THE BIOSYNTHESIS OF MANNOSYL-1-PHOSPHORYL-POLYISOPRENOL IN MICROCOCCUS LYSODEIKTICUS AND ITS ROLE IN MANNAN SYNTHESIS*

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Previous studies from this laboratory have shown that crude, cell-free extracts of *Micrococcus lysodeikticus* catalyze the incorporation of mannose from GDP-mannose into three lipids[1] and a mannan.[2] Two of the lipids have been identified as α-D-mannosyl-(1 → 3′)-diglyceride and α-D-mannosyl-(1 → 3)-α-D-mannosyl-(1 → 3′)-diglyceride.[1] In this communication, the results of studies on the structure of the third mannose-containing lipid are presented. All the available evidence indicates that the structure of this compound is mannosyl-1-phosphoryl-polyisoprenol (MPP). Preliminary experiments suggest that MPP serves as an intermediate in the enzymatic transfer of mannosyl groups from GDP-mannose to mannan.

Methods.—Enzyme preparation: A particulate preparation that catalyzes the synthesis of MPP, mannan, and mannosyldiglyceride was obtained by centrifugation of a crude, cell-free extract of *M. lysodeikticus*[1] at 100,000 × *g* for 1 hr. The MPP synthetase was prepared from the crude extract as follows: Crude extract (60 ml) was heated at 40° for 20 min. The pH of the solution (at 0°) was adjusted to 5.5 with 1 *N* acetic acid, and 15.7 gm of $(NH_4)_2SO_4$ was added with stirring. After 30 min, the precipitate was collected by centrifugation, dissolved in 0.02 *M* Tris-HCl, pH 7.6 (buffer A), and dialyzed overnight against 2 liters of buffer A. The dialyzed preparation (64 ml) was added with rapid stirring to 550 ml of acetone at −25°. The resultant precipitate was washed successively with 300 ml each of acetone and diethyl ether at −25° and then dried *in vacuo* for 2 hr (1.35 gm dry wt). Prior to use, 300 mg of acetone powder was suspended in 8.0 ml of buffer A and stirred at 0° for 45 min. Insoluble material was removed by centrifugation at 6000 × *g* for 5 min.

Preparation and purification of MPP: To 12.5 mmoles of Tris-maleate, pH 8.5; 10 mmoles of $MgCl_2$; and 0.5 mmole of Tris-HCl, pH 7.6, in 60 ml of H_2O were added 60 ml of a solution of acetone powder enzyme, 75 ml of a sonicated suspension of 1.45 gm of total lipids from *M. lysodeikticus*,[1] and 2 ml of GDP-mannose-U-C^{14} (14.72 μmoles, specific activity 2.45×10^5 cpm/μmole). After incubation at 37° for 2 hr, the reaction was terminated and the lipids were extracted as previousyl described.[1]

The lipid (2.0×10^6 cpm, 8.15 μmoles of mannose) was applied to a silicic acid column (60 gm) and eluted successively with 1000 ml of $CHCl_3$, 600 ml of acetone, and 600 ml $CHCl_3$–CH_3OH, 1:1. Crude MPP, quantitatively recovered in the last fraction, was dissolved in $CHCl_3$–CH_3OH, 2:1 (CM) and applied to a DEAE-cellulose column (4.5 × 30 cm) prepared in CM. The column was eluted with 2400 ml of CM, 700 ml of CH_3OH, and 700 ml of CM containing 84 ml of concentrated NH_4OH. All of the radioactive lipid was recovered in the acidic lipid fraction eluted with the last solvent. This fraction was evaporated to dryness and dissolved in 16 ml of $CHCl_3$–CH_3OH, 1:4. After addition of 1.5 ml of 1 *N* NaOH, the solution was incubated at 37° for 15 min. Then 1.5 ml of 1 *N* acetic acid, 30 ml of $CHCl_3$–CH_3OH, 9:1, 15 ml of isobutanol, and 30 ml of H_2O were added and the solution was mixed vigorously. The aqueous layer was discarded and the $CHCl_3$ layer was washed with 15 ml of H_2O-CH_3OH, 2:1, and then evaporated to dryness. The C^{14}-MPP (1.72×10^6 cpm, 7.0 μmoles) was applied to a DEAE-cellulose column and

