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Interaction of Soluble Glucosyl- and Mannosyl-Transferase Enzyme Activities in the Synthesis of a Glucomannan

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A neutral-detergent-solubilized-enzyme preparation derived from *Phaseolus aureus* hypocotyls contains two types of glycosyltransferase activity. One, mannosyltransferase enzyme activity, utilizes GDP-α-D-mannose as the sugar nucleotide substrate. The other, glucosyltransferase enzyme activity, utilizes GDP-α-D-glucose as the sugar nucleotide substrate. The soluble enzyme preparation catalyses the formation of what appears to be a homopolysaccharide when either sugar nucleotide is the only substrate present. A β-(1→4)-linked mannan is the only polymeric product when only GDP-α-D-mannose is added. A β-(1→4)-linked glucan is the only polymeric product when only GDP-α-D-glucose is added. In the presence of both sugar nucleotides, however, a β-(1→4)-linked glucomannan is formed. There are indications that endogenous sugar donors may be present in the enzyme preparation. There appear to be only two glycosyltransferases in the enzyme preparation, each catalysing the transfer of a different sugar to the same type of acceptor molecule. The glucosyltransferase requires the continual production of mannose-containing acceptor molecules for maintenance of enzyme activity, and is thereby dependent upon the activity of the mannosyltransferase. The mannosyltransferase, on the other hand, does not require the continual production of glucose-containing acceptors for maintenance of enzyme activity, but is severely inhibited by GDP-α-P-glucose. These properties promote the synthesis of β-(1→4)-linked glucomannan rather than β-(1→4)-linked glucan plus β-(1→4)-linked mannan when both sugar nucleotide substrates are present.

Particulate enzyme preparations from *Phaseolus aureus* exhibit GDP-D-glucose transglucosylase and GDP-D-mannose transmannosylase enzyme activities which result in the formation of polysaccharides *in vitro* (Barber *et al.*, 1964; Elbein, 1969). The functions of these enzyme systems *in vivo* have not been demonstrated, but they appear to have a part in the biogenesis of the plant cell wall. The synthesis of plant cell-wall polysaccharides apparently involves a complicated reaction sequence that has more enzymic components than sugar nucleotide–acceptor transglycosidases (Villemez & Clark, 1969; Kauss, 1969; Villemez, 1970). The examination of the details of polysaccharide biosynthesis is consequently difficult to approach with particulate enzymes. The reports of solubilized enzymes that are involved in polysaccharide biosynthesis have so far been confined to preparations that catalyse the formation of homopolysaccharides (Feingold *et al.*, 1958; Liu & Hassid, 1970; Stafford & Brummond, 1970; Tsai & Hassid, 1971; Heller & Villemez, 1972). Most plant cell-wall polysaccharides are heteropolysaccharides (Aspinall, 1970), and their formation, regardless of mechanism, must surely involve the co-operative interaction of individual glycosyltransferases at some point in the synthesis. The present paper describes the formation of a glucomannan, catalysed by a soluble enzyme preparation containing glucosyl- and mannosyl-transferases.

Materials and Methods

Materials

GDP-α-D-[U-^{14}C]glucose (sp. radioactivity 166 μCi/μmol) and GDP-α-D-[U-^{14}C]mannose (sp. radioactivity 55 μCi/μmol) was obtained from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A.

Methods

Preparation of particulate enzyme. The particulate enzyme from *P. aureus* seedlings was prepared in a manner similar to that described by Villemez & Clark (1969). Mung-bean hypocotyls (3–4cm long) were homogenized in a cold mortar with sand, in the presence of an equal weight of potassium phosphate buffer (0.05M, pH7.3, 0°C) containing 1% (w/v) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.), 5mM-dithiothreitol (Calbiochem, Los Angeles, Calif., U.S.A.) and 1mM-$MgCl_2$.

The homogenate was strained through two layers of Miracloth (Calbiochem) and the filtrate centrifuged at 1000*g* for 10min. The material sedimenting from the 1000*g* supernatant solution at 49000*g* in 15min was resuspended in 0.5ml (per 10g of hypocotyls) of 0.05M-potassium phosphate buffer, pH7.3, containing 0.01M-$MgCl_2$, 0.4M-sucrose, 5mM-dithiothreitol and 1% bovine serum albumin. The particulate enzyme was stored at −20°C for later use.

Preparation of Triton-solubilized enzyme. Frozen particulate enzyme (1ml) was thawed at 0°C, and 40μl of an aqueous solution of Triton X-100 (12.5%, w/v) was added. The mixture was stirred for 5min at 0°C and then centrifuged at 300000*g* for 40min. The pelleted material was discarded, and the supernatant solution was used as an enzyme source.

Enzyme reaction. Enzyme (50μl) was incubated in a water-bath at 22–24°C for 30s. Radioactive sugar nucleotides and any other additives (in 50μl of water) were added to produce a final volume of 100μl. After the desired time-interval, the reaction was terminated by the addition of 50μl of aq. 15% (w/v) trichloroacetic acid. Approx. 10mg of powdered cellulose (Sigma Chemical Co.) was added to each sample to provide a supporting medium. The insoluble material was extracted three times with 1ml of water to remove unchanged substrate and other water-soluble material. The water-insoluble residue was extracted with two 1ml portions of aq. 50% (v/v) butanol and then with two 1ml portions of aq. 45% (w/v) phenol. The insoluble residue was washed with 1ml of water, 1ml of acetone and then dispersed in 0.5ml of methanolic 1M-Hyamine. This suspension and the analysis tube containing it were placed in a counting vial for radioactivity determinations. Radioactivity was measured by liquid-scintillation counting and all samples were corrected for quenching by external standardization (Villemez, 1971).

Preparation of radioactive polysaccharides. [^{14}C]-Glucan for structural analysis was prepared by incubating 100μl of soluble enzyme and 100μl of GDP-α-D-[^{14}C]glucose (400000c.p.m., 1.5nmol) at 24°C for 15min.

