Biosynthesis of Acidic Glycolipids in Cotton Fibers. Possible Intermediates in Cell Wall Synthesis

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Summary

A particulate enzyme from cotton fibers tranfers mannose from GDP-^{14}C-mannose and glucose from UDP-^{14}C-glucose to a phosphate-containing lipid which is present in these membrane particles. Both glycolipids are retained on DEAE-cellulose and both have identical R_f values in three different solvent systems. The sugar moieties of these lipids are very acid labile so that 50% of the sugar is released in 0.001 - 0.003N HCl at 100° in 5 minutes. Both lipids are able to donate their sugar moieties to the respective nucleoside diphosphate to form sugar nucleotides (mannolipid to GDP to form GDP-mannose; glucolipid to UDP to form UDP-glucose). Thus, these lipids are similar in many respects to the mannosyl-phosphoryl-polyprenols synthesized from other sources.

During studies on the biosynthesis of cell-wall polysaccharides by particulate extracts of cotton fibers, it was observed that radioactive mannose from GDP-^{14}C-mannose was incorporated into chloroform:methanol (C:M) soluble products. Since the kinetics of incorporation suggested that the lipid might be turning over, we examined the properties of the mannolipid and found that it was quite similar to the mannosyl-phosphoryl-undecaprenol isolated by Scher et al (1) and shown to be an intermediate in mannan biosynthesis in Micrococci (2). Furthermore, the ^{14}C-mannolipid can donate its mannosyl moiety to GDP to form GDP-^{14}C-mannose. The particulate enzyme from cotton fibers also transfers glucose from UDP-^{14}C-

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glucose to a lipid which has similar properties to those of the mannolipid. The lipid moieties of these glycolipids have not yet been identified but both of these compounds have similar R_f values to those reported for mannosyl-phosphoryl-polyprenols from bacteria (2) and mammalian sources (3).

Materials and Methods

The enzyme preparation used for these studies was prepared from maturing cotton fibers. Ten to 12 day old bolls were obtained from greenhouse grown plants. Fibers were removed, cut into small sections with scissors, placed in 50mM Tris buffer, pH 7.2 containing 0.001M β-mercaptoethanol and gently blended in a Waring Blender for 1 or 2 min. The fibrous material was removed by filtering through cheesecloth and the homogenate was centrifuged at 20,000 X g to isolate the particulate enzyme. This residue was resuspended in the same buffer and used in the following experiments.

Incubation mixtures for the incorporation of mannose from GDP-^{14}C-mannose and glucose from UDP-^{14}C-glucose into C:M soluble lipids contained the following components in a final volume of 0.25 ml: GDP-^{14}C-mannose, 1 X 10^{-5} μmoles (25,000 CPM) or UDP-^{14}C-glucose, 2 X 10^{-4} μmoles (25,000 CPM); $MgCl_2$, 2.5 μmoles; Tris buffer, 12.5 μmoles; and an appropriate amount of enzyme (usually 50-100 ml). After incubation for 15 min. at room temperature, the reaction was stopped by the addition of 2.5 ml of chloroform: methanol (2:1) followed by 0.75 ml of water. The mixture was shaken for several min. and was centrifuged to separate the layers. The bottom layer was removed and saved and the upper layer was reextracted with 1 ml of C:M. Again the mixture was centrifuged and the lower layer was removed and combined with the first lower layer. The combined chloroform layers were extracted once with 1 ml of chloroform:methanol:water (3:48:47) and the lower layer was saved for the determiantion of radioactivity. Only one radioactive lipid was formed from GDP-^{14}C-mannose so the radioactivity in the C:M layer could be used as a measure of mannolipid formation. However,

UDP-^{14}C-glucose is incorporated into steroid glucosides as well as the glucolipid described in this communication. It was therefore necessary in this case to separate the radioactive lipids by thin layer chromatography before the radioactivity in the glucolipid could be determined. The glucolipid could be easily separated from steroid glucosides in chloroform:methanol:water (65:25:4) in which solvent it had a R_f of 0.42. No radioactivity was found in C:M when heat-killed enzyme was used or when ^{14}C-sugar nucleotide was added at the end of the incubation.

