Plant Physiol. (1972) 50, 473–476

The Role of a D-Mannosyl-Lipid as an Intermediate in the Synthesis of Polysaccharide in *Phaseolus aureus* Seedlings[1]

Received for publication March 6, 1972

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ABSTRACT

Particulate preparations from *Phaseolus aureus* produce a D-mannosyl-lipid when treated with GDP-D-mannose. This lipid complex appears to be an active D-mannose donor, and some investigators have proposed that its role might be an obligatory intermediate in mannan synthesis of higher plants. When the partially purified D-mannosyl-lipids, isotopically labeled in the D-mannose moiety, were treated with particulate enzymes under a variety of conditions, a negligible amount of material was produced that behaved as a polysaccharide. Endogenous, particle-bound D-mannosyl-^{14}C-lipid prepared from *P. aureus* particles readily transferred D-mannose to GDP to yield GDP-D-mannose and was hydrolyzed to free D-mannose when treated briefly with 0.01 N HCl at 100 C. The D-mannosyl-lipid, therefore, exhibits active D-mannose transfer potential in its endogenous state. When endogenous glycosyl-lipid was incubated in the absence of GDP-D-mannose-^{14}C, little or no polysaccharide was produced. It was, instead, slowly degraded to D-mannose. Addition of several different unlabeled sugar nucleotides had no effect on the results. Our studies to date, therefore, offer no evidence that the mannosyl-lipid is an obligatory precursor of polysaccharide.

Glycosyl-lipids have been established as intermediate compounds in the biosynthesis of polysaccharides (13, 14), glycopeptides (2), and lipopolysaccharides (18, 20) in microorganisms. Sugars are transferred reversibly from nucleoside diphosphate sugars to C_{55}-phosphoryl-polyisoprenol carriers and then donated by the lipid to growing polymer chains of the cell walls. In higher plants the participation of lipid intermediates for polymer formation has not been unequivocally demonstrated, although a D-mannosyl-lipid showing active D-mannose transfer potential has been reported (9, 17) in particulate enzyme preparations of *P. aureus* (mung bean). Kauss (9) showed that a glycolipid, which has been synthesized from GDP-D-mannose and an endogenous lipid acceptor by a particulate enzyme preparation obtained from mung bean shoots, exhibits a similar group transfer potential as in microorganisms (13). However, experiments undertaken by Kauss to demonstrate a direct transfer of the mannosyl groups from the isolated ^{14}C-mannosyl-lipid to polysaccharide produced only small amounts of what appeared to be polymeric material, which was not sufficient to identify the product. He thus concluded that "the problem in regard to the acceptor molecules for the mannosyl groups of the mannosyl lipid has, therefore, to await better methods for reconstitution of the complex membrane system."

Mung bean shoots incorporated mevalonic acid-5-^{3}H into the lipid portion of the D-mannosyl-lipid (9), suggesting that the lipid is an isoprenol. Also, recent work by Alam and Hemming (1) has demonstrated that exogenous betulaprenol can act as a D-mannosyl acceptor in mung bean preparations to produce a D-mannosyl-lipid which is chromatographically similar to that prepared from endogenous acceptor.

Chase experiments by Villemez and Clark (17) indicated that there was a turnover of a lipid material, formed from GDP-D-mannose and mung bean particles as source of enzyme; they therefore concluded that the glycosyl-lipid is a direct precursor of polysaccharide, presumably mannan. They state that other polysaccharides synthesized from UDP-D-galactose, UDP-glucuronic acid, and GDP-D-glucose can be synthesized from analogous glycosyl-lipid intermediates. However, turnover results do not constitute conclusive evidence that the glycosyl-lipid is a direct precursor to the polysaccharide.

Since the question regarding glycosyl-lipid formed from sugar nucleotides as a precursor to polysaccharides in plants is at present a moot question, an effort is being made in this report to throw some light on this problem.

MATERIALS AND METHODS

Materials. GDP-D-mannose-^{14}C, uniformly labeled in D-mannose (143 μc per μmole) was purchased from Amersham/Searle Corporation. The remaining chemicals were purchased from Sigma Chemical Company and Calbiochem.