[^{14}C]Glucomannan was prepared by incubating 100μl of either GDP-α-D-[^{14}C]mannose (500000 c.p.m., 6.2nmol) and GDP-D-glucose (0.62nmol) or 100μl of GDP-D-[^{14}C]glucose (400000c.p.m., 1.5nmol) and GDP-α-D-mannose (15nmol) with 100μl of soluble enzyme at 24°C for 15min.

[^{14}C]Glucomannan, with both D-glucose and D-mannose labelled, was prepared by incubating 500μl of GDP-α-D-[^{14}C]glucose (1.6×10^6c.p.m., 5nmol) and GDP-α-D-[^{14}C]mannose (1.0×10^6c.p.m., 12nmol) or 500μl of GDP-α-D-[^{14}C]glucose (1.28×10^6c.p.m., 6.2nmol) and GDP-α-D-[^{14}C]mannose (2.0×10^6c.p.m., 24nmol) at 24°C for 1h. Radioactive polysaccharides were isolated as described above and then extracted with three 1ml portions of acetic acid immediately before addition of the acetolysis medium.

Preparation of radioactive oligosaccharides. Oligosaccharides were obtained by acetolysis of the polysaccharide under the conditions described by Scher & Lennarz (1969). Sugars were deacetylated by dissolving them in dry methanol, adding a catalytic amount of barium methoxide, and leaving them to react for 30min at room temperature (Steward *et al.*, 1968). Barium was removed by bubbling CO_2 through the solution and filtering off the $BaCO_3$ precipitate.

Analytical methods. Periodate oxidations were performed by the modification of the procedure of Hay *et al.* (1965) described by Elbein (1969).

Radioactive oligosaccharides in 100μl of water were reduced by treatment with 1ml of 0.1M-$NaBH_4$ and stirred overnight at room temperature. The reaction mixture was adjusted to pH3.6 with Dowex 50 (H^+ form) and evaporated to dryness after removal of the Dowex 50. Boric acid was removed by repeated evaporation to dryness in the presence of acidic methanol. The reduced oligosaccharides were hydrolysed in 2M-HCl at 100°C for 2h. The hydrolysate was concentrated to dryness at reduced pressure over NaOH.

Chromatographic methods. Descending paper chromatography was performed with Whatman no. 1 and no. 4 paper. The following solvent systems were used: I, propan-1-ol–ethyl acetate–water (7:1:2, by vol.); II, butan-2-one–water–acetic acid (9:1:1, by vol.) saturated with boric acid; III, butan-1-ol–pyridine–0.1M-HCl (5:3:2, by vol.); IV, butan-1-ol–pyridine–water (10:3:3, by vol.). The mobility of the various oligosaccharides is compared with cellobiose (Sigma Chemical Co.), which was used as a reference compound. The sugar standards were detected by alkaline silver nitrate reagent.

Results

A previous report from this laboratory described a Triton X-100-solubilized-enzyme preparation that catalysed the synthesis of a β-(1→4)-linked linear mannan (Heller & Villemez, 1972). This enzyme preparation also catalysed the synthesis of a [^{14}C]-polysaccharide from GDP-α-D-[^{14}C]glucose, which appeared to be a β-(1→4)-linked glucan.

Structure of [^{14}C]glucan

The [^{14}C]polysaccharide formed from GDP-α-D-[^{14}C]glucose proved to be a β-(1→4)-linked linear glucan, as indicated by the results below. Examination of the partial acetolysis products in solvent I (Fig. 1*a*) indicated [^{14}C]oligosaccharide products

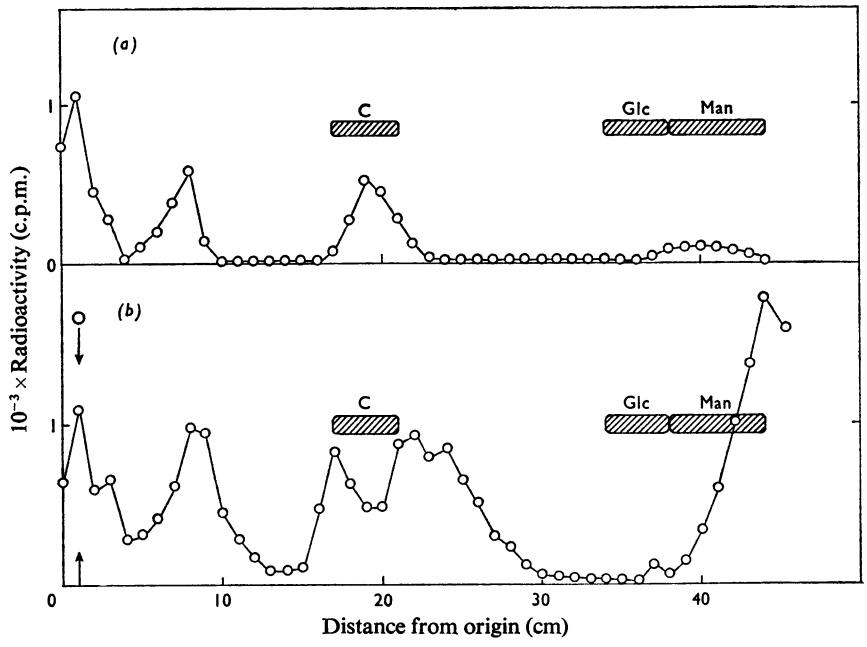

Fig. 1. *Paper chromatogram of soluble oligosaccharides produced by acetolysis of polysaccharides*

(*a*) The polymer was synthesized from GDP-D-[^{14}C]glucose (500000c.p.m., 1.9nmol); (*b*) the polymer was synthesized from GDP-D-[^{14}C]mannose (750000c.p.m., 9.3nmol) and GDP-D-glucose (1.4nmol). The soluble oligosaccharides were separated on Whatman no. 1 paper with solvent I as described in the text. Standard compounds shown are: C, cellobiose; Glc, glucose; Man, mannose. The arrows indicate the origin (O).

