The Formation of a β -(1 $\rightarrow$ 4)-D-Galactan Chain Catalysed by a *Phaseolus aureus* Enzyme

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With a particulate enzyme preparation from *Phaseolus aureus* hypocotyls, UDP- α -D-[U- 14 C]galactose served as a precursor for a number of products. One of these products was characterized as a β -(1 $\rightarrow$ 4)-linked galactan. The ADP-, GDP-, TDP- and CDP-derivatives of α -D-galactose did not serve as biosynthetic precursors for any products insoluble in 70% ethanol, nor as substrates for a sugar nucleotide 4-epimerase which is present in the particulate enzyme preparation. The 14 C-labelled β -(1 $\rightarrow$ 4)-galactan is alkali-insoluble and was characterized by analysis of partial acetolysis products. The labelling pattern of the [14 C]oligosaccharides derived from acetolysis indicates that (1) only slightly more than two [14 C]galactose moieties are added to the growing polysaccharide chain on average, and (2) these additions take place at the reducing end of the polysaccharide chain. The radioactive β -(1 $\rightarrow$ 4)-linked galactan chain represented 8.5% of the radioactivity initially added, and 20% of the water- and butanol-insoluble products derived from UDP- α -D-[14 C]galactose. Total hydrolysis of the alkali-insoluble fraction of *Phaseolus aureus* hypocotyl yielded D-glucose and D-mannose in a 5:1 ratio but no detectable quantities of D-galactose. A trace quantity of a radioactive disaccharide, identified as (1 $\rightarrow$ 3)-linked galactobiose, was isolated from the partial acetolysate of the alkali-insoluble [14 C]polysaccharide material. Also isolated from this partial acetolysate was a C-1 derivative of [14 C]galactose, which could not be identified. An alkali-soluble galactose-containing polysaccharide was also synthesized in this enzymic reaction, and represented 20% of the water- and butanol-insoluble products derived from UDP- α -D-[14 C]galactose. The spectrum of radioactive oligosaccharides produced by partial acetolysis of this alkali-soluble polysaccharide material was different from that obtained from the alkali-insoluble polysaccharide, indicating a different structure.

Galactose-containing polysaccharides are significant components of the plant cell-wall (Aspinall, 1970). In one model of cell-wall structure, a β -(1 $\rightarrow$ 3)-linked galactan backbone was proposed as the connecting structure joining the wall protein extensin and wall cellulose microfibrils (Lampert, 1970). A much more detailed model of sycamore primary cell wall, with considerable supporting data, was proposed recently (Talmadge *et al.*, 1973; Bauer *et al.*, 1973; Keegstra *et al.*, 1973). In this latter model β -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-linked galactan backbones serve to cross-link the polysaccharide and protein components of the wall.

Very little information is available about the biosynthesis of galactose-containing polysaccharides in higher plants. Using a particulate enzyme system from *Phaseolus aureus*, McNab *et al.* (1968) demonstrated the synthesis of a water-soluble galactan. However, the product was not characterized and sugar nucleo-

tide specificity was not investigated. The present report provides evidence that UDP- α -D-galactose is the sugar donor for the synthesis of at least two galactose-containing polysaccharides in *Phaseolus aureus* hypocotyls. One of these polysaccharides has been characterized as a β -(1 $\rightarrow$ 4)-linked D-galactan.

Materials and Methods

Preparation of radioactive substrates

Uniformly labelled D-[14 C]galactose was obtained through the courtesy of Dr. D. McBrien and Dr. S. Abraham. The specific radioactivity was 127 μ Ci/ μ mol, and it was prepared by a modification of the method of Bean *et al.* (1953). From this radioactive galactose, α -D-[U- 14 C]galactose 1-phosphate was obtained by reaction with ATP and galactokinase isolated from galactose-adapted yeast (Trucco *et al.*, 1948). The freeze-dried preparation of *Saccharomyces fragilis* (galactose-adapted), from which the galactokinase was isolated, was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

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To prepare ADP- α -D-[U- 14 C]galactose, GDP- α -D-[U- 14 C]galactose, CDP- α -D-[U- 14 C]galactose, TDP- α -D-[U- 14 C]galactose and UDP- α -D-[U- 14 C]galactose, the micro-scale method of Elbein (1966) was employed. The reaction involves a condensation between α -D-galactose 1-phosphate and the morpholine derivative of the respective nucleoside monophosphate. Morpholides of UMP, CMP, GMP, TMP and AMP were purchased from Calbiochem, La Jolla, Calif., U.S.A. The α -D-[U- 14 C]galactose 1-phosphate employed had a specific radioactivity of $4\mu\text{Ci}/\mu\text{mol}$, and was prepared by mixing the α -D-[U- 14 C]galactose 1-phosphate ($127\mu\text{Ci}/\mu\text{mol}$), described above, with the appropriate amount of α -D-galactose 1-phosphate (Sigma). These yields of radioactive sugar nucleotides were generally 3–13%, and the chromatographic mobilities in solvent *f* relative to UDP- α -D-galactose (Sigma) were: 0.56 for the guanine derivative, 0.73 for the adenosine and cytidine derivatives, 1.00 for the uridine derivative and 1.42 for the thymidine derivative. Under these conditions galactose 1-phosphate had a mobility 2.15 times, and D-galactose 4.50 times, that of UDP- α -D-galactose.

