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Inhibition of Glycosylphosphatidylinositol Biosynthesis in *Leishmania mexicana* by Mannosamine*

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In several *Leishmania* species, glycosylated inositol phospholipids exist as free lipids, as membrane protein anchors, and as the membrane-binding moieties of the lipophosphoglycans. Both the glycolipid-anchored cell surface metalloproteinase, gp63, and the lipophosphoglycans have been proposed to be involved in cell invasion. Moreover, the lipophosphoglycans have been implicated in the survival of *Leishmania* in the parasitophorous vacuole of the host macrophage. In this report we show that mannosamine effectively inhibits the biosynthesis of both free glycosylated inositol phospholipids and the lipophosphoglycans of *Leishmania mexicana*. [^{3}H]Mannosamine is incorporated into glycosylated inositol phospholipids, but not significantly into lipophosphoglycans when added as a radiochemical tracer at a subinhibitory concentration. The reversible inhibitory effect of mannosamine may be useful for studying precursor/product relationships during the biosynthesis of free glycosylated inositol phospholipids, glycolipid anchors, and the lipophosphoglycans. The implications of these data for the mode of action of mannosamine are discussed.

Leishmania species are digenetic parasitic protozoa that alternate between a uniflagellate (promastigote) form in the sandfly vector, and a nonflagellate (amastigote) form within the parasitophorous vacuole of the mammalian host's macrophages (Chang, 1983). The predominant parasitic determinants believed to be involved in invasion of the host macrophage are the 63-kDa metalloproteinase, gp63 (Russell and Wright, 1988), and the heterodisperse lipophosphoglycan (LPG)[1] (Talamas-Rohana *et al.*, 1990). The precise mechanism of invasion is incompletely defined, but almost certainly involves LPG and the complement receptor type 3 (CR3, CD11b/CD18), and *in vivo* may require prior opsonization with complement (Kelleher *et al.*, 1992; Mosser *et al.*, 1992). Moreover, several studies indicate that a direct interaction between saccharides of LPG and gp63 and a macrophage lectin is important (Blackwell *et al.*, 1985; Handman and Goding, 1985). LPG is also required for the continued survival of *Leishmania donovani* parasites within the parasitophorous vacuole (McNeely and Turco, 1990; McConville and Homans, 1992). Both of these molecules are highly expressed on the surface of all *Leishmania* spp. examined to date; LPG at ~6 × 10^6 and gp63 at ~5 × 10^5 copies/cell (McConville and Blackwell, 1991; Bouvier *et al.*, 1987).

LPG (Turco *et al.*, 1987; McConville *et al.*, 1990a; Thomas *et al.*, 1992; Ilg *et al.*, 1992), gp63 (Bordier, 1986), and a complex array of abundant small glycosylinositolphospholipids (GIPLs) (McConville *et al.*, 1990b; McConville and Blackwell, 1991), present at ~10^7 copies/cell, are linked to the plasma membrane of the promastigote by glycosylphosphatidylinositol (GPI) membrane anchors (Ferguson and Williams, 1988; Cross, 1990). The structures of these molecules have been determined in some detail. The GPI-anchor of *Lleishmania major* gp63 contains the consensus ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-1-PO_4 glycan core with a 1-alkyl-2-acyl glycerol lipid structure (Schneider *et al.*, 1990). A candidate precursor for this GPI-anchor, with the same structure, has been identified in *Leishmania mexicana* (Field *et al.*, 1991a). The GIPL and LPG structural homology with the protein-linked GPIs extends only to the α1-4-linked mannose in *L. major* (McConville *et al.*, 1990a, 1990b) but is more extensive in *L. donovani* (McConville and Blackwell, 1991). Although the LPG core and repeat structures are conserved between different species (McConville *et al.*, 1990a; Turco *et al.*, 1987; Ilg *et al.*, 1992), the biosynthetic relationship between the LPG/GIPL glycosylation pathway and that leading to protein anchors is not completely understood. A recently described *in vitro* system from *L. donovani*, capable of synthesis of LPG, may go some way to clarifying this issue (Carver and Turco, 1991).

Mannosamine (2-amino-2-deoxy-D-mannose; ManN) is an inhibitor of GPI biosynthesis (Lisanti *et al.*, 1991). The mode of action of this compound is not fully defined. Because the effects of ManN are reversed by low concentrations of glucose (Pan and Elbein, 1985), it is most likely that ManN is transported into the cell via the glucose transporter. In addition, ManN is ineffective *in vitro*, suggesting that it must be converted to an activated form before it can exert its effect (Pan and Elbein, 1985). At least three potential mechanisms of action can be considered: 1) the sugar is incorporated into glycoconjugates and acts as a chain terminator by preventing further elongation of the chain, due to the absence of a hydroxyl at the 2 position; 2) the presence of ManN at the nonreducing terminus of a growing glycan chain prevents further substitution by preventing recognition by glycosyltransferases, or 3) ManN acts as a competitive inhibitor of

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[1] The abbreviations used are: LPG, lipophosphoglycan; AHG, 2-anhydroglucose; AHM, 2-anhydromannose; Dol-P, phosphoryldolichol; GIPL, glycosyl-PI lipid; GPI, glycosylated PI; ManN, mannosamine; ManN(Ac), *N*-acetylmannosamine; PDE, phosphodiesterase; MDCK, Madin-Darby canine kidney cells; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; PAGE, polyacrylamide gel electrophoresis; CMW, chloroform/methanol/water; CM, chloroform/methanol.

glycosyltransferase activity by sequestering pools of nucleotide sugars and lipid-linked sugar donors as transfer-incompetent forms. These three possibilities are not mutually exclusive, and any or all could be acting to a lesser or greater extent, depending on the biosynthetic step under consideration.

We sought to use this compound to modify the surface of *L. mexicana*, specifically to assess its effect on gp63 and LPG biosynthesis. As ManN is also incorporated into *N*-linked glycans (Pan and Elbein, 1985; Pan *et al.*, 1992a), we anticipated that the structure of these moieties would also be altered. In this paper we describe the effects of ManN in *L. mexicana* on GPI-lipids, GPI-anchored protein, and LPG.

EXPERIMENTAL PROCEDURES

Parasites—Promastigotes of *L. mexicana* (strain MNYC/BZ/62/M379) were cultured in SDM79 (Brun and Schonenberger, 1979) supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA) at 26 °C. Wild-type and the ricin-resistant R2D2 clone of *L. donovani* were kindly provided by Dr. S. J. Turco (University of Kentucky College of Medicine, Lexington, KY) and were maintained as described (King and Turco, 1988). Bloodstream form *Trypanosoma brucei* were grown in rodents and isolated from whole blood as described (Field and Menon, 1992).

