A PARTICLE BOUND INTERMEDIATE IN THE BIOSYNTHESIS OF PLANT CELL WALL POLYSACCHARIDES[1]

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SUMMARY: Rate, isotope dilution, and direct incorporation studies provide evidence that a lipid soluble material is a direct precursor of higher plant cell wall polysaccharides. The intermediate is acid labile with the solubility properties of a complex glycolipid. It is tightly bound to the particulate enzyme system, and cannot be extracted with the usual lipid solvents until dissociated.

A decade ago, Colvin (1,2) suggested that complex glycolipids might serve as intermediates in the biosynthesis of structural polysaccharides. Glycolipids, the structures of which have been proposed (3-5), are now known to be intermediates in the synthesis of bacterial cell wall polysaccharides. A recent report (6) indicates that a similar intermediate is involved in the synthesis of yeast mannan. A number of plant cell wall polysaccharides have been synthesized with _in vitro_ systems (7-12), but there has been no evidence to indicate that intermediates other than sugar nucleotides are involved. This communication provides evidence that a glycolipid is a direct precursor.

The data presented here are mainly confined to polysaccharide synthesized from GDP-mannose by a _Phaseolus aureus_ enzyme system (7). However, we have similar data relating to polysaccharides (8-11) synthesized from UDP galactose, UDP-glucuronic acid, and GDP-glucose, from the same enzyme system. These latter results, along with those we are presently obtaining with polymers (11-12) synthesized from UDP-galacturonic acid and from UDP-glucose, will be presented in a full report at a later time.

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METHODS

Enzyme preparation: The particulate enzyme system was prepared in a manner similar to that described previously (11). The shoots, with hypocotyls 4-5 cm long, from dark germinated seeds were homogenized in a cold mortar with sand, in the presence of an equal weight of potassium phosphate buffer (0.05 M, pH 7.3, 0°) containing 1% bovine serum albumin and 0.001 M $MgCl_2$. The crude homogenate was strained through 2 layers of cheesecloth, and the filtrate centrifuged at 1000g for 10 min. The material sedimenting from the 1000g supernatant solution at 49,000g in 15 min was washed once with homogenizing buffer and resuspended in 0.5 ml (per 10g of shoots) of 0.05 M potassium phosphate buffer (pH 7.3) that contained 0.01 M $MgCl_2$, 0.4 M sucrose, and 1% bovine serum albumin. This suspension was used as enzyme immediately, or stored at -20° for later use.

Reaction conditions: The temperature of 50 µl of enzyme was raised from 0°to reaction temperature by immersing it in a water bath at reaction temperature for 1 min. GDP-mannose -^{14}C (65 µc/µmole) in 50µl water, at the reaction temperature, was added to the enzyme with sufficient velocity to cause essentially instantaneous mixing. The reaction temperatures were between 20-26°. Specific temperatures, as well as amounts of substrate employed, are indicated in the figure legends. The reactions were terminated by the addition of 50 µl of an aqueous solution of 15% trichloroacetic acid in the same manner as the substrate was added. Samples were maintained between 0-4° until that stage of the analysis where the trichloroacetic acid had been removed.

Analysis: Powdered cellulose (2-4 mg) was added to each sample to provide a supporting media. The insoluble material was extracted three times with 1 ml aliquots of cold water to remove unreacted sugar nucleotide and other side products of the reaction. The intermediate was then extracted from the water insoluble material with two 1 ml portions of 45% aqueous phenol for 10 min at 15-20° (13). The insoluble material remaining was washed with 1 ml of 1:1 chloroform-methanol, and this wash

solution was added to the phenol extract. The phenol extract was spotted on Whatman No. 1 paper strips, dried, and counted in a scintillation spectrometer system. The residual, insoluble material was dispersed in 0.5 ml of 10% methanolic Hyamine, and the precipitate and analysis tube placed in a counting vial for scintillation counting. All samples were corrected for quenching.