For the experiments other than the specificity studies, UDP- α -D-[U- 14 C]galactose of specific radioactivity $127\mu\text{Ci}/\mu\text{mol}$ was used. This was synthesized by a pyrophosphorylase-catalysed reaction between UTP (Sigma) and α -D-[U- 14 C]galactose 1-phosphate (sp. radioactivity $127\mu\text{Ci}/\mu\text{mol}$; prepared as described above). The pyrophosphorylase enzyme preparation was obtained from *Phaseolus aureus* by the method of Feingold *et al.* (1958b). Acid hydrolysis of the purified product gave [U- 14 C]-galactose as the only radioactive product.

Enzyme preparation and galactose transferase specificity reactions

The particulate enzyme preparation was obtained by the procedure described by Villemez & Clark (1969). The enzyme activity was very stable when stored at -20°C , and no difference in activity or specificity was observed after freezing or after storage for 1 week. For the specificity study several experiments were performed. Comparable amounts of each of the five sugar nucleotides were used in each experiment, but between experiments ranges in quantity of 1–2nmol (4050–8100c.p.m.) were used. For each sample a suitable quantity of one of the five radioactive galactose nucleotides and 0.1ml of a fresh preparation of particulate enzyme suspension [enzyme suspended in 0.05M-potassium phosphate buffer, pH 7.3, containing 0.01M-MgCl₂, 0.4M-sucrose and 1% (w/v) bovine albumin] containing approx. 1mg of plant protein were combined to produce a final volume of 0.2ml. The reaction mixtures were incubated for 30min at 25°C , then chilled in ice and 0.1ml of aq. 15% (w/v) trichloroacetic

acid was added to stop the reaction. Approx. 10mg of powdered cellulose (Sigma) or laminarin (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.) was added to provide a carrier, 1ml of water was added and the insoluble material was sedimented by centrifugation at 12000g for 10min. The supernatant solution was collected and the insoluble material extracted twice more with 0.7ml portions of water in the same manner. The water extracts were combined, neutralized by the addition of $9.2\mu\text{l}$ of 10M-NaOH, 10mg of powdered cellulose was added as a carrier, and ethanol was added to a final concentration of 70% (v/v). After 60min incubation at 40°C the 70%-ethanol-insoluble material was collected by centrifugation for 15min at 49000g and washed twice with aq. 70% (v/v) ethanol. The radioactivity in the ethanol-soluble and -insoluble fractions was determined by liquid-scintillation counting as described previously (Villemez & Clark, 1969).

The epimerase specificity reactions were carried out as described above with minor variations as noted below. In a typical experiment, approx. 1.2nmol (4700c.p.m.) of the radioactive sugar nucleotides was used, and the reactions were terminated by heating for 5min in boiling water. The reaction mixtures were extracted with water as described above, and the water-soluble material was hydrolysed with 1.8M-HCl at 100°C for 30min. The hydrolysate was subjected to chromatography in solvent *b*, and the areas corresponding to D-glucose and D-galactose were assayed for radioactivity by liquid-scintillation counting.

Preparation of [14 C]galactan

A reaction mixture consisting of 9×10^6 c.p.m. of UDP- α -D-[14 C]galactose ($127\mu\text{Ci}/\mu\text{mol}$; see above) and 0.3ml of the particulate enzyme suspension, in a final volume of 0.6ml, was incubated for 30min at 26°C . The reaction was stopped and the insoluble material extracted with water as described above. The water-insoluble pellet was suspended in butanol-1-ol by sonic oscillation and the insoluble material was collected by centrifugation. This extraction was repeated twice more. The water- and butanol-insoluble material was then extracted twice with 1ml portions of an aq. 45% (w/v) phenol emulsion and twice with 1ml portions of aq. 2% (w/v) NaOH at 100°C for 5min each. The insoluble material was washed with 1ml portions of water four times, and dried by repeated additions and evaporations of ethanol. This alkali-insoluble residue contained the [14 C]galactan, which was characterized as described below.

Partial acetolysis of the [14 C]galactan

Partial acetolysis was accomplished by using the conditions of Scher & Lennarz (1969), and allowing

the reaction to proceed for 62 h. Deacylation of the acetylated oligosaccharides was done by the procedure of Steward *et al.* (1968). For the reduction of the oligosaccharides, the procedure of Scher & Lennarz (1969) using NaBH_4 was followed, but the addition of acetone was omitted.