coinciding in chromatographic mobility with the partial acetolysis products of cellulose. Glucose was the only radioactive monosaccharide isolated, as demonstrated by elution and rechromatography. The radioactive peaks corresponding to cellobiose and cellotriose were eluted, reduced with $NaBH_4$ and hydrolysed with acid. The only radioactive products, [^{14}C]glucitol and [^{14}C]glucose, were separated by chromatography in solvent II. The ratio of radioactivity present as glucitol and glucose, 1:1 and 1:2, confirmed the indication from the chromatographic mobility that these two oligosaccharides were di- and tri-saccharides of glucose. Periodate oxidation, followed by reduction, hydrolysis and chromatography of the products in solvent III, resulted in the detection of [^{14}C]erythritol and [^{14}C]glycerol as reaction products. These products are expected from hexose-containing oligosaccharides linked β-(1→4). The [^{14}C]polysaccharide, from which these oligosaccharides were obtained, was resistant to solubilization by two treatments with 2% (w/v) NaOH at 100°C. The insolubility of the [^{14}C]polysaccharide, and the absence of partial acetolysis products other than cellodextrins, make the presence of other glycosidic linkages or of chain branching unlikely possibilities.

Structure of [^{14}C]*glucomannan*

When GDP-α-D-[^{14}C]mannose is used as a substrate, GDP-α-D-glucose is a strong competitive inhibitor of [^{14}C]polysaccharide synthesis with this enzyme system (Heller & Villemez, 1972). Examination of the structure of the [^{14}C]polysaccharide that is synthesized from GDP-α-D-[^{14}C]mannose under these inhibitory conditions indicates that it is not a mannan. Partial acetolysis produced a more complicated spectrum of oligosaccharides than would be expected from a mannan (Fig. 1*b*), but the only radioactive monosaccharide that could be isolated from acid hydrolysis of the oligosaccharides was [^{14}C]mannose. Reduction followed by acid hydrolysis of the isolated [^{14}C]oligosaccharides indicated the presence of a non-radioactive sugar as a component of some of these oligosaccharides. Two [^{14}C]oligosaccharides, particularly notable in this respect, produced only [^{14}C]mannose (from the oligosaccharide with $R_{cellobiose}$ 0.85) and only [^{14}C]-mannitol (from the oligosaccharide with $R_{cellobiose}$ 1.26) after reduction and acid hydrolysis. This indicated that [^{14}C]mannose occupied only the non-reducing position in the oligosaccharide with $R_{cellobiose}$ 0.85, and only the reducing position in the

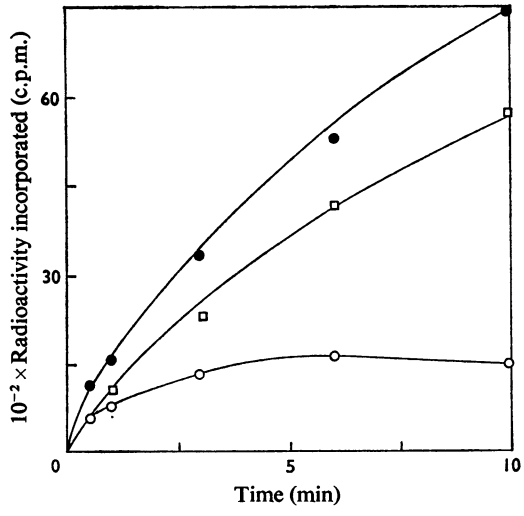

Fig. 2. *Enhancement of glucosyltransferase activity by GDP-α-D-mannose*

GDP-α-D-[^{14}C]glucose (39100c.p.m., 0.15nmol) was added to each sample alone (○), with 0.91nmol of GDP-α-D-mannose (●), or with 3nmol of GDP-α-D-mannose (□). Reaction conditions and analysis are described in the text.

oligosaccharide with $R_{cellobiose}$ 1.26. The [^{14}C]polysaccharide had been synthesized in the presence of GDP-α-D-glucose, so D-glucose was, most likely, the non-radioactive moiety in these oligosaccharides.

When GDP-α-D-[^{14}C]glucose and GDP-α-D-mannose were used as substrates for this enzyme preparation, it was observed that the amount of [^{14}C]polysaccharide that resulted was dependent on the relative concentration of GDP-α-D-mannose present (Fig. 2). This enhancement of [^{14}C]polysaccharide synthesis from GDP-α-D-[^{14}C]glucose in the presence of GDP-α-D-mannose was more a result of the extension of the time during which the reaction occurred than of an increase in rate. Little enhancement of the initial rate of [^{14}C]polysaccharide synthesis was observed. Also, at higher relative concentrations of GDP-α-D-mannose, a slight inhibitory effect on the rate and extent of polysaccharide synthesis was observed. The [^{14}C]polysaccharide that resulted from these reactions was not a [^{14}C]glucan. Partial acetolysis produced [^{14}C]oligosaccharides, with a chromatographic pattern (Fig. 3) similar to that produced from the [^{14}C]polysaccharide resulting from the use of GDP-α-D-[^{14}C]mannose and GDP-α-D-glucose as substrates (see Fig. 1*b*). Analysis of the oligosaccharides in a manner identical with that indicated in the previous section demonstrated that: (1) [^{14}C]glucose was the only radioactive monosaccharide that could be

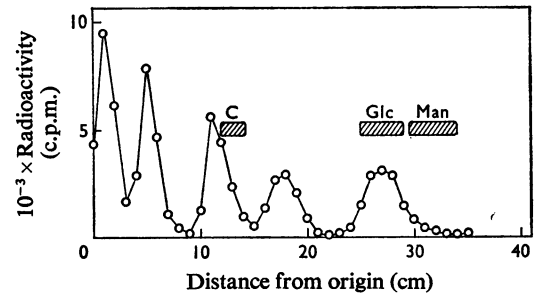

Fig. 3. *Paper chromatogram of soluble oligosaccharides produced by acetolysis of polysaccharide*

The polymer was synthesized from GDP-D-[^{14}C]-glucose (500000c.p.m., 1.9nmol) and GDP-D-mannose (19nmol). The soluble oligosaccharides were separated by paper chromatography on Whatman no. 1 paper in solvent I. Standard compounds shown are: C, cellobiose; Glc, glucose; Man, mannose.

obtained from the [^{14}C]oligosaccharides; (2) there were non-radioactive sugars present in the oligosaccharides. Particularly noticeable were oligosaccharides with $R_{cellobiose}$ 0.89 and 1.33. On reduction and hydrolysis treatment, only [^{14}C]glucitol was isolated from the oligosaccharide with $R_{cellobiose}$ 0.89 and only [^{14}C]glucose from the oligosaccharide with $R_{cellobiose}$ 1.33. This indicated that [^{14}C]glucose occupied only the reducing position of the oligosaccharide with $R_{cellobiose}$ 0.85, and only the non-reducing position of the oligosaccharide with $R_{cellobiose}$ 1.33 (note the complementary results obtained from the polysaccharide synthesized from GDP-α-D-[^{14}C]mannose and GDP-α-D-glucose, given in the preceding paragraph).

