Incubation of microsomal preparations from *Leishmania donovani* parasites with UDP-[3H]galactose or GDP-[14C]mannose resulted in incorporation of radiolabeled into an endogenous product that exhibited the chemical and chromatographic characteristics of the parasite’s major surface glycoconjugate, lipophosphoglycan. The [3H]galactose- or [14C]mannose-labeled product was (i) cleaved by phosphatidylinositol-specific phospholipase C; (ii) deaminated by nitrous acid; and (iii) degraded into radioactive, low molecular weight fragments upon hydrolysis with mild acid. Analysis of the products of mild acid hydrolysis revealed the presence of phosphorylated Gal-β-Man as the major fragment with lesser amounts of mono-, tri-, and tetrasaccharides. The incorporation of the two isotopic precursors was neither stimulated by the addition of dolichylphosphate nor inhibited by amphotericin B, indicating that dolichol-saccharide intermediates are not involved in assembly of the repeating units of lipophosphoglycan. Development of this cell-free glycosylating system will facilitate further studies on the pathway and enzymes involved in lipophosphoglycan biosynthesis.

LPG is the predominant surface glycoconjugate of the promastigote form of all *Leishmania* protozoan parasites. LPG is believed to be a multifunctional glycoconjugate for the organism (see Ref. 1 for a review). It has been implicated in the attachment of the parasite to epithelial cells of its insect vector’s midgut (2), in complement activation and resistance to complement-mediated damage (3), in attachment and entry into host macrophages (4, 5), and in enabling the parasite to survive in the phagolysosomal compartment (6, 7). Structurally, the LPG from *L. donovani* consists of a polymer of repeating PO4-6Gal(1,4)Manα1 units (average of 16 units) linked via a phosphosaccharide core to a novel lyso-1-O-alkyl phosphatidylinositol lipid anchor (8-10). The *L. donovani* LPG is capped at the nonreducing end with a saccharide with the generalized structure of (Gal)(β1-3)(Man). In all species of *Leishmania* examined thus far, the lipid anchor and phosphosaccharide core are structurally conserved whereas the repeating units display species variations. In *Leishmania major* (11, 12) and *Leishmania mexicana*, the LPGs contain additional sugar residues as side chains attached to the basic repeating PO4-6Gal(1,4)Manα1 backbone. Virtually nothing is known regarding the biosynthesis of LPG. *Leishmania* parasites have been reported to abundantly synthesize a family of molecules termed glycosolectin-phospholipids (13-15) or glycosylphosphoinositols (16, 17). The carbohydrate portions of these molecules are the same as the corresponding part of the phosphosaccharide core of LPG and are attached via an acetylated glucosamine to either alkylacyl-PI or lyso-1-O-alkyl-PI. These findings would suggest that at least some of the glycosolectin-phospholipids are precursors in the biosynthetic pathway leading to LPG.

In this paper, we describe the development of a cell-free particulate preparation of *L. donovani* that can be used to examine LPG biosynthesis in vitro. Incubation of the microsomal fraction with UDP-[3H]galactose or GDP-[14C]mannose results in the enzymatic radiolabeling of an endogenous product having chemical and chromatographic properties of LPG.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials were obtained as follows: Dulbecco’s modified Eagle’s medium from Gibco; UDP-galactose, GDP-mannose, dolichylphosphate, phenyl-coupled Sepharose CL-4B, alkaline phosphatase (Escherichia coli), and β-galactosidase (jack bean) from Sigma, UDP-[3H]galactose (25 Ci/ml) from American Radiolabeled Chemicals, Bio-Gel P-10 from Bio-Rad; DE52 cellulose from Whatman; and amphomycin from Bristol Laboratories. Man-P-Dol was donated by Jeffrey Rush and Charles J. Waeche (University of Kentucky). GDP-[U-14C]mannose (50-150 mCi/ml) was obtained as previously described (18). PI-PLC was prepared from *Bacillus thuringiensis* as previously described (19).

**Cell Culture**—*L. donovani* (promastigote form) were grown at 25°C in Dulbecco’s modified Eagle’s medium supplemented with adenine (0.05 mM), xanthine (0.05 mM), biotin (1 mg/ml), Tween 80 (40 mg/liter), hemin (5 mg/liter), triethanolamine (0.5 M/liter), and 0.3% bovine serum albumin.

