ABSTRACT A solubilized mannosyl transferase(s) was obtained by treatment of the pig aorta particulate enzyme with the nonionic detergent Nonidet P-40 followed by centrifugation at 100,000 × g for 60 min. This enzyme preparation catalyzed the transfer of mannose from GDP-[14C]mannose (but not from [14C]mannosylphosphoryldolichol) to form a heptasaccharide–lipid. The synthesis of this heptasaccharide–lipid required the addition of an acceptor lipid that was isolated from pig liver. The oligosaccharide portion of the acceptor lipid appeared to be a mixture of trisaccharide and pentasaccharide. The formation of heptasaccharide–lipid did not require divalent cation, was not inhibited by EDTA, and was not inhibited by the antibiotic amphotericin. The heptasaccharide portion of the heptasaccharide–lipid had the same migration properties on paper chromatograms in two different solvent systems as a previously characterized Man5GlcNAc2 oligosaccharide (Li & Kornfeld, S. 1970 J. Biol. Chem. 254, 2754–2758). It also had the same migration properties as the oligosaccharide that accumulates in the presence of amphotericin. All three of these oligosaccharides emerged from a Bio-Gel P-4 column in the same position. Radioactive mannosone was released from the heptasaccharide by digestion with α-mannosidase. These data demonstrate that at least some of the α-linked mannose residues in the heptasaccharide–lipid are donated directly from GDP-mannose.

The biosynthesis of the oligosaccharide chain of asparagine-linked glycoproteins takes place through the participation of lipid-linked oligosaccharide intermediates (1). An undetermined number of membrane-bound glycosyl transferases catalyze the sequential addition of sugars to lipid-linked saccharides to form a large oligosaccharide–lipid whose composition has been shown to be (GlcNAc)β(Man)α(GlcNAc)2–pyrophosphoryl dolichol (2). Because all of these enzymes are membrane bound, it is difficult to study the individual reactions in the dolichol cycle with these particulate enzyme systems.

In order to study this series of reactions, we have initiated a program to solubilize and isolate as many of the glycosyl transferases as possible. Many of these enzymes can be solubilized from the pig aorta particulate enzyme by treatment with the nonionic detergent Nonidet P-40. Previous reports have described the properties of the solubilized transferases that catalyze the formation of GlcNAc-pyrophosphoryl dolichol (3), mannosylphosphoryldolichol (4), and Man–β-GlcNAc–GlcNAC-pyrophosphoryldolicholy (5).

In this report, we describe some of the properties of another mannosyl transferase(s) that has been solubilized from the particulate fraction. This enzyme transfers mannose from GDP-mannose (but not from mannosylphosphoryldolichol) to a lipid-linked oligosaccharide acceptor to form a heptasaccharide–lipid. This heptasaccharide had the same chromatographic properties as a Man5GlcNAc2, previously characterized by Li and Kornfeld (6). The heptasaccharide is similar to that which accumulates when mannosyl phosphoryl dolichol formation is inhibited by EDTA (4) or the antibiotic amphotericin (7). The solubilized mannosyl transferase (3) is not inhibited by either EDTA or amphotericin.

**EXPERIMENTAL PROCEDURES**

Materials. GDP-[14C]mannose (270 µCi/μmol, 1 Ci = 3.7 × 10^10 becquerels) and Na[3H]H2O (25 mCi/4 mg) were purchased from New England Nuclear. DEAE-Cellulose and unlabeled sugar nucleotides were from Sigma. Man5GlcNAc2 was a generous gift of S. Kornfeld. The acceptor–lipid fraction was isolated from pig liver as described (8). Generally, this involved homogenizing pig liver in a Waring Blender to prepare a particulate fraction that was incubated with GDP-mannose. After this incubation, the mixture was extracted with CHCl3/CH3OH/H2O, 1:1:1 (vol/vol), to obtain lipid-linked monosaccharides. After removal of the CHCl3, the particles were isolated by centrifugation, washed several times with 50% CH3OH, and extracted with CHCl3/CH3OH/H2O, 10:10:3 (vol/vol), to obtain lipid-linked oligosaccharides. The lipid-linked oligosaccharides were purified by chromatography on DEAE-cellulose followed by saponification and rechromatography on DEAE-cellulose (9). The acceptor lipid was stored at −20°C in CHCl3/CH3OH, 1:1.