Periodate oxidation of oligosaccharides

In a modification of the procedure described by Elbein (1969), the ^{14}C -labelled oligosaccharide, with $2\mu\text{mol}$ of lactose as carrier, was placed in 1 ml of unbuffered 0.05 M-sodium metaperiodate and allowed to react at 5°C for 80–92 h. Reduction then took place after the addition of 1 ml of 0.1 M- NaBH_4 followed by the addition of a second 1 ml portion 30 min after the first. After 2 h at room temperature, the reaction mixture was passed through a column of Dowex 50-W (H^+ form) to destroy the excess of NaBH_4 and to remove cations. After removal of boric acid by repeated additions and evaporation of methanol, the mixture was hydrolysed with 2 M-HCl at 100°C for 2 h. After hydrolysis the solution was evaporated to dryness under reduced pressure, over solid KOH. Analysis of the non-volatile oxidation products was performed by paper chromatography with solvent *h*, followed by paper electrophoresis in buffer *e*.

Chromatography

Descending paper chromatography was performed on Whatman no. 1, 4, 40 or 3 MM paper with one of the following solvent systems (all by vol.): (a) ethyl acetate–propanol–water (1:7:2), (b) ethyl acetate–pyridine–water (10:4:3), (c) ethyl acetate–acetic acid–formic acid–water (18:3:1:4), (d) ethyl acetate–propanol–water (57:32:13), (e) propan-2-ol–water (4:1), (f) ethanol–1 M-ammonium acetate, pH 3.8 (5:2), (g) butan-2-one–acetic acid–water (9:1:1) saturated with boric acid, (h) butanol–pyridine–0.1 M-HCl (5:3:2). Paper electrophoresis was performed on Whatman 54 or 3 MM paper and with one of the following buffers: (i) 0.2 M-ammonium formate, pH 3.7, (j) 0.25 M-ammonium acetate, pH 3.6, (k) 0.05 M-sodium borate, pH 9.2, (l) 0.1 M-sodium molybdate adjusted to pH 5 with H_2SO_4 . The compounds were eluted from the paper with water containing 5% (v/v) ethanol. If necessary, before elution, the fluors used in liquid-scintillation counting were washed off with diethyl ether. Sugars were detected with the AgNO_3 reagent (Trevelyan *et al.*, 1950). Nucleotides were detected with u.v. light. Radioactive bands were located either by cutting the paper into 1 cm strips and counting them by liquid-scintillation counting, or by radioautography on Kodak NS-54T X-ray film (Eastman, Rochester, N.Y., U.S.A.). All liquid-scintillation counting was done with a Packard Tri-Carb model 3375 spectro-

meter, equipped with automatic external standardization.

Results

With this particulate enzyme from *Phaseolus aureus* hypocotyls, the only nucleoside diphospho derivative of galactose that shows significant biological activity is UDP- α -D-galactose (Table 1). The comparable adenosine, cytidine, guanosine and thymidine derivatives do not serve as substrates for the formation of polysaccharides, nor do they serve as substrates for the sugar nucleotide 4-epimerase also present in the particulate enzyme preparation. No water-soluble polysaccharide is synthesized by using any of the galactose nucleotides. After reaction with UDP- α -D- ^{14}C galactose, both electrophoretically mobile (in buffer *j*) and immobile radioactive material can be isolated from the water-soluble material. The only electrophoretically mobile components that could be detected on paper chromatography in solvent *f* were two with chromatographic mobilities identical with those of α -D-galactose 1-phosphate and UDP- α -D-galactose (it should be noted that the nucleotide and 1-phosphate derivatives of glucose and galactose are not separated by chromatography in this buffer). On chromatography of the electrophoretically immobile material in solvent *b* 63% of the radioactivity migrated with D-glucose and D-galactose, and 37% migrated with sucrose. Sucrose synthetase activity has been demonstrated in *Phaseolus aureus* extracts (Delmer & Albersheim, 1970).

Acid hydrolysis of the UDP- α -D-[U- ^{14}C]galactose used as substrate followed by chromatography in solvent *b* revealed no traces of D- ^{14}C glucose. All of the radioactivity was present as D- ^{14}C galactose. However, after reaction with the *Phaseolus aureus* enzyme, similar treatment of the entire reaction mixture resulted in about 16% as D- ^{14}C glucose and 84% as D- ^{14}C galactose. The distribution of D- ^{14}C glucose in the various fractions of the reaction mixture varies considerably. The water-soluble fraction has 17% of the radioactivity as D- ^{14}C glucose. However, the alkali-insoluble fraction has 25% as D- ^{14}C glucose, whereas the alkali-soluble ^{14}C polysaccharide material is only 5% D- ^{14}C glucose, and the water-soluble polysaccharide rendered soluble by extraction with 45% phenol is 90% D- ^{14}C glucose.

