Metabolic Relationships of the Isolated Fractions of the Pectic Substances of Actively Growing Sycamore Cells

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(Received 14 March 1967)

1. D-Glucose and L-arabinose serve as precursors of the pectic polysaccharides of sycamore suspension-callus tissue. 2. The rates and characteristics of the incorporation of radioactive sucrose, glucose and mesoinositol by sycamore callus tissue have been compared and shown to be different. 3. The time-course of the incorporation of radioactive glucose into the major fractions within the cells has been determined. Approx. 7–10% of the radioactivity incorporated is present in the whole pectin of the cells. 4. A study of the continuous incorporation of radioactive glucose showed that the neutral arabinan–galactan fraction of the pectin quickly became saturated with the radioactive label. During the incorporation of radioactivity from a pulse of radioactive glucose the neutral fraction became progressively less labelled, with a corresponding increase in the radioactivity of the weakly acidic pectinic acid, which is known to contain neutral sugars. 5. When the cells were exposed to a pulse of radioactive L-arabinose, the label accumulated first in the neutral fraction and then after 4 hr. it passed to the weakly acidic pectinic acid with a corresponding decrease in the radioactivity of the neutral fraction. 6. The product that was initially labelled during the first hour of exposure of the cells in the stationary phase to radioactive glucose was identified as an incompletely methylated galacturonan in which the radioactivity was present in the anhydrogalacturonide residues. This polysaccharide probably acts as the precursor of the polyuronide portions of both the strongly acidic and weakly acidic pectinic acids. 7. The observations are discussed in relation to the structure of the pectic substances and their function in cell growth and development. A tentative model for their metabolic relationship is put forward.

The pectic polysaccharides of higher plants are a distinctive component of primary, but not secondary, wall material (Thornber & Northcote, 1961a), and are probably present also in the cell plate and middle lamella (Kerr & Bailey, 1934; Albersheim, Mühlethaler & Frey-Wyssling, 1960; Albersheim & Killias, 1963). Since cell-wall extension is restricted to primary growth, theories of its mechanism have laid special stress on a possible interrelation of wall plasticity and pectin metabolism (Bonner, 1961).

An effect of auxin on the activity of pectinesterase and its binding to the cell wall was claimed by several workers (Glasziou, 1957; Glasziou & Inglis, 1958; Adamson & Adamson, 1958; Yoda, 1958), but has been shown by Jansen, Jang & Bonner (1960b) to be a non-specific adsorption of protein. Jansen, Jang, Albersheim & Bonner (1960a) found that auxin (indol-3-ylacetic acid) promoted the incorporation of methyl groups into *Avena* coleoptile pectin, but caused no overall change in the degree of methylation. They also suggested that their cold-

water-soluble pectin was the precursor of the cell-wall material. There is therefore no direct evidence for an effect of auxin on methylation and demethylation of pectinic acids that would enable its effects on wall plasticity to be explained.

Effects of auxin on net pectin synthesis are well established. Carlier & Buffel (1955) showed that it promoted hot-water-soluble pectin synthesis in the potato tuber, and a promotion of pectin synthesis relative to cellulose synthesis has been shown in tobacco pith (Wilson & Skoog, 1954; Wilson, 1961). Ordin, Cleland & Bonner (1955) found that indol-3-ylacetic acid promoted the incorporation of glucose and of methyl groups from methionine into the hot-water-soluble fraction of the *Avena* coleoptile wall, and that it also inhibited their loss from that fraction. Albersheim & Bonner (1959) examined this effect in more detail and found that indol-3-ylacetic acid promoted the incorporation of glucose into water-soluble pectins but had no effect on residual pectin. A parallel action of the auxin on the incorporation of methyl ester groups was reported

(Sato, Byerrum, Albersheim & Bonner, 1958). Albersheim (1963) also found an incorporation of *meso*inositol into pectin (similarly affected by indol-3-ylacetic acid) and postulated a derivative of methyl-esterified galacturonic acid as a precursor. Villemez, Swanson & Hassid (1966) and Kauss, Swanson & Hassid (1967), however, have found that the methyl ester of UDP-galacturonic acid is not an effective pectin precursor in a system *in vitro*.

The studies of Baker & Ray (1965*a,b*) and Ray & Baker (1965) show that auxin promotes the synthesis of all non-cellulose polysaccharides in *Avena* coleoptile by a direct action, and indirectly promotes the synthesis of cellulose after extension. It is therefore doubtful that cell-wall plasticity can be explained solely in terms of pectin metabolism, which may, however, have a specific role in new wall development.