**Preparation of Membranes**—Parasites (6 x 10⁸) were harvested at 10974
a density of 5-6 x 10^7 cells/ml, pelleted by centrifugation at 3,000 x g for 10 min, washed with phosphate-buffered saline (20), repelletted, and suspended in 10 ml of HEPES buffer (100 mM HEPES, pH 7.4, 50 mM KCl, 10 mM MnCl2, 10 mM MgCl2, 0.1 mM TLCK, and 1 mg/ ml leupeptin) containing 10% glycerol. The cells were disrupted in a Parr nitrogen cavitation bomb after dialibration at 1.50 psi for 25 min at 4 °C. The debris was removed by centrifugation at 30,000 x g for 7 min, and the supernatant was centrifuged at 100,000 x g for 1 h at 4 °C. The resulting membrane pellet was resuspended in 10 ml of HEPES buffer without glycerol and centrifuged again at 100,000 x g for 1 h at 4 °C. The membranes were then resuspended in 1 ml (15-17 mg/ml) of the HEPES buffer without glycerol, 0.5% CHAPS for 10 min, washed five times with the addition of 5 ml of 0.1 M acetic acid, 0.6 ml of H2O, and 3.6 ml of Solvent E. Fractions of 0.6 ml were collected and assayed for radioactivity.

**Identification of the Radiolabeled Product as LPG**

Samples were applied to a column of DE52 cellulose (0.5 x 2 cm) equilibrated in 1.0 mM Tris, pH 8.0. The fraction was then washed sequentially with 3.0 ml of 0.1 M NaCl and 0.1 M acetic acid, 1.2 ml of 0.1 M acetic acid, 0.6 ml of H2O, and 3.6 ml of Solvent E. Fractions of 0.6 ml were collected and assayed for radioactivity. Bio-Gel P-10 Gel Filtration Chromatography—Samples were applied to a column of Bio-Gel P-10 (1.0 x 10 cm) equilibrated in 0.1 M NaH2PO4, 0.02% sodium azide. Fractions of 0.6 ml were collected and assayed for radioactivity. Blue dextran was used as the void volume marker, and mannose was used as the retention volume marker.

**DEAE-Cellulose Ion-exchange Chromatography**—Samples were applied to a column of DE52 cellulose (0.5 x 2 cm) equilibrated in 1.0 mM Tris, pH 8.0. The fraction was then washed sequentially with 3.0 ml of 0.1 M NaCl and 0.1 M acetic acid, 1.2 ml of 0.1 M acetic acid, 0.6 ml of H2O, and 3.6 ml of Solvent E. Fractions of 0.6 ml were collected and assayed for radioactivity.

**High Pressure Liquid Chromatography—**Desalted neutral glycan were run on a Bio-Gel P-10 (1.0 x 30 cm) equilibrated in 0.1 M NaH2PO4, 0.02% sodium azide. Fractions of 0.6 ml were collected and assayed for radioactivity. Blue dextran was used as the void volume marker, and mannose was used as the retention volume marker.

**Paper Chromatography—**Samples were chromatographed on Whatman No. 3 paper in descending mode for 24 h using n-butanol/ pyridine/water (6:4.5:3) as the solvent system. Starchose, raffinose, lactose, Gal3(1,4)Man, galactose, glucose, and mannose were used as standards and were detected with silver nitrate and ethanolic NaOH (21). Sections of 1 cm were cut from the chromatogram, soaked in 0.6 ml of 1% sodium dodecyl sulfate, and assayed for radioactivity.

**Alkaline Phosphatase Digestion**—Incubation digestion (0.1-0.5 units) was done in 1 mM Tris, pH 8.0, for 16 h at 37 °C; PI-PLC digestion (0.1-0.1 units) in 25 mM HEPES, pH 7.4, 2.5 mM EDTA, 0.1% CHAPS for 1-2 d at 37 °C; and β-galactosidase digestion (0.1-0.2 units) in 0.05 M sodium citrate, pH 5, for 48 h at 37 °C. The incubation mixture for a final ratio of chloroform/methanol/water of 3:2:1 and sonicated. The membranes were sequentially extracted by an extraction protocol described elsewhere (8). One of the solvents (Solvent E) in the extraction protocol consisted of water/ethanol/diethylether/pyridine/NH40H (15:15:5:1:0.017) and was used to solubilize LPG. The Solvent E extract was dried under reduced pressure and was chromatographed over a column of phenyl-Sepharose as described below.