Partial acetolysis of alkali-soluble and alkali-insoluble polysaccharides

The oligosaccharide patterns produced by chromatography in solvent *b* of the partial acetolysates are given in Fig. 1. Similar oligosaccharide patterns are produced by partial acid hydrolysis, but the yields are lower. On inspection, it is obvious that the ^{14}C polysaccharide, or mixtures of ^{14}C polysaccharides,

Table 1. Sugar nucleotide specificity of galactosyltransferase and sugar nucleotide 4-epimerase enzyme activity

The enzyme suspension was prepared, made to react with substrate, and the products were analysed as described in the Materials and Methods section. For this experiment approx. 1.2nmol (4700c.p.m.) of each substrate, and 0.1 ml of enzyme suspension (approx. 1 mg of plant protein) in a final volume of 0.2 ml was allowed to react at 25°C for 30 min. To analyse for water-soluble [¹⁴C]polysaccharide, the water extract was made 70% (v/v) with respect to ethanol and the precipitate counted for radioactivity by liquid-scintillation counting. To determine the proportion of [¹⁴C]glucose residues, the water-soluble material was acid-hydrolysed and chromatographed in solvent *b*; all radioactivity appeared as [¹⁴C]galactose or [¹⁴C]glucose after this treatment. Substrates were prepared as described in the Materials and Methods section. Water/butanol-insoluble radioactivity reflects galactosyltransferase enzyme activity. The percentage is of the total amount of radioactivity added. Water-soluble –70%-ethanol-insoluble radioactivity reflects any water-soluble [¹⁴C]polysaccharide formed. Proportion of [¹⁴C]glucose reflects sugar nucleotide 4-epimerase enzyme activity. The percentage is of the total amount of radioactivity present in the water-soluble fraction. The corresponding value for the water-insoluble fraction, with UDP-α-D-[U-¹⁴C]galactose as substrate, was 15.0%. There was essentially no water-insoluble radioactivity for the other four sugar nucleotides.

Substrate	Water/butanol-insoluble radioactivity (%)	Water-soluble-70%-ethanol-insoluble radioactivity (%)	Proportion of [¹⁴ C]glucose (%)
UDP-α-D-[U- ¹⁴ C]galactose	25.8	0	17.0
ADP-α-D-[U- ¹⁴ C]galactose	0.0	0	3.3
GDP-α-D-[U- ¹⁴ C]galactose	0.8	1.4	3.9
CDP-α-D-[U- ¹⁴ C]galactose	0.3	0	3.8
TDP-α-D-[U- ¹⁴ C]galactose	0.4	0	—

Fig. 1. Radioactive bands resulting from partial acetolysis of alkali-insoluble and alkali-soluble [¹⁴C]polysaccharide

Alkali-insoluble and alkali-soluble [¹⁴C]polysaccharides were prepared from UDP-α-D-[U-¹⁴C]galactose as described in the Materials and Methods section. The bands resulting from partial acetolysis (for conditions of reaction, see the Materials and Methods section) were made visible by radioautography. The chromatographic mobility (not relative intensity; see Table 2) in solvent *b* is indicated relative to the mobility of D-galactose. Abbreviations for standard compounds are: Lac, lactose; Gal, D-galactose; Glc, D-glucose; Cello, cellobiose. Total hydrolysis of the two [¹⁴C]polysaccharide fractions produce only D-[¹⁴C]galactose and D-[¹⁴C]glucose, of which D-[¹⁴C]glucose made up 5% in the alkali-soluble and 25% in the alkali-insoluble polysaccharides. For further details of the bands see Table 2 and the text.

are different in the two fractions. The oligosaccharide pattern produced by partial acetolysis of the alkali-soluble fraction is too complex to result from breakdown of a homopolysaccharide with a single type of linkage. Therefore characterization of the [¹⁴C]-

oligosaccharides resulting from the alkali-insoluble [¹⁴C]polysaccharide was attempted first. The quantitative distribution of the radioactive oligosaccharides from the alkali-insoluble [¹⁴C]polysaccharide is given in Table 2.

Characterization of [^{14}C]oligosaccharides derived from alkali-insoluble [^{14}C]polysaccharide

The [^{14}C]monosaccharides present in the partial acetolysate are represented by bands 8 and 7, which are D- ^{14}C]glucose and D- ^{14}C]galactose respectively (see Fig. 1 and Table 2). Band 6, on further examination as described in a section below, was found to contain only a trace of a typical oligosaccharide. This oligosaccharide appears to be a (1 $\rightarrow$ 3)-linked galactobiose. The remainder of the band-6 material is an unfamiliar D- ^{14}C]galactose derivative, which remains unidentified. However, bands 1–5 appeared to be a homologous oligosaccharide series (Fig. 2).

Characterization of band 5. On total acid hydrolysis and chromatography with solvent *b*, the only radioactive components observed were D- ^{14}C]glucose and D- ^{14}C]galactose, in a ratio of 1:3. Electrophoresis of the components of band 5 in buffer *k* revealed only two radioactive components, also present in a radioactivity ratio of 1:3. The minor component, with an electrophoretic mobility exactly that of cellobiose, produced only D- ^{14}C]glucose on acid hydrolysis. The major electrophoretic component produced only D- ^{14}C]galactose on acid hydrolysis. Chromatography in solvents *c* and *d* of the major band resulting from electrophoresis revealed only one component. Only this major D- ^{14}C]galactose-containing component was characterized further.