RESULTS AND DISCUSSION

Using techniques applied previously, we could find no evidence of a lipid extractable component that had the properties of a precursor of polysaccharide synthesis. However, when the amount of incorporation of

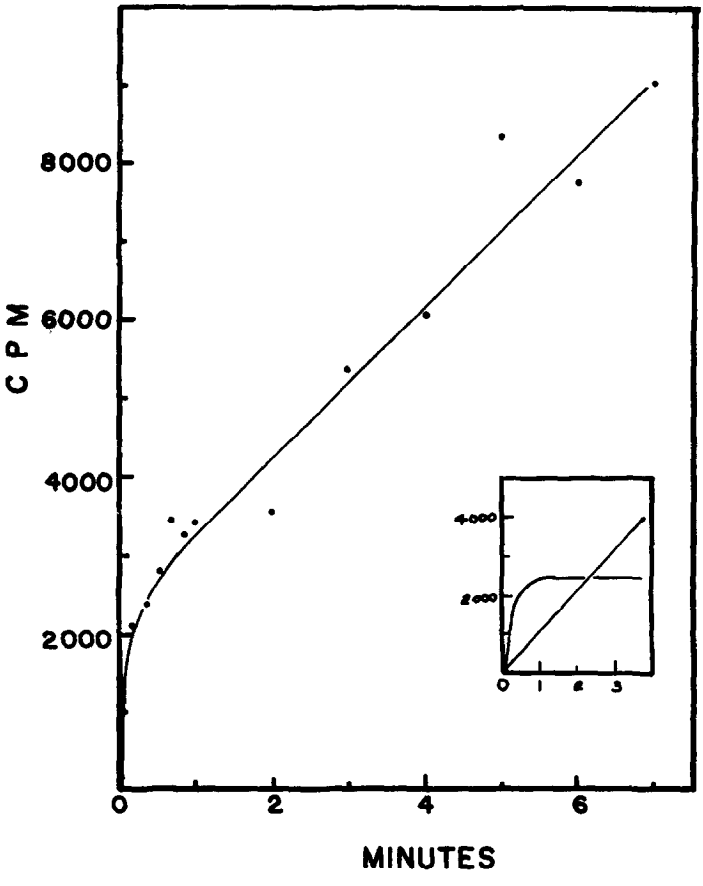

Fig. 1 Incorporation of ^{14}C from UDP-glucuronic acid-^{14}C into water insoluble material. 50,000 cpm (0.15 mμmoles UDP-glucuronic-^{14}C acid added to each sample and reacted at 20° for the indicated time. Reaction conditions and analysis as described in the text.

monosaccharide into the water and lipid insoluble residue, i.e. presumably exclusively polysaccharide material, was studied as a function of time, a biphasic curve resulted (Fig. 1). After about 1 min of reaction time, incorporation increased in a linear fashion, but at earlier times the rate of incorporation was noticeably faster. The biphasic character of the rate curve suggested the possibility that the material being measured consisted of another component in addition to the radioactive polysaccharide. Subtracting the linear (polysaccharide) rate of incorporation from the total, produced another curve (inset, Fig. 1) that presumably reflected the rate behavior of the contaminating component. This calculated rate of the