Enzyme Preparation. Mung bean (*P. aureus*) seedlings were grown in the dark for 3 or 4 days. The shoots were ground in 40-g portions with sand and 20 ml of 0.1 M tris-HCl buffer, pH 7.5, in 0.25% bovine serum albumin and 5 mM dithiothreitol. The homogenates were squeezed through cheesecloth and centrifuged at 1000g for 5 min to remove sand particles. The resulting supernatant solution was centrifuged at 30,000g for 30 min. The pellet obtained from this solution contained the active enzymes. It was resuspended in 1 to 2 ml of buffer. The preparations contained 33 to 53 μg per μl of protein.

Preparation of Partially Purified D-Mannosyl-Lipid. Isotopically labeled lipid was prepared from mung bean particles and GDP-D-mannose-^{14}C as in the following procedure: particulate enzyme, 15 ml, was incubated with 15 ml of 0.1 M tris buffer, 3 ml of 50 mM $MgCl_2$, 3 ml of 50 mM $MnCl_2$, and 21 nmoles (5.6×10^6 cpm) of GDP-D-mannose-^{14}C for 15 min at 25 C. The incubation was then extracted twice with 50-ml portions of 2:1 (v/v) chloroform-methanol. The lower, organic phases were

[1] This investigation was supported in part by Research Grant A-1418 from the National Institutes of Health, United States Public Health Service, and by Research Grant GB11819 from the National Science Foundation. Support of this work by the Agricultural Experiment Station is also acknowledged.

Table I. *Effect of Digitonin on the Incorporation of Radioactive D-Mannose from Exogenous D-Mannosyl-^{14}C-Lipids into Polysaccharide of Mung Bean Enzyme Particles*

Additions to Lipid Dispersion Medium	Enzyme Particles	Radioactivity at the Origins of the Paper Chromatograms		Unreacted D-Mannosyl-Lipid Observed at Solvent Front
		Before hydrolysis	After hydrolysis[1]	
		cpm		
None	Unboiled	905	215	23,900
None	Boiled	590	100	25,300
Digitonin (1%)	Unboiled	1410	205	24,800
Digitonin (1%)	Boiled	735	40	26,700

[1] Hydrolysis conditions were 0.01 N HCl at 100 C for 90 min.

separated by centrifugation. They were combined, extracted once with 50 ml of water, and evaporated *in vacuo* to yield 1.3 nmoles (3.5×10^5 cpm) of crude lipid. The D-mannosyl-lipid was partially purified by chromatography on silicic acid and DEAE[2]-cellulose acetate. The crude lipid, in chloroform, was applied to a 1×23 cm column of 13 g of silicic acid. The column was eluted with 230 ml of chloroform, 100 ml of acetone, and 120 ml of 2:1 chloroform-methanol. The radioactive lipid, 0.75 nmoles (2×10^5 cpm) was eluted by the last solvent. It was then applied in chloroform-methanol to a 3.6×44-cm column of DEAE-cellulose acetate (6) in the same solvent. The column was treated with 1500 ml of chloroform-methanol, and the D-mannosyl-lipid was then eluted with a 0.0 to 0.1 M linear gradient of ammonium acetate in the same solvent. Evaporation *in vacuo* followed by lyophilization to remove ammonium acetate gave 0.68 nmoles (1.8×10^5 cpm) of the lipid, which was then stored at −20 C in chloroform. This compound released D-mannose when treated briefly with 0.01 N HCl at 100 C.

Incubation of Partially Purified D-Mannosyl-Lipid with Particulate Enzyme. Mannosyl-lipid, 0.13 nmole (3.6×10^4 cpm), was dispersed in 300 μl of 0.1 M tris-HCl in 0.25% BSA and 5 mM dithiothreitol by sonication at 0 C at 60% maximum intensity using a microprobe on a Bronwill Biosonik sonicator. The probe was rinsed with 100 μl of buffer solution, and the milky suspension was mixed with 500 μl of particulate enzyme, 100 μl of 50 mM $MgCl_2$, and 100 μl of 50 mM $MnCl_2$. After preincubation at 0 C for 30 min, the mixtures were incubated at 25 C for 1 hr, then streaked on a wide sheet of Whatman No. 3 chromatography paper and irrigated with solvent 1 for 12 hr.

