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Biosynthesis of Galactan by a Particulate Enzyme Preparation from *Phaseolus aureus* Seedlings

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A particulate cell-free enzyme system was prepared from *Phaseolus aureus* shoots. This preparation was able to incorporate [^{14}C]galactose from UDP-[^{14}C]galactose into a water-soluble polysaccharide, which has a probable molecular weight of at least 4600. The only labelled component detectable in the polymer was shown to be [^{14}C]galactose; two labelled oligosaccharides containing only [^{14}C]galactose were isolated by partial hydrolysis. The galactan-synthesizing activity of this particulate preparation is maximal at 30° and pH 7·1 in the presence of 5·0mM-magnesium chloride and 0·2M-sucrose. Although 3-day-old seedlings were used as a source of enzyme, it appears that 4- or 5-day-old beans contain greater synthetase activity. The enzyme system has an apparent Michaelis constant of $5{\cdot}8 \times 10^{-6}$M, and will catalyse the polymerization of galactose residues at the rate of 7·5mμmoles/mg. of protein/min. at a substrate concentration of 9·6mM.

Evidence that has accumulated over the past 15 years emphasizes the importance of nucleoside diphosphate glycosyl compounds in many monosaccharide interconversions (Kalckar & Maxwell, 1958; Hassid, Neufeld & Feingold, 1959; Strominger 1960) and in the syntheses of oligo- and polysaccharides. This latter topic has been reviewed (Feingold, Neufeld & Hassid, 1961; Leloir, 1964*a*, *b*; Nordin & Kirkwood, 1965; Robbins, Wright & Dankert, 1966). Knowledge of the biosynthesis of the polysaccharide constituents of higher plants has not advanced at the spectacular rate that has been achieved with bacterial polysaccharides.

UDP-glucose is one of the crucial glycosyl donors involved in the biosynthesis of the glucose-containing polymers of higher plants. Many UDP-glucose–polysaccharide glucosyltransferases have been found; the syntheses of starch (de Fekete, Leloir & Cardini, 1960; Leloir, de Fekete & Cardini, 1961), callose (Feingold, Neufeld & Hassid, 1959) and several glucans, one of which may be cellulose (Brummond & Gibbons, 1964, 1965; Ordin & Hall, 1967; Villemez, Franz & Hassid, 1967), have been reported from this derivative. Villemez and coworkers have shown that UDP-galacturonic acid is the essential donor for pectin (Villemez, Lin & Hassid, 1965; Villemez, Swanson & Hassid, 1966) and it has been demonstrated that UDP-xylose is the precursor of the β-1,4-linked xylan that is present in corn-cobs (Bailey & Hassid, 1966). Previously, Pridham & Hassid (1966) had isolated a particulate enzyme from corn shoots, capable of transferring both D-xylose and L-arabinose from their UDP derivatives to polymers, but the products found were not characterized in detail.

Uridine nucleotides, however, are not unique as glycosyl donors in the plant world and it is some years now since it was shown that ADP-glucose was a more effective substrate for the synthesis of starch (Recondo & Leloir, 1961; Murata, Minamikawa & Akazawa, 1963). Also, the synthesis of cellulose by an enzyme from a higher plant with GDP-glucose as the glycosyl donor has been reported (Elbein, Barber & Hassid, 1964; Barber, Elbein & Hassid, 1964). These workers discovered that the presence of GDP-mannose enhanced the incorporation of [^{14}C]glucose from GDP-[^{14}C]glucose and altered the character of the products (Barber & Hassid, 1965). Elbein & Hassid (1966) have reported the partial characterization of a glucomannan when both GDP-glucose and GDP-mannose were present in the incubation mixture.

Although galactose is an important constituent of the cell wall in early stages of growth, second only in quantity to glucose (Bailey, Haq & Hassid, 1967; Nevins, English & Albersheim, 1967), no synthesis of a galactan or galactose-containing polysaccharide by an enzyme from a higher-plant source has been reported. Yet it has long been known that UDP-galactose is active as a galactosyl

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donor for several galactosidic compounds (Cleland & Kennedy, 1960; Watkins & Hassid, 1961). Sussman & Osborn (1964) showed the presence of a UDP-galactose–polysaccharide galactosyltransferase in cell-free extracts of the slime mould, *Dictyostelium discoideum*. These workers did not carry out a detailed analysis of the product, but did present some evidence for the existence of an acid mucopolysaccharide of fairly high molecular weight that contained galactose, galactosamine and galacturonic acid.