**RESULTS**

**In vitro Incorporation of [3H]Galactose- and [14C]Mannose into Endogenous Acceptors—**Microsomal membranes from L. donovani were incubated with UDP-[3H]galactose or GDP-[14C]mannose for up to 60 min. Radioactive glycoconjugates were prepared by extracting the membranes with organic solvents. One of the organic solvent extracts, the Solvent E extract, was subjected to hydrophilic and amphiphatic fractions by hydrophobic chromatography on phenyl-Sepharose. LPG radiolabeled in vivo and extracted by Solvent E is retained by the hydrophobic support and can be eluted with a polar organic solvent mixture (10). Up to 80% of the total in vitro [3H]galactose- or [14C]mannose-labeled material was similarly retained by chromatography on phenyl-Sepharose and eluted with an organic solvent (Solvent E). This amphipathic substance, characterized as LPG (see below), represented about 20-30% of the total [3H]galactose or [14C]mannose incorporated into macromolecules and was characterized further.

A time course for the glycosylation in vitro of LPG with UDP-[3H]galactose is shown in Fig. 1, left. Incorporation of the label into LPG was observed using particulate preparations of wild-type L. donovani, whereas very low rates of radiolabeling of LPG were observed with membranes prepared from an LPG-deficient variant line of L. donovani (22). Similar results were obtained when GDP-[14C]mannose was used as the radioactive nucleotide-sugar donor (Fig. 1, right).

**Identification of the Radiolabeled Product as LPG**

Analysis of the [3H]Galactose- or [14C]Mannose-labeled Glycoconjugate—The isolated [3H]galactose- or [14C]mannose-labeled glycoconjugate was subjected to digestion by several
types of hydrolyses, and the radioactive products were analyzed by chromatography on phenyl-Sepharose. As shown in Fig. 2A, the intact glycoconjugate was retained by the hydrophobic support equilibrated in an aqueous solvent, suggesting that it was a lipid-containing glycoconjugate. By analogy to in vivo labeled LPG (8), the in vitro [3H]galactose- and [14C]mannose-labeled glycoconjugates were subjected to digestion with the highly specific bacterial phospholipase PI-PLC. The enzyme-treated [3H] or [14C]glycoconjugate eluted quantitatively in the acetic acid/NaCl breakthrough (Fig. 2B), suggesting the removal of a phosphatidylinositol moiety from the glycoconjugates. Another aliquot of the intact radiolabeled glycoconjugate was subjected to nitrous acid deamination for 40 h at 25 °C. As shown in Fig. 2C, the labeled product was found to contain an unacetylated hexosamine (most likely glucosamine) since the HONO-treated glycoconjugate eluted in the breakthrough. In the absence of HONO, incubation of the glycoconjugate for 40 h at 25 °C resulted in no breakdown. One of the structural features of the LPG of L. donovani is its extreme lability to mild acidic conditions, which cleave the multiple phosphodiester linkages in LPG and result in the release of PO4-6Gal(pl,4)Man as the major fragment (9, 23). Of particular significance, pretreatment of the intact [3H]galactose- or [14C]mannose-labeled glycoconjugate with 0.02 N HCl for 5 min at 100 °C also resulted in radioactivity quantitatively eluting in the breakthrough, indicating the presence of acid labile bonds in the in vitro labeled product (Fig. 2D).

![Fig. 2](image)

**Fig. 2**. Hydrophobic chromatography of the [3H]galactose- and [14C]mannose-labeled glycoconjugate on phenyl-Sepharose. Samples were applied to a column of phenyl-Sepharose equilibrated in 0.1 N acetic acid and 0.1 N NaCl and eluted as described under "Experimental Procedures." The column solvent was changed as indicated in the figure as follows: a, 0.1 N acetic acid; b, water; and c, water. Panel A, intact radiolabeled glycoconjugate; panel B, glycoconjugate pretreated with PI-PLC; panel C, glycoconjugate pretreated with 0.02 N HCl, 5 min, 100 °C. Closed circles, [3H]galactose label; open circles, [14C]mannose label.

To obtain information regarding the relative size of the [3H]galactose-labeled glycoconjugate, it was first treated with PI-PLC to remove the lipid moiety, and the carbohydrate portion was subjected to gel filtration chromatography on Bio-Gel P-10. The PI-PLC-treated glycoconjugate eluted close to the void volume of the column (Fig. 3), an elution position comparable to PI-PLC-treated LPG labeled in vivo (24) and indicative of a relatively large molecular size. Hydrolysis of the PI-PLC-treated glycoconjugate with mild acid resulted in a major product that eluted close to the retention volume and a minor product in the retention volume of Bio-Gel P-10. Similar chromatographic results were obtained with the [14C]mannose-labeled glycoconjugate (data not shown).