Preparation of Radioactive Particle-bound D-Mannosyl-Lipids. Particulate enzyme, 500 μl; 0.1 M tris-HCl buffer containing 0.25% BSA and 5 mM dithiothreitol, 500 μl; 50 mM $MgCl_2$, 100 μl; 50 mM $MnCl_2$, 100 μl; and GDP-D-mannose-^{14}C, 0.7 nmoles (1.74×10^5 cpm), 10 μl, were incubated for 10 min at 25 to 26 C followed by cooling in ice for 2 min. The incubation was centrifuged at 30,000*g* for 10 min at 0 C. The supernatant solution was discarded. The pellet was resuspended in 200 μl of cold 0.1 tris buffer in 0.25 M BSA and 5 mM dithiothreitol.

Analytical Procedures. Incubations were analyzed by descending paper chromatography on Whatman No. 1, 3 MM, or 3 paper using ethyl acetate-1-butanol-water-acetic acid, 3:4:4:2.5 (solvent 1) or 2-propanol-ethyl acetate-water, 7:1:2 (solvent 2). Radioactivity on the chromatograms was located and measured by cutting the paper into 1-cm strips, which were counted in Liquifluor (New England Nuclear) in a Nuclear Chicago Mark I liquid scintillation counter. Quench corrections were applied.

Immobile radioactivity at the origins of chromatograms was considered to be polysaccharide, and that at the solvent front was D-mannosyl-lipid. The other components were identified by comparison of their mobilities with authentic materials. Authentic D-mannose was located on paper chromatograms with ammonia and silver nitrate reagent (12). D-Mannose 1-phosphate was located by the method of Bandurski and Axelrod (4).

Protein in the enzyme preparations was analyzed by the Lowry method (11).

RESULTS

Incorporation Experiments with Exogenous Lipid. Incorporation experiments were conducted along with boiled enzyme controls using two lipid dispersion media, 0.1 M tris-HCl buffer containing 0.25% BSA and 5 mM dithiothreitol and the same buffer with 1% digitonin added to solubilize the lipid. Incubation of the dispersions containing radioactive D-mannosyl-lipid with particulate enzymes produced some radioactive material that was immobile after paper chromatography of the incubations, 905 cpm with no digitonin present and 1410 cpm with digitonin. However, most of this radioactivity was lost when the particles at the origins were heated at 100 C in 0.01 N HCl for 90 min (Table I). Paper chromatographic analysis of the hydrolysates showed that most of the lost radioactivity was D-mannose. Since the mild acid treatment was insufficient to hydrolyze appreciably the polysaccharide (19), the radioactivity lost from the origin appeared to come from D-mannosyl-lipid which was physically adsorbed on the particles. If this is the case, the higher immobile radioactivity observed in the presence of digitonin suggests that digitonin facilitates the adsorption process.

After the first mild hydrolysis, the particles at the origins were heated in 1 N HCl at 100 C for 20 min. The remaining radioactivity in them was completely removed by the treatment. Paper chromatographic analysis of the hydrolysates showed only the presence of D-mannose. Oligomers of D-mannose, which would have been expected if radioactive polysaccharide was present, were not observed.

In addition to the experiments reported in Table I, the D-mannosyl-lipid was incubated with particulate enzyme in the presence of unlabeled GDP-D-mannose and GDP-D-glucose. Several experiments were also conducted using digitonin-solubilized enzyme (10). In all of these cases the synthesis of chromatographically immobile, radioactive product was negligible.