Biosynthetic Labeling of Leishmania Parasites—Parasites were washed three times in ice-cold PBS and resuspended (2×10^7 cells/ml) in 5 ml of methionine-free, glucose-free, or inositol-free RPMI medium (Specialty Media, Inc., Lavallette, NJ) supplemented with [^{35}S]methionine (1184 Ci/mmol), [^{3}H]ethanolamine (20 Ci/mmol), [6-^{3}H]glucosamine[2] (30 Ci/mmol), [6-^{3}H]galactose (30 Ci/mmol), [^{3}H]inositol (80 Ci/mmol) (all from Amersham Corp.) or [^{3}H]ManN (10–20 Ci/mmol) (American Research Chemicals, Inc), each at 50 μCi/ml. Parasites were labeled overnight at 26 °C.

Detergent and Solvent Extractions—For protein analysis, labeled cells were washed three times in ice-cold PBS and extracted with lysis buffer (20 mg/ml *n*-octyl D-glucopyranoside in PBS containing 100 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone and 25 μg/ml leupeptin (all from Sigma)). Lysates were clarified by centrifugation in an Eppendorf microcentrifuge for 30 min at 4 °C and the soluble extracts resuspended in SDS-PAGE sample buffer. Lipids and LPG were extracted from cell pellets as follows. The cells were lysed by adding chloroform/methanol (CM) (2:1, v/v), typically 1 ml, sonicating, pelleting, and re-extracting with the same solvent. The pellet was then extracted with chloroform/methanol/water (CMW) (10:10:3, v/v/v). The CM and CMW extracts were dried and partitioned between water and butanol-1 (Masterson *et al.*, 1989). For some experiments the cells were extracted with CMW only (Masterson *et al.*, 1989). The delipidated pellet was then extracted twice with water/ethanol/diethyl ether/pyridine/ammonium hydroxide (solvent E) (Orlandi and Turco, 1987). An aliquot of this extract and the butanol-1 phase from the CM and CMW extractions were taken for scintillation counting. The residue after the solvent E extraction was dried in a Speedvac and solubilized in 5% SDS overnight at room temperature (Field *et al.*, 1991b). For some experiments the solvent E extract was dried and resuspended in SDS-PAGE sample buffer (nonreducing, typically 100 μl), and stored at 4 °C. Aqueous-soluble metabolites were obtained either from solvent E extracts, or from the aqueous phase of CMW extracts following butanol-1 partitioning essentially as described (Field and Menon, 1992).

Triton X-114 Extraction—Radiolabeled proteins were extracted into Triton X-114 and subjected to PI-PLC treatment essentially as described by Orlean (1990). Proteins in the detergent and aqueous phases after PI-PLC treatment were precipitated with trichloroacetic acid, washed with acetone, resuspended in sample buffer, and analyzed by SDS-PAGE in a 11% polyacrylamide gel followed by autoradiography.

Chromatography—Ascending thin layer chromatography (TLC) was performed on Si60 thin layer plates (Merck) using CMW (10:10:3 or 10:10:2.7, v/v/v) as solvent (system 1), or on plastic-backed cellulose TLC plates (Chromogram, Eastman Kodak) with butanol-1, pyridine, 0.1 N HCl (5:3:2, v/v/v) as solvent (system 2) in pre-equilibrated tanks. For system 2, typically two developments were required for adequate resolution (this system, after two developments, efficiently resolves GlcNAc/ManNAc/AHM (2-anhydromannose) and AHG (2-anhydroglucose), but GlcN and ManN are poorly separated). Chromatography standards were derived from the same compounds used in the metabolic labeling experiments by *N*-acetylation or deamination. Bloodstream form trypanosome GPIs were prepared by *in vitro* labeling with [^{3}H]GDP-Mannose (Amersham) essentially as described by Masterson *et al.* (1989). [^{3}H]Glucitol, glucobiitol, and glucotriitol were prepared from a partial hydrolysate of dextran (Sigma) reduced with NaB[^{3}H]$_4$ (Du Pont-New England Nuclear, 12 Ci/mmol) (Ashford *et al.*, 1987). After chromatography, plates were dried in air and radioactivity located by scanning with a Berthold LB2845 linear scanner. Descending paper chromatography was performed on strips of Whatman 3MM developed in 1 M ammonium acetate, pH 3.5, ethanol (2:5, v/v) (Paladini and Leloir, 1952). Radioactivity was detected either with the linear scanner or by cutting the chromatogram into 0.5–1.0-cm strips and scintillation counting. Phenyl-Sepharose (Sigma) hydrophobic interaction chromatography was performed as described by Carver and Turco (1991), using a 0.5-ml column, and eluting in steps with ≥5 column volumes of each of four solvents (100 mM NaCl/HAc, 100 mM HAc, water, and solvent E). Gel filtration through Bio-Gel P-10 (Bio-Rad) was performed using a 85 × 1.5-cm column exactly as described by Carver and Turco (1991) controlled with an fast protein liquid chromatography system (Pharmacia LKB Biotechnology Inc.). The column void and included volume were calibrated with blue dextran and [^{3}H]mannose, respectively. Blue dextran was coinjected with each sample, and the eluate monitored by liquid scintillation counting.

Chemical and Enzyme Treatments—LPG was depolymerized by mild acid treatment using 40 mM trifluoroacetic acid at 100 °C for 8 min (McConville *et al.*, 1990a). Following the incubation, the sample was cooled, dried, and either partitioned between water and butanol-1 or resuspended in 100 mM NaCl, 100 mM acetic acid. Nitrous acid deamination and digestion with *Bacillus thuringiensis* PI-PLC (gift of Dr. M. Low, Columbia University, New York) was performed as described by Mayor *et al.* (1990). Efficiency of PI-PLC digestion on LPG samples was assessed by rechromatography on phenyl-Sepharose, with a typical conversion >80%. Acid hydrolysis of deaminated lipid head groups was achieved by incubation in 50 μl of 1 M HCl at 100 °C for 3 h (Ashford *et al.*, 1987). Samples were freed of HCl by evaporation in a SpeedVac, and lyophilized overnight. The hydrolysates were *N*-acetylated with acetic anhydride and desalted by passage through 1 ml of AG50 (H^+ form) (Bio-Rad) before analysis. For phosphodiesterase (PDE) treatment, samples were resuspended in 40 μl of 25 mM Tris-HCl, pH 8.8, containing 0.5 mM magnesium acetate, and 6 milliunits of *Crotalus durissus* PDE (Sigma) were added. The sample was incubated at 37 °C for 12–16 h (Dieckmann-Schuppert *et al.*, 1992). Alkaline phosphatase treatment was performed as for PDE, except that the buffer also contained 10 mM magnesium chloride and 0.5 mM zinc chloride. 10 units of enzyme (calf intestinal alkaline phosphatase) (Boehringer) were added, and the sample incubated for 2.5 h at 37 °C, after which time a second aliquot of enzyme was added and the incubation continued for a further 0.5 h (Dieckmann-Schuppert *et al.*, 1992).