To confirm the indication of the above results, that a [^{14}C]glucomannan was being synthesized when both substrates were present, [^{14}C]polysaccharide was synthesized with this enzyme preparation with both GDP-α-D-[^{14}C]mannose and GDP-α-D-[^{14}C]glucose as substrates. Two product characterizations were carried out, one by using the [^{14}C]polysaccharide that resulted from a concentration ratio of GDP-α-D-[^{14}C]mannose to GDP-α-D-[^{14}C]glucose of 2, and another with that from a concentration ratio of 5. There were essentially no differences in the results obtained from each characterization. Paper chromatography, in solvent I, of the partial acetolysis products resulted in an oligosaccharide spectrum that was similar to that presented in Figs. 1(*b*) and 3. The radioactive monosaccharides present were [^{14}C]glucose and [^{14}C]mannose, as expected. The radioactive oligosaccharides with a chromatographic mobility expected of disaccharides ($R_{cellobiose}$ 0.80–

1.6) were purified by paper chromatography in solvent IV. Only three disaccharides were present, and these were identified as mannosyl-β-(1→4)-mannose, glucosyl-β-(1→4)-mannose, and glucosyl-β-(1→4)-glucose in the following manner. (1) The chromatographic mobility of these three components in solvent IV coincides with those reported previously (Karacsonyi, 1969) for mannosyl-β-(1→4)-mannose ($R_{cellobiose}$ 1.1), glucosyl-β-(1→4)-mannose ($R_{cellobiose}$ 1.51) and glucosyl-β-(1→4)-glucose ($R_{cellobiose}$ 1.0). (2) Borohydride reduction followed by acid hydrolysis produced the predicted products for each, that is [^{14}C]-mannose and [^{14}C]mannitol from mannosylmannose, [^{14}C]glucose and [^{14}C]mannitol from glucosylmannose, and [^{14}C]glucose and [^{14}C]glucitol from glucosylglucose. The ratio of the radioactivity found in the [^{14}C]sugar and [^{14}C]sugar alcohol was 0.93 when derived from [^{14}C]glucosylglucose, and 0.80, 0.86 and 1.08 in three separate determinations on the [^{14}C]-mannosylmannose disaccharide. This ratio for the products from [^{14}C]glucosylmannose was 1.98 and 1.88 in two separate determinations. For this last result, the specific-radioactivity ratio of the two radioactive substrates utilized indicates that a ratio of 3.2 is to be expected for a 1:1 molar ratio of glucose to mannose. This discrepancy is also observed to exactly the same degree in the analysis of a radioactive trisaccharide, identified as β-(1→4)-linked mannobiosylglucose, reported below. (3) An enzyme preparation from *Penicillum ochro-chloron*, kindly provided by Dr. Elwyn Reese, containing only β-mannosidase and β-glucosidase activity (Reese & Shibata, 1965), quantitatively hydrolysed these oligosaccharides in 1h at 40°C. This indicates that the sugars are joined by β-linkages.

One of the trisaccharides expected to result from partial acetolysis of glucomannan, mannosylmannosylglucose, has been shown to have a chromatographic mobility in solvent I slightly less than that of cellobiose (Perila & Bishop, 1961). Therefore the peak with $R_{cellobiose}$ 0.85 (solvent I) obtained from the partial acetolysate was eluted from the chromatogram and subjected to further analysis. The radioactive trisaccharide was purified by paper chromatography in solvent IV with $R_{cellobiose}$ 0.86. Reduction of the [^{14}C]oligosaccharide, followed by acid hydrolysis and chromatographic separation of the products, resulted in the isolation of two products, [^{14}C]glucitol and [^{14}C]mannose. The [^{14}C]mannose/[^{14}C]glucitol radioactivity ratio was 0.98 and 1.01, in two separate determinations. The expected radioactivity ratio for [^{14}C]mannosylmannosylglucose, calculated from the specific radioactivities of the substrates, is 0.63. The radioactivity found in the [^{14}C]glucose moiety is only 62% of that expected for [^{14}C]mannosylmannosylglucose. However, the radioactivity in the [^{14}C]glucose moiety of [^{14}C]glucosylmannose, reported in the preceding paragraph, was also only 62% of that expected from the relative specific radioactivities of the substrates. An enzyme preparation containing β-mannosidase and β-glucosidase enzyme activity (Reese & Shibata, 1965), supplied by Dr. Elwyn Reese, catalysed the quantitative hydrolysis of this trisaccharide in 1h at 40°C. This indicates that the glycosidic linkages joining the sugar moieties of this trisaccharide are β-linkages.