Attempts to Incorporate Particle-bound D-Mannosyl-Lipids. Particle-bound D-mannosyl-lipid was prepared from mung bean particles by centrifugation in a manner similar to that described by Anderson and co-workers (2) for isolation of particle-bound 2-acetamido-2-deoxy-D-glucosyl-acetylmuramyl(-pentapeptide)-diphospholipid in *Staphylococcus aureus*. It was found that one centrifugation of the particles removed essentially all GDP-D-mannose-^{14}C, and identical results were obtained from experiments with twice-centrifuged particles. In order to minimize degradation of the particulate enzyme, the particles were, therefore, centrifuged only once and resuspended in buffer. The compositions of particles recovered by this treatment are illustrated in Figures 1 and 2 and Table II. Besides polymer and D-mannosyl-lipid, other major radioactive components, D-mannose 1-phosphate, D-mannose, and a series of unidentified compounds, were obtained. The latter appeared on the paper chromatograms between D-mannose and the lipid. The nature of these substances is now being investigated.

[2] Abbreviations: BSA: bovine serum albumin; DEAE: diethylaminoethyl.

Experiments were conducted to establish whether the endogenous D-mannosyl-lipid in the particles possessed active D-mannose transfer potential. Figure 1 demonstrates that the lipid rapidly transfers D-mannose to GDP to form GDP-D-mannose. The solvent-front portions of paper chromatograms of particles containing the endogenous D-mannosyl-lipid were heated at 100 C in 0.01 N HCl for 30 min. More than 95% of the radioactivity was released as D-mannose, which was identified by paper chromatography.

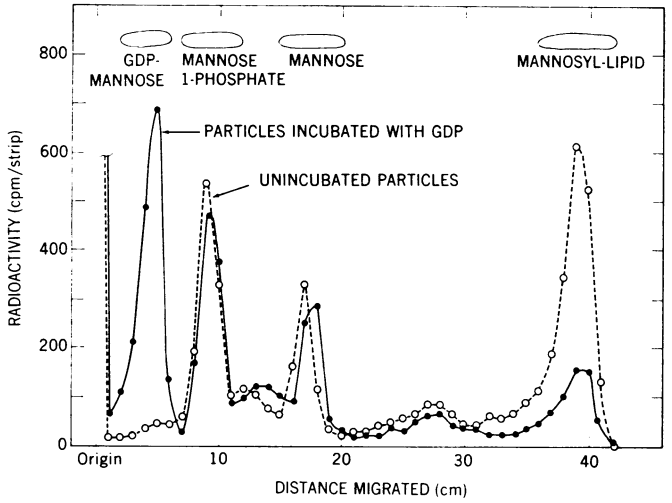

FIG. 1. Paper chromatograms of particle-bound D-mannosyl-lipids before and after reaction with GDP. The particles were prepared as described in "Materials and Methods;" 100 μl of the suspended particles were quenched in 50 μl of acetic acid. Another 100 μl were incubated with 10 μl of 50 mM $MgCl_2$, 10 μl of 50 mM $MnCl_2$, 15 μl of saturated NaF (9), and 10 μl of 0.2 M GDP at 25 C for 5 min and then quenched with 50 μl of acetic acid. The incubations were streaked on Whatman No. 1 paper and irrigated for 12 hr with solvent 1. GDP-D-mannose-^{14}C was eluted from the chromatograms and analyzed by electrophoresis using ammonium formate buffer, pH 3.6. Its mobility coincided with that of authentic GDP-D-mannose.