eluted with CM and CH_3OH as described above. The column was then eluted with 0.005 *M* ammonium acetate in 99% CH_3OH and 10-ml fractions were collected. MPP was eluted in a single radioactive peak between fractions 144–160. The pooled fractions were evaporated to dryness, dissolved in 2 ml of 1% CH_3OH in $CHCl_3$, and applied to a Unisil silicic acid column (1 × 7 cm). The column was eluted with 10 ml each of 1% CH_3OH in $CHCl_3$, 5% CH_3OH in $CHCl_3$, and 50% CH_3OH in $CHCl_3$. The last effluent contained 1.28 × 10^6 cpm (5.2 μmoles) of MPP. Examination of MPP by thin-layer chromatography on silica gel (eluent: $CHCl_3$, CH_3OH, H_2O, 12:6:1) revealed the presence of one compound that was positive in tests for PO_4 and lipid (Rhodamine). This compound (R_f = 0.22) contained greater than 95% of the radioactivity. Two other minor components (R_f = 0.48, 0.58) were detected with Rhodamine; both were free of PO_4 and radioactivity.

MPP was further purified by gel filtration on a Sephadex column. To a column (2.5 × 81 cm) of LH-20 Sephadex prepared in 0.001 *M* ammonium acetate in $CHCl_3$-CH_3OH, 1:1, was added 0.643 × 10^6 cpm (2.62 μmoles) of MPP. The column was eluted with the above solvent at a flow rate of 0.5 ml/min and 2-ml fractions were collected. The MPP was eluted in a single radioactive peak in fractions 100–108 and 0.575 × 10^6 cpm (2.43 μmoles of MPP) was recovered. Mannose and phosphate determinations, as well as NMR and mass spectral analyses, were performed on MPP purified in this manner.

Chromatographic procedures: The following solvents were employed in paper chromatography: (*A*) Butanol, pyridine, 0.1 *N* HCl, 5:3:2. (*B*) Ethyl acetate, pyridine, H_2O, 120:50:40. (*C*) Isopropanol, pyridine, H_2O, 120:40:40. (*D*) Isobutyric acid, NH_4OH, H_2O, 57:4:39. (*E*) Ethanol, 1 *M* ammonium acetate, pH 7.3, 5:2. (*F*) Isopropanol, H_2O, 80:20.

Assay methods: MPP synthesis with the acetone powder enzyme was determined either by extraction of the incubation mixture with CM[1] or by paper chromatography. Incubation mixtures were spotted directly on Whatman 3-MM paper and chromatographed overnight in solvent system D.

Mannan and MPP synthesis by the particulate enzyme were assayed separately in paired incubation mixtures. After termination of the reaction by heating at 100°, one incubation mixture was extracted with CM and the MPP was isolated by silicic acid chromatography.[1] The second incubation mixture was spotted on S & S green ribbon paper and run in solvent E at 30° for 18 hr. The mannan remained at the origin, whereas GDPM migrated 14–15 cm; MPP and mannosyldiglyceride were carried off the paper.

Results.—Properties of MPP synthetase: The experiments shown in Table 1, expt. A, illustrate the requirements for MPP synthesis using the acetone powder preparation. Optimum synthesis of MPP from GDP-mannose (GDPM) requires Mg^{++}, and a lipid or lipids in the total lipid extract. As shown in Table 1,

TABLE 1. *Requirements for the synthesis of MPP from GDPM.*

Expt.	Conditions	MPP (mμmoles)
A	Complete system*	7.15
	" " , minus Mg^{++}	0.09
	" " , minus lipid	0.44
B	Complete system*	7.98
	" " , plus 250 mμmoles GMP	7.81
	" " , plus 100 mμmoles GDP	3.92
	" " , plus 250 mμmoles GDP	2.04
	" " , plus 100 mμmoles GDP, plus additional 1.0 mg lipid	6.00
	" " , plus 250 mμmoles GTP	1.80

