Mannosyltransferase Activity in Calf Pancreas Microsomes

FORMATION FROM GUANOSINE DIPHOSPHATE-D-[14C]MANNOSE OF A C-LABELED MANNOLIPID WITH PROPERTIES OF DOLICHYL MANNOPYRANOSYL PHOSPHATE*

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SUMMARY

Calf pancreas microsomes incorporated radioactive mannose from GDP-D-[14C]mannose into products precipitated by 6% trichloroacetic acid-0.5% phosphotungstic acid. This incorporation was maximally stimulated by 10 mM Mn²⁺ ions and showed a broad pH optimum of 6.0 to 6.7 in Tris-maleate buffer. Between 70 and 90% of the radioactivity was found in products soluble in chloroform-methanol (2:1, v/v). The rates of labeling of the chloroform-methanol extract and of the residual precipitate were constant for 10 and 30 min, respectively.

The chloroform-methanol extract from rough microsomes incubated with GDP-D-[14C]mannose contained a single 14C-labeled product, which was stable under weak alkaline conditions and yielded [14C]mannose upon mild acid hydrolysis and some [14C]mannosyl phosphate upon hot alkaline treatment. It co-chromatographed on silica gel thin layer plates with authentic dolichyl α-D-mannopyranosyl phosphate in seven solvent systems, and could be clearly separated from authentic P⁴-dolichyl P⁴-α-D-mannopyranosyl pyrophosphate and from authentic ficiprenyl α-D-mannopyranosyl phosphate, a mannolipid having a shorter polyprenyl moiety. The [14C]mannolipid did not undergo hydrogenolysis during catalytic hydrogenation under conditions which released [14C]mannosyl phosphate from allylic polyisoprenyl mannosyl phosphates. Thus the mannolipid formed from endogenous lipid is very similar to authentic dolichyl α-D-mannopyranosyl phosphate.

Dolichyl phosphate, ficiprenyl phosphate, and solanesyl phosphate (and to a limited extent retinyl phosphate) acted as exogenous acceptors of D-mannose from GDP-D-[14C]mannose in the presence of 0.1% Triton X-100 to yield the corresponding labeled polyprenyl mannosyl phosphates. The lipid formed from dolichyl phosphate could not be separated from that formed from endogenous lipid, and, like the pancreatic mannolipid, it did not undergo hydrogenolysis. The different mannolipids formed from the exogenous polyprenyl phosphates could be clearly distinguished from one another on thin layer chromatograms, which separated polyprenyl mannosyl phosphates according to the length of their lipid moieties.

The participation of polyprenyl sugar phosphates as intermediates in the biosynthesis of several types of complex carbohydrates has been clearly established in microorganisms (5). In 1969, Caccam et al. (6) reported that particulate fractions from several animal tissues that secrete mannos-containing glycoproteins synthesize 14C-labeled mannolipid when incubated with GDP-D-[14C]mannose. The radioactive labeled lipid formed in these tissues observed the possibility that polyprenyl sugar derivatives are involved in the biosynthesis of animal glycoproteins. Since then, reports describing the biosynthesis of mannolipids in various biological systems have appeared (6-17). It has been demonstrated that a mannolipid formed in normal liver is a derivative of dolichol (12, 13), a term applied to a class of polyisoprenoids containing from 16 to 21 isoprene units, including a saturated α-isoprenyl residue (18). In the liver of vitamin A-deficient animals a mannolipid derived from retinol is also formed (9, 17).

The transfer of mannose from labeled mannolipid to endogenous acceptors has been demonstrated (12, 15, 18), but so far these mannolipids have not been shown to serve as intermediates in the biosynthesis of specific glycoproteins using exogenous acceptors. The possibility that different mannolipids may be involved in the formation of different glycoproteins and even in the attachment of different mannose residues to the same glycoprotein molecule has been discussed (20).

Several glycoproteins that contain an N-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-L-asparaginyl type of linkage are
formed in the pancreas, namely, lipase (21), deoxyribonuclease (22), and ribonuclease B, C, and D (23–25). Although the biosynthesis of glycoproteins with this type of linkage has been intensively investigated in recent years (for review see Ref. 26), little is known concerning the enzymic synthesis of the mannose-containing inner core.

A study of mannosyltransferase activity in calf pancreas was undertaken to determine whether this tissue is capable of synthesizing polypropyl mannose compounds and to investigate the possible function of this type of lipid in the biosynthesis of glycoproteins and other complex carbohydrates. The results presented here demonstrate that pancreatic microsomes incubated with GDP-α-[U-14C]mannose synthesize a radioactive labeled manno-lipid with properties similar to those of authentic dolichyl α-D-mannopyranosyl phosphate.