Perchloric Acid and Trichloroacetic Acid Precipitations—Uptake of radiolabel was analyzed by precipitation of total cell protein as follows. The cell pellet was washed extensively with cold PBS and then resuspended in 1 ml of ice-cold 0.9 M perchloric acid. The cell debris was pelleted in a microcentrifuge and the supernatant (water-soluble metabolites) was analyzed by scintillation counting. Precipitation with trichloroacetic acid was performed as follows. Cells were pelleted and washed with PBS. The cells were then pipetted onto a pre-wetted glass-fiber filter (GF/B, Whatman) and washed with 5% trichloroacetic acid. The filter was washed with 100% ethanol and then taken for scintillation counting. Alternatively, total cell protein was precipitated with 10% trichloroacetic acid, and the pellet then transferred to a GF/B filter for washing and counting. Some lipid extracts were also analyzed by glass-fiber filter binding. The extract was pipetted directly onto a water-washed GF/B membrane, the solvent allowed to evaporate, and then water-soluble material was washed off with 3 × 5 ml of distilled water. The filter was then taken for scintillation counting.

Gel Electrophoresis/Immunoprecipitation—SDS-PAGE analysis was carried out on 11, 12.5, or 15% gels (Laemmli 1980). gp63 was immunoprecipitated from lysed cells with either monoclonal antibody TüL3.8 or a rabbit serum antirecombinant gp63 (Medina-Acosta *et al.*, 1989). Radioactivity in SDS-PAGE bands was quantitated using Protosol (Du Pont) following the manufacturer's instructions. Pro-

[2] All monosaccharides discussed in this paper are assumed to be in the D-pyranose form.

tein molecular weight standards were from Amersham International, Inc.

Scintillation Spectroscopy and Autoradiography—Radioactivity in liquid samples was determined by liquid scintillation counting using Ready-Safe™ scintillation mixture (Beckman). Gels were processed for autoradiography by impregnation with Autofluor™ (Du Pont) or ENH³ANCE™, dried, and exposed to Kodak X-Omat AR film at −80 °C using two intensifying screens.

RESULTS

Effect of Mannosamine on GPI-anchor Precursor Synthesis—In wild-type *L. mexicana* incubated overnight in 1 mM ManN in glucose-free medium, incorporation of [³H]ethanolamine into GPI-anchor precursor (LP-1) (Field *et al.*, 1991a) was decreased by ~90% compared with cells grown in the same medium without ManN (Fig. 1, *panel A*). Synthesis of [³H]ethanolamine-labeled phosphatidylethanolamine (PE) was unaffected at this concentration of inhibitor. At 10 mM ManN, synthesis of [³H]ethanolamine-labeled lipids was decreased by 50% (Fig. 1), due to growth inhibition. In addition to the inhibition of synthesis of LP-1, we also observed a large decrease in the incorporation of [³H]galactose (~80%) into CMW extractable material when cells were labeled with [³H] galactose in the presence of 10 mM ManN (data not shown), reflecting the toxicity of the ManN.

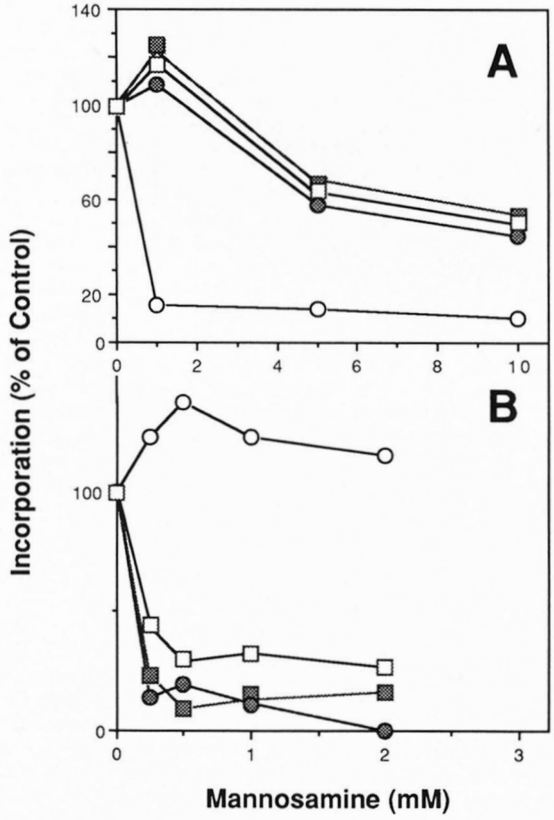

FIG. 1. **Effect of mannosamine on biosynthesis of GPI lipids.** *L. mexicana* promastigotes were metabolically labeled with [³H]ethanolamine (*panel A*) or [³H]inositol (*panel B*) in the presence of the indicated concentration of mannosamine, and the lipids extracted as described under "Experimental Procedures." Lipid extracts were analyzed by scintillation counting with TLC (system 1) resolution of the various species. Assignments were made based on comparison to migration positions of authentic standards derived from bloodstream and procyclic form *T. brucei* (see Fig. 4). *Panel A*: *open circles*, LP-1; *closed circles*, phosphatidylcholine/*lyso*-PE; *open squares*, PE; *closed square*, total incorporation; *panel B*: *open circles*, PI; *closed circles*, GPI-I; *open squares*, GPI-II; *closed squares*, GPI-III. GPI-I, -II, and -III were assigned as GPI species based on PI-PLC sensitivity.

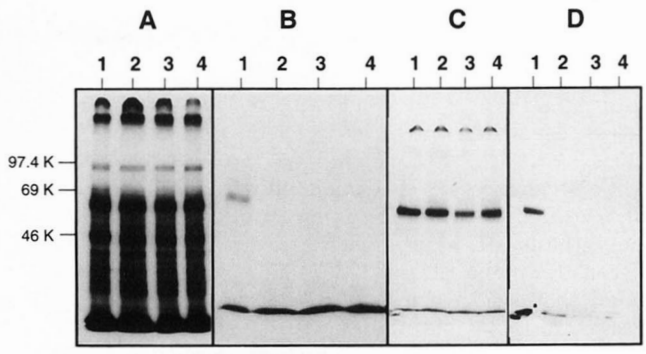

FIG. 2. **Effect of mannosamine on biosynthesis of gp63.** Autoradiograms of SDS-PAGE gels containing material from promastigotes metabolically labeled with [³⁵S]methionine (*panels A-C*) or with [³H]ethanolamine (*panel D*). Labeling was performed in the presence of mannosamine at (*1*) 0, (*2*) 0.25, (*3*) 0.50, and (*4*) 1 mM. *Panels A* and *D*, total cell lysates; *panel B*, proteins bound to immobilized ConA; *panel C*, gp63 immunoprecipitates.