* Complete system contained (in μmoles): Tris-maleate, pH 8.5, 25; $MgCl_2$, 20; Tris-HCl, 2; total lipids of *M. lysodeikticus*, 2.0 mg (expt. A) or 1.5 mg (expt. B); enzyme, 1.87 mg (A) or 2.4 mg (B); GDP-mannose-C^{14}, 7.9 mμmoles (A) or 36.8 mμmoles (B); and H_2O to a final volume of 0.41 ml. Incubation at 37° for 60 min (A) or 30 min (B).

expt. B, MPP synthesis is markedly inhibited by GDP, but not by GMP. This inhibition by GDP is reversed by an excess of total lipid. The inhibition observed by GTP presumably is due to its conversion to GDP by a GTPase activity detected in the enzyme preparation.

To investigate the possibility that the apparent inhibition by GDP is due to the reversible nature of the reaction leading to MPP from GDPM and lipid, purified C^{14}-MPP was added to the acetone powder enzyme in the presence and absence of GDP. As shown in Figure 1, addition of GDP but not GMP results in formation of GDPM-C^{14} from C^{14}-MPP. As above, GTP is also effective in this reversal, presumably owing to its hydrolysis to GDP. The radioactive product was identified as GDPM by paper chromatography and by degradation to C^{14}-mannose after sequential treatment with phosphodiesterase and alkaline phosphatase, using the DEAE disc assay method.[3] Thus, the above data suggest that MPP is formed in a reversible reaction in which the mannosyl moiety of GDPM is transferred to a lipid, with concomitant production of GDP. In preliminary experiments not shown, direct evidence for the production of GDP was obtained using guanosine-labeled GDPM as a susbstrate.

The structure of MPP: Although MPP was found to be stable to treatment with dilute alkali (see *Methods*), it was readily attacked by dilute acid. Treatment with 0.01 N HCl at 100° for 10 min released C^{14}-mannose, which was identified by paper chromatography in systems A, B, C, and D and by gas chromatog-

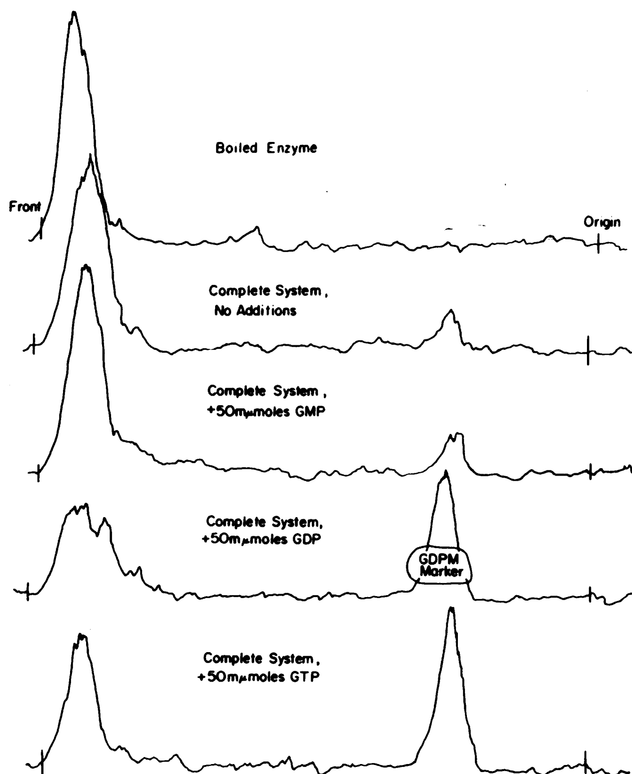


FIG. 1.—Incorporation of C^{14}-mannose from MPP into GDPM.

The complete system contained: Tris-maleate, pH 8.5, 12.5 μmoles; $MgCl_2$, 10 μmoles; acetone powder enzyme, 1.6 mg; MPP, 14.2 mμmoles (480 cpm/mμmole); and the indicated derivatives of guanosine in a final volume of 0.15 ml.

After incubation at 30° for 30 min, the incubation mixture was spotted on Whatmann 3-MM paper and chromatographed as indicated in the text.