**EXPERIMENTAL PROCEDURE**

**Materials**

The following compounds were obtained from the companies indicated: α-mannose, methyl α-D-mannopyranoside, and l-fucose, Pfannstiehl Laboratories, Inc., Waukegan, Ill.; α-D-mannopyranosyl phosphate, GMP, GDP, and Escherichia coli alkaline phosphatase, Sigma Chemical Co., St. Louis, Mo.; GDP-α-[U-14C]mannose (154 to 160 Ci per mmol), New England Nuclear Corp., Boston, Mass.; ultrapure sucrose, Schwartz-Mann, Orangeburg, N.Y.; soybean trypsin inhibitor, Worthington Biochemical Corp., Freehold, N.J.; bovine serum albumin, Miles Laboratories, Inc., Elkhart, Ind.; bovine lecithin, Analabs, Inc., North Haven, Conn.; Triton X-100 (B grade), Calbiochem, La Jolla, Calif.; chlortetracycline and sodium borohydride, Ventron Corp., Beverly, Mass. Silicic acid, Grade 940, 60 to 200 mesh, was purchased from W. A. Grace and Co., Davison Chemical Division, Baltimore, Md., and fibrous DEAE-cellulose was supplied by Schleicher & Schuell, Inc., Keene, N.H. Separate publications describe the chemical synthesis of dolichyl α-D-mannopyranosyl phosphate and citronellyl α-D-mannopyranosyl phosphate (1), fucaprenyl α-D-mannopyranosyl phosphate (27), α-D-dolichyl α-D-mannopyranosyl pyrophosphate,1 dolichyl phosphate (16), fucaprenyl phosphate (28), solanesyl phosphate, farnesyl phosphate (same method as Ref. 28), citronellyl phosphate (1), and retinyl phosphate (29). All other chemicals were reagent grade commercial products.

**Analytical Methods**

Protein was estimated by the method of Lowry et al. (30), with bovine serum albumin as a standard. Lipid phosphate was determined by the procedure of Shin (31).

Thin layer chromatography was performed on Merck precoated plates of Silica Gel G (0.25-mm thick, without fluorescence indicator, Brinkmann Instruments, Inc., Westbury, N.Y.) in the following solvent systems: A, chlortetracycline-water (65:25:4); B, 2,6-dimethyl-4-heptanone-acetic acid-water (20:15:2); C, chlortetracycline-water (60:25:1); D, benzene-chloroform-water (4:1:1); E, chlortetracycline-methanol 1/5 M ammonium hydroxide-water (80:30:0.5:3); F, chlortetracycline-methanol-acetic acid-water (30:15:4:2); G, l-propanol-water (7:3); and H, l-butanol-acetic acid-water (3:1:1).

Unsaturated lipids were detected on the plates with the anisaldehyde reagent (52). Descending paper chromatography was performed on Whatman No. 1 paper (H. Reeves, Engel and Co., Clifton, N.J.) in the following solvent systems: I, ethyl acetate-95% ethanol-1 M ammonium hydroxide-water (8:2:1:1); J, 2-methylpropanoic acid-15 M ammonium hydroxide-water (57:4:39); K, 1-butanol-acetic acid-water (2:1:1); and L, 95% ethanol-1 M ammonium acetate, pH 7.5 (7:3). The composition of each solvent system is expressed as a volume ratio. Carbohydrate standards were located with the periodate-benzidine test (53).

**Preparation of Microsomes and Microsomal Subfractions**

Microsomes—Calf pancreata were placed on ice immediately upon their removal from slaughtered animals. All subsequent procedures were performed at 4°. The tissue was rinsed with a 0.3 M sucrose solution and trimmed free of extraneous material. It was then minced with scissors, finely chopped with razor blades, and suspended in a 0.3 M sucrose solution containing 0.01% (w/v) soybean trypsin inhibitor (5 ml of solution per g of tissue). This suspension was homogenized with five strokes of a glass-Teflon Potter-Elvejem homogenizer (Kontes Glass Co., Vineland, N.J.; Size 24) driven by a motor at 8,000 rpm. After filtration of the crude homogenate through four layers of cheesecloth, the suspension was centrifuged for 10 min at 2,000 rpm (650 × g max) in the GSA rotor of a Sorvall RC2 centrifuge. The resulting supernatant was centrifuged for 15 min at 9,000 rpm (9,700 × g max) in the SS 34 rotor of the same centrifuge. The 9,700 g supernatant was then centrifuged at 30,000 rpm (105,600 × g max) for 60 min in a No. 30 rotor of a Beckman model L2-65B ultracentrifuge to obtain a pellet of microsomes.

**Microsomal Subfractions**—Microsomes were fractionated into rough and smooth microsomes by centrifugation on discontinuous gradients in a Beckman No. 30 rotor. Each microsomal pellet was suspended in 9.5 ml of a 0.3 M sucrose solution by gentle homogenization and 9 ml of this microsomal suspension was deposited as a layer on a 14 ml of 1.3 M sucrose solution. During centrifugation for 15 hours at 30,000 rpm, rough microsomes sedimented to the bottom of the tube as a tightly packed, tan pellet, whereas smooth microsomes formed a fluffy, white band at the interphase of the two sucrose solutions. In some cases, this band was drawn off, diluted with a 0.3 M sucrose solution, and sedimented at 30,000 rpm for 60 min. Usually, the supernatant was decanted and the tubes were allowed to drain to obtain the rough microsomal pellet.

**Electron Microscopy**—For examination of the smooth and rough microsomal subfractions with the electron microscope, pellets were fixed in 2.5% (v/v) glutaraldehyde and then treated with osmium tetroxide. Sections from Epon-embedded pellets were stained with uranyl acetate and lead citrate (34).