Effect of ManN on gp63 Biosynthesis—The observation that the incorporation of [³H]ethanolamine into LP-1 was inhibited by ~90% at 1 mM ManN suggested that synthesis of GPI-anchored gp63 would also be altered, as described for MDCK cell GPI-anchored proteins and for the procyclic acidic repetitive protein (procyclin) of *T. brucei* (Lisanti *et al.*, 1991). When we analyzed detergent extracts of *L. mexicana* promastigotes labeled with [³H]ethanolamine in the presence of increasing concentrations of ManN, the incorporation of [³H]ethanolamine into gp63 was greatly diminished (Fig. 2, *panel D*). Maximal effect was obtained at 250 μM, with no further reduction when the concentration of the inhibitor was increased to 1 mM. We always observed residual incorporation of [³H]ethanolamine into gp63 (<10% of the control level). This effect was due to inhibition of GPI biosynthesis and not protein synthesis as total incorporation of radioactivity into trichloroacetic acid-precipitable material from [³⁵S]methionine-labeled *L. mexicana* promastigotes was unaltered in cells labeled in the presence of up to 2 mM ManN (data not shown, and Fig. 2, *panel A*), and there was no reduction of incorporation of radioactivity into the gp63 band immunoprecipitated with the TüL3.8 antibody (Fig. 2, *panel C*). By SDS-PAGE analysis, the gp63 labeled overnight with [³⁵S]methionine was indistinguishable whether ManN was present or not. The kinetics of incorporation of [³⁵S]methionine into trichloroacetic acid-precipitable material were unaltered by the addition of 250 μM ManN but, under the conditions used here, incorporation reached a plateau after ~8 h, due to depletion of [³⁵S]methionine from the medium. As gp63 has a half-life greater than 72 h,[3] the [³⁵S]methionine-labeled gp63 recovered by immunoprecipitation after an overnight labeling (Fig. 2, *panel C*) represents protein synthesized during the first 6–8 h of culture. These data indicate that the inhibitory effect of mannosamine on the biosynthesis of LP-1 does not significantly alter either turnover or the apparent molecular weight of gp63. These results can be interpreted to mean that a pool of unlabeled GPI-anchor precursor (LP-1) may be present which could be sufficient to allow continued anchor addition in the absence of *de novo* GPI synthesis. Alternatively, residual LP-1 biosynthesis may be sufficient for adequate GPI-anchor to gp63, *i.e.* LP-1 may be synthesized in great excess under normal conditions.

We examined the solubility of gp63 from control and ManN-treated promastigotes after detergent-phase separation using Triton X-114 (Fig. 3). In cells incubated with

[3] E. Medina-Acosta, unpublished observation.

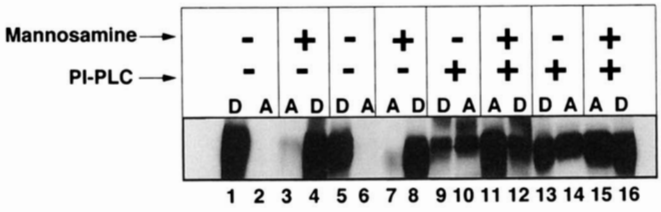

FIG. 3. **gp63 contains a GPI-anchor following ManN-treatment.** Promastigotes were incubated for 2 (*lanes 1–4* and *9–12*) or 24 h (*lanes 5–8* and *13–16*) in the presence (*lanes 3, 4, 7, 8, 11, 12, 15,* and *16*) or absence (*lanes 1, 2, 5, 6, 9, 10, 13,* and *14*) of 0.25 mM mannosamine prior to labeling with [^{35}S]methionine for 2 h (maintaining the presence or absence of the ManN). gp63 was immunoprecipitated with a rabbit serum antirecombinant gp63. Equal aliquots of gp63 were incubated with (*lanes 9–16*) or without PI-PLC (*lanes 1–8*) at 37 °C. The aliquots were then extracted with Triton X-114. Phases were separated and the radiolabeled gp63 in each phase (detergent, *D*; aqueous, *A*) was precipitated, resuspended in sample buffer, and analyzed by SDS-PAGE and fluorography.

ManN for 2 or 24 h prior to labeling with [^{35}S]methionine, gp63 remained sensitive to PI-PLC, as demonstrated by the recovery of the protein in the aqueous phase following treatment with PI-PLC (Fig. 3, compare *lanes 11* and *12* (2 h) and *15* and *16* (24 h)), whereas the protein was retained in the detergent phase of the non-PI-PLC treated samples (Fig. 3, *lanes 3* and *4* and *7* and *8*, respectively). Identically, in the non-ManN control group, the same effect of PI-PLC on the phase partitioning of gp63 was observed. This observation demonstrates that even following a block in GPI-biosynthesis for 24 h, gp63 is still synthesized with a GPI-anchor, and suggests that residual LP-1 biosynthesis is sufficient for GPI-anchor addition. LP-1 synthesized before the ManN addition is unlikely to still be present in the endoplasmic reticulum. Similar observations have been made in *T. brucei* using fluoroglucose (Schwarz *et al.*, 1989).

We studied the effect of ManN on *N*-glycan structure by lectin binding. We observed that gp63 synthesized by cells grown in the presence of ManN was no longer retained by immobilized concanavalin A (ConA), whereas in control cells gp63 was the major protein species bound by the lectin (Fig. 2*B*). This result indicates that ManN also affects the biosynthesis of *N*-linked glycans in *L. mexicana*, as well as the GPI pathway, and taken together with the data presented below, probably reflects incorporation of ManN into the oligomannose *N*-glycans of gp63 (Olafson *et al.*, 1990; Pan *et al.*, 1992a), preventing their recognition by ConA.