In general, metabolic studies on the pectic substances have been restricted to examination of methyl group and anhydrouronide incorporation: little attention has been given to the neutral sugars now known to be present in many pectinic acids (Barrett & Northcote, 1965; Gould, Rees, Richardson & Steele, 1965; Rees & Richardson, 1966; Stoddart, Barrett & Northcote, 1967).

Stoddart *et al.* (1967) have found a general similarity between the pectic substances of several growing tissues and a marked difference between these and mature tissue. The present paper reports a re-examination of the possible metabolic interrelation of these various components of the pectic complex of sycamore.

MATERIALS AND METHODS

The methods of pectinesterase (pectin pectyl-hydrolase, EC 3.1.1.11) preparation, enzymic de-esterification of pectinic acid, ethylene oxide esterification of pectinic acid, hydrolysis with HNO_3 and sugar detection have been described (Barrett & Northcote, 1965), as have the methods of growth and maintenance of the callus tissues, zone electrophoresis, diazomethane esterification of pectinic acids, transelimination degradation, hydrolysis with H_2SO_4 and paper chromatography (Stoddart *et al.* 1967).

Growth media. The variations of the growth media used for the sycamore suspension-callus tissue in the metabolic experiments are shown in Table 1.

Handling of tissue. All operations requiring a high degree of sterility were carried out in a glove-box, the atmosphere within which could be sterilized by ultraviolet irradiation. All apparatus used within the box was sterilized by autoclaving for 25 min. at 15 lb./in.² (120°).

Stock liquid suspension-callus cultures were maintained in 11. roller bottles as previously described (Jeffs & Northcote, 1966; Stoddart *et al.* 1967). For incorporation experiments smaller (15 ml.) cultures were used. These were grown in 125 ml. bottles rotated at 78 rev./min. at 25° in the dark.

The cells were separated from the growth medium by filtration through a no. 3 porosity sintered-glass crucible. Suction was provided by means of a rubber pro-pipette. No cells passed through the sinter and very few entered it. They were washed with medium *in situ* and transferred to new medium by means of a sterile spatula.

For time-course experiments samples of tissue and medium were withdrawn by means of sterilized wide-bore (3 mm.) Pasteur pipettes, plugged with non-absorbent cotton wool. The operation was conducted in the glove-box.

Sycamore suspension-callus tissue was used for all the metabolic studies. Bean suspension-callus tissue was used only for the analysis of the growth medium.

Testing of sterility. Samples of both stock and experimental cultures were examined microscopically at each subculture. Contamination by bacteria or yeasts was easily detectable.

Periodically, samples of culture medium were plated out on nutrient agar in Petri dishes, which were then incubated at 25° and 37° for 78 hr. and examined for microbiological growth.

Collecting and washing of samples. Samples removed from the liquid suspension cultures were transferred to 1 cm. x 7.5 cm. test tubes. Strict asepsis was not maintained beyond this point. The cells were collected by slow centrifugation and the medium was pipetted off and stored at -15° with a drop of toluene. The cells were washed by resuspension three times in non-radioactive medium and twice in glass-distilled water. They were then suspended in a little glass-distilled water, transferred to a weighed glass specimen tube and rapidly frozen (-40°) as a suspension. These tubes were closed with plastic caps and stored at -15°.

Freeze-drying and extraction of tissue. The plastic caps of the specimen tubes containing the frozen tissue were perforated and the cells freeze-dried. After weighing (wt. of

Table 1. *Compositions of media used for metabolic studies*

The composition of the salts and organic components have been described elsewhere (Jeffs & Northcote, 1966).

Medium	Inorganic salts	Organic components	2,4-Dichloro-phenoxylacetic acid (6 p.p.m.)	Coconut milk (20%)	Sucrose (2%)	Other components
H2	+	+	+	+	+	—
BH2	+	+	—	—	—	—
BH2/24D	+	+	+	—	—	—
BH2/24D/Suc	+	+	+	—	+	—
BH2/24D/Glc	+	+	+	—	—	D-Glucose (1%)
BH2/24D/Inos	+	+	+	—	—	<i>meso</i> Inositol (0.01%)

freeze-dried tissue 4–12 mg.), the tissue was quantitatively transferred to test tubes (6 in. $\times$ 1 in.) with ground-glass necks and 4–5 ml. of 2% (w/v) sodium hexametaphosphate, adjusted to pH 3.7 with HCl, was added (Stoddart *et al.* 1967). The tubes were then heated under reflux in a water bath for 4 hr. at 100°.

Removal of starch from the extract. After cooling, each extract was filtered through a filter-cell (Johns-Manville Co.) and adjusted to pH 6.5–7.0 with NaOH, and a few crystals of NaCl were added. One drop of human salivary α -amylase (EC 3.2.1.1) (Olaitan & Northcote, 1962) was added followed by a drop of toluene, and digestion was allowed to proceed at 25° for at least 1 hr. The activity of the α -amylase was confirmed with a solution of starch, by using iodine. After digestion the extracts were dialysed against several changes of distilled water at 4° for 24 hr.