DEAE-cellulose was prepared for the purification of acidic lipids as described by Rouser (4). The glucolipids were purified on DEAE-cellulose and subjected to saponification as described by Scher et al (1). However, in the final purification on DEAE-cellulose, lipids were eluted with a gradient of 0 - 0.05M ammonium acetate. Acid hydrolysates on the glycolipids were done in 50% n-propanol in the appropriate concentration of HCl. At various times, samples were withdrawn and partitioned between 2.5 ml of C:M (2:1) and 1 ml of water and the radioactivity in the aqueous phase was determined. Mannose and other hexoses were determined by the anthrone method (5) and phosphorus by the method of Chen et al (6).

Results and Discussion

When GDP-^{14}C-mannose was incubated with the particulate enzyme, radioactivity was incorporated into C:M soluble products (Figure 1). After 15 min., the particles were centrifuged and washed to remove any remaining GDP-^{14}C-mannose and resuspended either in the presence or absence of additional of GDP-^{14}C-mannose. When more GDP-^{14}C-mannose was added, there was additional incorporation of radioactivity into C:M. However, when no GDP-^{14}C-mannose was added there was a gradual decline in the radioactivity in the lipid fraction suggesting that the lipid was turning over. The incorporation of radioactivity from GDP-^{14}C-mannose was fairly linear with time and protein concentration and had an almost complete dependence on the presence of Mg^{++}.

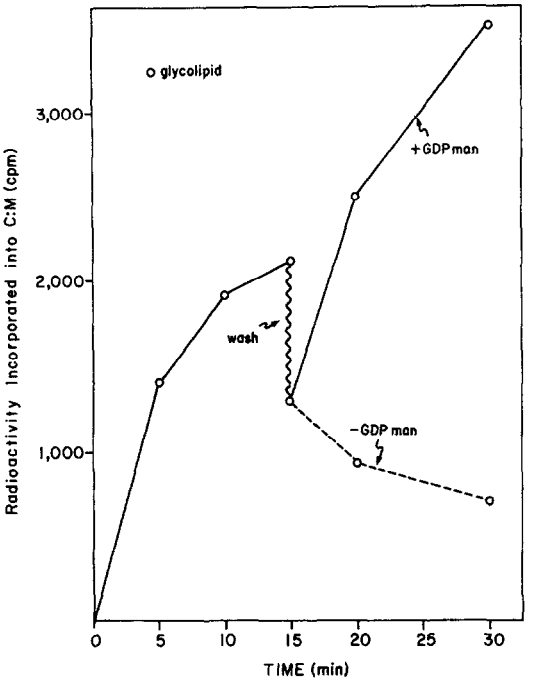

Figure 1. Incorporation of GDP-^{14}C-mannose into mannolipid by the particulate enzyme from cotton fibers. After incubation at room temperature for 15 min., the particulate fraction was isolated by centrifugation and washed to remove GDP-^{14}C-mannose (see arrow). The particles were then resuspended and incubated without GDP-^{14}C-mannose or with additional GDP-^{14}C-mannose. At various times, aliquots were removed and extracted with C:M as described. O - O radioactivity into C:M from GDP-^{14}C-mannose; O --- O radioactivity remaining in C:M after removal of GDP-^{14}C-mannose.

The radioactive product formed from GDP-^{14}C-mannose was isolated by extraction with C:M and was purified on DEAE-cellulose. From 1 X 10^6 CPM of GDP-^{14}C-mannose in a large scale incubation, 1 X 10^5 CPM were incorporated into C:M. Ninety % of this radioactivity was retained on DEAE-cellulose and was eluted with 0.1M ammonium acetate. After saponificatio to cleave acyl groups, the mannolipid was placed on DEAE-cellulose and was