The purified D- ^{14}C]galactose-containing component of band 5 was reduced with NaBH_4 , then acid-

hydrolysed. Chromatography in solvent *g* indicated the presence of D- ^{14}C]galactose and D- ^{14}C]galactitol in a 1:1 ratio as the only radioactive products. Combined with the chromatographic mobility, this indicates that the major component of band 5 is a D-[U- ^{14}C]galactosyl-D-galactose.

The purified D- ^{14}C]galactose-containing component of band 5 was subjected to periodate oxidation, followed by reduction and acid hydrolysis. Chromatography in solvent *h* of the reaction products, which represented 50% of the initial radioactivity, resulted in only two radioactive components corresponding in mobility to D-threitol (37%) and glycerol (63%). The presence of D- ^{14}C]threitol indicates a (1 $\rightarrow$ 4)-glycosidic linkage, implying that the disaccharide is a (1 $\rightarrow$ 4)-linked D-[U- ^{14}C]galactosyl-D-galactose. The yield of [^{14}C]threitol is about 61% of the theoretical yield. This low yield is most likely due to the conditions under which the oxidation was performed, causing an overproduction of [^{14}C]glycerol at the expense of [^{14}C]threitol.

The major component of band 5 was mixed with D-galactosyl- β -(1 $\rightarrow$ 4)-D-galactose (provided by Professor T. E. Timell; see Gillham *et al.*, 1958) and

Table 2. *Distribution of radioactivity from chromatography of partial acetolysis products of alkali-insoluble [^{14}C]polysaccharide*

Procedure was as described in Fig. 1. The number assigned to each radioactive band is that depicted in Fig. 1; bands are numbered in sequence from the origin; see Fig. 1. The total radioactivity recovered as soluble oligosaccharides represents approx. 60% of the starting [^{14}C]polysaccharide. The results are rounded to the nearest hundred.

Band (no.)	Standards	Radioactivity recovered (c.p.m.)
0	Origin	63 500
1		32 800
2		16 700
3		21 500
4		16 000
5	Lactose	18 000
No visible band*	Cellobiose	11 700
6		34 000
7	D-Galactose	36 000
8	D-Glucose	6 800

* The area (3.8 cm wide) between bands 5 and 6.

Fig. 2. *Homologous oligosaccharide series plot*

R_m , i.e. $\log[(1 - R_F)/R_F]$, was calculated for oligosaccharides nos. 1–5 and 7 (D-galactose) obtained from partial acetolysis of alkali-insoluble [^{14}C]polysaccharide (see Fig. 1 for numbering code). This was then plotted against *n*, which is the degree of polymerization for the [^{14}C]oligosaccharides.

incubated with β -D-galactosidase (from *Escherichia coli*; Sigma). Complete hydrolysis was effected, with D-[14 C]galactose as the only radioactive product and only D-galactose detectable with the AgNO_3 reagent. Under conditions resulting in 50% hydrolysis of authentic D-galactosyl- β -(1 $\rightarrow$ 4)-D-galactose, no hydrolysis was observed to be catalysed by this enzyme with melibiose [D-galactosyl- α -(1 $\rightarrow$ 6)-D-glucose] as substrate. These results indicate that the linkage configuration joining the two galactose moieties of the disaccharide is β . Therefore the major component of band 5 is D-[U- 14 C]galactosyl- β -(1 $\rightarrow$ 4)-D-galactose.

Characterization of band 4. Like band 5, chromatography of band 4 (in solvent *c* for 4 days, and solvent *d* for 8 days) produced two radioactive components. The major component (75% of the total radioactivity), on acid hydrolysis, produced only D-[14 C]galactose, as demonstrated by chromatography in solvent *b*. Reduction and hydrolysis, as described above, of this major component yielded D-[14 C]galactose and D-[14 C]galactitol, in a 1.2:1 ratio, as the only radioactive products. Periodate oxidation, followed by reduction and hydrolysis, resulted in a recovery of slightly more than 50% of the starting radioactivity. The only radioactive products detectable on chromatography in solvent *h* were two, one with a mobility corresponding to D-threitol (53%), and the other with a mobility corresponding to glycerol (47%). The oxidation products of the disaccharide (see above) indicate that, under our conditions of oxidation, a considerable portion of the radioactivity that would be expected to appear as [14 C]threitol is actually appearing as [14 C]glycerol. Applying a correction factor indicates that the radioactive oxidation products of this compound should be 80–90% [14 C]threitol and 10–20% [14 C]glycerol. Treatment with an α -D-galactosidase from *Aspergillus giganteus* (supplied by Dr. E. T. Reese; see Reese & Shibata, 1965) catalysed no hydrolysis of this compound under conditions in which melibiose was hydrolysed completely. In contrast, an *E. coli* β -D-galactosidase (Sigma) catalysed the complete hydrolysis of this compound, under conditions in which no hydrolysis of melibiose was detected. The above evidence indicates that the major component of band 4 is β -D-(1 $\rightarrow$ 4)-[14 C][D-galactosyl- β -D-(1 $\rightarrow$ 4)-galactosyl]-D-galactose.