The dialysis residues were quantitatively transferred to round-bottomed flasks and reduced to small bulk by rotatory evaporation under reduced pressure below 40°. Absolute ethanol (in excess of 85%, v/v) was added to each sample to precipitate the polysaccharide and the bulk of the organic phase was removed. Residual solvent was removed by further rotatory evaporation to dryness.

Electrophoresis of pectin. The pectin samples from the extraction were each dissolved in a little water and transferred to glass-fibre paper (Whatman GF81) for electrophoresis (Barrett & Northcote, 1965; Stoddart *et al.* 1967). Markers of sycamore suspension-callus whole pectin were used.

After electrophoresis the papers were dried first in air and then at 50–60°. Strips 4 cm. $\times$ 1 cm. were then cut with a sharp razor-blade, down the direction of the run of each sample, such that the long axis of each strip was at right angles to the direction of electrophoretic movement of the sample and the origin line of each sample lay on the division between two strips. By this means all polysaccharide was cut out of the sheet.

Liquid-scintillation spectrometry (Wang & Jones, 1959). The strips were transferred to 2 in. $\times$ $\frac{1}{4}$ in. glass specimen tubes and 0.5 ml. of scintillation fluid [2,5-diphenyloxazole, 3.5 g.; 1,4-bis-(5-phenyloxazol-2-yl)benzene, 50 mg.; sulphur-free toluene, 1 l.] was added to each. The tubes were stoppered and placed in 20 ml. Packard liquid-scintillation spectrometer tubes. Counting was carried out in a Packard Tri-Carb model 314 DC liquid-scintillation spectrometer, with the samples at 1°.

The efficiency of counting remained constant at 57–58% throughout the range of loadings used. Orientation effects were not observed. Background counts varied a little between batches of paper (Stoddart & Northcote, 1967).

Gas-flow Geiger counting. Samples of whole freeze-dried cells were transferred to weighed acid-washed aluminium planchets, silicone-greased along the inside of the rim. The planchets were reweighed, and a few drops of a solution of cetyltrimethylammonium bromide (0.5%, w/v) and polyvinyl alcohol (0.25%, w/v) in water were applied to the cells until they dispersed. The planchets were then heated to dryness under an infrared lamp. Samples of whole pectin were applied in aqueous solution to planchets and dried to a hard film under the infrared lamp. Samples of media were similarly prepared, but a drop of the cetyltrimethylammonium bromide–polyvinyl alcohol solution was added to give a glazed surface. All counts were corrected to infinite thinness.

Radioactive chemicals. D-[U-¹⁴C]glucose, (123 mc/m-mole), [U-¹⁴C]sucrose (10 mc/m-mole) and [U-¹⁴C]meso-inositol (approx. 160 mc/m-mole) were purchased from The Radiochemical Centre, Amersham, Bucks.; L-[1-¹⁴C]-arabinose (10.0 mc/m-mole) was purchased from Calbiochem, Los Angeles, Calif., U.S.A.

All radioactive solutions were sterilized by autoclaving before addition to the cultures.

Electrophoresis of sugars and uronic acids. In addition to chromatographic separation, sugars and uronic acids were separated by zone electrophoresis on Whatman no. 1 paper with 0.05 M-sodium tetraborate buffer (pH 9.2), with monosaccharides, sucrose, glucuronic acid and galacturonic acid as markers. The effective length of the paper was 45 cm. and the starting line was 18 cm. from the cathode. The electrodes were of platinum. The tank (Durrum, 1950) was filled with 'white spirit' (Esso Petroleum Co. Ltd., Cambridge) and cooled by water in a glass coil. Electrophoresis was carried out at 1800 v and 10 ma for 1.5–2 hr.

Sugars and uronic acids were developed by the aniline–hydrogen phthalate method of Wilson (1959). Sucrose was detected by overspraying with urea–HCl (Bell & Northcote, 1954).

Fractional extraction of whole tissue. Washed sycamore callus cells that had been 'fed' with radioactive precursors were heated under reflux in redistilled analytical-grade methanol (Hopkin and Williams Ltd., Chadwell Heath, Essex) (about 15 ml./ml. of packed cells) for 15 min. After cooling they were filtered through a no. 3 porosity sinter, washed with methanol and transferred to a weighed specimen tube for freeze-drying. The methanolic extract was reduced to dryness by rotatory evaporation and the yield of freeze-dried methanol-extracted cells was weighed and transferred to redistilled analytical-grade peroxide-free dry ether (May and Baker Ltd., Dagenham, Essex) (6 ml./ml. of packed cells). After reflux extraction for 2 hr. the ethereal solvent was pipetted off and the cells washed two or three times with more ether. The ethereal solution was reduced to dryness by rotatory evaporation. The residual ether was evaporated from the cell residue, which was retained for the extraction of pectin.