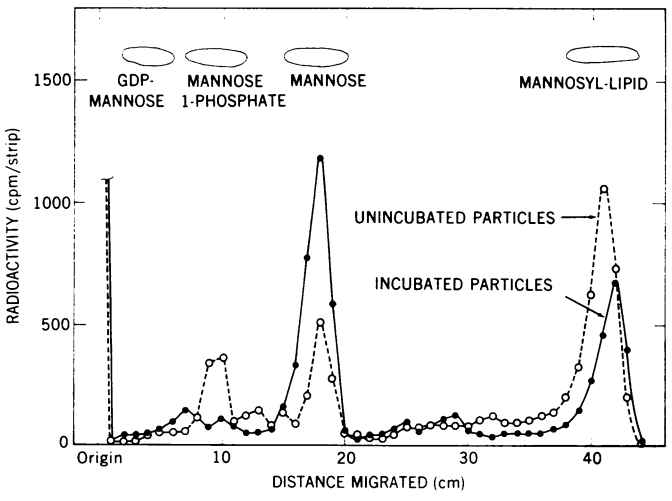

FIG. 2. Paper chromatograms of particle-bound D-mannosyl-lipids before and after incubation. The particles were prepared as described in "Materials and Methods;" 100 μl of the suspended particles were quenched with 50 μl acetic acid, and another 100 μl were incubated for 1 hr at 25 C as described in Table II. The preparations were streaked on Whatman No. 1 paper and irrigated for 12 hr with solvent 1.

Table II. *Paper Chromatograms of Particle-bound D-Mannosyl-Lipids from Mung Bean Preparations Before and After Incubation with Sugar Nucleotides*

The particle-bound lipid was prepared as described in "Materials and Methods;" 100 μl was quenched with 50 μl of acetic acid. Another 100 μl was incubated for 1 hr at 25 to 26 C with 20 μl of 50 mM $MgCl_2$, 20 μl of 50 mM $MnCl_2$, and 50 μl of fresh enzyme preparation and then quenched with 50 μl of acetic acid. Both samples were streaked on Whatman No. 1 chromatography paper and irrigated for 12 hr with solvent 1. The different experiments are not comparable, as they were conducted using different enzyme preparations. Normalization and quench corrections were applied to the data.

Additional Incubation Components	Radioactivity in Unincubated Particles		Radioactivity in Incubated Particles	
	Origin	Mannosyl-lipid at solvent front	Origin	Mannosyl-lipid at solvent front
	cpm			
None	14,700	3610	14,800	2610
0.7 nmole GDP-D-mannose-^{12}C	12,000	3130	12,100	1975
0.32 nmole GDP-D-mannose-^{12}C + 0.49 nmole GDP-D-glucose-^{12}C	12,400	4020	12,400	2740
Sugar nucleotide mixture[1]	11,200	2180	11,200	1590

[1] The sugar nucleotide mixture consisted of a 10 μl solution containing the following unlabeled materials: 0.15 nmole GDP-D-mannose, 0.23 nmole GDP-D-glucose, 0.23 nmole UDP-D-glucose, 0.46 nmole UDP-D-galactose, 0.76 nmole UDP-D-glucuronic acid, 0.076 nmole UDP-*N*-acetyl-D-glucosamine, 0.14 nmole ATP, and 2 μg of yeast extract.

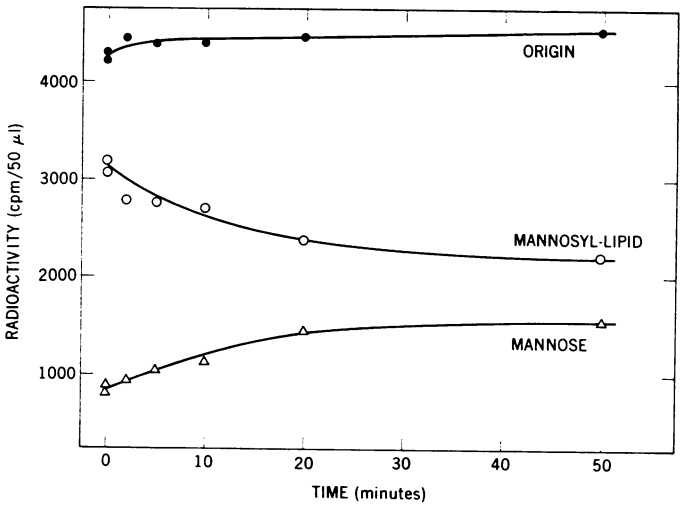

FIG. 3. Time-course study of the incubation of particle-bound mannosyl-lipid. The suspended particles were prepared as described in "Materials and Methods." They were mixed with 50 μl of fresh enzyme particles, 20 μl of 50 mM $MgCl_2$, and 20 μl of 50 mM $MnCl_2$, and 50 μl aliquots were incubated at 26 C for the indicated time intervals. The incubations were terminated with 50 μl of acetic acid, streaked on Whatman No. 1 paper, and irrigated 12 hr with solvent 1.