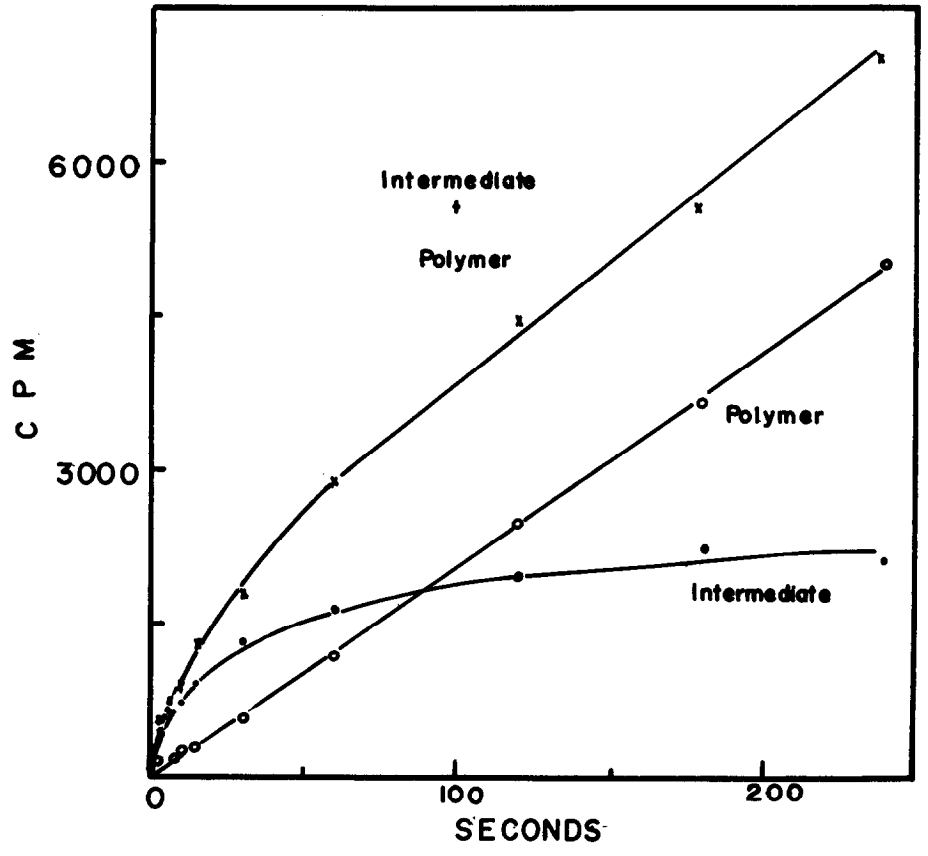

Fig. 2 Incorporation of ^{14}C from GDP-mannose-^{14}C into polysaccharide and intermediate. 25,000 cpm (0.35 mμmoles) GDP-mannose-^{14}C added to each sample and reacted at 25° for the indicated time. Reaction conditions and analysis as described in the text. x-x water insoluble material; •-• intermediate; o-o polysaccharide.

contaminating component was consistent with that of a precursor of the polysaccharide. Consequently, the isolation of this component was attempted. Lipid solvents such as chloroform, ether, ethanol, methanol, or mixtures of these, butanol-6 M acetic acid (3), ethyl acetate-butanol-water-acetic acid (6), extracted essentially no radioactivity from the mixture. However, the extractive procedure described by Westphal (13) succeeded in separating completely the two components. The polysaccharide was totally insoluble when treated with phenol-water mixtures, whereas the material with the faster initial rate of formation was completely extracted. The experimental curves coincided with those calculated above (inset, Fig. 1). The data for such an experiment utilizing GDP-mannose as substrate are given in Fig. 2.

To demonstrate that the phenol extractable material was being converted into polysaccharide, isotope dilution experiments were performed, an example of which is given in Fig. 3. During the period following the addition of a large quantity of non radioactive substrate, both polysaccharide and phenol extractable material adjust to the new conditions (larger substrate concentration of much lower specific radioactivity) in exactly the manner that would be predicted for a precursor-product relationship. In addition, radioactive intermediate has been isolated and added to the enzyme system. Direct incorporation of intermediate has been low in most experiments, but incorporation as high as 50% has been observed occasionally. Surface active agents must be used to solubilize the intermediate, and these disrupt the particulate enzyme system. Therefore, there is little doubt that the radioactive intermediate incorporates into polysaccharide directly, but these data are not completely satisfactory yet. We are developing a soluble enzyme system with which we expect to obtain better data.

