chromatography (i.e. they were distributed in the various oligosaccharide peaks). All of the 14C as expected was in mannose and all of the 3H in glucosamine as shown by complete acid hydrolysis and paper chromatography.

Partial Characterization of Glycoprotein

The insoluble residues isolated from large scale incubations with UDP-[3H]GlcNAc and unlabeled GDP-mannose or with GDP-[14C]mannose alone were used for characterization of the glycoprotein. Essentially all of the radioactivity incorporated from UDP-[3H]GlcNAc could be rendered water-soluble by treatment of the residue with Pronase. However, only about 10% of the activity incorporated into this residue from GDP-
\[ ^{[1]C} \text{mannose could be solubilized with Pronase; the remaining 90\% is apparently in polysaccharide (23). Ten per cent of the radioactivity from GDP-\[^{[1]C}\]mannose could also be solubilized by treatment of the residue with 1.0\% sodium dodecyl sulfate.} \]

Both the \[^{[1]}H\]GlcNAc labeled glycopeptides and the \[^{[1]}C\]mannose-labeled glycopeptides obtained by Pronase digestion emerged from Sephadex G-25 in the same area and both ran slightly ahead of the oligosaccharides from the oligosaccharide-lipids (see Fig. 12). The two glycopeptides also eluted from Sephadex G-50 in the same area which was about midway between cytochrome c and stachyose. On the other hand, the sodium dodecyl sulfate solubilized material was excluded from the Sephadex G-50 column and emerged with blue dextran. The glycopeptides were also subjected to paper electrophoresis in 1.5 M formic acid as described by Lucas et al. (9). Essentially all of the GlcNAc-labeled glycopeptide and much of the mannose-labeled glycopeptide moved toward the negative pole whereas the sodium dodecyl sulfate solubilized "glycoprotein" remained at the origin.

In order to determine the sugar that was involved in linkage to the protein, the \[^{[1]}C\]mannose-labeled glycopeptides were treated with 1 N NaOH in the presence of 1 M NaBH\(_4\) at 100° for 6 hours. Following this treatment samples were neutralized with acetic acid and concentrated to dryness. Borate was removed by repeated addition and evaporation of CH\(_2\)OH. The sample was then passed through a Sephadex G-25 column to remove contaminating \(^{[1]}H\). A peak of \(^{[1]}H\) and \(^{[1]}C\) emerged from the column in the same general area as the original glycopeptide and this was followed by a large peak of \(^{[1]}H\). The \(^{[1]}C,^{[1]}H\) peak was purified further by chromatography on SS-589 green ribbon paper in Solvent C. Essentially all of the radioactivity remained at the origin. The origins were eluted and hydrolyzed in 4 N HCl at 100° for 4 hours. Neutral sugars were then separated from amino sugars on Dowex 50-H\(^+\). Both the neutral fraction (3.7 x 10\(^6\) cpm of \(^{[1]}H\), 100,590 cpm of \(^{[1]}C\)) from the column and the HCl eluate (1.5 x 10\(^6\) cpm of \(^{[1]}H\), 11,000 cpm of \(^{[1]}C\)) contained \(^{[1]}H\), but essentially all of the \(^{[1]}C\) was in the neutral fraction. The small amount of radioactivity detected as \(^{[1]}C\) in the HCl eluate is probably due to incomplete separation of isotopes, since 0.5 to 1\% of the \(^{[1]}H\) counts in the \(^{[1]}C\) channel. Most of the \(^{[1]}H\) in the neutral sugar fraction chromatographed in the mannitol-xylitol areas of the papers while the \(^{[1]}C\) was found in mannose. On the other hand, much of the \(^{[1]}H\) in the HCl fraction migrates with authentic glucosaminol in Solvents C and D. However, a peak of \(^{[1]}H\) remained at the origins of the chromatograms. N-Acetylation of the material migrating with glucosaminol by the method of Roseman and Ludwieg (24) yielded a neutral sugar which migrated with N-acetylglucosaminol in Solvent C. These data imply that plants contain glycopeptides having GlcNAc \(\rightarrow\) protein linkages and these compounds may be the final acceptors for the oligosaccharide-lipids.

**DISCUSSION**

The studies described in this paper show that cotton fibers contain enzymes which catalyze the incorporation of mannose and GlcNAc into both lipid and glycoprotein. The data are consistent with the following mechanism of glycoprotein biosynthesis.

\[
\begin{align*}
\text{GDP-Man} & \quad \text{UDP-GlcNAc} \\
\text{Man-P-polyisoprenol} & \quad \text{GlcNAc-GlcNAc-(P)-P-lipid} \\
\text{(Man)}_n\text{-GlcNAc-GlcNAc (P)-P-lipid} & \quad \text{(Man)}_n\text{-GlcNAc-GlcNAc-protein}
\end{align*}
\]