There are eight possible combinations of glucose and mannose in a β-(1→4)-linked trisaccharide, including glucotriose and mannotriose. One of these, β-(1→4)-linked [^{14}C]mannosylmannosylglucose (reported in the preceding paragraph), was easily isolated because of its relatively rapid mobility on paper chromatography in solvents I and IV. The other possible trisaccharides proved difficult to obtain in pure form, and consequently a complete structure analysis on each was not attempted. However, indications that at least most of the seven remaining possibilities are present in the partial acetolysate of the [^{14}C]glucomannan are given in Fig. 4. This chromatogram, developed in solvent IV for 11 days at room temperature, represents a partial separation of the material eluted from the trisaccharide region of a paper chromatogram developed in solvent I. At least five, and possibly six, components are evident in the chromatographic spectrum of these [^{14}C]trisaccharides. Since [^{14}C]cellobiose was present in only minor quantities, we did not expect to find a detectable amount of [^{14}C]cellotriose. The two major areas of radioactivity were eluted separately, reduced with $NaBH_4$ and hydrolysed with acid. The products, from each area, were [^{14}C]glucitol, [^{14}C]glucose, [^{14}C]-mannitol and [^{14}C]mannose. This indicates that each major area consists of at least two different oligosaccharides of [^{14}C]glucose and [^{14}C]mannose. The ratios of radioactivity found in the four products of reduction and hydrolysis were considerably different for the two major areas of radioactivity, and this confirms that there is a different mixture of [^{14}C]-oligosaccharides present in each of the two major peaks. Enzymic-hydrolysis studies (with enzymes described by Reese & Shibata, 1965) on the mixture indicate that all the glycosidic linkages in the mixture of oligosaccharides are β-linkages. Periodate oxidation of the chromatographically immobile (solvent I) radioactivity produced [^{14}C]erythritol and [^{14}C]-glycerol, the oxidation products expected (Hay *et al.*, 1965) of (1→4)-glycosidic linkages joining hexoses.

Accurate determination of the relative quantities of the [^{14}C]oligosaccharides present in the acetolysate was impossible. However, whenever [^{14}C]glucomannan was synthesized [^{14}C]mannobiosylglucose, [^{14}C]-glucosylmannose and [^{14}C]mannosylmannose were present in the partial acetolysate in approximately equivalent amounts, and together constituted a major portion of the oligosaccharides. In contrast, [^{14}C]-cellobiose was a very minor constituent of the partial

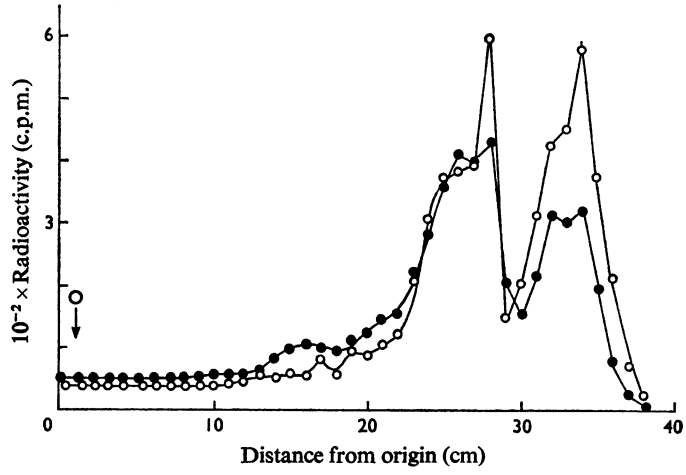

Fig. 4. *Chromatography of trisaccharides obtained from partial acetolysis*

The trisaccharides were chromatographed in solvent IV for 11 days on Whatman no. 1 paper. ● and o, Oligosaccharides obtained from [^{14}C]polysaccharide synthesized from a 1:2 and a 1:5 molar ratio of GDP-α-D-[^{14}C]glucose to GDP-α-D-[^{14}C]mannose respectively. The arrow indicates the origin (O).

acetolysate whenever [^{14}C]glucomannan was synthesized, and [^{14}C]cellotriose could not be detected. There was no indication of substantial glucose→glucose linkages in the polysaccharide material under conditions when the ratio of the glucosyltransferase activity to the mannosyltransferase activity would lead one to predict that one-third to one-half of the linkages in the [^{14}C]polysaccharide material would be glucose→glucose linkages.

Properties of the enzyme preparation

We found that diluting the Triton-solubilized-enzyme preparation with the aqueous solution in which it was dissolved produced a non-linear decrease in enzyme activity/unit volume (Fig. 5). Dilution to decrease the mannosyltransferase concentration to 70% results in an enzyme with 69% of the original activity/unit volume of preparation. In contrast, dilution to decrease the mannosyltransferase concentration to 50% results in an enzyme with only 15.6% of the original activity/unit volume of preparation. The curve produced by plotting glucosyltransferase activity as a function of dilution was much flatter than that for the mannosyltransferase activity (Fig. 5). However, when glucosyltransferase activity was measured in the presence of GDP-α-D-mannose, the same relationship produced a curve with the same general shape as that for the mannosyltransferase activity. But the dilution that produced half-maximum activity for glucosyltransferase activity in the presence of GDP-α-D-mannose (about 54% dilution) was less that that for mannosyltransferase activity (about 63% dilution), and was very close to the comparable value for glucosyltransferase activity in the absence of GDP-α-D-mannose (about 53% dilution).

Passage of the soluble-enzyme preparation through a Bio-Gel A 15 m column decreased the activity of both the glucosyltransferase and the mannosyltransferase enzymes. However, the mannosyltransferase enzyme was inactivated to a much greater extent, with only traces of activity detectable. The only glycosyltransferase activity observed in the fractions obtained from the column was a single peak occurring near the total elution volume of the column. This peak contained all the glucosyltransferase activity and the traces of mannosyltransferase activity remaining. Consequently, a small amount of glucosyltransferase activity was obtained almost free of mannosyltransferase activity. This purified enzyme fraction was assayed for glucosyltransferase activity in the presence and absence of GDP-α-D-mannose (Table 1) and no stimulation was observed as a result of the presence of GDP-α-D-mannose.