**Anion-exchange Chromatographic Analysis of the Mild Acid-generated Products—**The products of mild acid hydrolysis of the [3H]galactose-labeled glycoconjugate were further analyzed by anion-exchange chromatography on DEAE-cellulose. As shown in Fig. 4A, three peaks of radioactive fragments were resolved. One product (Peak I) did not bind the ion-exchange support, whereas two products (Peaks II and III) did bind and were resolved with a NaCl gradient. Peak III was the major mild acid-generated product and eluted in a position identical to monophosphorylated saccharides. Pre-treatment of the fragments with alkaline phosphatase resulted in a quantitative loss of Peak III and a concomitant increase in radioactivity associated with Peak II, suggesting that Peak III was indeed a monophosphorylated saccharide.

Peak II was found to be a saccharidyl cyclic phosphate from the following observation: the isolated Peak II was subjected to a stronger acidic condition of 0.1 N HCl for 15 min at 100 °C which is known to hydrolyze cyclic phosphate (25, 26). Following acid hydrolysis of [3H]galactose-labeled Peak II, the hydrolysate was reapplied to a column of DEAE-cellulose, and it was resolved into two products (Fig. 4C). One neutral fragment (42% of recovered radioactivity) eluted in the breakthrough of the column and the other (58% of recovered radioactivity) eluted in a position similar to Peak III, indicative of a saccharidyl monophosphate ester. Indeed, the latter was found to be completely susceptible to alkaline phosphatase in contrast to the isolated Peak II which was resistant to hydrolysis by the enzyme. These results were consistent with the proposal that Peak II was a saccharidyl cyclic phosphate; further characterization of the sugar portion is discussed.

![Fig. 3](image)

**Fig. 3**. Bio-Gel P-10 chromatography of PI-PLC-treated [3H]galactose-labeled glycoconjugate. Circles, PI-PLC-treated [3H]glycoconjugate not hydrolyzed with mild acid; triangles, PI-PLC-treated [3H]glycoconjugate hydrolyzed with mild acid.
Cell-free Biosynthesis of LPG

Fig. 4. DEAE-cellulose chromatography of the mild acid hydrolysis products of ['H]galactose-labeled glycoconjugate. The ['H]glycoconjugate was hydrolyzed with 0.02 N HCl for 5 min at 100 °C, dried by evaporation under N2, resuspended in 1 mM Tris-HCl, pH 8, and applied to a column of DE52 cellulose as described under "Experimental Procedures." Panel A, ['H]glycoconjugate pretreated with mild acid; panel B, ['H]glycoconjugate pretreated with mild acid, dried under N2 to remove the acid, and then treated with alkaline phosphatase; and panel C, isolated Peak II from panel A treated with 0.1 N HCl for 15 min at 100 °C, and reapplied to DEAE-cellulose. Standard monophosphorylated saccharides (e.g., PO4-Gal-Man and galactose 6-phosphate) eluted in fractions 28-34. Similar chromatographic results were obtained with the [14C]mannose-labeled glycoconjugate (data not shown).

Paper Chromatographic Analysis of the ['H-Gal- and ['C-Mann]-labeled Peak III—The identity of major radioactive peak (Peak III) derived by the DEAE-cellulose chromatography (Fig. 4A) was determined by sequential hydrolase degradation of the isolated fragment, followed by chromatographic analysis of the products. As expected, the untreated ['H]galactose-labeled fragment remained close to the origin (Fig. 5A) as did a sample pretreated with β-galactosidase (data not shown). However, treatment of the ['H]galactose-labeled fragment with alkaline phosphatase resulted in comigration of the material with the standard disaccharide Gal(β1,4)Man on paper (Fig. 5B) and by high performance liquid chromatography (Fig. 6). Furthermore, digestion of the alkaline phosphatase-treated fragment with β-galactosidase yielded a single radioactive peak that comigrated with standard galactose (Fig. 5C). Similar treatments of [14C]mannose-labeled Peak III resulted in identical observations except that β-galactosidase digestion of the alkaline phosphatase-treated Peak III yielded a radioactive peak that comigrated with standard mannose (data not shown). Thus, the major mild acid hydrolysis frag-
Paper Chromatographic Analysis of the [\(^{3}H\)Gal- and \[^{14}C\)Man]-labeled Peaks I and II—[\(^{3}H\)Galactose-labeled Peaks I and II, isolated by DEAE-cellulose chromatography, were also analyzed by paper chromatography. The \([^{3}H]\)galactose-labeled Peak I, which consisted of fragments that did not bind to DEAE-cellulose, was resolved into at least three entities on paper (Fig. 7A). One species in Peak I comigrated with the standard disaccharide (Gal-Man). Several other species appeared to be tri- and tetrasaccharides since they migrated similar to such standards. Analysis of \([^{14}C]\)mannose-labeled Peak I revealed \([^{14}C]\)mannose as the main component, with lesser amounts of the Gal-Man disaccharide, tri- and tetrasaccharides (Fig. 7C).