Preparation and Assay of Soluble Mannosyl Transferase(s). The intimal layer of pig aorta was removed, ground in a meat grinder, and homogenized in Tris-HCl (pH 7.5) in a Waring Blender. The particulate enzyme was isolated by centrifugation and mixed vigorously in 0.5% Nonidet P-40 to solubilize the glycosyl transferases. The solubilized enzyme was centrifuged for 1 hr at 100,000 × g to remove the particulate material, and the supernatant liquid was used as the source of enzyme. Incubation mixtures were prepared in the following way. Acceptor lipid was added in various amounts to assay tubes and the solvent was removed under a stream of nitrogen. Nonidet P-40 was added to give a final concentration of 0.25% and the mixture was stirred vigorously on a Vortex mixer to suspend the lipid. pH 7.5 Tris buffer (5 µmol) and the other components (as indicated in the figures and tables) were added, followed by GDP-[14C]mannose and solubilized enzyme. The final volume of the incubation mixture was usually 0.4 ml. Incubations were at 37°C for various periods of time, and the reactions were stopped by the addition of 2 ml of CHCl3/CH3OH, 1:1 (vol/vol), and 0.5 ml of H2O. Generally, 0.1 ml of the particulate enzyme (1–2 mg of protein) was added after the CHCl3/CH3OH in order to aid in the extraction procedure. After vigorous mixing, the chloroform layer was removed and the multiple extraction procedure was followed (10). The heptasaccharide–lipid was extracted with CHCl3/CH3OH/H2O, 10:10:3 (vol/vol).

The heptasaccharide–lipid was subjected to mild acid hy-
RESULTS

Properties of the Solubilized Mannosyl Transferase(s).

Previous studies (1, 4) with the particulate enzyme from pig aorta showed that either EDTA or the antibiotic amphotericin inhibited the transfer of mannose from GDP-[14C]mannose to dolichyl phosphate, preventing the formation of mannosylphosphoryldolichol. However, in these experiments [14C]mannose was still found in the lipid-linked oligosaccharides, strongly suggesting that some of the mannoses were donated directly from GDP-mannose. The major oligosaccharide synthesized in those reactions appeared to be a heptasaccharide. The results described in this paper show that the heptasaccharide–lipid is also formed when the solubilized enzyme is incubated with GDP-[14C]mannose and an acceptor lipid from liver.

A typical assay for the formation of the heptasaccharide–lipid by solubilized enzyme is presented in Fig. 1. In these experiments, the radioactive oligosaccharide–lipids were extracted from the incubation mixtures with chloroform/methanol/water, 10:10:3, and the lipids were subjected to mild acid hydrolysis to liberate the oligosaccharides. The oligosaccharides were separated by paper chromatography and their radioactive content was measured by scintillation counting. The heptasaccharide migrated somewhat slower than the stachyose standard with an RF of 0.7. In the presence of GDP-[14C]mannose and the acceptor-lipid from liver, the solubilized enzyme produced substantial amounts of heptasaccharide–lipid (Fig. 1B). When acceptor–lipid was omitted from the incubation mixture, little or no heptasaccharide was observed (Fig. 1A). Small amounts of other radioactive peaks were detected in areas corresponding to hexasaccharide, pentasaccharide, and tetrasaccharide (Fig. 1B and D). These oligosaccharide–lipids are probably intermediates on the route to synthesis of the heptasaccharide–lipid. In time-course experiments, the appearance and disappearance of these oligosaccharides were consistent with the hypothesis that they are intermediates in synthesis.

Fig. 1C shows that GDP-[14C]mannose could not be replaced by [14C]mannosylphosphoryldolichol, indicating that GDP-[14C]mannose was directly donating the [14C]mannose seen in the heptasaccharide. This was also indicated by the experiment shown in Fig. 1D. In this case, GDP-[14C]mannose, acceptor lipid, and dolichyl phosphate were incubated with the solubilized mannosyltransferase(s) and the radioactive oligosaccharides were examined. If the mannose from GDP-[14C]mannose was first being transferred to dolichyl phosphate and then to lipid-linked oligosaccharide, the addition of dolichyl phosphate should increase the amount of radioactivity in the heptasaccharide. Instead, there was a decrease in the radioactive heptasaccharide, probably because the presence of dolichyl phosphate resulted in the trapping of some mannose as mannosylphosphoryldolichol. This is evidenced by the fact that a large peak of radioactive mannose was found in Fig. 1C and D. Because the transfer of mannose from mannosylphosphoryldolichol and the transfer from GDP-mannose have somewhat different conditions for optimal activity, these experiments were done with different detergent concentrations and with

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![Fig. 1](image1.png)