Effect of Mannosamine on GIPL Biosynthesis—We chose to limit our analysis to detection of PI-PLC and nitrous acid-sensitive species, and not to attempt further structural dissection of this complex family of glycolipids. When we labeled *L. mexicana* promastigotes with [^{3}H]inositol, several ^{3}H-labeled species were recovered in a CMW extract (Fig. 4*A*). The most prominent species was PI (R_F 0.69). Several polar lipids were also labeled (I, II, and III in Fig. 4), including one which co-chromatographed with LP-1 (R_F 0.16). Peaks I-III, LP-1 and PI were sensitive to *B. thuringiensis* PI-PLC (not shown). Peaks I-III and LP-1, but not PI, were also sensitive to nitrous acid cleavage. Addition of ManN (0.25–2.0 mM) to the cells at the start of the labeling experiment did not affect the total incorporation of [^{3}H]inositol (data not shown, and Fig. 1*B*), demonstrating that ManN does not compete with inositol for entry into the cell. A small stimulation in the radioactivity incorporated into PI (~25%, Fig. 1*B*) may reflect the inhibition of glycosylation of PI in the ManN-treated cells. ManN inhibited the synthesis of peaks I-III (Figs. 1*B* and 4*B*) and caused the appearance of several new peaks in the polar region of the TLC plate (Fig. 4*B*, *inset*, R_F = 0.03–0.38). LP-1 could not be quantitated from the [^{3}H]inositol labeling experiment as the new species in the extracts from the mannosamine-treated cells obscured the LP-1 region (Fig. 4*B*). These species were also sensitive to bacterial PI-PLC and to nitrous acid deamination (data not shown), suggesting that either ManN, a metabolite of ManN, or another compound that was not utilized in the control cells had been incorporated into the GPI species.

We also labeled *L. mexicana* promastigotes with [6-^{3}H] galactose and analyzed the lipid species by extraction with CM followed by CMW. This radiolabel was selected as ManN and glucose possibly use the same transporter to enter the cell (Pan and Elbein, 1985), whereas galactose could be efficiently epimerized at the 4′-hydroxyl to glucose once within the parasite. Similarly to the inositol labeling experiment, there was essentially no difference in the total incorporation of radiolabel when we included ManN up to 5 mM (data not shown). Analysis of the CM extract by TLC system 1 resolved several peaks that co-chromatographed with the inositol-labeled species I-III, as well as PI. Similarly a peak which co-chromatographed with LP-1, as well as a major species at R_F 0.56 (Fig. 4, *panels C* and *D*), was seen in the CMW extract. Peaks I-III, LP-1, PI, and the vast majority of the material in the CMW extract were sensitive to bacterial PI-PLC (data not shown), suggesting that the [^{3}H]galactose had been converted to inositol and probably other sugars. These data strengthen the assignment of peaks I-III and LP-1 as GIPLs. Synthesis of peaks I-III and LP-1 were also sensitive to ManN (Fig. 4, *panels E* and *F*), confirming the observations made with the [^{3}H]inositol-labeled lipids. We also saw new polar species similar to the inositol labeling (Fig. 4, *panels B* and *F*).

L. mexicana promastigotes were labeled with [^{3}H]ManN, and the CM and CMW extracts were analyzed by TLC (system 1). Incorporation of [^{3}H]ManN into lipids was approximately 5-fold less than [6-^{3}H]galactose (Table I). Several species were labeled with ManN in both extracts (Fig. 5), some of which chromatographed in the same region of the plate as the new species generated by ManN in the [^{3}H] inositol and galactose labeling experiments (Fig. 4). The peaks in this region were sensitive to nitrous acid deamination and to bacterial PI-PLC (Fig. 5).

The PI-PLC resistant peak in the CM extract (Fig. 5, *panels A* and *C*) migrated very close to position of dolichol phosphomannose from *T. brucei* (Low *et al.*, 1991), suggesting that it may correspond to Dol-P-ManN as Dol-P-Man is recovered in CM extracts. Because of the small amount of material present we were unable to characterize this species further. The PI-PLC-resistant peak in the CMW extract (Fig. 5, *panels B* and *D*) was sensitive to nitrous acid, suggesting that it contained ManN. This peak is tentatively assigned as a possible dolichol-containing *N*-glycosylation pathway intermediate based on its R_F being similar to that for the dolichol-linked oligosaccharide P1 ($Man_5GlcNAc_2$-PP-Dol) reported by Low *et al.* (1991). It should be noted that because the incorporation of ManN leads to the presence of additional free amines in the GPI head group, direct comparison of the migration positions of the [^{3}H]ManN-labeled species with the [^{3}H]inositol or galactose-labeled GPIs is not possible.

To show that the GPIs contained ManN and not a metabolic product, the aqueous phase from the CM extract deamination was analyzed by acid hydrolysis followed by *N*-acetylation. If the ManN had been *N*-acetylated *in vivo*, we would expect the amine to be protected from deamination, so that, following the procedure, we would recover ManNAc. On the