As expected, it was found that \([^{3}H]\)galactose or \([^{14}C]\)mannose-labeled Peak II hardly migrated on paper in the solvent system of n-butanol/pyridine/water (6:4:3) due to the presence of the cyclic phosphate. Therefore, the radiolabeled Peak II was pretreated with acid conditions to hydrolyze the cyclic phosphates and then with alkaline phosphatase to cleave the resultant saccharidyl monophosphate esters. Upon paper chromatography, the majority (60%) of the \([^{3}H]\)galactose radioactivity comigrated with the Gal-Man disaccharide standard (Fig. 7B). Lesser amounts of other saccharides were also observed. Similarly, the acid hydrolyzed, alkaline phosphatase-treated \([^{14}C]\)mannose-labeled Peak II yielded a variety of small sized components on paper (Fig. 7D). Like the \([^{3}H]\)galactose-labeled counterpart, the main \([^{14}C]\)component was a species that comigrated with the standard Gal-Man disaccharide.

Identification of the Radiolabel—To determine the identity of the radiolabel, the \([^{3}H]\)galactose- and \([^{14}C]\)mannose-labeled glycoconjugates were subjected to strong acid hydrolysis (2 N trifluoroacetic acid, 2.5 h, 100 °C) and analyzed by descending paper chromatography. Strong acid hydrolysis of the \([^{3}H]\)galactose-labeled glycoconjugate revealed that approximately one-third of the radioactivity comigrated with standard galactose and the remainder hardly migrated (data not shown). However, pretreatment of the strong acid hydrolyzed \([^{3}H]\)glycoconjugate with alkaline phosphatase prior to chromatography on paper resulted in all of the radioactivity comigrating with galactose. These results suggested that a phosphate ester of galactose was generated by strong acid hydrolysis of the glycoconjugate which was then susceptible to alkaline phosphatase. Similar observations were reported with LPG labeled in vivo with \([^{3}H]\)galactose (23). Strong acid hydrolysis of the \([^{14}C\text{-Man}]\)glycoconjugate yielded a single radioactive peak that comigrated with standard mannose.

Partial Characterization of the Glycosyltransferases

Effect of Various Reagents on \([^{3}H]\)Galactose and \([^{14}C]\)Mannose Incorporation—To obtain information on the requirements of the galactosyltransferase and mannosyltransferase activities, various reagents were either deleted or added to the micromones, and the effect on LPG biosynthesis was measured. The absence of GDP-Man from the incubation mixture significantly lowered \([^{3}H]\)galactose incorporation from UDP-\([^{3}H]\)Gal into LPG; likewise, the absence of UDP-Gal resulted in decreased \([^{14}C]\)mannose incorporation from GDP-\([^{14}C]\)Man (Table I). The activities of both transferases were adversely affected by removing the divalent cations Mg\(^{2+}\) and Mn\(^{2+}\) from the incubation mixture, whereas the absence of either ATP or dithiothreitol had no significant effect. The addition of the zwitterion detergent CHAPS inhibited the activity of the galactosyltransferases while the addition of the nonionic detergent Triton X-100 had a mild inhibitory effect on the mannosyltransferase at the concentration used.

It was important to determine whether the mannose and galactose residues were added sequentially to LPG or added as a Gal-Man disaccharide unit donated from a preformed precursor. To address this aspect, LPG labeled with GDP-\([^{14}C]\)mannose in the presence or absence of unlabeled UDP-Gal was hydrolyzed with acid (0.1 N HCl), and the fragments were analyzed. In the absence of UDP-Gal, the monosaccharide \([^{14}C]\)mannose should be obtained in appreciable amounts upon acid hydrolysis of endogenously labeled LPG if the...
Effect of various metabolites on incorporation of \(^{3}H\)galactose and \(^{14}C\)mannose into endogenous LPG