**Washing and Storage**—For mannosyltransferase studies, pellets of microsomes or microsomal subfractions were washed by gentle homogenization in 50 ml Tris-maleate buffer, pH 6.3, followed by centrifugation at 30,000 rpm for 60 min. The washed pellets could be stored at −85° for several months without appreciable loss of transferase activity. Each pellet was thawed only once, immediately before use.

**Mannosyltransferase Activity**

**Conditions of Incubation**—Standard mixtures of incubation for the determination of mannosyltransferase activity with endogenous acceptors consisted of Tris-maleate buffer, pH 6.3 (40 mM), microsomes or microsomal subfractions (2 to 3 mg of protein per ml), MnCl2 (10 mM), and GDP-α-[U-14C]mannose (0.05 to 0.15 mM) in a final volume of 0.3 ml. In experiments with exogenous polypropyl phosphates as acceptors, a procedure similar to that of Behrens and Leloir (35) was employed for the preparation of reaction mixtures. Appropriate quantities of the polypropyl phosphates dissolved in organic solvents were mixed with 5-μl portions of a solution containing equal parts of 0.2 M MnCl2 and 0.1 M sodium EDTA, pH 6.4. Each mixture was dried under N2 and then suspended in 10 μl of 0.5% (v/v) Triton X-100. Tris-maleate buffer, pH 6.3 (40 mM), rough microsomes (2 mg protein per ml), MnCl2 (10 mM), and GDP-α-[U-14C]mannose (0.075 μCi per ml) were added in a final volume of 0.25 ml. When necessary, larger reaction mixtures were prepared, maintaining the concentrations present in the standard mixtures. Incubations were performed at 30° for 30 min, unless otherwise stated.

**Mannosyltransferase Determinations**—Incubation mixtures were prepared in duplicate; controls containing microsomes that had been boiled for 5 min before the addition of radioactive precursor were also processed. At the end of the incubation period, the reactions were stopped by cooling to 4° and by the addition of 1 volume of ice-cold 12% trichloroacetic acid-1% phosphotungstic acid solution, followed by 2 volumes of ice-cold 6% trichloroacetic acid-0.5% phosphotungstic acid. After 20 to 30 min at 4°, the mixtures were centrifuged and the supernatants were discarded. Each precipitate was washed three to four times with 2 ml of ice-cold 6.9% trichloroacetic acid-0.5% phosphotungstic acid each time, and was then extracted twice at room temperature with 2 ml of chloroform-methanol (2:1, v/v). The chloroform-methanol extracts from a given sample were pooled and the radioactivity was
measured. The residual precipitate was dried at room temperature and then dissolved in 2 ml of 0.5 M NaOH at 90° for 30 min and the radioactivity was determined. The results in the tables and figures are expressed as averages of duplicate determinations after subtraction of the boiled control values. The chloroform-methanol extract from boiled controls contained only 1% of the radioactive activity found in the extract of a standard reaction mixture after 30 min of incubation, and the precipitate from boiled controls contained approximately 10% of the radioactivity in the precipitate obtained from a standard reaction mixture after a 30-min incubation.

Direct Extraction of Mannolipids—For the characterization of labeled mannolipids formed from endogenous lipid and from exogenous polypropyl phosphates, direct extraction of the reaction mixture was performed without prior precipitation with trichloroacetic acid-phosphotungstic acid, since traces of acid in the extracts degraded the labeled products. The amount of radioactivity extracted in chloroform-methanol was about the same with either direct extraction of the incubation mixture or extraction of the precipitate obtained after acid treatment. The reaction mixture was vigorously shaken at room temperature with 5 volumes of chloroform-methanol (2:1, v/v). After 30 to 60 min at room temperature, the suspension was centrifuged to separate the two phases and an insoluble material which was obtained at the interface. The chloroform-methanol phase was removed and filtered through glass wool, and in some cases, was washed with 0.2 volume of chloroform-methanol-water (3:48:47, v/v) (36).

Characterization of Mannolipid

Strong Acid Hydrolysis—A sample of the 4C-containing chloroform-methanol extract was evaporated to dryness under N2. After addition of 1.0 M HCl (100 ml) and of D-galactose, D-glucose, D-mannose, L-glucose, and methyl a-D-mannopyranoside (50 mg each), the mixture was heated at 100° for 30 min, and then diluted with 4 volumes of H2O and passed through a column of AG 1-X8 resin (150 mm, 100-200 mesh, formate form, Bio-Rad Laboratories, Richmond, Calif.). The resin was washed with water (4 ml), and the eluted material was lyophilized and analyzed by paper chromatography.

Mild Acid Hydrolysis—Equal volumes of radioactive chloroform-methanol extract were dried under N2. To each residue cooled to 4°, ice-cold 10 mM HCl (200 ml) was added and the mixtures were incubated at 105° or 100° for various periods of time; after incubation the samples were cooled to 4°, neutralized with ice-cold 10 mM NaOH (200 ml), and then shaken at room temperature with 5 volumes of chloroform-methanol (2:1, v/v) and centrifuged. The lower phase was washed twice with chloroform-methanol-water (0.5 ml, 3:48:47, v/v), and the washings were combined with the corresponding aqueous phase. The radioactivity in each phase was measured.