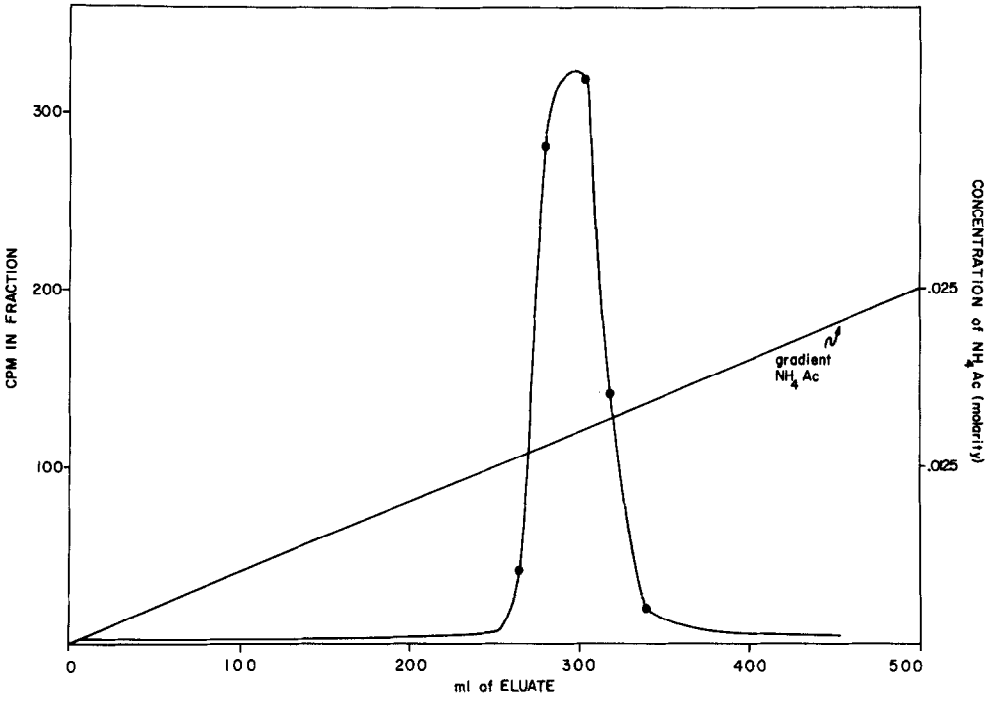

Figure 2. DEAE-cellulose chromatography of radioactive mannolipid. The lipid was eluted with a linear gradient of 0 - 0.05M ammonium acetate.

eluted with a linear gradient of 0 - 0.05M ammonium acetate. As shown in Figure 2, the radioactivity eluted in a symmetrical peak at about 0.02M ammonium acetate. About 90% of the radioactivity placed on the column was eluted in this peak. The lipid eluted from this column had a mannose to total phosphate ratio of 0.85:1. The radioactive sugar in this lipid was very labile to acid hydrolysis as shown in Figure 3. Thus, the sugar was completely released into the aqueous phase in 1 minute when the mannolipid was placed in 0.01N HCl at 100°. In 0.001N HCl, 50% of the mannose was released in 5 minutes at 100°. Almost all of the radioactivity was identified as mannose by paper chromatography in several different solvent systems, although small amounts of a material which migrated like mannose-phosphate were observed.