After extraction with 45% phenol, and separation into aqueous and phenolic phases by cooling to 5°, the intermediate appears exclusively in the phenolic phase if the extraction takes place at 5°. Upon removal of

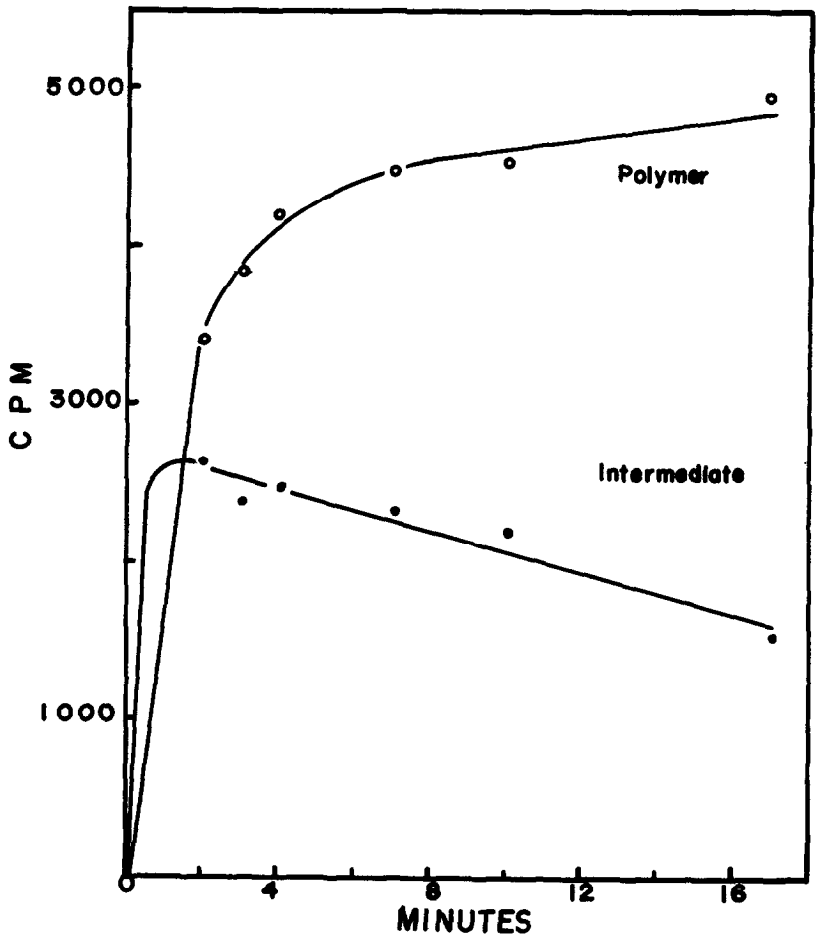

Fig. 3 Isotope dilution of GDP-mannose-^{14}C. Fifty µl of enzyme preincubated for 1 min with 10 µl 0.001 M GDP-mannose at 24°. 125,000 cpm GDP-mannose-^{14}C (1.75 mµmoles) added and incubation continued for 2 minutes, at which point 50 µl of 0.01 M GDP-mannose was added. Time zero is the point at which the radioactivity was added. Other reaction conditions and analysis as described in the text.

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the phenol by dialysis, the intermediate precipitates and is henceforth soluble in typical lipid solvents such as 1:1 chloroform-methanol.

No greater quantity of intermediate is extracted at higher temperatures than 5-10°, but increasing quantities of radioactivity from intermediate appear in the water phase as a function of increasing extraction temperature. This radioactivity remains water soluble and resides in what appears, upon paper chromatography, to be low molecular weight oligosaccharides. These

small oligosaccharides apparently result from the hydrolytic cleavage, at higher temperatures, of the linkage that attaches the saccharide moiety of the intermediate to the lipophilic moiety, since these mild conditions do not hydrolyze sugar-sugar glycosidic linkages. The individual sugars which make up these oligosaccharides are representative of the polymer being formed. For example, from UDP-galactose only galactose is found, but from UDP-glucuronic acid, xylose, arabinose, and glucuronic acid result.

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