Weak Alkaline Hydrolysis—The procedure of Dawson (37) as described by Lahav et al. (38) was employed. Equal volumes of radioactive chloroform-methanol extract were first dried under N2, and then each residue was dissolved in 0.5 ml of chloroform-methanol (1:4, v/v) and cooled to 4° and 1.0 M NaOH (0.05 ml) was added. The mixtures were incubated for various periods at 37°, cooled to 4°, and neutralized with ice-cold 1.0 mM acetic acid (0.05 ml). Chloroform-methanol (1 ml, 9:1, v/v) was added, followed by 2-methyl-1-propanol (0.5 ml) and water (1 ml). After mixing and centrifugation, the lower phase was washed with 0.5 ml of methanol-water (1:2, v/v), and this washing was combined with the corresponding aqueous phase. The radioactivity in each of these phases was determined.

Hot Alcohol Hydrolysis—Equal volumes of radioactive chloroform-methanol extract were dried under N2. Each residue was dissolved in 1-propanol (0.45 ml) and cooled in an ice bath, and 1 ml NaOH (0.05 ml) was added. The mixtures were heated in a water bath at 90° for various periods of time, and subsequently cooled and neutralized with 0.1 M acetic acid (0.5 ml). After vigorous shaking at room temperature with 1 ml of water and 5 ml of chloroform-methanol (2:1, v/v), the mixtures were centrifuged, and the lower phase was rinsed with 1 ml of chloroform-methanol-water (3:48:47, v/v). The rinses were combined with the corresponding aqueous phase and the radioactivity in each phase was determined.

Silicic Acid Column Chromatography—The chloroform-methanol extract was evaporated to dryness in vacuo, and the residue was dissolved in chloroform and passed through a column of silicic acid (0.6 X 13.5 cm) suspended in chloroform. The radioactivity was eluted successively with 5 bed volumes of chloroform, 5 bed volumes of acetone, and 10 bed volumes of chloroform-methanol (1:1, v/v). The radioactivity in each eluate was determined.

DEAE-cellulose Chromatography—The radioactive labeled mannolipid that had been partially purified from a chloroform-methanol extract by weak alkaline treatment and silicic acid chromatography was placed on a column of DEAE-cellulose (2.5 X 20 cm) prepared by the procedure of Rouser et al. (39). The column was eluted sequentially with chloroform, chloroform-methanol (4:1, v/v), and chloroform-methanol (4:1, v/v) containing 50 mM ammonium acetate. The radioactivity in each eluate was determined.

Catalytic Hydrogenation—The platinum catalyst was prepared by reduction of chloroplatinic acid with sodium borohydride (40). Chloroform-methanol extracts were evaporated in vacuo, and the residues were dried by the addition and evaporation of toluene and dissolved in 1-butanol. After addition of 1.4 mg of catalyst to a 1-ml portion of the lipid solution, the mixtures were shaken for 4 hours at room temperature in the presence of H2 at 1.4 atm in a Parr apparatus or in the presence of air (control). Each sample was then recently evaporated at room temperature and the chloroform-methanol isolobutanol (2:1:1, v/v) and 1 ml of H2O were added with vigorous shaking. The phases were separated by centrifugation, and the radioactive activity in each phase was determined. Complete recovery of the radioactivity was obtained. In some cases, the aqueous phases were freeze-dried and examined by paper chromatography. When hydrogenation of authentic fecaprenyl a-D-mannopyranosyl phosphate was performed, the reduced a-D-mannopyranosyl phosphate was converted to a-D-mannose and determined by gas-liquid chromatography (41).

Radioactivity Measurements

The radioactivity was quantitatively determined with a Packard liquid scintillation spectrometer, model 3075, with 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) for solutions, plates from paper chromatograms, and scrapings from silica gel plates. The radioactive components were located on thin layer chromatograms, with Kodak No-Screen medical x-ray film (N3-27) after a 3 to 7-day exposure.

RESULTS

Mannosyltransferase Activity of Unfractionated Microsomes—Incubation of unfractionated, calf pancreas microsomes with GDP-[3H]mannose resulted in the incorporation of radioactivity into endogenous acceptors. The labeled products were divided into two classes. The chloroform-methanol extract contained products that were insoluble in 6% trichloroacetic acid 0.5% phosphotungstic acid and ming were soluble in chloroform-methanol (2:1, v/v), whereas the precipitate consisted of products insoluble in both trichloroacetic acid-phosphotungstic acid and in chloroform-methanol.