RESULTS

Incorporation of D-[U-¹⁴C]glucose. Callus cells, harvested in the exponential phase of growth (6 days from subculture), were resuspended and cultured under the standard conditions for 4 days in BH2/24D/Glc medium. They were then harvested again and transferred to bottles containing BH2/24D/Glc medium and similar media of lower glucose concentrations, all of which contained known amounts of radioactive glucose. Samples were withdrawn aseptically at intervals and the cells were washed and freeze-dried. Weighed portions were applied to planchets and counted in the gas-flow Geiger counter. A correction curve for self-absorption was obtained with D-[U-¹⁴C]glucose and non-radioactive freeze-dried tissue. From the corrected counts the weight of glucose taken up and not metabolized as volatile products, per unit dry weight of cells, was calculated (Fig. 1).

Fig. 1. Time-course of incorporation of D-[U- 14 C]glucose into sycamore callus cells in BH2/24D/Glc medium. ●, 1% glucose; ▲, 0.5% glucose.

Fig. 2. Time-course of incorporation of [U- 14 C]sucrose (2%) into sycamore callus cells in BH2/24D/Suc medium.

At glucose concentrations below 0.5% the uptake curves were markedly non-linear. Initial rates of incorporation were found to be proportional to the glucose concentration.

Incorporation of [U- 14 C]sucrose. An experiment was carried out to determine the progress of sucrose incorporation by using a procedure similar to that described above, except that BH2/24D/Suc medium was used. A longer overall time was found necessary because of the low specific activity and final uptake rate of the sucrose. A larger standard error in the counting procedure was also found. The results are shown in Fig. 2.

Incorporation of [U- 14 C]mesoinositol. The uptake

Fig. 3. Time-course of incorporation of [U- 14 C]mesoinositol (0.01%) into sycamore callus cells in BH2/24D/Inos medium.

of mesoinositol was investigated with BH2/24D/Inos medium (0.08 μC in a 15 ml. culture). Cells were taken after 2 days in BH2/24D medium. Samples were withdrawn and treated as described above. The results are shown in Fig. 3.

Sucrose and glucose incorporation followed by incubation in non-radioactive media. The steady-state sucrose uptake experiment (Fig. 2) indicated that the process of sucrose incorporation was biphasic and that the more rapid process was an exchange of unlabelled sucrose within the cell with radioactive sucrose outside. The size of this internal sucrose pool is represented by the intercept of the final steady-uptake rate curve with the ordinate.

Radioactive sucrose was supplied to cells in BH2/24D/Suc medium for 1 hr. and the cells were then harvested, washed and resuspended in BH2/24D medium for further culture. Samples of cells were periodically withdrawn, washed and counted. The results are shown in Fig. 4(a). About half the radioactivity present in the cells after 1 hr. was lost by 2 hr. The effect was more pronounced if resuspension was in BH2/24D/Glc medium (Fig. 4b). Samples of the resuspension medium in each case showed only a single labelled spot with the electrophoretic mobility of sucrose. All activity was lost on dialysis.

Similar experiments were performed with glucose as the source of label (Figs. 4c and 4d), with BH2/24D/Suc medium for resuspension in one case (Fig. 4d). Where no sucrose was added to the resuspension medium an initial loss followed by an uptake of radioactivity was always observed (Fig. 4c). If sucrose was present a loss of radioactivity took place (Fig. 4d). Electrophoretically the radioactive material in the resuspension medium again moved with sucrose, and was lost during dialysis.

Distribution of D-[U- 14 C]glucose within the cells.

Fig. 4. Incorporation of radioactivity of [U-¹⁴C]sucrose (*a* and *b*) and D-[U-¹⁴C]glucose (*c* and *d*) into sycamore callus cells incubated for 1 hr. with the radioactive substance and subsequently chased by growing the cells in BH2/24D medium (*a* and *c*) or BH2/24D/Glc medium (*b*) or BH2/24D/Suc medium (*d*). The arrow on each abscissa indicates the time the cells were placed in the non-radioactive medium.

A culture (70 ml.) was grown in a 500 ml. bottle until early stationary phase and D-[U-¹⁴C]glucose was added (100 μ C, 0.22 mg.) under sterile conditions. After 1 hr. of exposure to the radioisotope the cells were harvested, washed and resuspended in fresh H2 medium (60 ml.). Culture was continued and aseptic samples were taken at intervals.