All reaction mixtures consisted of membranes (2–2.5 mg of protein), 50 mM HEPES, pH 7.4, 25 mM KCl, 5 mM MnCl\(_2\), 5 mM MgCl\(_2\), 0.1 mM TLCK, 1 \(\mu\)g/ml leupeptin, 0.8 mM ATP, and 0.4 mM dithiothreitol in a total volume of 250 \(\mu\)l. When \(^{3}H\)galactose incorporation into LPG was examined, the incubation mixture contained 2 \(\mu\)M UDP-(\(^{3}H\)galactose (1.7 \(\mu\)Ci) and 10 \(\mu\)M GDP-mannose. When \(^{14}C\)mannose incorporation was examined, the incubation mixture contained 2 \(\mu\)M GDP-[\(^{14}C\)]mannose (0.4 \(\mu\)Ci) and 10 \(\mu\)M UDP-galactose. After 20 min at 25 \(^\circ\)C, the reactions were terminated, and incorporation of radioactivity into LPG was determined as described under “Experimental Procedures.” Values shown are average of three experiments. The values in parentheses are the percentage incorporation relative to the “Complete” value.

| Reaction mixture | \(^{3}H\)Galactose incorporation into LPG | \(^{14}C\)Mannose incorporation into LPG |
|------------------|----------------------------------------|---------------------------------------|
| Complete         | 2,280 \((55\%\))                         | 730 \((30\%\))                        |
| Minus companion  | 950 \((42\%\))                          | 310 \((43\%\))                        |
| nucleotide-sugar |                                        |                                       |
| Minus Mg/Mn      | 650 \((29\%\))                          | 30 \((4\%\))                          |
| Minus ATP        | 2,430 \((108\%\))                       | 540 \((73\%\))                        |
| Minus dithiothreitol | 2,460 \((114\%\))                  | 710 \((97\%\))                        |
| CHAPS (0.1%)     | 890 \((39\%\))                          | 600 \((83\%\))                        |
| Triton X-100 (0.1%) | 2,550 \((112\%\))                  | 490 \((67\%\))                        |

TABLE II

Proportion of \(^{14}C\)mannose-labeled fragments derived from LPG

All reaction mixtures consisted of membranes (2–2.5 mg of protein), 50 mM HEPES, pH 7.4, 25 mM KCl, 5 mM MnCl\(_2\), 5 mM MgCl\(_2\), 0.1 mM TLCK, 1 \(\mu\)g/ml leupeptin, 2 \(\mu\)M GDP-[\(^{14}C\)Man (0.4 \(\mu\)Ci), 0.8 mM ATP, and 0.4 mM dithiothreitol in a total volume of 250 \(\mu\)l. As indicated in the table, UDP-Gal was added to the incubation mixture at a final concentration of 30 \(\mu\)M. After 20 min at 25 \(^\circ\)C, the reactions were terminated, \(^{14}C\)Man-LPG was isolated, hydrolyzed with 0.1 N HCl for 15 min at 100 \(^\circ\)C, and incorporation of radioactivity into various acid-generated fragments was determined by paper chromatography as described under “Experimental Procedures.” Values shown are representative of two experiments. The values shown are the percentage of radioactivity relative to the total radioactivity recovered following paper chromatography of the fragments.

| \(^{14}C\)Man | % | % |
|--------------|---|---|
| Mannose      | 35 | 18 |
| Gal-Man      | 33 | 61 |
| Trisaccharide| 11 | 11 |
| Tetrasaccharide | 19 | 10 |

Effect of amphomycin on \(^{14}C\)mannose incorporation into Man-P-Dol and LPG

All reaction mixtures consisted of membranes (2–2.5 mg), 50 mM HEPES, pH 7.4, 25 mM KCl, 5 mM MnCl\(_2\), 5 mM MgCl\(_2\), 0.1 mM TLCK, 1 \(\mu\)g/ml leupeptin, 10 \(\mu\)M UDP-Gal, 2 \(\mu\)M GDP-[\(^{14}C\)Man (0.4 \(\mu\)Ci), 0.8 mM ATP, and 0.4 mM dithiothreitol in a total volume of 250 \(\mu\)l. As indicated in the table, CaCl\(_2\) (final concentration of 10 mM) and 25 \(\mu\)g of amphomycin were added to the incubation mixture. After 20 min at 25 \(^\circ\)C, the reactions were terminated and incorporation of radioactivity into Man-P-Dol and LPG was determined as described under “Experimental Procedures.” Values shown are representative of three experiments. The values in parentheses are the percentage incorporation relative to the control value.

| Reaction mixture | Man-P-dolichol | LPG |
|------------------|---------------|-----|
| Control          | 370 \(\pm\) 1,300 |
| Plus Ca\(^{2+}\) | 420 \((115\%)\) | 1,490 \((115\%)\) |
| Plus Ca\(^{2+}\) and amphomycin | 30 \((8\%)\) | 1,560 \((120\%)\) |

FIG. 8. Effect of exogenously added Dol-P on \(^{14}C\)mannose incorporation into Man-P-Dol and LPG. Membranes were incubated with GDP-[\(^{14}C\)mannose as described under “Experimental Procedures” except that Triton X-100 was added at a concentration of 0.04%. Dol-P was also added at concentrations as indicated. After an incubation period of 20 min, the membranes were extracted and \(^{14}C\)LPG in the Solvent E extract was quantitated as described under “Experimental Procedures.” Solid bars: \(^{14}C\)Man-P-Dol; cross hatched bars: \(^{14}C\)LPG.