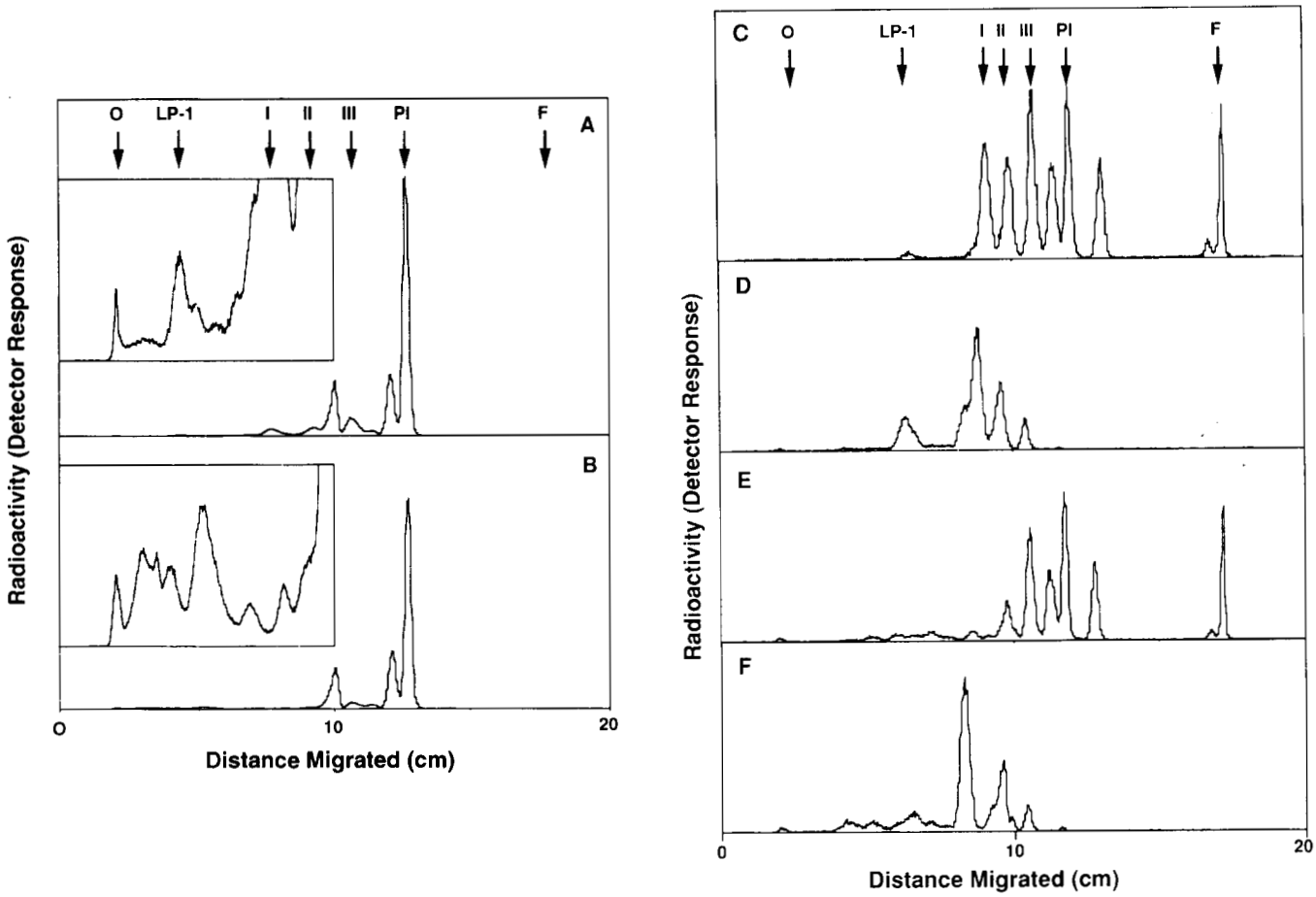

FIG. 4. **Effect of mannosamine on biosynthesis of [^{3}H]inositol and galactose-labeled lipid.** Labeled cells were extracted and lipids analyzed by TLC system 1. *Panels A* and *B*, lipids from CMW extracted cells labeled with [^{3}H]inositol in the absence (*A*) or presence (*B*) of 250 μM mannosamine. Extracts from the same number of cells were run to allow semiquantitative comparison of the lipid species. *Insets* show the region of the TLC plate corresponding to 0–10 cm with the *y* axis expanded to allow visualization of the low abundance polar lipids. These data are quantitatively displayed as part of Fig. 1. *Panels C-F*, lipids from CM (*C* and *E*) followed by CMW (*D* and *F*) extracted cells labeled with [6-^{3}H]galactose in the presence (*E* and *F*) or absence (*C* and *D*) of 500 μM mannosamine. Note that in the extracts from the ManN-treated cells, several new polar species are produced, which are PI-PLC sensitive (data not shown, see text). Migration positions of PI, LP-1, and the PI-PLC-sensitive species GPI-I, -II, and -III are indicated by *arrows*. *O* is the origin; *F* is the front.

TABLE I

Incorporation of radiolabel into extracts from L. mexicana promastigotes

Cells were labeled for 16 h and extracted as described under "Experimental Procedures."

Radiolabel	CM	CMW	CMW[a]	Solvent E[b]
		cpm/10^8 cells		
[^{3}H]Inositol			5.6×10^6	8.2×10^4
[6-^{3}H]Galactose[c]	1.4×10^5	2.7×10^4		1.0×10^5
[^{3}H]Mannosamine	2.9×10^4	8.6×10^3		7.5×10^6[d]

[a] Single step extraction performed (CM extraction omitted).

[b] Not all radioactivity recovered in the solvent E extraction is LPG. Typically about 50% of the radioactivity runs at the front of an SDS-PAGE gel, suggesting that it is residual phospholipid or water-soluble metabolite.

[c] Note that considerable interconversion of the [6-^{3}H]galactose occurs during this time.

[d] In this case, essentially none of the radioactivity recovered in the solvent E extract was LPG as determined by SDS-PAGE, glass-fiber filter binding, and gel filtration.

other hand, if the ManN was not *N*-acetylated, we would recover AHG. If ManN had been epimerized to GlcN, we would recover AHM. We observed that the radioactivity co-chromatographed with the AHG (~65%) and AHM (~35%) standards and not with the untreated or *N*-acetylated ManN or GlcN standards (data not shown), demonstrating both that ManN had been incorporated into GPIs and was not *N*-acetylated. Some conversion of the ManN to GlcN appeared to have occurred. This observation is consistent with those of Pan *et al.* (1992a), who found basic oligomannose-type glycans in cells grown in the presence of ManN, and with our observations of new polar lipids produced in the presence of inhibitory concentrations of ManN. We did not attempt to define further the structures of the GPI species labeled with ManN as the presence of variable numbers of free amines in the head group glycans would have made the analysis too complex and beyond the scope of this report. These data confirm that the [^{3}H]ManN is incorporated into GPI species.

Effect of Mannosamine on LPG Biosynthesis—Biosynthesis of LPG was assessed by analysis of solvent E extracts. Incorporation of [^{3}H]inositol and [6-^{3}H]galactose was efficiently inhibited by ManN at 250 μM (Fig. 6, *panels A* and *B*). We also analyzed the effect of ManN on biosynthesis of LPG in *L. donovani* wild-type and LPG-deficient (R2D2) promastigotes (King and Turco, 1988). 1 mM ManN prevented the incorporation of [6-^{3}H]galactose into LPG (Fig. 6*B*). No corresponding [6-^{3}H]galactose-labeled band was observed in the R2D2 extracts as expected. We also observed that [^{3}H]ManN, at subinhibitory levels, was incorporated into LPG in

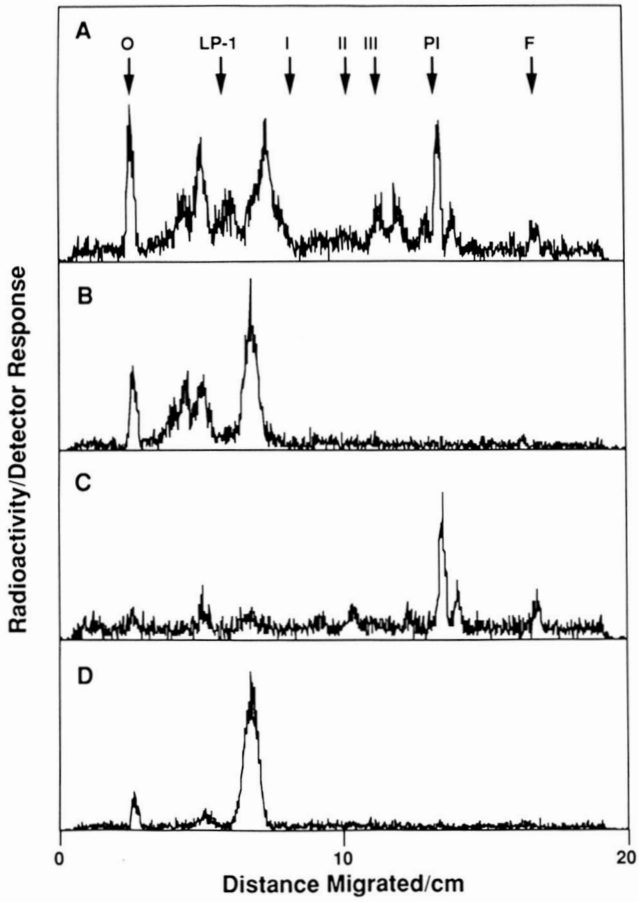

FIG. 5. **GPI species are labeled with [^{3}H]mannosamine.** TLC profiles of CM (*panels A* and *C*) and CMW (*panels B* and *D*) extracts of *L. mexicana* promastigotes were metabolically labeled with [^{3}H] mannosamine. Lipids were chromatographed using system 1. *Panel A* and *B*, untreated; *panels C* and *D*, following digestion with *B. thuringiensis* PI-PLC. Migration positions of LP-1, PI, GPI-I, -II, and -III and the origin (*O*) and front (*F*) are indicated as in Fig. 1.