Cell samples were collected as described above, washed in glass-distilled water and transferred to methanol. They were extracted under reflux, filtered, washed with methanol and dried. The methanolic extracts were evaporated to dryness, dissolved in a little water, applied to planchets, dried and counted.

The yields of methanol-extracted cells were weighed and then further extracted with ether. The ethereal extracts and washings were applied to washed aluminium planchets and evaporated to dryness. The residual methanol- and ether-extracted cells were then extracted with 2% sodium hexametaphosphate (pH 3.7) and the pectin was purified and isolated. Residual cell walls, filtered out on a glass-fibre disk (GF 81), were washed and

Fig. 5. Distribution of radioactivity in various fractions of the sycamore cells after they had been incubated in D-[U-¹⁴C]glucose for various times. L, Fraction soluble in ether; H, fraction soluble in sodium hexametaphosphate (2%); M, fraction soluble in methanol; R, insoluble residue.

dried, and known weights were applied to planchets, dried and counted. The results are shown in Fig. 5.

It was noted in preliminary studies that the

Fig. 6. Distribution of radioactivity in the electrophoretically separated zones (A, B, C and D) of the whole pectin extracted from sycamore cells after (a) 2.5 hr. and (b) 20 hr. of exposure to D-[U- 14 C]glucose. The electrophoresis was carried out on glass-fibre paper (GF 81) in pyridine-acetate buffer, pH 6.5, with 10 mM-EDTA. The paper was cut into 1 cm. strips from the origin (each strip 1 cm. $\times$ 4 cm.), and the strips were counted in a scintillation counter.

methanol and ether extraction procedure led to marked shrinkage of the cell wall, such that after ether extraction the cells would enter a no. 3 porosity sintered-glass filter. Hexametaphosphate treatment led to a re-swelling of the cell walls. Microscopic examination showed that the residual material retained the form of the cell wall, but was far more transparent.

Samples of media were retained and it was found that radioactivity was lost from the cells to the media in a manner similar to that shown in Fig. 4; no high-molecular-weight (i.e. non-diffusible) radioactive material was found in the media up to 72 hr.

Continuous incorporation of D-[U- 14 C]glucose into pectin. Cells were harvested near the end of their exponential phase of growth, washed and cultured for 2 days in BH2/24D medium. They were harvested again and transferred to 15 ml. of BH2/24D medium to which 0.1 mg. of glucose/ml. (9.35 μ C/15 ml.) had been added. Samples were withdrawn, washed and freeze-dried. A portion of the cells was counted and the remainder extracted for pectin. The extracted pectin was analysed by zone electrophoresis and the radioactive zones were detected by scintillation counting. Results are shown in Figs. 6(a) and 6(b). The radioactivity present in each region of the electrophoretogram was calculated and corrected relative to a constant rate of glucose incorporation by the cells. The results are shown in Fig. 7. The radioactivity present in the neutral fraction rapidly tended to a

constant maximum value unlike that of the pectinic acid fractions (Figs. 7a-c).

Pulse 'feeding' of D-[U- 14 C]glucose. In the pulse-labelling experiments cultures were taken late in their exponential phase or early in their stationary phase of growth, and supplied with D-[U- 14 C]glucose for 1 hr. The cells were then harvested, washed and cultured in fresh medium devoid of radioactivity. In general the radioactive glucose was added to the H2 medium in which the cells had been growing and chasing was provided by fresh H2 medium. By this means the cells were kept in an environment similar to that of their normal growth. Experiments were also performed in which non-radioactive glucose was used for chasing and in which coconut milk was absent from the medium. These last-named experiments gave similar results to those obtained with the whole H2 medium.

In stationary-phase cultures, the product labelled after 1 hr. of exposure to radioactive precursor (first labelled product) showed a characteristic electrophoretic mobility corresponding to zone B (Fig. 8a). A chemical study was made of this material. After 3 hr. of subsequent chasing with unlabelled H2 medium, the radioactivity corresponded to the weakly acidic pectinic acid (zone C) (Fig. 9a) and not to the first labelled product. At times between 12 hr. and 24 hr. from the start of chasing, the neutral (zone D) and strongly acidic (zone A) components of the pectin became labelled in addition to the weakly acidic pectinic acid (zone C) (Fig. 9b). In pulse

Fig. 7. Time-course of incorporation of radioactivity into the four zones (A, B, C and D) of the extracted whole pectin of sycamore cells: (a) zone D, (b) zone C, (c) zone B, (d) zone A.

experiments radioactivity was quickly lost from the neutral component; most of it disappeared within 12 hr. of the first appearance of label in this component and after 72 hr. of chasing only the weakly acidic pectinic acid (zone C) and the strongly acidic pectinic acid (zone A) were labelled (Fig. 9c).