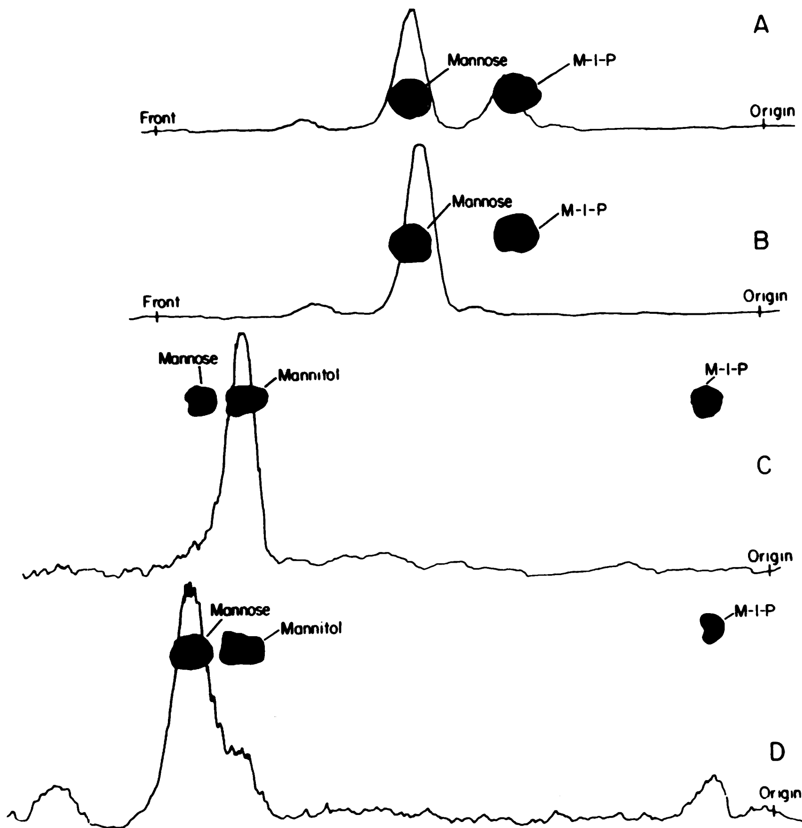


FIG. 2.—Characterization of the water-soluble products of mild acid hydrolysis of MPP. Approximately 15,000 cpm of MPP was mixed with 25 μl of CH_3OH and 100 μl of 0.01 *N* HCl and heated at 100° for 1 min. The hydrolysate was neutralized with NaOH and analyzed by paper chromatography in system D (*A*). A second hydrolysate was evaporated to dryness after neutralization, and the residue was dissolved in 75 μl of H_2O and treated with 50 μg of alkaline phosphatase for 30 min at 37°. The results of paper chromatographic analysis are shown in (*B*). In a separate experiment, 75,000 cpm of MPP was hydrolyzed and the C^{14}-mannose-PO_4 (30,000 cpm) was isolated by preparative paper chromatography. One aliquot (10,000 cpm) was reduced with $NaBH_4$ after acid hydrolysis (0.1 *N* HCl at 100° for 10 min), and a second was subjected to $NaBH_4$ before acid hydrolysis as described by Ishihara and Heath.[4] A paper chromatogram (solvent B) of the products of the former treatment is shown in (*C*); that of the latter is shown in (*D*).

raphy of the trimethylsilyl derivative.[1] Milder conditions of hydrolysis produced C^{14}-mannose and a substance with the mobility of C^{14}-mannose-1-P (Fig. 2*A*). Treatment of this substance with alkaline phosphatase results in the formation of C^{14}-mannose (Fig. 2*B*). The phosphate was assigned to the 1 position of mannose by virtue of the resistance of the mannose phosphate to reduction by $NaBH_4$. When the C^{14}-mannose phosphate, obtained free of mannose by preparative chromatography, was treated with acid and then subjected to $NaBH_4$ reduction, the product was mannitol (Fig. 2*C*). When the sequence was reversed and the mannose phosphate was treated with $NaBH_4$ prior to acid hydrolysis, the

major product was mannose (Fig. 2*D*). Traces of mannitol, mannose-1-P, and α-methylmannose were also evident. Thus the ability of the phosphate linked to mannose to protect the molecule from reduction clearly indicates that mannose-1-P is a product of the mild acid hydrolysis of MPP.