Frydman & Neufeld (1963) synthesized galactinol by transfer of UDP-galactose to *myo*inositol. Workers in several Laboratories (Bourne, Walter & Pridham, 1965; Pridham & Hassid, 1965; Gomyo & Nakamura, 1966) have reported the synthesis of raffinose by the transfer of galactose from its uridine nucleotide to sucrose, catalysed by enzymes present in cell-free extracts of different plants.

We have now synthesized a galactan with particulate enzyme preparations from *Phaseolus aureus* shoots. The properties of this enzyme system and some evidence on the nature of the polysaccharide formed are presented.

MATERIALS AND METHODS

UDP-[^{14}C]galactose (uniformly labelled in the sugar moiety) of specific activity 111mc/m-mole was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. UDP-[^{14}C]galacturonic acid was synthesized as described by Villemez *et al.* (1965). UDP-galactose was purchased from Calbiochem, Los Angeles, Calif., U.S.A., and all other chemicals used were analytical-grade reagents.

Preparation of enzyme. Mung-bean seeds (*Phaseolus aureus*) were soaked overnight in water and then grown in the dark at room temperature in moist vermiculite for 3 days. In a typical preparation, 10g. of shoots was chilled and ground for a few minutes with 10g. of sand in a cold mortar. All subsequent operations were carried out at 0–4°. The crude slurry was strained through two layers of muslin and the resultant liquid (5–7ml.) centrifuged at 500*g* for 15min. This pellet was discarded and the supernatant liquid centrifuged at 34000*g* for 1hr. The supernatant liquid was discarded and the pellet resuspended in buffer (5ml.) with a loose-fitting ground-glass Potter-type homogenizer. The buffer consisted of 0·05M-sodium cacodylate, pH7·3, containing 0·4M-sucrose. The pH of the suspension was 7·0–7·1. The suspension contained about 0·5mg. of protein/ml.

Assay. A 10μl. portion of this particulate preparation containing about 5μg. of protein was added to approx. 10000 counts/min. (0·123mμmole) of UDP-[^{14}C]galactose (10μl.). The mixture was incubated at 25° for 2–30min. To stop the reaction, 20μl. of 0·05M-sodium tetraborate, pH9·5, was added, producing a final pH of about 8·6. The suspension was immediately frozen until it was analysed. Analysis was carried out by subjecting the reaction mixture to paper electrophoresis (45v/cm.) in 0·05M-sodium tetraborate buffer, pH9·5, for the length of time required for the picric acid reference spot to migrate 15–20cm. from the origin. The paper was air-dried and the spot at the origin was cut out. Radioactivity was determined at about 40% efficiency in a Packard Tri-Carb liquid-scintillation counter at 0°, with 15ml. of a counting medium consisting of 0·5% (w/v) 2,5-diphenyloxazole and 0·03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene. Under these conditions the radioactivity that remained at the origin resided in the [^{14}C]galactan.

Analytical methods. Paper chromatograms were developed in one of the following solvent systems: (*a*) ethyl acetate–pyridine–water (8:2:1, by vol.); (*b*) butan-1-ol–ethanol–water (1:1:1, by vol.); (*c*) butan-2-one–acetic acid–water (9:1:1, by vol.) saturated with boric acid. Untreated Whatman no. 1 filter sheets were used for both chromatography and electrophoresis. Reducing sugars were located on chromatograms with the alkaline $AgNO_3$ spray reagent (Trevelyan, Procter & Harrison, 1950), but alcohols were generally detected more readily with the alkaline periodate/permanganate spray (Lemieux & Bauer, 1954). Radioactive areas were located on paper with a Packard radiochromatogram scanner (model 7200) at about 4% efficiency. Occasionally the areas were located by cutting the paper strips into 0·5–1cm. pieces and counting at 0° in the Packard Tri-Carb scintillation counter as described above. Sugars and polysaccharides were recovered from these pieces of paper by elution with water, after removal of the counting fluid by washing with toluene followed by chloroform.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Sugars were measured by the anthrone procedure (Dische, 1962). [^{14}C]Oligosaccharides in 100μl. of water were reduced by the addition of $NaBH_4$ (2mg.). After 2hr. at room temperature the excess of borohydride was destroyed and the Na^+ removed by the simple addition of Dowex 50W (X4) cation-exchange resin. Boric acid was removed as its volatile methyl ester by repeated evaporation to dryness in the presence of methanol at 40°.