As noted previously (Heller & Villemez, 1972), digitonin treatment of the *Phaseolus aureus* particulate enzyme, under the conditions of Liu & Hassid (1970), results in the solubilization of only trace amounts of mannosyltransferase enzyme activity. In contrast, the amount of glucosyltransferase activity solubilized by the digitonin treatment is comparable

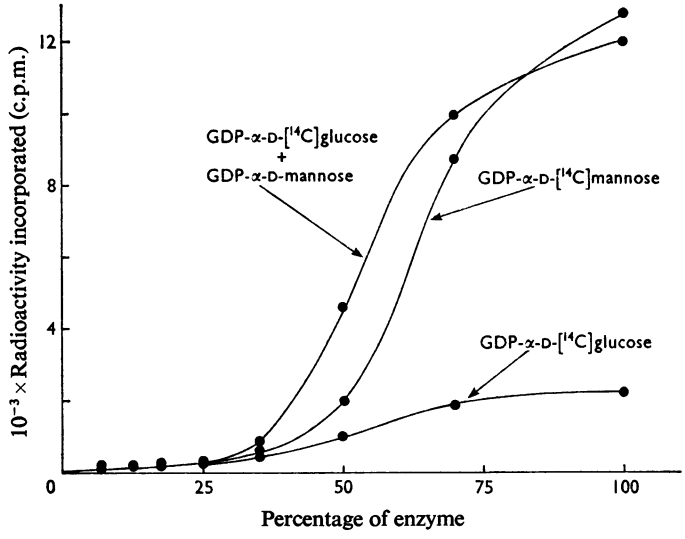

Fig. 5. *Glucosyl- and mannosyl-transferase activity as a function of enzyme concentration*

The soluble enzyme was diluted with increasing amounts of 0.05 M-potassium phosphate buffer (pH 7.2) containing 0.01 M-$MgCl_2$, 0.5% Triton X-100, 0.4 M-sucrose, 1% bovine serum albumin and 5 mM-dithiothreitol so that the abscissa represents the percentage of enzyme in the reaction mixture. The diluted enzyme was treated with GDP-α-D-[^{14}C]glucose (34000 c.p.m., 0.13 nmol), GDP-α-D-[^{14}C]mannose (46000 c.p.m., 0.57 nmol) or GDP-α-D-[^{14}C]glucose (34000 c.p.m., 0.13 nmol) and GDP-α-D-mannose (1.0 nmol). Reaction conditions and analysis are described in the text.

Table 1. *Fractions from a Bio-Gel A 15 m column containing glycosyltransferase activity*

Soluble-enzyme preparation was chromatographed on a Bio-Gel A-15 m column (1 cm × 20 cm) equilibrated with the resuspension buffer (see the text) containing 0.5% Triton X-100. Fractions (1 ml) were collected at a flow rate of 20 ml/h, and 50 μl of each fraction was tested with GDP-α-D-[^{14}C]glucose (32000 c.p.m., 0.12 nmol), GDP-α-D-[^{14}C]mannose (46000 c.p.m., 0.57 nmol), or GDP-α-D-[^{14}C]glucose (32000 c.p.m., 0.12 nmol) and GDP-α-D-mannose (1.0 nmol). The products were analysed as described in the Materials and Methods section. Only those fractions that contained enzymic activity are presented.

	Radioactivity incorporation into [^{14}C]polysaccharide (c.p.m.)		
Fraction no.	From GDP-[^{14}C]mannose	From GDP-[^{14}C]glucose	From GDP-[^{14}C]glucose +GDP-mannose
10	8	14	8
11	7	25	32
12	10	78	80
13	29	143	153
14	27	122	124
15	21	74	84
16	11	37	40

with that solubilized by Triton X-100 (Fig. 6). This provided another enzyme preparation with which to determine the effects of GDP-α-D-mannose on the glucosyltransferase activity in the near-absence of mannosyltransferase activity. No stimulation of glucosyltransferase activity was observed when GDP-α-D-mannose was added to this enzyme preparation. Rather, GDP-α-D-mannose has the effect expected

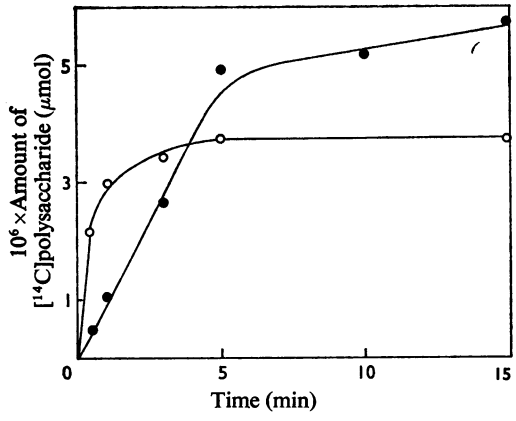

Fig. 6. *Glucosyltransferase activity in Triton X-100-solubilized- and digitonin-solubilized-enzyme preparations*

GDP-α-D-[^{14}C]glucose (44000c.p.m., 0.17nmol) was added to each sample. The reaction temperature was 24°C, and other conditions are described in the text. ●, Digitonin-solubilized enzyme; ○, Triton X-100-solubilized enzyme. The enzyme activity represents that derived, by each method, from a comparable quantity of plant material.

of a competitive inhibitor with a K_i much greater than the K_m of the glucosyltransferase enzyme. When GDP-α-D-mannose and GDP-α-D-[^{14}C]glucose were present in a ratio of 18:1, the glucosyltransferase activity was decreased to 55% of the control rate. The glucosyltransferase activity that should result from adding this relative quantity of GDP-α-D-mannose was calculated by using the kinetic parameters determined for the *Phaseolus aureus* particulate glucosyltransferase enzyme (Villemez, 1971). The calculation indicates that this relative concentration of GDP-α-D-mannose should decrease glucosyltransferase activity to 52.5% of the control rate, which agrees well with the observed value reported above.