The ratio of total PO_4 to mannose (determined by radioactivity) in intact MPP was 1.00:0.97. When MPP was subjected to mild acid hydrolysis for 10 min followed by extraction with ether, all the radioactivity was found in the aqueous phase. The ratio of PO_4 to mannose in the aqueous hydrolysate was total P:mannose (C^{14}):mannose (anthrone) = 1.00:1.08:0.98. The ether phase obtained by extraction of the hydrolysate was subjected to analysis by mass spectrometry. The major constituent of the ether extract was an undecaprenol containing an olefinic group in each isoprene residue. A weak molecular ion was observed for this component at m/e 766, and it was accompanied by a much stronger ion at m/e 748, corresponding to the loss of water from the molecular ion. Further losses of ω-terminal isoprene residues by successive cleavages of allylic bonds in the chain gave a regular sequence of ions at m/e 679, 611, 543, 407, 339, 271, 203, and 135. The locations and relative intensities of these ions were the same as those in a castaprenol with 11 isoprene residues[5] and in a ficaprenol with 11 isoprenes.[6] The mass spectrum was also practically identical with those of polyisoprenoid alcohols involved in the biosynthesis of O-antigen of *Salmonella*[7] and bacterial cell-wall peptidoglycans.[8] There was no evidence in the mass spectrum for any saturated isoprene units. The location of the most intense ion in the spectrum at m/e 69 and the relative strength of $(M - 18)^+$ compared with $(M)^+$ agree with previous work with fully unsaturated polyprenols, whereas substantially different mass spectra were found with polyprenols containing a saturated hydroxy-terminal isoprene residue.[9] A weak ion at m/e 816 was attributed to $(M - 18)^+$ for a small amount of a dodecaprenol in the sample, and a comparison of the relative intensities of m/e 697 and 680 with those of individual ficaprenols[6] indicated that m/e 680 consisted partially of $(M - 18)^+$ of a decaprenol. Based on the relative intensities of these three $(M - 18)^+$ at m/e 816, 748, and 680 (corrected), the proportions of docecaprenol, undecaprenol, and decaprenol were 5, 91, and 4 per cent, respectively.

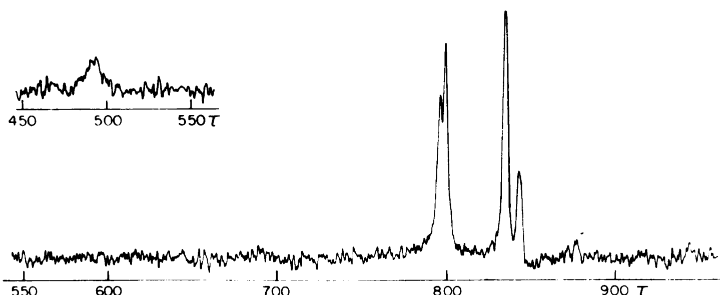


FIG. 3.—NMR spectrum of MPP. The spectrum of 1.5 μmoles of MPP, dissolved in 0.4 ml of $CDCl_3$ (containing 1% tetramethylsilane), was obtained with a Varian HA-100 NMR spectrometer.

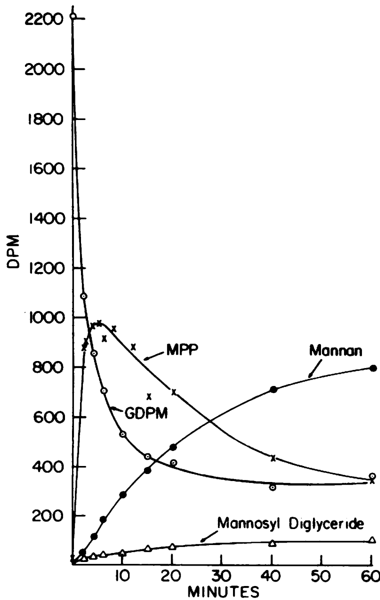


FIG. 4.—Kinetics of MPP, mannosyldiglyceride, and mannan synthesis from GDPM. Duplicate incubation mixtures for each time point contained Tris-maleate, pH 8.5, 40.0 μmoles; Tris-HCl, pH 7.6, 1.8 μmoles; GDPM, 3 mμmoles (936 dpm/mμmole); dialyzed charcoal-treated particulate enzyme, 1.6 mg; in a final volume of 0.175 ml. After incubation for the indicated time, one of each pair of tubes was assayed for MPP and mannosyldiglycerides, and the other for GDPM and mannan, as indicated in the text.