RESULTS AND DISCUSSION

[^{14}C]*Galactan.* The labelled product, formed from UDP-[^{14}C]galactose in the presence of mung-bean particulate enzyme, which was immobile on borate electrophoresis, was eluted from the Whatman no. 1 filter paper with water. All the radioactivity was eluted, whereas none of the radioactivity could be removed with chloroform–methanol (1:1, v/v). The aqueous eluate was concentrated by evaporation under reduced pressure at 40° and the resulting solution was freed from contaminating ions by dialysis against distilled water for 24hr. at 0–4° with two changes of water. No loss of radioactivity was observed after dialysis. The product was chromatographically immobile in solvent systems (*a*) and (*b*) after an irrigation period of 24hr. These results indicate that the [^{14}C]galactose from the UDP-[^{14}C]galactose is incorporated into a water-soluble polymer by this cell-free particulate preparation from mung beans.

[^{14}C]Galactan (30000counts/min.) was chromatographed on a column (30cm. × 1·5cm.) of Bio-Gel

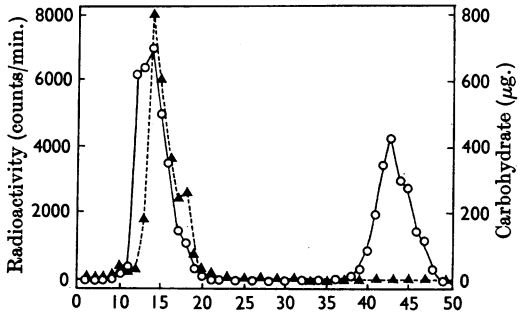

Fig. 1. Elution pattern obtained from Bio-Gel P6 chromatography of a mixture of [^{14}C]galactan, polysaccharide and galactose. ------, Radioactivity; ——, carbohydrate (determined with anthrone).

P6. The same solution contained the sycamore extracellular polysaccharide (3·5mg.) (Becker, Hui & Albersheim, 1964) and galactose (2·5mg.) as 'markers'. The column was eluted with water at a rate of 3ml./hr. Samples (1ml.) were analysed for radioactivity on the scintillation counter and for sugar content. Fig. 1 illustrates the elution pattern obtained. The radioactive galactan was eluted within the void volume as was the sycamore extracellular polysaccharide. This gel has an exclusion limit for compounds with a molecular weight of 4600, so it seems probable that the galactan has a molecular weight of at least this value.

The only labelled sugar in the polymer is [^{14}C]-galactose: this was illustrated by complete hydrolysis of the polysaccharide with 2·0N-trifluoroacetic acid in a sealed tube at 100° for 1hr. (Albersheim, Nevins, English & Karr, 1967) and examination of the hydrolysis products. The only radioactive compound that was detected had a chromatographic mobility identical with that of authentic galactose in both solvent systems. Hydrolysis of the polymer was repeated under milder conditions, with 0·5N-trifluoroacetic acid at 100° for 30min. A radioactive compound with chromatographic mobility identical with that of authentic galactose was detected as described above, in addition to two slower-moving constituents, oligosaccharide O1 and oligosaccharide O2. The former had R_{Gal} 0·56 (solvent *a*) and R_F 0·42 (solvent *b*), suggesting it was a disaccharide. Oligosaccharide O2, on the other hand, had R_{Gal} 0·26 (solvent *a*) and could be either a di- or a tri-saccharide. However, oligosaccharide O2 had R_F 0·28 (solvent *b*), suggesting that it was a trisaccharide. In addition to these products of partial hydrolysis, radioactivity was still detectable at the base line, presumably owing to undegraded polymer or higher oligosaccharides.