Characterization of band 3. Paper chromatography of band 3 in solvent *c* for 4 days resulted in only one radioactive band, although some streaking was apparent. Prolonged chromatography (10 days) in solvent *d* produced one major radioactive band (77% of the original material) plus a few faster-moving minor bands. The major band, however, yielded both D-[14 C]glucose (37%) and D-[14 C]galactose (63%) from acid hydrolysis. Reduction with NaBH_4 followed by hydrolysis gave D-[14 C]glucose, D-[14 C]-

galactose, D-[14 C]glucitol and D-[14 C]galactitol. The [14 C]galactose/[14 C]galactitol ratio was 1.1:1. More than one type of reducing group indicates that at least two oligosaccharides are present in the major band, one with a reducing-terminal D-[14 C]glucose moiety and the other with a reducing-terminal D-[14 C]galactose moiety. Periodate oxidation, followed by reduction and hydrolysis, resulted in the isolation of [14 C]glycerol, [14 C]threitol and [14 C]erythritol as the only non-volatile products. To separate [14 C]threitol and [14 C]erythritol, chromatography in solvent *h* was not sufficient. To separate these compounds, the band with the same mobility as that of D-threitol/D-erythritol in solvent *h* was eluted and subjected to electrophoresis in buffer *l*. The presence of D-[14 C]threitol and D-[14 C]erythritol as periodate oxidation products is indicative of D-[14 C]galactose moieties and D-[14 C]glucose moieties respectively, with the 4-position involved in a glycosidic linkage. The D-[14 C]threitol represented 62% of the D-[14 C]threitol/D-[14 C]erythritol mixture. The D-[14 C]galactitol represented 64% of the radioactivity found in the D-[14 C]galactitol/D-[14 C]glucitol mixture resulting from reduction of band 3 followed by acid hydrolysis. Also, the D-[14 C]galactose resulting from reduction and hydrolysis of band 3 represented 61% of the radioactivity in the radioactive sugars (D-[14 C]galactose/D-[14 C]glucose) that result from this treatment. That is, the proportions of reducing D-[14 C]galactose moieties to reducing D-[14 C]glucose moieties, of non-reducing D-[14 C]galactose moieties to non-reducing D-[14 C]glucose moieties, and of 4-linked D-[14 C]galactose moieties to 4-linked D-[14 C]glucose moieties and of total [14 C]galactose to total [14 C]glucose is, within error, the same. This suggests that the D-[14 C]galactose residues are components of one oligosaccharide and the D-[14 C]glucose residues are components of a different oligosaccharide. Therefore the D-[14 C]galactose-containing oligosaccharide is most probably β -(1 $\rightarrow$ 4)-linked [14 C]galactotriosyl-D-galactose.

Characterization of band 6. Total acid hydrolysis of band 6 followed by chromatography in solvent *b* resulted in a single radioactive component with a mobility exactly that of D-galactose. However, electrophoresis of the band 6 material in buffer *k* revealed two radioactive components: a major component (94%) with an electrophoretic mobility like that of galactosyl- β -D-(1 $\rightarrow$ 4)-galactose (further results indicate that this compound is not galactosyl- β -D-(1 $\rightarrow$ 4)-galactose; see below), and a minor component (6%) with an electrophoretic mobility like that of β -D-(1 $\rightarrow$ 3)-galactosylgalactose. Reduction of band 6 with NaBH_4 , followed by acid hydrolysis, resulted in approx. 3% as D-[14 C]galactitol and 97% as D-[14 C]galactose. Repetition of this experimental procedure, by using controls to ensure that the results were not due to incomplete reduction, verified

the above results. Consequently, it appears that the great majority of the D-[^{14}C]galactose moieties are non-reducing. The quantity of D-[^{14}C]galactitol obtained after the reduction-acid-hydrolysis procedure was consistent with the proposition that the minor component of band 6 was a D-[U- ^{14}C]galactobiose, and the major component consisted of a D-[^{14}C]galactosyl derivative of a non-radioactive compound. Periodate oxidation of band 6, followed by reduction and hydrolysis, resulted in only a 2.9% yield of non-volatile radioactive compounds. The periodate oxidation products were identified as [^{14}C]arabitol (1%) and [^{14}C]glycerol (1.9%) by comparison chromatography with authentic standards in solvent *h*. The presence of [^{14}C]arabitol indicates D-[^{14}C]galactose moieties linked 1 $\rightarrow$ 3. The yield and identity of the periodate oxidation products support the electrophoretic indication that the identity of the minor component of band 6 is probably [^{14}C]galactosyl- β -D-(1 $\rightarrow$ 3)-galactose. This oligosaccharide is present in small quantities. For example, it represents 0.6% of the partial acetolysis products in comparison with band 5 { [^{14}C]galactosyl- β -D-(1 $\rightarrow$ 4)-galactose}, which represents 7% of the partial acetolysis products.