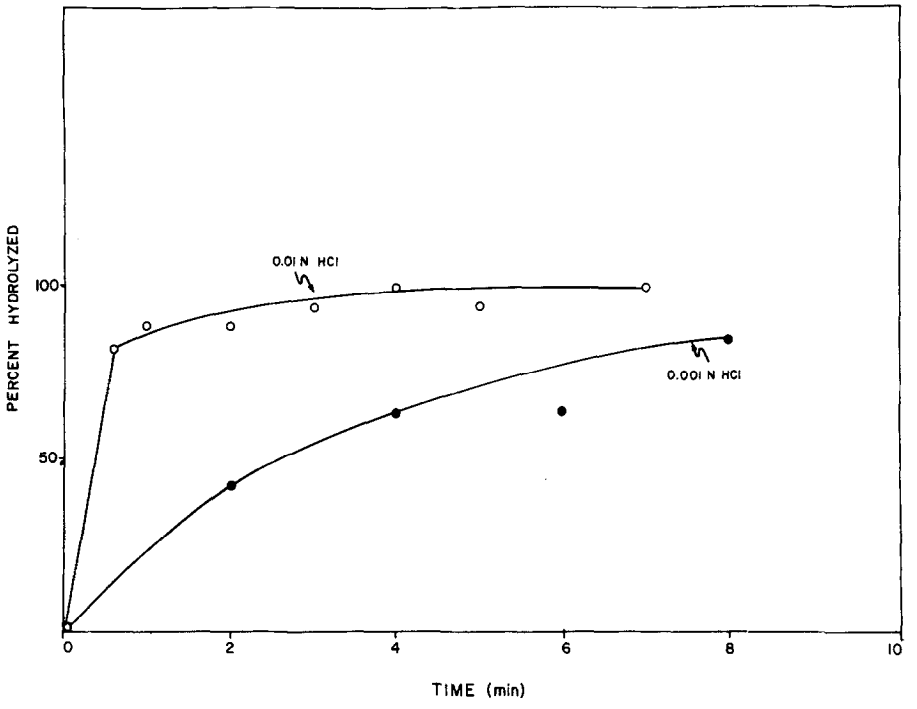

Figure 3. Acid hydrolysis of purified ^{14}C-mannolipid in 0.01N HCl and 0.001N HCl in 50% propanol. After hydrolysis at 100°, aliquots were partitioned between 2.5 ml C:M (2:1) and 1 ml water. Radioactivity in the aqueous phase was determined.

The reaction for the synthesis of the mannolipid is readily reversible as shown in Figure 4. In this experiment, the particulate enzyme was incubated with GDP-^{14}C-mannose to synthesize the ^{14}C-mannolipid and the particulate enzyme was then isolated by centrifugation and washed with buffer to remove GDP-^{14}C-mannose. The particles were then resuspended and incubated with GDP. At various times, the entire incubation mixture was streaked on Whatman 3MM paper and chromatographed in ethanol:1M ammonium acetate, pH 7.4. At zero time (upper tracing) all of the radioactivity was in the lipid, whereas after 1 minute in the presence of GDP, radioactivity decreased in the lipid and appeared in GDP-^{14}C-mannose. The radioactivity in the GDP-^{14}C-mannose was even greater in 5 minutes indicating that the