The time dependency for the formation of radioactive products is shown in Fig. 1. The products soluble in chloroform-methanol accounted for 70 to 90% of the incorporation. The incorporation of radioactivity into both the chloroform-methanol extract and the precipitate was greatly stimulated by addition of Mn2+, the concentration optimum being 10 mM in both cases (Fig. 2). Addition of Mg2+ at a concentration of 10 mM was less effective; the incorporation was only 30 and 50% of that observed with 10 mM Mn2+ for the precipitate and the chloroform-methanol extract, respectively. The incorporation of radioactivity into both fractions exhibited a broad optimum between pH 6.0 and 6.7 in Tris-maleate buffer. Triton X-100, which enhances mannosyltransferase activity in other systems (42, 43), did not stimulate the incorporation into endogenous acceptors of calf pancreas microsomes. In fact, Triton X-100 inhibited the incorporation of the radioactivity into endogenous acceptors, even at very low concentrations (Table I).
FIG. 1. Incorporation of radioactivity from GDP-n-[14C]mannose into endogenous acceptors. The size of the standard incubation mixture containing unfractionated microsomes was increased 30 times that described in the experimental section. At various times, duplicate aliquots of 0.5 ml were assayed for mannosyltransferase activity. □, radioactivity in precipitate; □, radioactivity in chloroform-methanol extract.

FIG. 2. Effect of MnCl2 ions on the transfer of radioactivity from GDP-n-[14C]mannose into endogenous acceptors. Standard incubation conditions were used with unfractionated microsomes and varying amounts of MnCl2. □, radioactivity in precipitate; □, radioactivity in chloroform-methanol extract.

TABLE I
Effect of Triton X-100 on the transfer of radioactivity from GDP-n-[14C]mannose into endogenous acceptors

<table>
<thead>
<tr>
<th>Triton X-100</th>
<th>Chloroform-methanol extract</th>
<th>Precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>cpm</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>15,100</td>
<td>1,190</td>
</tr>
<tr>
<td>0.062</td>
<td>13,600</td>
<td>1,330</td>
</tr>
<tr>
<td>0.125</td>
<td>12,300</td>
<td>1,300</td>
</tr>
<tr>
<td>0.500</td>
<td>9,440</td>
<td>665</td>
</tr>
<tr>
<td>1.000</td>
<td>4,480</td>
<td>620</td>
</tr>
</tbody>
</table>

TABLE II
Transfer of radioactivity from GDP-n-[14C]mannose into endogenous acceptors of microsomal subfractions

Standard incubation conditions were used, the amounts of protein per 0.5-ml incubation mixtures were 2.28, 1.42, and 1.36 mg for the unfractionated, smooth, and rough microsomal fractions, respectively.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Chloroform-methanol extract</th>
<th>Precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated microsomes</td>
<td>10,400</td>
<td>1,310</td>
</tr>
<tr>
<td>Smooth microsomes</td>
<td>1,230</td>
<td>510</td>
</tr>
<tr>
<td>Rough microsomes</td>
<td>7,210</td>
<td>2,170</td>
</tr>
</tbody>
</table>

Mannosyltransferase Activity in Microsomal Subfractions—Rough and smooth microsomal fractions were prepared and examined in the electron microscope. The rough microsomal fraction appeared fairly homogeneous and consisted of membrane vesicles with attached ribosomes; in contrast, the smooth microsomal fraction contained a mixture of membranous elements and very few ribosomes. The rough microsomal fraction was about 4 times more active, per mg of protein, than the smooth microsomal fraction in the incorporation of radioactivity from GDP-n-[14C]mannose into endogenous acceptors (Table II). The rough microsomal fraction was also more effective than unfractionated microsomes in the incorporation into the precipitate, but less active in the incorporation into chloroform-methanol-soluble material. Thin layer chromatography (Solvent A) of these chloroform-methanol extracts showed that both contained a radioactive component having an RF of approximately 0.5. The chloroform-methanol extract from unfractionated microsomes contained additional radioactive material which remained at the origin, whereas that obtained from the rough microsomal fraction had negligible radioactivity at the origin. To simplify the characterization of the radioactive component having an RF of about 0.5, rough microsomal fractions were used in subsequent experiments.

Properties of Mannosylipid—Since direct extraction with chloroform-methanol of an incubation mixture with rough microsomes yielded a single radioactive compound (Fig. 3), it was possible to characterize this labeled product without further purification. After strong acid treatment, the only radioactive product detected by paper chromatography (Solvents I and K) migrated as n-mannose (70 to 80% recovery of radioactivity), demonstrating that the labeled compound synthesized by rough microsomes from GDP-n-[14C]mannose contained n-mannose.

On silicic acid column chromatography of the components of the chloroform-methanol extract (Table III), the radioactive compound was recovered from the column in the chloroform-methanol eluate along with phospholipids. DEAE-cellulose chromatography of the partially purified labeled compound provided additional evidence for its acidic character, since the radioactivity was eluted with chloroform-methanol containing...
FIG. 3. Radioautograph of a two-dimensional thin layer chromatogram of chloroform-methanol extract. A standard incubation mixture containing rough microsomes and 0.15 μCi per ml of GDP-α-[^14C]mannose was extracted directly as described under "Experimental Procedure." An aliquot was reduced in volume under N₂ and chromatographed in 1, Solvent A (Rf 0.29), and 2, Solvent B (Rf 0.51).

TABLE III
Silicic acid column chromatography of chloroform-methanol extract
A chloroform-methanol extract obtained by direct extraction of a standard incubation mixture with rough microsomes was chromatographed as described under "Experimental Procedure;" 75% of the applied radioactivity was recovered.