The two radioactive areas corresponding to oligosaccharides O1 and O2 were eluted from the paper with water and the eluates concentrated to a small volume. Both compounds yielded only [^{14}C]-galactose after hydrolysis with 1·0N-trifluoroacetic acid for 1hr. at 100°. Treatment of the two oligosaccharides with sodium borohydride was followed by acid hydrolyses of the derived glycitols. Chromatographic analysis of the products in solvent (*c*) yielded labelled galactitol (R_{Gal} 2·8) as well as [^{14}C]galactose in each case. From radioactivity measurements, it was determined that oligosaccharide O1 had a galactose/galactitol ratio 1:1·6, whereas oligosaccharide O2 had a galactose/galactitol ratio 2·2:1, a value that is consistent with the idea that oligosaccharide O2 is a trisaccharide of galactose. The ratio obtained for oligosaccharide O1 is lower than that expected for a disaccharide. This could be explained by partial hydrolysis of the disaccharide before reduction, thereby increasing the amount of galactitol formed. The fact that labelled galactitol was obtained from these oligosaccharides after reduction with sodium borohydride shows that the biosynthesis was not the result of a single transglycosylation between non-radioactive polymer and labelled substrate. It seems reasonable to assume that these partial-hydrolysis products represent a picture of the whole molecule and that the polysaccharide is labelled uniformly throughout the chain. The only other labelled product present after reaction was UDP-galactose. Occasionally after long reaction times a trace of galactose 1-phosphate could be detected.

Properties of the enzyme preparation. The incorporation of radioactivity into galactan by this particulate preparation increased linearly for 20–30min. at 25°; the incorporation rate then decreased and became almost zero after 3hr. (Fig. 2). The possible reasons for the inactivation of the enzyme system have been considered by Villemez *et al.* (1966). Under optimum conditions the incorporation of galactose into polymer could have as high a value as 65%. No incorporation of radioactivity was observed either when [^{14}C]galactose or [^{14}C]galactose 1-phosphate was employed as the labelled substrate. Also, an enzyme preparation that had been boiled for 5min. was completely inactive. The dependence of the reaction rate on enzyme concentration is illustrated in Fig. 3. However, at a protein concentration of 45μg. and above the relationship is no longer linear. Further increases of enzyme do not enhance the rate proportionally.

The double reciprocal plot (Fig. 4) illustrates the influence of substrate concentration on rate of reaction. From these data an apparent Michaelis constant of $5·8 \times 10^{-6}$M was calculated. The highest rate observed was 7·52mμmoles/mg. of protein/min.

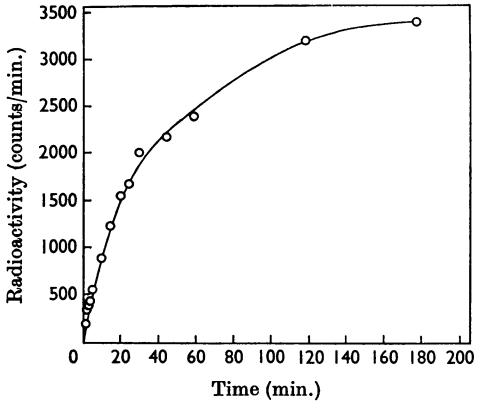

Fig. 2. Time-course of [^{14}C]galactan synthesis. Each sample contained 10μl. of particulate enzyme preparation, as described in the Materials and Methods section, 10μl. of 3mM-Mg^{2+} and 5200counts/min. of UDP-[^{14}C]galactose (5·3μmoles), in a total volume of 30μl. They were incubated for the indicated times and assayed as described in the Materials and Methods section.

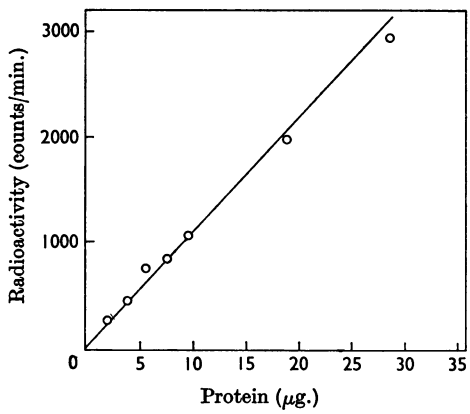

Fig. 3. Effect of enzyme concentration on [^{14}C]galactan synthesis. The particulate enzyme was prepared according to the procedure described in the Materials and Methods section. Different concentrations were obtained by dilution with appropriate volumes of buffer. Each reaction mixture contained 10μl. of each enzyme suspension and 11000 counts/min. of UDP-[^{14}C]galactose (11·0μmoles), in a total volume of 20μl. The reaction time was 30min.