TABLE III

Effect of exogenously added Dol-P on \(^{14}C\)mannose incorporation into Man-P-Dol and LPG

All reaction mixtures consisted of membranes (2–2.5 mg), 50 mM HEPES, pH 7.4, 25 mM KCl, 5 mM MnCl\(_2\), 5 mM MgCl\(_2\), 0.1 mM TLCK, 1 \(\mu\)g/ml leupeptin, 10 \(\mu\)M UDP-Gal, 2 \(\mu\)M GDP-[\(^{14}C\)Man (0.4 \(\mu\)Ci), 0.8 mM ATP, and 0.4 mM dithiothreitol in a total volume of 250 \(\mu\)l. As indicated in the table, CaCl\(_2\) (final concentration of 10 mM) and 25 \(\mu\)g of amphomycin were added to the incubation mixture. After 20 min at 25 \(^\circ\)C, the reactions were terminated and incorporation of radioactivity into Man-P-Dol and LPG was determined as described under “Experimental Procedures.” Values shown are representative of three experiments. The values in parentheses are the percentage incorporation relative to the control value.

| Reaction mixture | Man-P-dolichol | LPG |
|------------------|---------------|-----|
| Control          | 370 \(\pm\) 1,300 |
| Plus Ca\(^{2+}\) | 420 \((115\%)\) | 1,490 \((115\%)\) |
| Plus Ca\(^{2+}\) and amphomycin | 30 \((8\%)\) | 1,560 \((120\%)\) |
ration into endogenous LPG was slightly stimulated. In a related observation, amphotericin and calcium addition to the incubation mixture had no effect on ['H]galactose transfer from labeled UDP-Gal to endogenous LPG (data not shown).

**DISCUSSION**

This paper presents the first in vitro studies on the enzymatic synthesis of LPG. A membrane preparation from *L. donovani* was used as a source of glycosyltransferases catalyzing the transfer of [14C]mannose and [3H]galactose and [23]mannose from their respective nucleotide donors to an endogenous LPG acceptor that was previously described (8-10, 23). The high molecular weight product enzymatically glycosylated in vitro was judged to be LPG based on the following criteria. (i) The radiolabeled product was extracted from the membranes with the identical solvent mixture that extracts LPG from in vivo labeled parasites or from membranes derived from the parasites, indicating that the solubility properties are very similar. (ii) The radiolabeled product was susceptible to both nitrous acid and PI-PLC, demonstrating the presence of an unacetylated hexosamine, and that the lipid anchor was a phosphatidylinositol. (iii) The PI-PLC-cleaved product was found to have a similar size to PI-PLC-treated LPG obtained from in vivo labeled parasites. (iv) The radiolabeled product was cleaved into low molecular weight products upon mild acid hydrolysis, which is one of the structural features of LPG (23), and (v) membranes from an LPG-deficient line of *L. donovani* did not incorporate either radioactive galactose or mannose from their respective nucleotide-sugars into endogenous material that exhibited the general properties of LPG. Collectively, these results indicated that the microsomal galactosyl- and mannosyltransferases were able to transfer their respective sugars to an endogenous LPG. Since LPG is highly heterogeneous in the number (average of 16) of repeating PO4-Gal-Man1 units, it is highly probable that the transferases elongated endogenous LPG by transferring additional galactose and mannose residues.

When either radiolabeled UDP-Gal or GDP-Man was added to microsomes along with the corresponding unlabeled nucleotide-sugar, the major low molecular weight fragment derived by mild acid hydrolysis of the radiolabeled LPG product was found to be PO4-Gal-Man. Lesser amounts of small neutral saccharides and saccharidyl cyclic phosphates were also detected following hydrolysis. These results can be explained by an examination of a section of LPG’s repeating phosphorylated disaccharide units below:

A B C D E F G H
Gal-Man-PO4-Gal-Man-PO4-Gal-Man-PO4-Gal-Man-PO4 —— LPG

Mild acid hydrolysis with 0.02 M HCl would preferentially cleave the mannosylphosphate bonds at mannose residues D, E, F, H, etc., generating the phosphorylated Gal-Man disaccharide as the main anionic product. The neutral saccharides most likely were obtained by mild acid hydrolysis of the mannosylphosphate bond at mannose residue B. A mixture of Gal-Man and Man would be expected to be liberated if LPG were incompletely terminated and, indeed, were obtained. Small amounts of neutral radiolabeled saccharides containing three to four terminations were also observed. These probably represent the “cap” structures that have been found on the terminating, nonreducing end of LPG.