<table>
<thead>
<tr>
<th>Eluent</th>
<th>Counts per min eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform (5 bed volumes)</td>
<td>34</td>
</tr>
<tr>
<td>Acetone (5 bed volumes)</td>
<td>72</td>
</tr>
<tr>
<td>Chloroform-methanol (1:1) (10 bed volumes)</td>
<td>3620</td>
</tr>
</tbody>
</table>

Ammonium acetate, and not with chloroform or chloroform-methanol.

Weak alkaline treatment of the labeled compound, under conditions that cause the deacylation of glycosyl diglycerides with the release of water-soluble carbohydrate derivatives (37), did not release the radioactivity into the aqueous phase (Fig. 4A), and did not alter the chromatographic behavior of the labeled compound on Silica Gel G (Solvent A). In contrast, the labeled compound was extremely unstable in dilute acid (Fig. 4B); approximately 85% of the radioactivity was transferred to a water-soluble labeled product within 15 min at 80°C in 0.01 M HCl.

Paper chromatography (Solvent J) of the water-soluble products, obtained after only 1 min of mild acid treatment, gave no evidence for the release of labeled mannosyl phosphate, but showed radioactive spots migrating like D-mannose and methyl α-D-mannopyranoside (Fig. 5).

Treatment of the labeled compound with 0.1 M NaOH in 1-propanol at 90°C resulted in quantitative conversion of the radioactivity into water-soluble components within 5 min. Several labeled products were observed by paper chromatography (Solvents J and L). One, which co-chromatographed with D-mannosyl phosphate in both solvent systems, accounted for 20 to 30% of the radioactivity. After elution from the paper and treatment with alkaline phosphatase, this radioactive compound migrated with the same mobility as D-mannose, thereby confirming that some mannosyl phosphate had been formed during the alkaline treatment. The other products were not identified, but one of them corresponded to the product obtained when D-[^14C]-mannose was treated under the same alkaline conditions; none of them, however, corresponded to D-mannose itself.

These results suggest the presence of a D-mannosyl residue linked to a lipid residue through an acid-labile phosphate or pyrophosphate diester linkage.

Chromatographic Comparison of Pancreatic Mannolipid with Dolichyl α-D-Mannopyranosyl Phosphate and P-Dolichyl P-α-D-Mannopyranosyl Pyrophosphate—The pancreatic mannolipid was 2 Herscovics, A., unpublished data.

Fig. 4. Stability of the [^14C]mannolipid. Chloroform-methanol extracts obtained by direct extraction of standard incubation mixtures with rough microsomes were treated as described under "Experimental Procedure": A, weak alkaline hydrolysis at 37°C; B, mild acid hydrolysis at 80°C. ○, radioactivity in aqueous phase; □, radioactivity in chloroform-methanol phase.

Fig. 5. Paper chromatography of products released by acid. The aqueous phase obtained after 1 min of treatment of chloroform-methanol extract with 0.01 M HCl at 100°C was chromatographed in Solvent J. Man-1-P, α-mannopyranosyl phosphate; Man, D-mannose; Me-Man, methyl α-D-mannopyranoside.
Effect of nucleotides on the amount of radioactivity in the chloroform-methanol extract

Standard conditions of incubation were used with rough microsomes. After 5 min of incubation, one sample was assayed, and either GMP or GDP was added to the other samples, at a final concentration of 50 μM, as indicated. Incubation was continued for another 10 min before assay.

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Time of addition (min)</th>
<th>Total incubation (min)</th>
<th>Radioactivity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td></td>
<td>3830</td>
</tr>
<tr>
<td>None</td>
<td>15</td>
<td></td>
<td>5590</td>
</tr>
<tr>
<td>GMP</td>
<td>5</td>
<td>15</td>
<td>5110</td>
</tr>
<tr>
<td>GDP</td>
<td>5</td>
<td>15</td>
<td>1150</td>
</tr>
</tbody>
</table>

Addition of Exogenous Polypropyl Phosphates—The effect of various polypropyl phosphates on the incorporation of radioactivity from GDP-[14C]mannose into chloroform-methanol-soluble products was investigated in the presence of 0.1% Triton X-100. Dolichyl phosphate greatly stimulated this incorporation at very low concentrations (Fig. 7). In the absence of detergent, no stimulation was observed even at a high concentration of dolichyl phosphate (up to 0.3 mM). The chloroform-methanol extract obtained from an incubation with dolichyl phosphate contained a single radioactive spot, which migrated the same distance as the mannolipid formed from endogenous lipid (Fig. 8).

Other long-chain polypropyl phosphates, such as ficaprenyl phosphate and solanesyl phosphate, which have 11 and 9 isoprene residues, respectively, also greatly stimulated the incorporation of radioactivity into the chloroform-methanol extract (Table V). The extent of incorporation with these polypropyl phosphates varied to a greater degree than did the stimulation obtained with dolichyl phosphate. The chloroform-methanol extracts obtained from reaction mixtures stimulated with ficaprenyl phosphate or solanesyl phosphate contained two radioactive components each: one corresponding to that formed from the endogenous acceptor and a second radioactive compound having a lower mobility (Fig. 8). There was a 30 to 45% decrease in the amount found to co-chromatograph with authentic dolichyl α-D-mannopyranosyl phosphate upon thin layer chromatography in each of seven solvent systems (A to H). The results obtained in one system are presented in Fig. 6. In contrast, the pancreatic mannolipid (Rf 0.52) could be clearly separated in Solvent C from authentic [14C]mannose-phosphate (Rf 0.20). These results demonstrate that the pancreatic mannolipid is very similar to dolichyl α-D-mannopyranosyl phosphate.