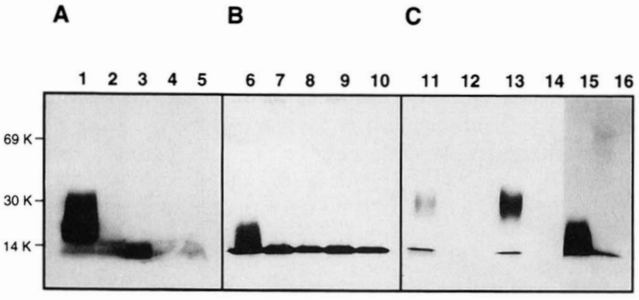

FIG. 6. **Inhibition of lipophosphoglycan biosynthesis by mannosamine.** Autoradiograms of 12.5% SDS-PAGE analysis of solvent E extracts from metabolically labeled promastigotes. *Panel A*, [^{3}H]inositol-labeled LPG of *L. mexicana* at 0, 0.25, 0.5, 1.0, and 2.0 mM mannosamine (*lanes 1–5*, respectively). *Panel B*, [6-^{3}H]galactose-labeled LPG of *L. mexicana* at 0, 0.5, 1.0, 2.0, and 5.0 mM mannosamine (*lanes 6–10*, respectively). *Panel C*, [^{3}H]ManN-labeled solvent E extracts of *L. donovani* wild-type (*lane 11*), LPG-deficient mutant strain R2D2 (*lane 12*), and *L. mexicana* (*lane 16*); [6-^{3}H] galactose-labeled solvent E extracts of *L. donovani* wild-type (*lane 13*), LPG-deficient mutant strain R2D2 (*lane 14*), and *L. mexicana* (*lane 15*).

wild-type, but not R2D2, *L. donovani* (Fig. 6*C*). Unexpectedly, when we repeated the [^{3}H]ManN labeling with *L. mexicana* promastigotes and analyzed the solvent E extract by SDS-PAGE, we observed no significant incorporation into LPG (Fig. 6*C*). Radiolabeled material behaving as LPG was not present in either the initial CMW lipid extracts, or in the solvent E extracted pellet, confirming that LPG was indeed not labeled in this *Leishmania* species (data not shown).

Following the SDS-PAGE analysis (Fig. 6*C*) we observed that the intensity of the band running at the front in the [^{3}H] ManN-labeled solvent E extract from *L. mexicana* could not account for the amount of radioactivity loaded onto the gel. This suggested that the majority of the radiolabel in the solvent E extract was low molecular weight, water soluble, and had been washed out of the gel after fixation (we observed that after butanol-1/water extraction, the lipid extracts labeled with ManN were more heavily contaminated with aqueous-soluble radioactivity than lipid extracts labeled with galactose or inositol). This made quantitation of data from SDS-PAGE highly inaccurate, and we therefore further analyzed the solvent E extracts by phenyl-Sepharose chromatography and gel filtration. When [6-^{3}H]galactose or glucosamine-labeled solvent E extracts were chromatographed on phenyl-Sepharose, a significant amount of radioactivity was retained and eluted by solvent E (typically 20–40% of total radioactivity), suggesting that this was authentic LPG (Carver and Turco, 1991). Following mild hydrolysis with trifluoroacetic acid, this material no longer bound to phenyl-Sepharose, as expected for LPG (Carver and Turco, 1991). Analysis of this material by SDS-PAGE confirmed this assignment (data not shown). In contrast, the [^{3}H]ManN-labeled extracts contained only small amounts of material that eluted from the phenyl-Sepharose column with solvent E (0.3% for *L. mexicana* and ~3% for *L. donovani*). Aliquots of [^{3}H]ManN-labeled solvent E extracts were analyzed by gel filtration to provide an upper limit estimate of the levels of LPG incorporating [^{3}H]ManN (data not shown). Essentially all radioactivity eluted as a single peak from the *L. mexicana* extract, while in the *L. donovani*, extract material also eluted at the void volume (the elution position of [6-^{3}H]galactose-labeled LPG from *L. mexicana* and *L. donovani*, data not shown; Carver and Turco (1991)). Less than 0.1% of the *L. mexicana* solvent E extractable radioactivity was voided by the column equivalent to ~2500 cpm/10^8 cells, indicating that there was essentially no incorporation of ManN into LPG in this species. In *L. donovani*, ~30,000 cpm/10^8 cells were recovered in the column void, suggesting that more [^{3}H]ManN could be incorporated by *L. donovani*, consistent with the SDS-PAGE analysis. These data indicate that incorporation of ManN into LPG is very inefficient for *L. mexicana*, while there is some labeling in *L. donovani*. However, even in the latter case the efficiency of incorporation is low.

Analysis of Aqueous-soluble ManN Metabolites—Gel filtration on Bio-Gel P-10 indicated that a significant amount of a low molecular weight metabolite was synthesized in the presence of [^{3}H]ManN. Analysis of the solvent E extract by paper chromatography and PDE/CIP digestion suggested the presence of ManN-phosphate and GlcNAc-phosphate, in a ratio of 4:1 (data not shown). We analyzed the aqueous-soluble metabolites from an overnight [^{3}H]ManN labeling of *L. mexicana* by descending paper chromatography. The vast majority of the material was identified as the phosphates of ManN and GlcNAc (in approximately equal amounts). This assignment was made based on the following analysis. We isolated the putative sugar phosphates from the relevant regions of the paper chromatogram by elution with water, followed by treatment with calf intestinal alkaline phosphatase. All the radioactivity then co-migrated with the respective monossacharide, *i.e.* ManN or GlcNAc on re-chromatography (we ran ManN, GlcN, ManNAc, and GlcNAc as standards on an adjacent paper strip). Therefore we were able to assign the original

major species as ManN-P and GlcNAc-P. Small amounts of the corresponding free sugars were also detected in the original extract. Very little of the radioactivity was recovered in the region where the nucleotide sugars GDP-Man and UDP-GlcNAc migrated. Analysis of the material in these regions demonstrated that ~10% of the radioactivity was sensitive to PDE and produced a species that co-chromatographed with ManN-P (assigned as described above). Subsequent digestion with calf intestinal alkaline phosphatase produced the free sugar. These data are consistent with the presence of a small amount of nucleotide-ManN and are in agreement with the recovery of both AHG and AHM from the deaminated GPI head groups, as the core GlcN is derived from UDP-GlcNAc (Doering *et al.*, 1989). No ManNAc was recovered, also in agreement with the GPI head group analysis.