Exponential-phase cultures showed a similar, but rather more rapid, labelling sequence. The first labelled product was seldom detectable. An incubation of 1 hr. with radioactive glucose led to labelling of the weakly acidic pectinic acid (zone C).

Chasing with unlabelled medium for a further 3 hr. led to labelling of the neutral and strongly acidic components (zones D and A). Subsequent changes were similar to those seen in the stationary-phase cultures.

Characterization of the first labelled product. A stationary-phase culture of cells was supplied with D-[U- 14 C]glucose for 1 hr.; it was then harvested and the pectin was extracted. It was analysed electrophoretically and shown to contain a single radioactive zone (Fig. 8a). Another sample developed visually contained all the normal components of sycamore pectin (Stoddart *et al.* 1967).

Treatment of a sample of the radioactive material with pectinesterase led to a shifting of the labelled material into the region corresponding to galacturonan (Fig. 8b), and a control sample developed visually showed a sharpening of the strongly acidic band (Stoddart *et al.* 1967). An enzymically demethylated sample of the material was dialysed against distilled water for 24 hr.; it was applied to a planchet and counted in the gas-flow counter, and a similar untreated sample was also dialysed and counted. No significant difference in amount of radioactivity between the two samples was found.

Hydroxyethylation of the radioactive pectin with ethylene oxide moved the zone of radioactivity towards the region of low acidity corresponding to apple-fruit pectinic acid (Barrett & Northcote, 1965; Stoddart *et al.* 1967) (Fig. 8c), and likewise the strongly acidic region of a visual marker was no longer present. Methylation of the material by diazomethane gave a similar result.

When the neutralized (hydroxyethylated) pectin was heated at 105° in phosphate buffer (pH 6.7) for 12 hr. in a sealed tube it underwent transelimination degradation. A marker of the degraded pectin showed strongly acidic and neutral components on electrophoresis. Only the acidic zone was radioactive (Fig. 8d).

The radioactive pectin (1 mg.) was dissolved in water (0.1 ml.) and hydrolysed by nitric acid-urea in sealed tubes at 100° for 18 hr. The hydrolysates were neutralized with Amberlite IR-400 (CO $_3^{2-}$ form) (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) and freeze-dried. Another sample was hydrolysed with sulphuric acid and neutralized by *NN*-bis-*n*-octylmethylamine. Each sample was dissolved in 0.15 ml. of saturated benzoic acid and 50 μ l. of this solution analysed chromatographically. Marker hydrolysate material and standard sugar solutions were run and developed by aniline phthalate in all the chromatograms and compared with a portion of the paper that carried the hydrolysate and was not coloured with aniline phthalate. This latter portion was cut into strips 1 cm. $\times$ 4 cm. and the radioactivity located by the method of Wang & Jones (1959).

Fig. 8. Characterization of the first labelled product in the whole pectin of sycamore cells. Stationary-phase cells were incubated with D-[U- ^{14}C]glucose for 1 hr. and the pectin was extracted. (a) Electrophoretic distribution of radioactivity of the pectin as extracted. (b) Electrophoretic distribution of radioactivity of the pectin after enzymic demethylation. (c) Electrophoretic distribution of the radioactivity of the pectin after esterification with ethylene oxide. (d) Electrophoretic distribution of the pectin after transelimination degradation of the esterified material. The shaded areas under the histogram represent the position of the zones made visible on the electrophoretogram by sulphonated α -naphthol.

Fig. 9. Distribution of radioactivity in the electrophoretically separated zones (A, B, C and D) of the whole pectin extracted from sycamore cells after 1 hr. exposure to D-[U- ^{14}C]glucose and subsequent incubation in non-radioactive H₂ medium: (a) for 3 hr.; (b) for 12 hr.; (c) for 72 hr.

All the radioactivity remained near the origin; none was associated with galactose or arabinose. Neither radioactivity nor a spot was found that corresponded to ribose or deoxyribose.

A sample of pectin (13.0 mg.) was esterified with ethylene oxide and dissolved in water (0.6 ml.). The solution was cooled to 1° and an excess of aqueous potassium borohydride solution (5.68 g./100 ml.) was slowly added with vigorous stirring over a period of 30 min. Stirring was continued for 12 hr. The product was mixed with acidified ethanol and the excess of borate removed by rotatory evaporation at 35°. The residue was freeze-dried, dissolved in water, dialysed against water at 0° for 24 hr. and again freeze-dried. Electrophoretic analysis showed that a considerable amount of non-reduced material remained. A portion of the partially reduced pectin was hydrolysed with nitric acid-urea and analysed chromatographically. The marker hydrolysate showed a slightly intensified galactose spot, and a little radioactivity (about 10% of that applied) was found in this region.