In order to obtain corroborative evidence for the polyisoprenoid nature of the lipid moiety of the mannosyl lipid, intact MPP was analyzed by NMR spectroscopy. The results of this analysis are shown in Figure 3. The principal features of the spectrum are two peaks at 8.42 and 8.34 τ, a complex peak centered at 9.98 τ, and a broad peak at 4.94 τ. The positions of these peaks are in exact agreement with those found for the internal repeating isoprenyl units of polyisoprenols by Feeney and Hemming[10] and by Bates *et al.*[11] Not evident were two very low-intensity peaks at 6.10–6.02 τ (doublet), and at 4.74–4.58 τ (triplet) reported to arise from the single hydroxy-terminal isoprenol unit of polyprenols.[10] Also not evident in the spectrum of MPP were the broad resonance peaks in the 6.3–6.7-τ region owing to the various protons of the mannosyl unit. However, when the spectrum was time-averaged by means of a C-1024 computer to increase the signal-to-noise ratio, a series of broad peaks was seen in the 6.5-τ region. Feeney and Hemming have demonstrated that one can estimate the proportion of *trans* versus *cis* double bonds in polyprenols by measurement of the areas under the peaks at 8.34 τ (*cis*) and 8.42 τ (*trans*).[10] The relative areas in MPP were found to be 3.0 (*cis*) to 1.0 (*trans*). Taking into account the fact that there are 12 CH_3-groups (11 double bonds) in the C_{55} alcohol, the above ratio becomes 9 (*cis*):3 (*trans*). Inasmuch as the methyl-terminal isoprenoid

unit, $CH_3—C(CH_3)=CH—CH_2—$, has one methyl group in the *trans* relationship, as well as one in the *cis*, it is evident that two internal *trans* double bonds are present per molecule of MPP.

Role of MPP in mannan synthesis: The particulate cell fraction isolated from crude extracts of *M. lysodeikticus* previously had been shown to catalyze the incorporation of mannose from GDPM into a mannan.[2] In order to investigate the possibility that MPP serves as an intermediate in this process, the kinetics of MPP, mannosyldiglyceride, and mannan synthesis from GDPM were studied. The results (Fig. 4) indicate that the initial rates of MPP synthesis and GDPM disappearance are rapid, and after much of the GDPM has been consumed, the level of MPP declines markedly. In contrast, the relative rate of C^{14}-mannan synthesis is low, and a small but detectable lag is evident during the early stages

TABLE 2. *Mannan, GDPM, and mannosyldiglyceride synthesis from MPP.*

Time (min)	Substrate remaining (dpm) MPP	Products Formed (dpm) Mannan	GDPM	Mannosyl-diglycerides
0	5600	62	50	4
30	3410	1640	740	100
60	2650	2021	1150	158
60, + GDP	930	800	4890	595
60, + alkaline phosphatase	3120	4550	559	97

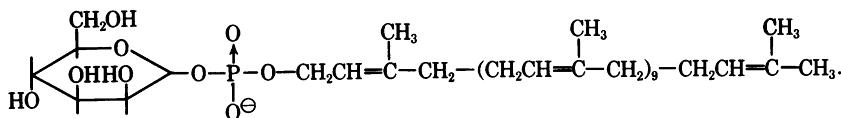
Duplicate incubation mixtures for each time point contained Tris-maleate, pH 8.5, 40 μmoles; Tris-HCl, pH 7.6, 1.8 μmoles; MPP, 9.2 mμmoles (936 dpm/mμmole); dialyzed charcoal-treated particulate enzyme, 2.0 mg in 0.205 ml. After incubation for the indicated time, one of each pair of tubes was assayed for MPP and mannosyldiglycerides, and the other for GDPM and mannan as indicated in the text. The results obtained in identical 60-min incubations in which 250 μg of alkaline phosphatase or 100 mμmoles of GDP was included are also shown.

when MPP synthesis is maximal. The rate of mannosyldiglyceride synthesis is low throughout the incubation.