DISCUSSION

In this study we document the action of ManN on *L. mexicana* promastigotes. The amino sugar has been previously shown to inhibit the attachment of GPI-anchors to proteins in mammalian cells (MDCK) and in the African trypanosome (*T. brucei*) (Lisanti *et al.*, 1991), and also to affect *N*-linked glycosylation in mammalian cells (Pan and Elbein, 1985; Pan *et al.*, 1992a). Previous studies in procyclic *T. brucei* indicated that premature chain termination is a likely consequence of ManN action, as inhibition of incorporation of [^{3}H]ethanolamine into PP1, the precursor of the PARP GPI-anchor (Field *et al.*, 1991b) is observed (Lisanti *et al.*, 1991). We have extended these results to show that a similar effect is seen for LP-1, a candidate precursor for the gp63 GPI-anchor (Field *et al.*, 1991a). That LP-1 is the gp63 precursor cannot be further strengthened by the observations made here because the structure of the anchor for *L. mexicana* gp63 is not known. In *T. brucei,* the presence of a nucleotide sugar form of ManN was suggested, as well as a putative phosphoryldolichol-ManN (Dol-P-ManN) structure (Lisanti *et al.*, 1991), but these species were not rigorously characterized.[4]

In this report we show that ManN can be incorporated into GPI lipid species in *Leishmania*, as determined by their PI-PLC and nitrous acid sensitivities and chromatographic and solvent extraction properties. At concentrations that inhibit GPI biosynthesis (~250 μM), ManN is efficiently incorporated into GPI lipids. ManN is also converted to a sugar phosphate and to GlcNAc-P. ManNAc-P could not be detected, suggesting that *N*-acetylation is closely coupled to 2′-epimerization of the ManN to GlcN.

It is probable that ManN is not *N*-acetylated before addition to glycosyl-PI, and certainly is not when present as part of the GPI glycan. *N*-Acetylation is a major metabolic path of ManN in mammalian cells, where ManNAc is converted into NeuNAc. Sialic acid synthesis has not been demonstrated in trypanosomatids. Some epimerization of ManN to GlcN does take place, as we detected GlcN in the GPI head groups. Evidence against an *N*-acetylation step is supported by our observations of ManN, not ManNAc, in the GPI head groups. [^{3}H]GlcNAc-phosphate was also detected in the solvent E extract, providing an explanation for the incorporation of GlcN into the GPI from ManN via UDP-GlcNAc and subsequent de-*N*-acetylation (Doering *et al.*, 1989).

The presence of ManN in the GPI-glycan suggests that one functional route of ManN action is through a Dol-P pathway, as Dol-P-Man has been shown to be the mannosyl donor for GPI lipids in a number of species (Menon *et al.*, 1990; Orlean, 1990; Sugiyama *et al.*, 1991). This is also a potential route for the incorporation of ManN into *N*-linked glycans (Pan *et al.*, 1992a), and also strongly suggests that ManN is first metabolized to nucleoside diphosphate-ManN (GDP-Man is required for the mannosylation of Dol-P) as suggested in *T. brucei* (Lisanti *et al.*, 1991). This hypothesis is supported by our detection of ManN-phosphates, and by the observation of a putative Dol-P-ManN species in *L. mexicana* promastigotes. The failure of ManN to act *in vitro* (Pan and Elbein, 1985) is attributable to the inefficient production of nucleotide sugar *in vitro*. ManN is less efficiently incorporated into GPI lipid than either inositol or galactose, which suggests that the activated forms of ManN are utilized less efficiently by the glycosyltransferases involved. This is consistent with the fact that ManN has a comparatively high ID_{50}.

ManN also provides the first example of a reversible inhibitor of LPG biosynthesis *in vivo*. The concentration required to effect >90% inhibition of LPG in *L. mexicana* is ~250 μM, a concentration well below the toxic dose (>5 mM) for this organism. Because the action of ManN is prevented by high concentrations of glucose present in culture media, it is not easy to clear the parasite surface of LPG and gp63 with ManN using conventional growth conditions. We are currently attempting to adapt and grow *L. mexicana* in low-glucose or glucose-free media to achieve this aim.

ManN cannot act only as a chain terminator as LPG does not contain a 2-linked mannose, either in the core or in the repeats. The lack of significant incorporation of [^{3}H]ManN into LPG of *L. mexicana* suggests that the amino sugar is not added as part of the repeat. Data from *in vitro* studies indicate that the mannose is derived from GDP-Man (Carver and Turco, 1991). It is possible that the LPG precursor cannot be elongated due to poor recognition of a ManN containing donor, but further experimentation will be required to dissect the mechanism of inhibition of LPG biosynthesis. The curious difference in the efficiency of incorporation of [^{3}H]ManN into the LPG of *L. donovani versus L. mexicana* cannot be explained by the data presented here, nor can it be accounted for by the difference in the structures of the respective LPGs (Turco *et al.*, 1987; McConville *et al.*, 1990b; Ilg *et al.*, 1992; Thomas *et al.*, 1992). One possible explanation is that the substrate requirements of the LPG mannosyltransferase in *L. donovani* are less stringent than the corresponding activity in *L. mexicana*, and that this observation represents a simple evolutionary difference between the two species.

Our data suggest that the mode of action of ManN is quite complex, involving the synthesis of NDP-ManN and Dol-P-ManN. These activated metabolites are able to become incorporated into GPI structures where they can act as chain terminators. Additional mechanisms possibly include kinetic effects on the transferases, both in their ability to transfer activated ManN intermediates and to recognize ManN at the nonreducing terminus as an acceptor. While ManN is a relatively nonspecific inhibitor, we suggest that it may be a useful tool to investigate GPI biosynthetic pathways and to generate GPI precursors *in vivo* that would be suitable for detailed structural analysis.

While this paper was in review another study was published on the effect of ManN on GPI biosynthesis in MDCK cells by Pan *et al.* (1992b). We note that the conclusions of their paper are in good agreement with the study reported in the present article.

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[4] M. C. Field and A. K. Menon, unpublished data.

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