Fig. 10. Distribution of radioactivity in an electrophoretogram on Whatman no. 1 paper (0.05 M borate buffer, pH 9.2) of a digest with crude *Aspergillus* pectinase of the first labelled product in the whole pectin of sycamore cells.

An attempt was made to reduce the radioactive pectin by the method of Zitko & Bishop (1966). Ethereal diazomethane was added to 2 mg. of the dry pectin and esterification was continued for 24 hr. at 0°. The excess of diazomethane was destroyed with a drop of 0.1 N-hydrochloric acid, and the polysaccharide was recovered by evaporation of the ether. It was dissolved in aqueous 80% dimethyl sulphoxide and solid potassium borohydride was added with stirring. After the solution had been stirred for 18 hr. the excess of borohydride was destroyed with acid, and evaporation with methanol removed the borate. The residue was hydrolysed. Again reduction was incomplete, though some radioactive galactose was obtained.

Radioactive pectin (2.7 mg.) was dissolved in 0.5 ml. of 0.1 M-tris hydrochloride buffer (pH 7.3) and 0.2 ml. of a solution (1 mg./ml. of buffer) of crude *Aspergillus* polygalacturonase (pectinase; Koch-Light Laboratories Ltd., Colnbrook, Bucks.) was added with a drop of toluene. Digestion was allowed to proceed at 26° overnight. Ethanol (0.5 ml.) was added and the precipitate was centrifuged. The supernatant solution was evaporated to dryness, dissolved in saturated benzoic acid solution and analysed by zone electrophoresis in borate buffer at pH 9.2. A spot corresponding in colour and mobility to galacturonic acid was seen; all the radioactivity detected corresponded to this spot (Fig. 10).

Characterization of the final labelled products. A stationary-phase culture of cells was supplied with D-[U-¹⁴C]glucose for 156 hr.; it was then harvested and the pectin extracted and analysed electrophoretically. Three radioactive zones corresponding to zone A, the strongly acidic pectinic acid, zone C, the weakly acidic pectinic acid, and zone D, the arabinan-galactan, were found. The pectin was hydrolysed by sulphuric acid, pectinesterase and crude *Aspergillus* pectinase by similar methods to those described for the first labelled material.

There was no loss of radioactivity when the methyl ester groups were removed; and radioactive galactose, arabinose and galacturonic acid were found in the acid and pectinase hydrolysates. The pectin was esterified with ethylene oxide and subjected to transelimination degradation. The degraded material gave two radioactive zones (zones D and A) on electrophoresis.

Continuous incorporation of L-[1-¹⁴C]arabinose. A solution (0.2 ml.) of the arabinose (10 μ C/ml.) was diluted with BH2/24D/Suc medium (1 ml.), autoclaved and added to a culture of sycamore cells (15 ml.) that had been grown into the late exponential phase. Samples were withdrawn at intervals and the pectin was extracted and analysed.

After 2.5 hr., radioactivity was found predominantly in the weakly acidic zone, with some also in the neutral zone; this gave a shoulder on the

Fig. 11. Distribution of radioactivity in the electrophoretically separated zones (A, B, C and D) of the whole pectin extracted from sycamore cells after (a) 5 hr., (b) 45 hr., (c) 1 hr. and (d) 2 hr. exposure to L-[1- 14 C]arabinose.

peak of the weakly acidic zone. After 5 hr. the shoulder was a little more pronounced (Fig. 11a) and thereafter a distinct neutral peak was evident that overlapped the weakly acidic peak (Fig. 11b). No radioactivity was found in the strongly acidic zone even after 45 hr. from the start of the arabinose incubation (Fig. 11b).

Arabinose (3 μ C, 0.3 ml.) was added in BH2/24D/Suc medium (1 ml.) to a culture of sycamore cells (15 ml.) in stationary phase (about 2 weeks' growth). Samples were removed at shorter time-intervals than before, and bench-top sterile techniques only were used. The pectin was extracted and analysed electrophoretically. At times varying from 15 min. to 1 hr. from the start of the experiment the labelling pattern of the material was that shown in Fig. 11(c). All of the radioactivity was present in the neutral component; very little was found in the weakly acidic material. After 2 hr. more radioactivity was found in the weakly acidic region, while the total radioactivity in the neutral region declined (Fig. 11d).

Arabinose incorporation followed by incubation in non-radioactive media. A culture of cells (15 ml., H2 medium) in the exponential phase of growth was supplied with L-[1- 14 C]arabinose (2 μ C, 0.2 ml.).