The saccharidyl cyclic phosphates were possibly derived by sporadic cleavage of phosphorylglactose bonds at galactose residues C, E, G, etc. However, the formation of an α-mannose 1,2-cyclic phosphate during mild acid catalyzed hydrolysis of LPG is unlikely because of a trans diaxial arrangement of the phosphate and C-2 hydroxyl (29). Alternatively, these substances may have arisen by hydrolysis of the mannosylphosphate bonds at mannose residues B, D, F, etc., resulting in occasional cyclization of the phosphate group on C-3,4 of the galactose moiety. Interpretations are complicated by the fact that phosphate groups undergo intramolecular migrations in acid solutions (30).

The data presented in this report are consistent with the proposal that the galactose and mannose residues of the LPG-repeating units are added from their respective nucleotide-sugar donors sequentially and to LPG. Of particular importance, it was found that in the absence of unlabeled UDP-Gal, radioactive mannose could be incorporated from GDP-[14C]mannose into endogenous LPG. Upon fragmentation of the LPG product with mild acid and subsequent analysis, [14C]mannose could be recovered as one of the main radioactive fragments. Moreover, in the presence of UDP-Gal, the proportion of [14C]mannose that could be recovered decreased, and the proportion of Gal-[14C]Man disaccharide increased when UDP-Gal was added to the incubation mixture. This indicates that the mannose and galactose residues are added sequentially to LPG.

Since Leishmanial parasites are known to synthesize ManP-Dol (31), the possible involvement of the mannosylipid intermediate as the direct mannose donor in the assembly of the repeating units was examined. On the basis of two different approaches, it was concluded that the repeating unit synthesis does not involve Man-P-Dol. First, the addition of Dol-P to the incubation mixture increased Man-P-Dol synthesis, but did not stimulate radioactive mannose incorporation into LPG. Conversely, when the formation of Man-P-Dol was blocked by the addition of amphotericin and calcium (27, 28), the transfer of radioactive mannose from the labeled nucleotide-sugar to LPG was slightly stimulated. Our results do not exclude the possibility of the participation of Man-P-Dol as a direct precursor of the 2 mannose residues located in the phosphosaccharide core (10) of LPG. This region of LPG is analogous to the carbohydrate portion of the glycosylphosphatidylinositol anchor of trypanosomal variant surface glycoproteins (32; see Ref. 33 for a review of its biosynthetic pathway). In the anchor of this protein, Menon and coworkers (34, 35) have recently demonstrated that Man-P-Dol functions as the direct donor of mannose residues present in the core structure. Man-P-Dol has also been implicated as a mannosyl donor in the glycan portion of the THY-1 glycolipid anchor (36-38) and glycolipid-anchored proteins of yeast (39).

The origin of the phosphate groups that bridge the GalMan disaccharides in LPG is not yet known. It is possible that mannose 1-phosphate rather than mannose is donated from GDP-Man to a nonreducing galactose residue in LPG during polymerization of the repeating units. Such a transfer would result in the retention of the α-anomeric configuration of the mannosylphosphate bond in LPG derived from the GDP-Man donor. This would be comparable to the transfer of N-acetylglucosamine 1-phosphate from its nucleotide-sugar to Dol-P to form GlcNAc-P-P-Dol in the dolichol oligosaccharide pathway (40) and the transfer of mannose-1-phosphate from GDP-Man in the biosynthesis of yeast mannans (41). In essence, the repeating units of LPG might be synthesized by the individual alternating transfer of galactose and mannose 1-phosphate residues from their nucleotide derivatives.

The development of an in vitro system capable of LPG biosynthesis should facilitate efforts to elucidate the enzymatic details of this assembly process. Further work is currently underway in our laboratory to characterize fully these
envelopes with the use of exogenous acceptors. Ultimately, detailed information on the biosynthetic pathway of LPG might be exploited in the design of chemotherapeutic agents that could inhibit synthesis of this unique Leishmanial macromolecule.

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