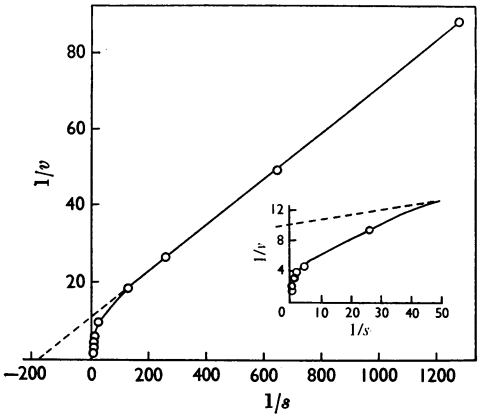

Fig. 4. Effect of substrate concentration on [^{14}C]galactan biosynthesis. These samples were assayed as described in the Materials and Methods section. The incubation time was 2min. Velocity, v, is expressed as 10^6counts/min./mg. of protein/min.; substrate concentration, s, is expressed as mM.

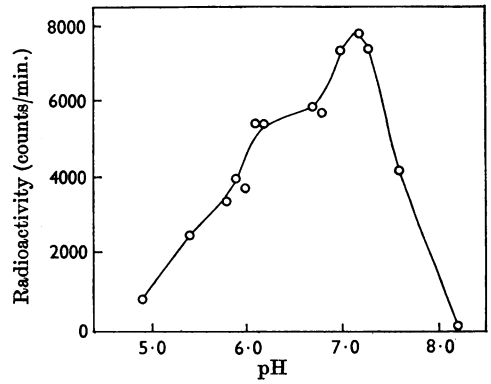

Fig. 5. Effect of pH on [^{14}C]galactan biosynthesis. Assayed as described in the Materials and Methods section, the curve is compiled with two 0·05M-buffers: phosphate, pH6·0–7·9, and cacodylate, pH4·9–7·0. The reaction mixtures contained 20000counts/min. of UDP-[^{14}C]galactose (20μmoles) in 10μl. as substrate. The reaction time was 30min.

at a concentration of 9·6mM of UDP-galactose. This value is appreciably greater than that obtained for the biosynthesis of cellulose from GDP-glucose (Barber *et al.* 1964) and does compare favourably with the value calculated for polygalacturonic acid (Villemez *et al.* 1966). It will be noticed that at high substrate concentration the rate of reaction increases disproportionately. Another polysaccharide synthetase has been observed to produce the same effect (Villemez *et al.* 1966). This can be explained by postulating the existence of an allosteric effect (co-operative substrate activation), or simply by the presence of another enzyme that uses UDP-galactose less efficiently in making a similar product. The effect of pH (Fig. 5) would support this latter viewpoint, as a definite shoulder exists at pH6·1–6·4; this could be the maximum value for a second enzyme.

Table 1. *Effect of various cofactors on the incorporation of* [^{14}C]*galactose into galactan*

The substances indicated were present at a final concentration of 5·0 μM. The assay conditions were those described in the Materials and Methods section except that 3000 counts/min. of UDP-[^{14}C]galactose (3·1 μmoles) was used as substrate. The reaction time was 15 min.

Addition	Incorporation (counts/min.)	Addition	Incorporation (counts/min.)
None	610	$MgCl_2$	952
$CaCl_2$	380	$MnCl_2$	900
$CoCl_2$	415	$MnSO_4$	810
$CuSO_4$	130	NaCl	788
$FeCl_2$	728	NH_4Cl	550
$FeSO_4$	702	$ZnCl_2$	160
$FeCl_3$	115	Mercaptoethanol	355
$HgCl_2$	80	GSH	580
KCl	160	Cysteine	345

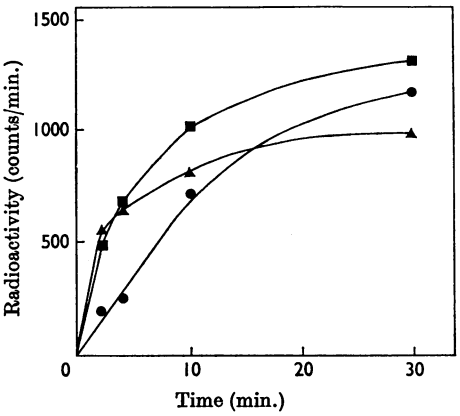

Fig. 6. Effect of temperature on reaction rate. The experimental procedure was the same as given in Fig. 2, except that the temperature of incubation was: ●, 24°; ■, 30°; ▲, 36°.