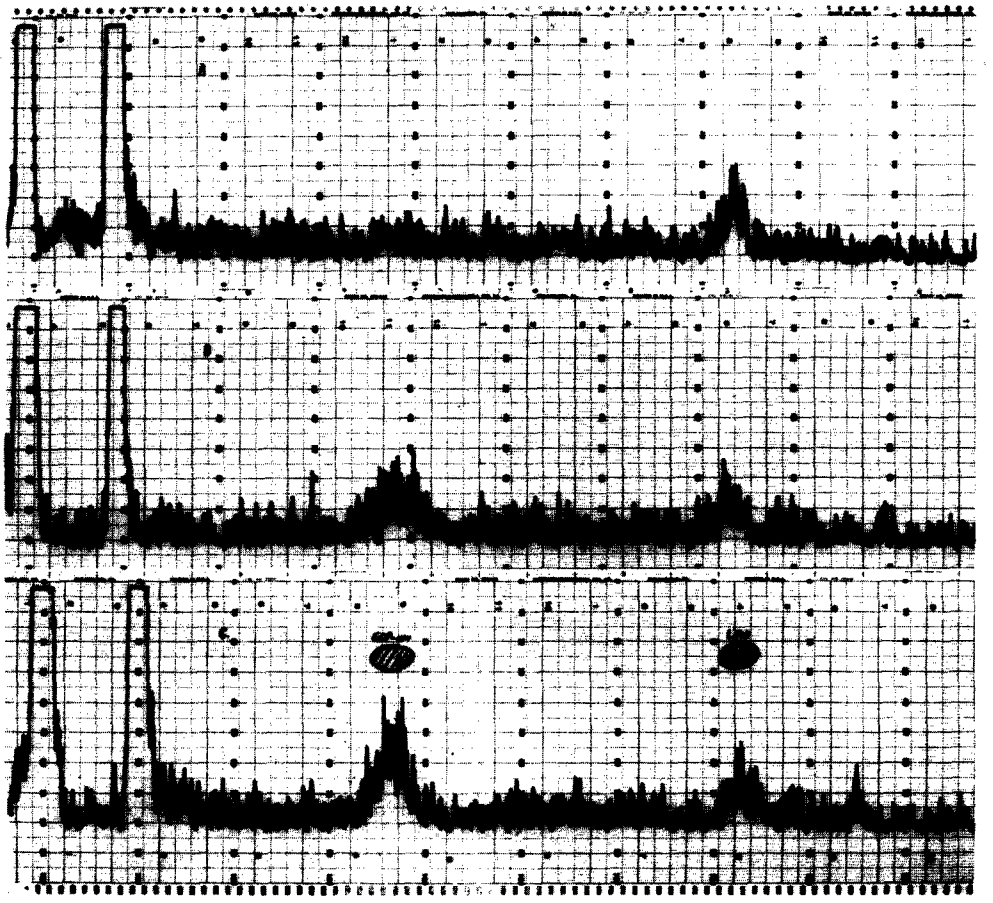

Figure 4. Transfer of ^{14}C-mannose from mannolipid to GDP. Mannolipid was synthesized by incubation of particulate enzyme with GDP-^{14}C-mannose for 10 min. Particles were then centrifuged and washed to remove GDP-mannose and reincubated with GDP (5 X 10^{-4}M). The reaction was stopped by the addition of 3 volumes of ethanol and the entire mixture was chromatographed in ethanol:1M ammonium acetate, pH 7.2 (7:3). Upper scan:0 time or minus GDP; middle scan:plus GDP, 1 min. incubation; lower scan, plus GDP, 5 min. incubation. Starting from left, peaks correspond to marker dye, polysaccharide (orgin), GDP-mannose and lipid.

mannolipid could transfer mannose to GDP to form GDP-mannose. No formation of GDP-mannose was observed when GDP was omitted in the second incubation or when GDP was replaced by GMP.

TABLE I

Thin Layer Chromatographic Mobility of Mannolipid and Glucolipid

SOLVENTS*	R_f of GLUCOLIPID	MANNOLIPID
A	0.42	0.35
B	0.89	0.88
C	0.21	0.18

*Solvents were as follows: A, chloroform:methanol:H_2O (65:25:4); B, chloroform:methanol:acetic acid:water ( ); C, chloroform:methanol:ammonium hydroxide (75:25:4).

A glucolipid was also formed by the particulate enzyme from UDP-^{14}C-glucose. This glucolipid was retained on DEAE-cellulose and eluted with ammonium acetate. As shown in Table I, the glucolipid and the mannolipid had identical R_f values upon thin layer chromatography in three different solvent systems. These solvents included an acidic, a basic and a neutral solvent and R_f values exhibited by these two lipids correspond to those reported for mannosyl-phosphoryl-polyprenols from other systems (2,3). The glucose moiety of the glucolipid was very labile to acid hydrolysis such that 50% of the radioactivity was released in about 2 minutes in 0.003N HCl. Most of the radioactivity released by hydrolysis was identified as glucose by paper chromatography in several different solvent systems.

The reaction for formation of the glucolipid was reversible as was the synthesis of the mannolipid. As shown in Table II after synthesis of the glucolipid and then removal of excess UDP-^{14}C-glucose by centrifugation and washing of the particles, the addition of UDP resulted in the formation of UDP-^{14}C-glucose, whereas in the absence of UDP or when UDP was replaced by UMP, no UDP-^{14}C-glucose was found.

TABLE II

Reversal of Glucolipid Accumulation by UDP

Additions to 2nd Incubation	Incubation Time (min)	CPM in Glucolipid	CPM in UDP-glu*
NONE≠	0	630	56
NONE	5	559	60
UMP	5	581	62
UDP	5	239	410

*Incubations were as described in the text for synthesis of glucolipid. After incubation for 10 min., reaction mixtures were centrifuged to remove UDP-^{14}C-glu. The pellet was resuspended in buffer and UDP (5×10^{-3}M) or UMP (5×10^{-3}M) were added as indicated. After incubation for an additional 10 min., 2.5 ml of C:M and 0.75 ml H_2O was added and extraction was done as described. The C:M layer was subjected to thin layer chromatography in solvent A to isolate the glucolipid whereas the aqueous phase was chromatographed in ethanol:1M ammonium acetate, pH 7.4 (7:3) to isolate UDP-glu. Both glucolipid and UDP-glu were then counted.

≠Only the first incubation was done with this reaction mixture.

Although the lipid moieties of these glucolipids have not yet been identified, these two compounds are similar in a number of respects to glycolipids which are intermediates in polymer synthesis (2,3). Thus these two lipids are acidic glycolipids containing phosphorus in which the sugars are attached by acid-labile linkages. Further, the reactions for synthesis of these lipids are readily reversible indicating that these lipids are easily able to donate their sugar moieties. Villamez (7) and Kauss (8) presented prelininary evidence for lipid intermediates in mung bean seedlings but did not isolate these materials. Alam and Hemming (9) reported the transfer of mannose from GDP-^{14}C-mannose to betulaprenol phosphate in mung beans.

Acknowledgements

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