**Fig. 1.** Paper chromatographic analysis of products formed by solubilized enzyme. After extraction with organic solvents, the lipid-linked saccharides were hydrolyzed and chromatographed on Whatman no. 1 paper in solvent A. (A) Soluble enzyme (300 µg of protein) plus GDP-[14C]mannose (50,000 cpm) without acceptor lipid. (B) Soluble enzyme plus GDP-[14C]mannose plus acceptor-lipid (6 µg of phosphorus). (C) Soluble enzyme plus [14C]mannosylphosphoryldolichol (32,000 cpm) plus acceptor lipid. (D) Soluble enzyme plus GDP-[14C]mannose plus acceptor lipid plus dolichyl phosphate (2 µg). Standards are stachyose (S), maltose (MALT), and mannose (MAN).

![Fig. 2](image2.png)

**Fig. 2.** Effect of time and enzyme concentration on the formation of heptasaccharide–lipid. Assays were with GDP-[14C]mannose and acceptor-lipid (6 µg of phosphorus). Heptasaccharide–lipid was isolated by solvent extraction and heptasaccharide was isolated by paper chromatography. O, 1 mg of protein; •, 400 µg of protein; x, 100 µg of protein.
and without cations. The results were essentially the same as those shown in Fig. 1, verifying the direct transfer.

The formation of the heptasaccharide–lipid was somewhat proportional to time and increased with increasing amounts of solubilized enzyme (Fig. 2). In these experiments it was also necessary to add acceptor lipid to the incubations in order to form the heptasaccharide–lipid. Fig. 3 demonstrates the effect of adding increasing amounts of acceptor lipid to these incubation mixtures. In the absence of acceptor lipid, little heptasaccharide–lipid was formed; increasing amounts of acceptor lipid resulted in the synthesis of increased amounts of heptasaccharide–lipid. Because these incubation mixtures contained divalent cation, some radioactivity was also incorporated into lipid-linked monosaccharides (i.e., mannosylphosphoryldolichol), probably because the acceptor-lipid fraction also contained small amounts of dolichyl phosphate. However, in other experiments in which divalent cation was omitted, only the lipid-linked oligosaccharides were formed (see below).

The transfer of mannose from GDP-[14C]mannose was not stimulated by the addition of divalent cation to the incubation mixtures, nor did the presence of EDTA inhibit this activity (Table 1). Divalent cations are required for the synthesis of mannosylphosphoryldolichol, and this reaction has been shown to be inhibited by EDTA (4). In fact, the solubilized enzyme was still active in forming heptasaccharide–lipid even after dialysis against 1 mM EDTA, although this procedure resulted in a 30–40% loss in activity. This activity could not be restored by the addition of Mn²⁺ or Mg²⁺. Table 1 also demonstrates that the antibiotic amphotycin had no effect on the formation of heptasaccharide–lipid. Even at 25 μg of antibiotic per ml, a concentration that causes an 85–90% decrease in the synthesis of mannosyl phosphoryldolichol, there was no decrease in the amount of radioactivity in the heptasaccharide–lipid. These data support the idea that GDP-[14C]mannose, rather than mannosylphosphoryldolichol, is the mannosyl donor in these reactions.

### Characterization of the Acceptor Lipid

In order to characterize the acceptor lipid used in these studies, an aliquot of the lipid was subjected to mild acid hydrolysis to release the oligosaccharides, and the sugars were reduced with NaBH₄. After this reduction, the mixture was acidified with HCl and passed through a column of Dowex 50 (H⁺). The eluate from the column was taken to dryness several times in the presence of methanol to remove borate and was then subjected to paper electrophoresis in pH 3.5 formate buffer to remove contaminating radioactivity. The origin from the electrophoretogram was eluted and chromatographed on Whatman no. 1 paper. A number of radioactive peaks were observed on these papers,

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**Table 1. Effect of various additions on formation of heptasaccharide–lipid**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
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<td></td>
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<td>4089</td>
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<tr>
<td>Exp. II</td>
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</tr>
<tr>
<td>Amphotycin</td>
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</table>

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**Fig. 3.** Effect of acceptor lipid concentration on the incorporation of mannose from GDP-[14C]mannose into lipid-linked monosaccharide (mannosylphosphoryldolichol) (●) and lipid-linked oligosaccharide (heptasaccharide–lipid) (O). Acceptor-lipid phosphorus content was 0.3 mg/ml; 300 μg of soluble enzyme was used in each assay.