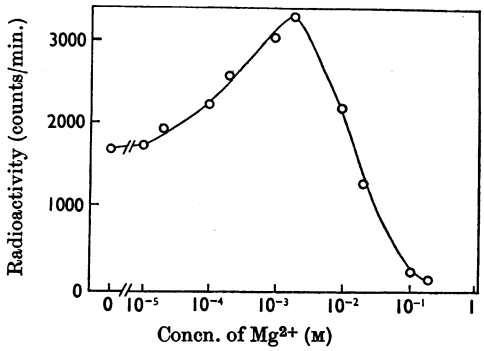

Fig. 7. Effect of Mg^{2+} concentration on [^{14}C]galactan formation. Experimental procedure was that described in Fig. 2 with variations in the $MgCl_2$ concn.

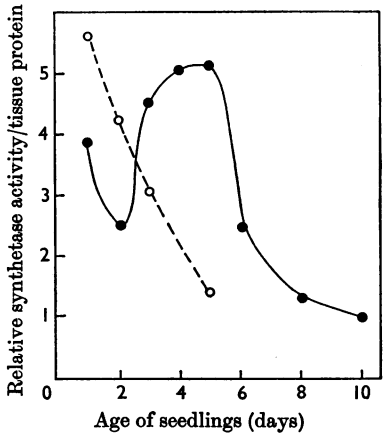

Fig. 8. Effect of bean maturation on [^{14}C]galactan synthetase (●) and [^{14}C]polygalacturonate synthetase (○) activities. The enzyme suspensions were prepared as described in the Materials and Methods section.

Although effective galactan synthetase activity could be obtained by this mung-bean enzyme preparation, prepared and assayed in the absence of sucrose, the presence of this compound in the resuspension medium improved the rate of polymer formation threefold. However, unlike the xylan synthetase (Bailey & Hassid, 1966) and the polygalacturonate synthetase (Villemez *et al.* 1966), the presence of bovine serum albumin has no effect on the [^{14}C]galactan formation. The enzyme involved in the galactan synthesis may not be as easily inactivated as the other two.

However, this particulate preparation, like the other two enzyme systems, seems to possess all the factors required for galactan biosynthesis, with the possible exception of substrate. As in the biosynthesis of pectin with a similar system, these factors cannot be washed away from the particles. Even when the particles are washed with EDTA, there is no loss in synthetase activity. Nevertheless, the incorporation of [^{14}C]galactan can be stimulated by the presence of certain salts in the incubation mixture (Table 1). The greatest stimulation was observed when Mg^{2+} was present, but a slight stimulation was also observed when Mn^{2+}, Na^+ or Fe^{2+} was present during reaction. Polysaccharide synthesis is inhibited greatly by Fe^{3+}, Cu^{2+}, Zn^{2+} and K^+ and to a smaller extent by Ca^{2+} and Co^{2+}. A slight inhibition is also noticed when cysteine, GSH or mercaptoethanol is introduced into the reaction mixture.

The optimum conditions for the enzyme appear to be a pH range 7·0–7·2 (Fig. 5), a temperature of 30° (Fig. 6) and a magnesium chloride concentration of 5 mM (Fig. 7).

A study was also made to determine how the time of germination affects the galactan synthetase activity (Fig. 8). There are two peaks of activity. A maximum is found in beans that have been germinated for 4 or 5 days. The activity is also high in enzyme extracted from 1-day-old beans, and the two stages may represent the activity of different enzymes. These results can be compared with those obtained from a similar study on the biosynthesis of polygalacturonic acid, where the specific activity of the pectin synthetase enzyme decreases with increasing age of the shoots.

It seems likely, from these results and from the synthesis of other plant polysaccharides *in vitro*, that enzymes associated with the same type of particle are responsible for cell-wall polysaccharide biosynthesis (Villemez, McNab, Grimes & Albersheim, 1967). However, it appears that the various polysaccharide synthetase activities are at maximum values at different stages of bean maturity. This seems a reasonable postulate, since the different polysaccharides appear at different stages in bean cell-wall growth (D. J. Nevins, P. D. English & P. Albersheim, unpublished work).

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