A more direct test of the hypothesis that MPP serves as an intermediate in mannan synthesis was accomplished by incubation of partially purified C^{14}-MPP with the particulate enzyme in the absence of GDPM. As shown in Table 2, the C^{14}-mannosyl moiety of MPP is enzymatically transferred to mannan. However, it is also evident that GDPM is formed from MPP, presumably because of the presence of endogenous GDP in the crude enzyme. Because GDPM is formed during the synthesis of mannan from MPP, these results alone do not exclude the possibility that GDPM, not MPP, is the immediate precursor of mannan.

The results of two additional experiments make this possibility unlikely. First, addition of exogenous GDP stimulates GDPM synthesis by shifting the equilibrium in the MPP synthesis reaction. At the same time, however, mannan synthesis is inhibited. The latter result is explicable only if MPP, not GDPM, is the immediate precursor of mannan. Secondly, the addition of alkaline phosphatase, which would be expected to destroy endogenous GDP and thus inhibit GDPM formation from MPP, stimulates mannan synthesis and, as predicted, inhibits GDPM synthesis.

Discussion.—On the basis of the foregoing chemical and enzymatic studies, we propose that the structure of MPP is:



Mass spectrometric and NMR analyses on the lipid moiety of MPP indicate that the major polyisoprenoid component is 55 carbons in length and that it contains 11 double bonds; two of the internal double bonds are *trans* and eight are *cis*. The reversible enzymatic formation of MPP involves the transfer of the mannosyl moiety from GDPM to a lipid which, on the basis of its chromatographic properties, is presumed to be a C_{55} polyisoprenyl phosphate. Further studies on the structure of the mannosyl acceptor lipid are in progress.

Recently, lipid intermediates have been implicated in bacterial peptidoglycan biosynthesis[8] and in O-antigen biosynthesis.[5,12] The lipid moiety of these intermediates and the lipid moiety of MPP appear to be similar, if not identical, in structure. Moreover, in all of these studies, as well as in the present investigation, the initial reaction leading to formation of these intermediates has been shown to be freely reversible (cf. Struve and Neuhaus[13]). However, in the case of MPP, the hexose (mannose) is linked to the lipid via a phosphodiester bridge, whereas in the investigations cited above the hexose units are linked by a pyrophosphate ester.

Previous studies have shown that *M. lysodeikticus* contains a mannan associated with the cell membrane[14] and that GDPM can serve as a precursor in its synthesis *in vitro.*[2] The results of the present investigation suggest that MPP serves as an intermediate in the incorporation of mannosyl residues from GDPM into mannan. Although further studies will be necessary to clarify the details of this process, these findings suggest that isoprenoid lipids may function as "coenzymes" in the biosynthesis of the relatively simple homopolysaccharides, as well as in the formation of the complex heteropolysaccharides of the bacterial cell envelope.

Summary.—The structure of an alkali-stable, acid-labile lipid formed from GDP-mannose and an endogenous lipid in crude, cell-free preparations of *Micrococcus lysodeikticus* has been shown to be mannosyl-1-phosphoryl-polyisoprenol. This compound can serve as a mannosyl donor in the enzymatic synthesis of a homopolysaccharide of mannose.

Note added in proof: Further results provide strong additional support for the proposal that MPP serves as an intermediate in mannan synthesis according to the following sequence: GDPM $\overset{A}{\rightleftarrows}$ MPP $\overset{B}{\rightarrow}$ mannan. Using the crude particulate enzyme in the presence of 8×10^{-3} M EDTA, reaction A is inhibited completely, whereas reaction B proceeds normally. Thus, when MPP synthesis from GDPM is inhibited, mannan synthesis is abolished. However, under these conditions the ability to synthesize mannan from exogenous MPP is retained.

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