**Fig. 4.** Identification of oligosaccharides on Bio-Gel P-4 column (2.1 × 93 cm). Oligosaccharides were placed on the column and eluted in 0.15% acetic acid. Fractions (4 ml) were collected and 0.2-ml aliquots of even-numbered fractions were removed for the determination of radioactivity. (Top) Oligosaccharide peaks A and B from acceptor lipid. (Middle) Heptasaccharide product formed from soluble enzyme. (Bottom) Man₅GlcNAc₂ (●) obtained from S. Kornfeld and oligosaccharide (O) made in the presence of amphotycin. Standards: BD, blue dextran; S, stachyose; MAN, mannose.
indicating that the acceptor lipid contained a mixture of lipid-linked saccharides (data not shown). A peak migrating with the mannose standard probably came from mannosyl-
phosphoryldolichol; a rapidly migrating peak and one at the origin probably resulted from contaminants in the NaN3H4. The two peaks that appeared to be of major interest as acceptors migrated in an area indicating that they were trisaccharide or tetrasaccharide (peak B) and pentasaccharide (peak A).

Peaks A and B were chromatographed on a calibrated column of Bio-Gel P-4 (Fig. 4 Top). Peak A emerged from the column before the stachyose marker and in an area suggesting that it was a pentasaccharide; peak B eluted just after stachyose and is probably a trisaccharide. After digestion with α-mannosidase, peak A migrated on paper with the trisaccharide Man-β-GlcNAc-GlcNAc. Peak B also migrated with this standard trisaccharide and its mobility was not altered by treatment with α-mannosidase. Thus, the acceptor lipids are probably Man-β-GlcNAc-GlcNAc-pyrophosphoryldolichol and Manα-α-Man-β-GlcNAc-GlcNAc-pyrophosphoryldolichol. We are not certain at this point how many mannose residues are being added.

Characterization of the Heptasaccharide–Lipid. The oligosaccharide–lipid formed in these reactions was obtained by extraction of incubation mixtures with CCl4/CH3OH/H2O, 10:10:3. The radioactive material bound to columns of DEAE-cellulose (acetate) in CHCl3/CH3OH, 1:1, and was eluted with a gradient of ammonium acetate in 99% CH3OH. The oligosaccharides were released from the lipid by mild acid hydrolysis and were purified by paper chromatography (Fig. 1). The major mannose-labeled oligosaccharide migrated slower than the stachyose standard (RF = 0.7) and had the same mobility as the oligosaccharide that accumulates in the presence of amphomycin (7). Both of these oligosaccharides also had the same mobility as a ManαGlcNAc2 oligosaccharide in two different solvent systems. The ManαGlcNAc2 standard was previously characterized by Li and Kornfeld (6) from Chinese hamster ovary cells.

The 14C-labeled product isolated by paper chromatography (RF = 0.7) was chromatographed on a Bio-Gel P-4 column along with the ManαGlcNAc2 oligosaccharide and the amphomycin oligosaccharide (Fig. 4). All three of these oligosaccharides eluted from the column in the same position and in the area expected from a heptasaccharide. The [14C]heptasaccharide product was reduced with NaN3H4 to label the reducing end and then was purified by paper electrophoresis and paper chromatography. This [3H, 14C]heptasaccharide was treated with α-mannosidase and the products were identified by paper chromatography. Two peaks of radioactivity were observed, corresponding to [14C]mannose and the [3H]trisaccharide, Man-β-GlcNAc-GlcNAc. Thus, the heptasaccharide–lipid has been partially characterized as Manα-Man-β-GlcNAc-Glc-NAc-pyrophosphoryldolichol.

The heptasaccharide isolated from Chinese hamster ovary cells and used as a standard in these studies has the structure (6):

![Diagram](https://example.com/diagram.png)

Because the heptasaccharide described in this paper has the identical migration on paper in two solvents and elutes in the same position from a Bio-Gel P-4 column, it probably has a structure similar to that of the above oligosaccharide. However, there could be differences in the linkages of the mannose residues, and more detailed studies will be necessary to establish its exact structure. It is clear from these studies that at least some of the α-linked mannose residues are donated directly from GDP-[14C]mannose without the participation of mannosylphosphoryldolichol. This study confirms earlier studies with EDTA (4) and amphomycin (7) which suggested a direct transfer of mannose from GDP-mannose to heptasaccharide–lipid. Forsee et al. (8) presented evidence for a direct transfer of mannose from GDP-mannose to lipid-linked oligosaccharide but no characterization of the product was given.

This research was supported by grants from the National Institutes of Health (HL 17783) and the Robert A. Welch Foundation.