The major component of band 6 has not yet been identified, but all of the radioactivity present as a D-[^{14}C]galactosyl derivative in this compound results in volatile products on periodate oxidation. This was determined by a check of radioactivity yield at each step of the periodate-oxidation-reduction-acid-hydrolysis reaction sequence. The loss of radioactivity occurred when the sample was concentrated under vacuum, and was not the result of random losses during the work-up of the reaction products.

Discussion

McNab *et al.* (1968) demonstrated that D-[^{14}C]galactose could be incorporated into polymeric structures. The substrate used in that study was UDP- α -D-[^{14}C]galactose and the [^{14}C]polysaccharide isolated was water-soluble. The substrate specificity of the galactosyl transferase was not determined, nor was the [^{14}C]polysaccharide characterized beyond demonstrating that more than one [^{14}C]galactosyl moiety was incorporated per polysaccharide chain. The results in the present report demonstrate that UDP- α -D-galactose is the preferred D-galactosyl donor in the formation of D-galactan chains by using enzyme preparations from *Phaseolus aureus* (Table 1). Further, UDP- α -D-galactose is the preferred D-galactosyl donor for a 4-epimerase which is present in the particulate enzyme preparation (Table 1; also see the Results section). It is notable that McNab *et al.* (1968) had no evidence indicating the presence of a 4-epimerase in their enzyme preparation. The

most likely explanation for this difference appears to be the slightly different means of enzyme preparation (see the Materials and Methods section, and McNab *et al.*, 1968) which results in the 4-epimerase enzyme activity in our enzyme preparation. Although there has been no report of a water-soluble glucan produced from UDP- α -D-glucose with *Phaseolus aureus* enzymes, there have been reports of alkali-soluble and alkali-insoluble glucans derived from UDP- α -D-glucose catalysed by *Phaseolus aureus* enzymes (Feingold *et al.*, 1968a; Villemez *et al.*, 1967; Flowers *et al.*, 1968; Chambers & Elbein, 1970; Clark & Villemez, 1972). Although McNab *et al.* (1968) examined only the water-soluble polysaccharide, they also reported that no water-insoluble [^{14}C]polysaccharide was detectable. Should any quantity of UDP- α -D-[^{14}C]glucose have been formed during their reactions, it is very likely that water-insoluble [^{14}C]polysaccharide would have been formed in their reaction system.

In addition to the presence of 4-epimerase, which was not observed in the previous study of [^{14}C]galactose polymerization (McNab *et al.*, 1968), the enzyme preparation in the present work contains more than one type of galactose transferase enzyme activity. The partial acetolysis products derived from alkali-soluble and alkali-insoluble [^{14}C]polysaccharide exhibit considerably different [^{14}C]oligosaccharide patterns (Fig. 1), indicating that at least two different polysaccharides are formed from UDP- α -D-galactose by using this particulate enzyme preparation. In contrast, the [^{14}C]oligosaccharides produced by partial acid hydrolysis of the [^{14}C]galactan reported by McNab *et al.* (1968) did not indicate more than one type of [^{14}C]galactose-containing polysaccharide. That this is not due exclusively to the different means of producing the oligosaccharides is shown by the fact that partial acid hydrolysis of the [^{14}C]polysaccharides in the present work produces the same [^{14}C]oligosaccharide patterns but in lower yield (see the Results section). It appears, consequently, that the slight differences in particulate enzyme preparation (see the Materials and Methods section, and McNab *et al.*, 1968) results in different and additional types of galactosyl transferase enzyme activity.

The structure of one chain of the alkali-insoluble [^{14}C]polysaccharide formed from UDP- α -D-[^{14}C]galactose was deduced by elucidating the structure of [^{14}C]oligosaccharides that resulted from partial acetolysis. The following evidence (see the Results section) indicates that a β -D-(1 $\rightarrow$ 4)-[^{14}C]galactan chain is formed from UDP- α -D-[^{14}C]galactose by *Phaseolus aureus* particulate enzymes. (1) A radioactive di- and tri-saccharide were isolated, purified, and characterized as being, respectively, [U- ^{14}C]galactosyl- β -D-(1 $\rightarrow$ 4)-galactose and β -D-(1 $\rightarrow$ 4)-[^{14}C]galactosyl-(1 $\rightarrow$ 4)-galactosyl]-D-[^{14}C]galactose. (2) A radioactive galactose tetrasaccharide, characterized