As noted above, characterization of the [^{14}C]-glucomannan formed by the soluble-enzyme preparation revealed that only small amounts of [^{14}C]-cellobiose could be detected in the partial-acetolysis products. Assuming that the glucosyltransferase activity which produces [^{14}C]glucan (in the absence of GDP-D-mannose) is expressed similarly in the presence of GDP-D-mannose, considerably more [^{14}C]cellobiose should have been evident. To obtain further information on this matter, the [^{14}C]polysaccharide material formed when GDP-D-[^{14}C]-glucose and GDP-D-[^{14}C]mannose were both present in the reaction mixture was examined by solubilization and enzymic-hydrolysis experiments. For these experiments, the [^{14}C]mannan (formed from GDP-D-[^{14}C]mannose alone) and [^{14}C]glucan (formed from GDP-D-[^{14}C]glucose alone) were used as comparison standards. The solubilization comparison was performed by using a borate-containing alkaline solution, which has been reported to facilitate the solubilization of mannose-containing polysaccharides (Timell, 1965). The enzymic-hydrolysis comparison was performed by using a *Bacillus subtilis* mannanase preparation (Reese & Shibata, 1965) supplied by Dr. Elwyn Reese. Two alkali-insoluble [^{14}C]polysaccharide preparations made by using both nucleotide substrates were examined, i.e. that formed in 2min and that formed in 10min at 25°C. Assuming that the glucosyltransferase activity is expressed in the same manner in the presence and in the absence of GDP-D-mannose, the [^{14}C]polysaccharide formed in 2min should be more than half [^{14}C]glucan, whereas after 10min of reaction only about 15% [^{14}C]glucan is to be expected (see Fig. 6, and also Heller & Villemez, 1972). The [^{14}C]mannan is much more rapidly solubilized than is the [^{14}C]glucan (Fig. 7). In 2h of extraction, approx. 87% of the [^{14}C]mannan was dissolved, as compared with approx. 62% of the [^{14}C]-glucan. The solubilization pattern of the [^{14}C]-polysaccharide material formed in 2min with both substrates is extremely similar to that of the [^{14}C]-mannan. The [^{14}C]polysaccharide material formed in 10min with both substrates is somewhat more difficult to solubilize than the [^{14}C]mannan, but not nearly as resistant to solubilization as the [^{14}C]glucan.

The results of catalysed hydrolysis with a β-mannanase, as measured by the appearance of soluble radioactivity, paralleled those of the solubilization experiment. The [^{14}C]mannan was hydrolysed much more rapidly than the [^{14}C]glucan. The hydrolytic pattern for the [^{14}C]polysaccharide material synthesized in 2min was very close to that of the [^{14}C]-mannan. Also, the [^{14}C]polysaccharide material synthesized in 10min was hydrolysed at a somewhat slower rate than the [^{14}C]mannan, but faster than the [^{14}C]glucan. For example, the amount of water- and alkali-soluble radioactivity produced after 43h of enzyme treatment was 48.9% for the [^{14}C]mannan, 48.7% from the [^{14}C]polysaccharide material synthesized in 2min, 38.8% from the [^{14}C]polysaccharide material synthesized in 10min, and 28.7% from the [^{14}C]glucan.

Discussion

An earlier report (Heller & Villemez, 1972) demonstrated that the Triton X-100-solubilized-enzyme preparation, which is the subject of the present paper, catalysed the synthesis of a β-(1→4)-linked [^{14}C]-mannan from GDP-α-D-[^{14}C]mannose. The results in

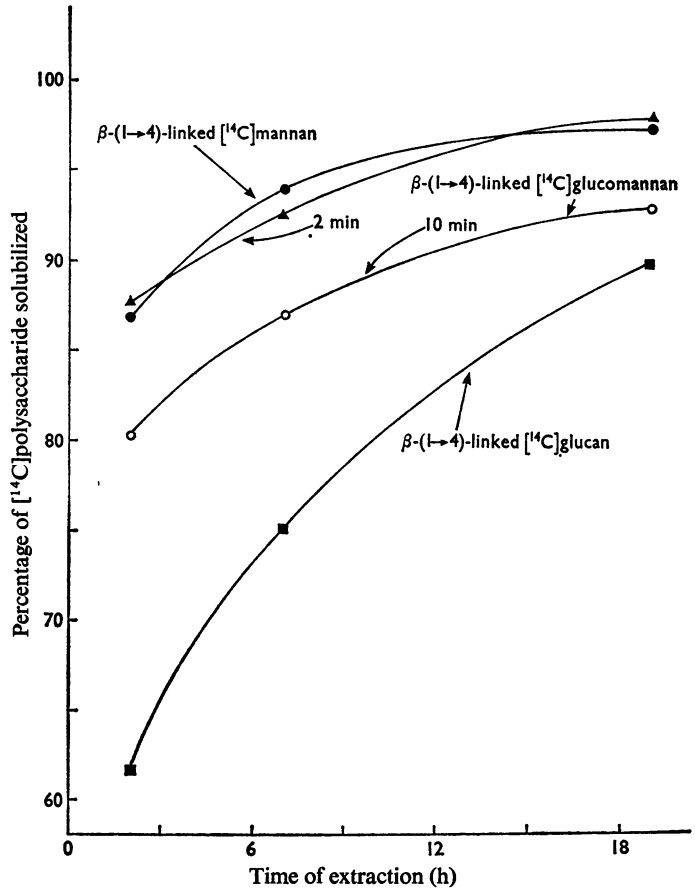

Fig. 7. *Fractional extraction of* [^{14}C]*polysaccharides*

The [^{14}C]mannan (●) and [^{14}C]glucan (■) were prepared by using Triton X-100-solubilized enzyme and either GDP-α-D-[^{14}C]mannose or GDP-α-D-[^{14}C]glucose as substrates. Reaction temperature was 23°C, reaction time 10min, and the other reaction conditions are stated in the text. The [^{14}C]glucomannan samples were synthesized in exactly the same manner by using both GDP-α-D-[^{14}C]mannose and GDP-α-D-[^{14}C]glucose in a molar ratio of 5:1. Two samples of [^{14}C]glucomannan were tested; one synthesized in 2min (▲) and the other in 10min (○) of reaction. The [^{14}C]polysaccharides were isolated as described in the text, all samples being extracted with 2% NaOH at 100°C as a final step. The [^{14}C]polysaccharide was stirred with 1ml of 20% NaOH–4% boric acid solution at 23°C for the time indicated. The quantity of [^{14}C]polysaccharide in solution was determined by liquid-scintillation counting.