Effect of GMP and GDP on Mannolipid Formation—The time course for the synthesis of the mannolipid by rough microsomes was very similar to that observed for the formation of chloroform-methanol-soluble labeled compounds by unfractionated microsomes (see Fig. 1); the initial rate of incorporation of radioactivity was constant for 10 min. The addition of GDP, 5 min after the beginning of incubation, caused the subsequent disappearance of about 70% of the mannolipid that had accumulated (Table IV). In contrast, the addition of an equivalent amount of GMP had little effect; the amount of radioactivity incorporated at the end of the incubation was about the same in the presence of GMP as in the control without nucleotide. These observations suggest that the pancreatic mannolipid, like the hepatic mannolipid studied by Richards and Hemming (11), is formed by the following reversible reaction:

\[ \text{polypropyl-P} + \text{GDP-[14C]mannose} \rightleftharpoons \text{polypropyl-[14C]mannosyl-P} + \text{GDP} \]
TABLE V
Effect of exogenous lipids on the incorporation of radioactivity from GDP-[14C]mannose into the chloroform-methanol extract

The conditions of incubation are described under "Experimental Procedure." The concentration of polyenyl phosphates was determined by phosphate analysis.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Concentration</th>
<th>Incorporation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µCi</td>
<td>cpm</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>4,294</td>
<td>1,250</td>
</tr>
<tr>
<td>Dolichyl phosphate</td>
<td>92</td>
<td>33,500</td>
<td>3,500</td>
</tr>
<tr>
<td>Dolichyl phosphate</td>
<td>258</td>
<td>3,000</td>
<td>31,500</td>
</tr>
<tr>
<td>Ficaprenyl phosphate</td>
<td>376</td>
<td>31,300</td>
<td>3,450</td>
</tr>
<tr>
<td>Solanesyl phosphate</td>
<td>402</td>
<td>33,500</td>
<td>3,450</td>
</tr>
<tr>
<td>Farnesyl phosphate</td>
<td>512</td>
<td>428</td>
<td>1,680</td>
</tr>
<tr>
<td>Citronellyl phosphate</td>
<td>428</td>
<td>1,680</td>
<td>1,680</td>
</tr>
<tr>
<td>Retinyl phosphate</td>
<td>184</td>
<td>33,500</td>
<td>3,450</td>
</tr>
<tr>
<td>+ lecithin (184 µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Incubations and thin layer chromatography were performed in the dark.

Dolichyl phosphate always stimulated the incorporation of radioactivity from GDP-[14C]mannose, the extent of which was variable. Hydrogenation of Labeled Mannolipids—Hydrogenation pro-
phosphate, or solanesyl phosphate were incubated with endogenous acceptor, and with exogenous dolichyl prenyl phosphate, which contain allylic phosphate groups, gave water-soluble radioactive products (Fig. 10) that showed no release of radioactivity into the aqueous phase. In contrast, the labeled compounds obtained from both dolichyl phosphate, which has a saturated α-isoprene residue, and from the endogenous lipid as acceptors showed no evidence of hydrogenolysis, since no significant amount of radioactivity became water-soluble during catalytic hydrogenation (Fig. 10).

The extent of hydrogenolysis of the mannolipids obtained from incubation with the allylic polyprenyl phosphates could not be increased by lengthening the hydrogenation period to 8 hours. When a sample (0.1 mg) of authentic ficaprenyl α-D-mannopyranosyl phosphate was hydrogenated for 4 hours under identical conditions, mannoseyl phosphate was formed in an 88% yield. The difference between the extent of hydrogenolysis of the labeled mannolipids (50 to 60%) and that of the authentic compound results partly from the presence, in the chloroform-methanol extracts, of 10 to 15% of the mannolipid derived from endogenous lipid that does not undergo hydrogenolysis. Additional factors, including the possible enzymic saturation of the α-isoprene units of the added polyprenyl phosphates, may also be responsible for the difference in the extent of hydrogenolysis.