by chromatographic mobility, was shown to contain only (1→4) glycosidic linkages between the [^{14}C]-galactose moieties by periodate oxidation, reduction, acid hydrolysis and identification of the radioactive products. The structure of this compound could not be conclusively demonstrated, since it could not be completely separated from a [^{14}C]-glucose-containing oligosaccharide, but (a) reduction and hydrolysis studies (see the Results section) indicate the presence of more than one [^{14}C]-oligosaccharide; (b) a β -D-(1→4)-linked alkali-insoluble linear [^{14}C]-glucan is known to be synthesized from UDP- α -D-[^{14}C]-glucose by this particulate enzyme system (Villemez *et al.*, 1967; Clark & Villemez, 1972); (c) the contaminating [^{14}C]-glucose moieties are linked β -(1→4); (d) a [^{14}C]-glucose disaccharide with the chromatographic mobility of cellobiose was purified away from the [^{14}C]-galactose disaccharide; (e) a radioactive trisaccharide containing only [^{14}C]-glucose was purified away from the [^{14}C]-galactose trisaccharide; and (f) no di- or tri-saccharides containing both [^{14}C]-galactose and [^{14}C]-glucose were isolated. Consequently, it seems likely that the tetrasaccharide mixture that could not be completely purified contains [^{14}C]-cellotetraose as a minor component, and β -D-(1→4)-[^{14}C]-[galactosyl - (1→4) - galactosyl - (1→4)-galactosyl]-D-[^{14}C]-galactose as the major component. (3) An indication of a homologous series of [^{14}C]-galactose-containing oligosaccharides of one to six units was obtained (Fig. 2). Three members of this series were identified unequivocally as D-galactose, β -(1→4)-linked galactobiose and β -(1→4)-linked galactotriose. Evidence was presented suggesting that the fourth member of the series is β -(1→4)-linked galactotetraose. It seems likely that the fifth and sixth members of the series are β -(1→4)-linked galactopentaose and galactohexaose respectively. This evidence demonstrates that a β -(1→4)-linked galactan chain is synthesized from UDP- α -D-galactose in this enzyme system. On the basis of the available evidence, it cannot be determined if the product *in vivo* is a pure β -(1→4)-linked linear galactan, since a small amount of what appeared to be [^{14}C]-galactosyl- β -(1→3)-galactose was isolated, as well as an unidentified [^{14}C]-galactosyl derivative of an unlabelled compound, from the partial acetolysis products of the alkali-insoluble [^{14}C]-polysaccharide (see the Results section).

A noteworthy observation resulted from the reduction and acid-hydrolysis studies of the [^{14}C]-oligosaccharides obtained from the alkali-insoluble [^{14}C]-polysaccharide. The radioactivity ratio between the reducing and non-reducing components of the [^{14}C]-galactobiose was approximately 1:1, indicating uniform labelling. However, the corresponding ratio for [^{14}C]-galactotriose and [^{14}C]-galactotetraose was only slightly greater, that is 1.2:1 and 1.1:1 respectively (see the Results section). Assuming that the acetolysis

reaction split glycosidic linkages in a random fashion, these results indicate that: (1) only two and a fraction radioactive galactosyl moieties, on the average, are added to the growing polysaccharide chain; and (2) the additions take place at the reducing end of the polysaccharide chain. This is an indication of consequence, since direct transglycosylation reactions result in addition to the non-reducing end of polysaccharide chains. In contrast, polysaccharide-synthesizing reaction sequences involving lipid intermediates such as that in O-antigen biosynthesis (Osborn, 1969) result in chain growth from the reducing end. There have been other more direct indications that plant cell-wall polysaccharide biosynthesis involves glycosyl-lipid intermediates (Villemez & Clark, 1969; Kauss, 1969).

In a recent model of sycamore cell primary wall structure (Talmadge *et al.*, 1973; Bauer *et al.*, 1973; Keegstra *et al.*, 1973), both β -(1→4)-linked galactan chains and β -(1→3)-galactan chains are important components. The isolation of [^{14}C]-galactosyl- β -(1→3)-galactose (see the Results section) implies the existence of β -(1→3)-galactose-to-galactose glycosidic linkages in *Phaseolus aureus* polysaccharides. It is possible that the alkali-soluble [^{14}C]-galactose-containing polysaccharide material (see Fig. 1), which has not yet been characterized, contains a β -(1→3)-linked galactan chain. The trace amount of β -(1→3)-linked galactobiose obtained from the alkali-insoluble polysaccharide material may have resulted from the incomplete extraction of β -(1→3)-linked galactan, which is alkali-soluble.

An examination of sugars present in the alkali-insoluble residue from *Phaseolus aureus* hypocotyls indicates the presence of D-glucose and D-mannose moieties in a 5:1 ratio (see the Results section). No D-galactose could be detected. It therefore seems likely that the β -(1→4)-linked galactan chain that was synthesized by this particulate enzyme system represents only one component of a finished product. For example, the presence of side-chain sugar residues would certainly increase the solubility of β -(1→4)-linked galactans. With L-arabinosyl or other sugar side chains attached, the β -(1→4)-linked [^{14}C]-galactan chain that we observe in the alkali-insoluble fraction would certainly appear in one of the soluble polysaccharide fractions. The presence of D-galactose-containing soluble polysaccharides in *Phaseolus aureus* has been demonstrated (Bailey *et al.*, 1967). Also, the sycamore primary cell-wall galactan chains isolated by Keegstra *et al.* (1973) had arabinosyl or araban side chains.

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