This pathway of biosynthesis of glycoproteins was first postulated by Hsu et al. (11) and also by Waechter et al. (8) and Lucas et al. (9) based on their studies in animal systems. Parodi et al. (25) and Behrens et al. (26) have presented evidence for a similar pathway in the formation of a glycoprotein in liver. However, in the animal systems which synthesize glycoproteins containing mannose, only one lipid-linked oligosaccharide was observed and this oligosaccharide was 7 to 9 glucose units in length. In the experiments described here, a series of oligosaccharides linked to lipid were observed and these oligosaccharides appear to range in size from 3 to probably 8 or 10 glucose units. In addition the oligosaccharides appear to be similar regardless of whether they are labeled with \[^{[1]}C\]mannose or with \[^{[1]}H\]GlcNAc. In fact, both the mannose-labeled and the GlcNAc-labeled oligosaccharides were shown to have GlcNAc at the reducing end and some \(\alpha\)-linked mannose at the nonreducing end. Further, these studies show that following an incubation with either GDP-\[^{[1]}C\]mannose or with UDP-\[^{[1]}H\]GlcNAc, a chase with cold GDP-mannose leads to a buildup of radioactivity in larger oligosaccharides while the radioactivity in the smaller oligosaccharides decreases. This would suggest that the smaller-sized oligosaccharides are the precursors for the larger oligosaccharides and that sequential addition of mannose units to the small oligosaccharides results in the formation of the larger oligosaccharides. Since we have been able to isolate a trisaccharide which apparently has the structure Man \(\rightarrow\) GlcNAc-GlcNAc, the cotton system may allow us to study the mechanism for the sequential addition of mannose.

Unfortunately it has not been possible to do stoichiometric studies in this system and show the stepwise conversion of one oligosaccharide to the next higher homolog. The problem is that the particulate enzyme apparently contains a whole series of endogenous oligosaccharide-lipids and each of these is an acceptor for mannose units. Therefore even in short time incubations one observes label in the larger-sized oligosaccharides. In fact, based on the finding that most of the \[^{[1]}H\]glucosaminol formed by NaBH\(_4\), reduction of the oligosaccharides comes from the larger-sized oligosaccharides, one must assume that the particulate enzyme contains much higher amounts of these larger-sized oligosaccharide-lipids. The fact that more than one oligosaccharide-lipid has not been observed in some of the animal systems may be due to more rapid synthesis in these systems whereas the cotton system may be more sluggish in this regard. One mammalian system which does show a series of oligosaccharide-lipids is that of aorta (12). In this case mannose from GDP-\[^{[1]}C\]mannose is found in a series of peaks which have been characterized partially as oligosaccharide-lipids.

Although we do not have any direct evidence for the transfer of mannose from the mannosylphosphorylpolyisoprenol to the
oligosaccharide-lipids, the experiments with p-hydroxymercuribenzoate indicate that the mannolipid is the precursor for the oligosaccharide-lipids. In these experiments, p-HMB was found to inhibit the synthesis of the mannosylphosphoryl-polysaccharide to continue. However, when the inhibition was reversed by the addition of dithiothreitol in the presence of an excess of cold GDP-mannose, radioactivity rapidly disappeared from the mannosylphosphoryl-polysaccharide and appeared in the oligosaccharide-lipids. The presence of excess GDP-mannose assured that oligosaccharide-lipid formation could not come from GDP-\[^{14}C\]mannose but must be coming from the mannosylphosphoryl-polysaccharide. Unfortunately, it was not possible to show stoichiometric conversion in these experiments, possibly because the oligosaccharide-lipids are quite unstable in these incubations and breakdown occurs. We observed this in other cases such as in the experiments showing the effect of GDP-mannose on \[^{3}H\]GlcNAc incorporation into the oligosaccharide-lipids. Similar findings were previously reported by Lucas et al. (9). Other studies notably those from the laboratories of Heath (10) and Lennarz (8) have shown the direct transfer of mannose from mannosylphosphoryl-polysaccharide into the \[^{14}C\]mannose-labeled oligosaccharide-lipids in cell-free extracts of mammalian tissues.

There are still a number of unanswered questions with regard to the oligosaccharide-lipids in this and other systems. For example, in our system we are not certain that mannose and GlcNAc are the only sugars present in these oligosaccharides nor what the ultimate size of the oligosaccharide chain is. We also do not know the glycosidic linkages of any of the mannose units. In terms of anomeric configuration, the first mannose which is attached to GlcNAc is most certainly \(\beta\) and some (15 to 25\%) of the terminal mannoses are \(\alpha\). However, the others remain unknown at this time.

In mammalian systems, the ultimate acceptor of the oligosaccharide portion of these lipids is a protein (8, 10, 12). This also appears to be the case in cotton, although it is based on circumstantial evidence. In our studies, both \[^{3}H\]GlcNAc and \[^{14}C\]mannose are incorporated into an insoluble material which is apparently a mixture of glycoprotein and polysaccharide. About 10\% of the \[^{14}C\]mannose and virtually all of the \[^{3}H\]GlcNAc could be solubilized by treatment of this residue with the proteolytic enzyme, Pronase. The solubilized material, labeled with either of these isotopes, was slightly larger in size than the oligosaccharides obtained from the oligosaccharide-lipids, and in both cases, the glycopeptides migrated toward the cathode upon paper electrophoresis in a formic acid solution indicating that at low pH they both contained a positive charge. Finally, the mannose-labeled glycopeptide gave rise to \[^{3}H\]glucosaminol when treatment with 1N NaOH containing 1 M NaB\(_3\)H\(_4\), suggesting the presence of GlcNAc \(\rightarrow\) peptide linkages. Recently, Roberts and Pollard (27) have shown the incorporation of GlcNAc from UDP-GlcNAc into glycolipids and glycoproteins in mung bean seedlings. Although these products were not completely characterized, they may be similar to the GlcNAc compounds reported here. While the nature or function of the glycoprotein synthesized in cotton is not known, Ericson and Chrispeels (28) have reported the presence of GlcNAc containing glycoproteins in plants. Based on solubility, the glycoprotein in cotton would appear to be a membrane component.

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