When particles containing endogenous D-mannosyl-lipid were incubated with GDP-D-mannose-^{14}C, the amount of radioactive polysaccharide increased considerably. This showed that the polysaccharide-synthesizing system in the particles re-

mained active after incubation for 10 min with substrate, centrifugation, and resuspension.

Incubation of the particle-bound glycosyl-lipid in buffer at 25 to 27 C for 1 hr resulted in a negligible increase in polymer. Figure 2 illustrates that the lipid was primarily degraded to D-mannose. The material was confirmed to be D-mannose by elution from the paper and rechromatography with solvent 2. Addition of 0.25% BSA and 5 mM dithiothreitol to the enzyme preparation and to the incubation mixtures had no effect on the fate of endogenous lipids; nor did addition of various sugar nucleotides, ATP, and yeast extract have any effect. Table II summarizes the results of the experiments. In order to insure that polymer was not being formed very rapidly initially and then slowly hydrolyzed enzymatically, a kinetic experiment was conducted with particle-bound lipid. Figure 3 shows that the glycosyl-lipid was degraded directly to D-mannose over a period of 50 min. The slight, initial increase (100–150 cpm) in polymer was apparently due to traces of GDP-D-mannose remaining in the particles.

DISCUSSION

In this study an attempt was made to determine whether the D-mannosyl-lipid prepared *in vitro* in mung bean extracts functions as an intermediate in polysaccharide synthesis. The partially purified glycosyl-lipid was treated with enzyme preparations under a variety of conditions. The results show that negligible quantities of polymer were produced. It should be noted that incubation systems utilizing exogenously added lipid reactants suffer some inadequacies, chiefly because of the lack of lipid dispersion due to aggregate formation (5) and problems arising from the impermeability of enzyme particles to exogenous substrates (6).

In an attempt to avoid these difficulties, the reaction of endogenous particle-bound D-mannosyl-lipids in the absence of GDP-D-mannose-^{14}C was examined. Rather than forming polysaccharide, however, the lipids were hydrolyzed slowly to D-mannose. It has been speculated that the D-mannosyl-lipid, instead of being a direct intermediate in mannan formation, might be the precursor of a series of glycosyl-lipid intermediates in a glucomannan synthesis. True mannans are rare in land plants (3), but glucomannan is formed in mung bean extracts from GDP-D-mannose and GDP-D-glucose substrates (7, 8). Kinetic studies have suggested that an intermediate containing both D-glucose and D-mannose is involved in the synthesis (16). Thus, the initially formed D-mannosyl-lipid might react further with GDP-D-glucose and GDP-D-mannose to produce a polyisoprenoid derivative of a mixed D-glucose-D-mannose oligomer. The lipid might then donate the oligosaccharide unit to the growing glucomannan chain. The mechanism would be comparable to that observed in heteropolysaccharide synthesis in bacteria (2, 14, 20). When the particle-bound lipids were incubated with GDP-D-glucose-^{12}C and GDP-D-mannose-^{12}C, no additional radioactive polymer was produced. Likewise, a mixture of several other different sugar nucleotides had no observable effect. Therefore, there is no evidence for this assumption.

In summary, techniques used successfully in establishing the obligatory intermediacy of glycosyl-lipids in polysaccharide synthesis in bacteria did not demonstrate a similar role for the D-mannosyl-lipid in mung beans. The only rapid enzymatic reaction that the lipid displayed was reversible exchange of D-mannose with GDP. Future investigations by these authors and by others might reveal the function of this active glycosyl-lipid in higher plants. In the meantime it is felt that, in the absence of convincing experimental data, claims that it is an obligatory intermediate in polysaccharide synthesis (15, 17) are unfounded.

Acknowledgment—The authors thank Raymond Jing for his technical assistance.

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