These results demonstrate that calf pancreas rough microsomes incubated with GDP-[14C]mannose in the presence of Mn2+ synthesize a [14C]-mannolipid very similar to authentic dolichyl α-D-mannopyranosyl phosphate chemically synthesized from pig liver dolichol. The labeled mannolipid has properties characteristic of polyprenyl sugar phosphates: it is extremely labile to mild acid, resistant to weak alkali, and acidic in nature as evidenced by its behavior on silicic acid and DEAE-cellulose. The formation of mannoseyl phosphate as one of the products of hot alkaline treatment indicates that the mannose residue is linked to the lipid residue through a phosphate group. The lipid residue of the mannolipid appears to be a dolichol, as co-chromatography with authentic dolichyl α-D-mannopyranosyl phosphate was observed in several solvent systems, including one which separates polyprenyl mannoseyl phosphates according to chain length (Fig. 9). The labeled mannolipid contains a phosphate diester, and not a pyrophosphate linkage, since it is clearly separated from P1-dolichyl P2-α-D-mannopyranosyl pyrophosphate upon thin layer chromatography. The mannolipid does not undergo hydrogenolysis during catalytic hydrogenation, which causes the release of mannoseyl phosphate from polyprenyl mannoseyl phosphates having unsaturated α-isoprene units (Fig. 11); this indicates that the mannolipid has a saturated α-isoprene residue.

The labeled mannolipid from calf pancreas is not necessarily identical with the authentic dolichyl α-D-mannopyranosyl phosphate. The authentic compound was prepared from pig liver dolichol, which is a mixture of isoprenologs: 35 to 40% of the C9, 20 to 25% each of the C10 and C11, and 8 to 12% each of the C8 and C10 homologs (18). The composition of calf pancreas dolichol has not been reported and may have a different distribution of isoprenologs. In addition, variations in the cis-trans-isomerism of the two mannolipids may exist.

Exogenous, long chain polyprenyl phosphates greatly stimulate the incorporation of radioactivity from GDP-[14C]mannose into lipid. Exogenous retinyl phosphate results in a slight stimulation. The addition of solanesyl phosphate, ficaprenyl phosphate, and retinyl phosphate produced radioactive compounds clearly different from that obtained from endogenous acceptor. Furthermore, the product obtained from ficaprenyl phosphate co-chromatographs with authentic ficaprenyl α-D-mannopyranosyl phosphate. These observations demonstrate that the polyprenyl phosphates stimulate the incorporation by acting as substrates rather than by a nonspecific stimulation of the transferase activity. Moreover, dolichyl phosphate is by far the most effective polyprenyl phosphate to be utilized; in this case, the radioactive product cannot be separated from the mannolipid formed from

**DISCUSSION**

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**Fig. 10.** Catalytic hydrogenation of labeled mannolipids. Chloroform-methanol extracts obtained from standard incubations with endogenous acceptor, and with exogenous dolichyl prenyl phosphate, ficaprenyl phosphate, or solanesyl phosphate were hydrogenated as described under "Experimental Procedure."
endogenous lipid. This observation provides additional evidence that the mannosid lipid formed from endogenous lipid is a dolichyl derivative. The mannosid lipid formed from exogenous polypropenyl phosphates appear to be monophosphates since they exhibit the same chromatographic behavior as the authentic polypropenyl mannose mannosid phosphates: their mobility is a logarithmic function of the number of isoprene units (Fig. 9).

While this work was in progress, the formation of polypropenyl mannose mannosid phosphates similar to the one described in this study has been reported in several animal tissues (6, 7, 10-11). In some cases, the transfer of the mannosid residue from mannosid lipid to endogenous peptide was observed, and it has been suggested that polypropenyl phosphates function in the transfer of mannose from GDP-mannose to both soluble (12, 19) and membrane glycoproteins (15). Studies where UDP-glucose and UDP-N-acetylglucosamine were glycoconjugate donors have demonstrated the formation of the corresponding dolichyl derivatives (10, 35, 44). In liver, dolichyl glucosid phosphate transfers its glucose residue to a lipid-linked oligosaccharide, which has been partially identified as a dolichyl oligosaccharide pyrophosphate containing about 20 monosaccharide residues (45-47). The oligosaccharide can then be further transferred to an endogenous peptide moiety (48). The transfer of mannose in liver to a similar lipid-linked oligosaccharide has also been reported (49). Leloir et al. (50) have suggested that a O-2-acetamido-2-deoxy-β-D-glucopyranosyl (1→4)-2-acetamido-2-deoxy-β-D-glucose derivative linked to dolichol via a pyrophosphate diester linkage is formed. These observations raised the possibility that the oligosaccharide core region of certain glycoproteins may be formed as a dolichyl derivative first, and then may be transferred to a protein chain as an oligosaccharide unit.

In calf pancreas microsomes, several other products besides the mannosid lipid investigated in this report are formed from GDP-[14C]mannose. Preliminary experiments indicate that the labeled mannosid lipid can transfer its radioactivity to products which have not yet been characterized. The labeled mannosid lipid is probably an intermediate in the formation of other carbohydrate-containing materials in calf pancreas. Furthermore, when calf pancreas microsomes are incubated with UDP-N-acetyl-3H]glucosamine, a lipid having the properties of P-dolichyl P4-acetamido-2-deoxy-a-D-glucopyranosyl pyrophosphate is formed (44). It remains to be determined whether these products are related to the biosynthesis of glycoproteins or to the formation of other types of complex carbohydrates in the pancreas.

**Note Added in Proof**—The [14C]mannosid lipid formed from GDP-[14C]mannose in calf pancreas microsomes has since been shown to contain a β-linked d-mannose residue (51).

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Jan S. Tkacz, Annette Herscovics, Christopher D. Warren and Roger W. Jeanloz


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