the present paper demonstrate that this same solubilized-enzyme preparation catalyses the formation of a β-(1→4)-linked [^{14}C]glucan from GDP-α-D-[^{14}C]-glucose, and the formation of a β-(1→4)-linked [^{14}C]glucomannan when both radioactive substrates are present. Characterization of the [^{14}C]polysaccharides was accomplished by analysis of partial acetolysis products by using periodate oxidation to determine the linkage position (Hay *et al.*, 1965), and stereospecific enzymes to determine the linkage configuration (Reese & Shibata, 1965). There were only two unexpected observations made during the characterization of the [^{14}C]polysaccharides. First, from the partial acetolysis of the [^{14}C]glucomannan no trace of [^{14}C]mannosylglucose was observed. The only disaccharides detected were, primarily, [^{14}C]glucosylmannose, [^{14}C]mannosylmannose and, in lesser quantity, [^{14}C]glucosylglucose. That β-(1→4)-mannose → glucose linkages occur in the [^{14}C]polysaccharide is demonstrated by the isolation of

[^{14}C]mannosylmannosylglucose in relatively plentiful quantities. Although the reasons are not apparent from the available results, it seems that the conditions of partial acetolysis are unfavourable to the production of the mannosylglucose disaccharide. The second unexpected observation was a disparity in the specific-radioactivity ratio between [^{14}C]glucose and [^{14}C]-mannose in the polysaccharide. The quantity of [^{14}C]polysaccharide available makes a direct determination of specific radioactivity impossible. However, the isolation of [^{14}C]glucosylmannose and [^{14}C]mannosylmannosylglucose in pure form allows a comparison between the specific radioactivities of the different sugar moieties, since the molar ratios are known. In both cases, the radioactivity in the [^{14}C]-glucose moiety was only 63% of that expected (relative to the [^{14}C]mannose moieties) from a consideration of the specific radioactivities of the substrate sugar nucleotides. If the radioactivity dilution were always found to occur in the reducing sugars of the oligosaccharides, it could be explained as resulting from the non-radioactive sugar moieties in the acceptor molecule, assuming that only two or three radioactive sugars were added to the non-reducing end of a long-chain acceptor polysaccharide. However, this explanation is untenable, since the [^{14}C]-glucose moieties of both oligosaccharides are diluted to the same extent (relative to the [^{14}C]mannose moieties), and [^{14}C]glucose occupies the reducing position in one and the non-reducing position in the other. Therefore it seems that non-radioactive sugars present in the enzyme preparation contribute to the growing chain of the [^{14}C]polysaccharide being formed, with more glucose positions being derived from this source than mannose positions. Knowing the nature of the material which serves as the donor of non-radioactive sugar could be of importance in understanding the mechanism of polymerization, but that information will have to wait for further experiments.

It seems likely that the GDP-α-D-glucose glucosyltransferase enzyme activity that is responsible for the production of [^{14}C]glucan is the same enzyme activity that catalyses the glucosyl-transfer reactions involved in [^{14}C]glucomannan production. The evidence which indicates this is as follows. (1) A kinetic study (Villemez, 1971) indicated that there was only one type of GDP-α-D-glucose transferase activity in *Phaseolus aureus* particulate-enzyme preparations. This Triton X-100-solubilized enzyme was derived from that particulate-enzyme preparation. It seems unlikely that a different enzyme activity would be generated in the solubilization process. (2) The quantity of [^{14}C]glucan that should be present, if it were being synthesized simultaneously with [^{14}C]glucomannan, is not found. A complete separation of [^{14}C]glucan and [^{14}C]glucomannan has not been achieved. But there are three types of experiment which indicate that [^{14}C]glucan formation is, at the minimum, decreased considerably when [^{14}C]glucomannan is being formed: (a) the quantity of [^{14}C]cellobiose found in the partial acetolysis products from [^{14}C]-polysaccharide synthesized with both substrates is much less than that expected from considerations of glucosyltransferase activity in the enzyme preparation (see the Results section); (b) fractional extraction of the insoluble [^{14}C]polysaccharide synthesized with both substrates indicates that most, if not all, of the [^{14}C]polysaccharide material contains substantial quantities of mannose (Fig. 7); (c) enzymic-hydrolysis studies with β-mannanase give the same indication, that most, if not all, of the [^{14}C]polysaccharide synthesized with both substrates contains substantial quantities of mannose (see the Results section). (3) The glucosyltransferase enzyme activity is not affected, directly, to any large extent by the concentrations of GDP-α-D-mannose used. Kinetic studies (Villemez, 1971) demonstrated that GDP-α-D-mannose was a relatively weak ($K_i = 10K_m$) competitive inhibitor of the glucosyltransferase enzyme activity. This lack of large effect is confirmed with two glucosyltransferase preparations relatively free of mannosyltransferase enzyme activity (Table 1; also see the Results section). Therefore the effect on glucosyltransferase enzyme activity produced by adding GDP-α-D-mannose results from a product of mannosyltransferase activity and not from the sugar nucleotide itself. This is supported by results presented previously (Villemez, 1971), and by the experiments presented in Fig. 5.

It also seems possible that the soluble GDP-α-D-mannose mannosyltransferase enzyme activity that is responsible for the production of [^{14}C]mannan, is the same enzyme activity that catalyses the mannosyl-transfer reactions involved in [^{14}C]glucomannan production. However, there is not as much evidence supporting this possibility. Kinetic studies of the soluble mannosyltransferase enzyme activity (Heller & Villemez, 1972) produce reciprocal plots, in the presence of GDP-α-D-glucose, that exhibit no anomalies and indicate that GDP-α-D-glucose is a strong competitive inhibitor of the mannosyltransferase enzyme activity. In contrast, kinetic studies on the particulate mannosyltransferase enzyme activity (Villemez, 1971) indicate the presence of more than one enzyme system. Further studies are necessary before this matter can be resolved.

The enzymic properties that promote the synthesis of [^{14}C]glucomannan, rather than [^{14}C]mannan plus [^{14}C]glucan, when both [^{14}C]sugar nucleotides are present appear to be: (1) the ability of the glucosyl- and mannosyl-transferases to use the same type of acceptor molecule; (2) the requirement for the presence of a mannose-containing acceptor for maintenance of glucosyltransferase enzyme activity; (3) the direct modulation of mannosyltransferase

enzyme activity in the presence of GDP-α-D-glucose, since it is a strong competitive inhibitor; (4) the relatively small direct effect of GDP-α-D-mannose, a weak competitive inhibitor, on glucosyltransferase enzyme activity.

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