After 15 min. incubation at 25°, a sample was withdrawn and the remaining tissue was collected on a sterile glass filter. The filtered cells were washed and resuspended in the non-radioactive H2 medium in which they had previously grown and samples were withdrawn at intervals during a period of 12 hr. The pectin was extracted from the samples and analysed. The results are shown in Fig. 12. The specific radioactivity of the pectin (135 counts/min./mg.) of the cells remained constant throughout the period. However, after the first 15 min. exposure of the cells to the arabinose all the radioactivity was found in the neutral (arabinan-galactan) component of the pectin (Fig. 12a). During the subsequent incubation in the non-radioactive medium, the radioactivity in the pectin appeared in the weakly acidic pectinic acid and was lost from the neutral fraction. (Figs. 12b and 12c).

Pectin in the medium of callus cultures. The non-radioactive media in which cells were incubated after exposure to radioactive glucose, sucrose and arabinose contained no radioactive pectin or other radioactive high-molecular-weight material (dialysis residue). However, non-radioactive polysaccharide was present in the media of cultures grown well into the stationary phase. Cultures at the

Fig. 12. Distribution of radioactivity in the electrophoretically separated zones (A, B, C and D) of the whole pectin extracted from sycamore cells after exposure to L-[1- 14 C]arabinose for: (a) 15 min.; (b) 15 min. followed by incubation in non-radioactive H₂ medium for 1 hr.; (c) 15 min. followed by incubation in non-radioactive H₂ medium for 4 hr.

logarithmic phase of growth contained little of this material. It was always associated with those cultures in which extensive necrosis was visible microscopically.

Samples of the media of aged cultures of sycamore and bean were dialysed for 24 hr. against distilled water and the dialysis residues were brought to 85% (v/v) with ethanol. The gelatinous precipitates that formed were centrifuged, dissolved in a little water and freeze-dried. Samples were analysed

by zone electrophoresis; the results are shown in Fig. 13.

Chromatographic analysis of the acid hydrolysates of the materials isolated from the media showed that they contained uronic acids and neutral sugars. The composition of the neutral sugars was for sycamore: glucose 28%, galactose 21%, arabinose 23%, xylose 28%, rhamnose trace; and for bean: glucose 40%, galactose 25%, arabinose 16%, xylose 19%, rhamnose trace.

Fig. 13. Electrophoretograms of whole pectin extracted from cells and growth media. (a) Sycamore suspension-callus pectin; (b) sycamore suspension-callus growth-medium pectin; (c) bean suspension-callus pectin; (d) bean suspension-callus growth-medium pectin; (e) apple-fruit pectinic acid.

Polygalacturonase activity in the media of sycamore callus cultures. A sterile sample of medium (1 ml.) from an old (26 day) culture was added to a sterile sample of whole sycamore suspension-callus pectin (Stoddart *et al.* 1967). The mixture was incubated with a drop of toluene for 18 hr. at 25°. The pectin was precipitated from the mixture by addition of ethanol (85%, v/v) and analysed electrophoretically. The strongly acidic component was no longer found but both the weakly acidic and neutral fractions were present. The pectin recovered from the incubation had the same electrophoretic pattern as that found in the media of cultures grown into the stationary phase.

DISCUSSION

The uptake of radioactive glucose into non-volatile cellular material appears to be of a type different from that of the sucrose. Initial rates of glucose incorporation are a function of the initial glucose concentration and can vary over a wide range. At a glucose concentration of 1% the rate of incorporation is 8.7 $\mu\text{g.}$ of glucose/mg. dry wt. of cells/hr., which was the maximum found. The sucrose incorporation, however, was markedly biphasic. This was interpreted as resulting from the summation of two processes, a relatively rapid exchange of a pool within the cells and a slower steady rate of incorporation of sucrose into cellular material. This second rate was found to be 2 $\mu\text{g.}$ of sucrose/mg. dry wt. of cells/hr. The existence of a pool of sucrose (approx. 190 $\mu\text{g.}$ /mg. of cells, calculated from the uptake curve) was further supported by the experiments in which radioactivity, identifiable as sucrose, could be chased from the

cells by incubation in non-radioactive media. Humphreys & Garrard (1966) have reported a sucrose leakage effect in corn scutellum somewhat like that found here.

Albersheim, Hui & Chang (1964) have reported the presence of an extracellular pectic substance in the media of sycamore suspension-callus tissues. We have also found this in cultures grown well into the stationary phase. Becker, Hui & Albersheim (1964) have claimed that this extracellular polysaccharide is very rapidly labelled if the cells are supplied with radioactive glucose and that it is chemically like that of the wall. We have not found any significant labelling of a high-molecular-weight extracellular material in our cultures. We have found that the pectic polysaccharides present in the medium are always associated with old cultures (in which cell necrosis is widespread) and that the strongly acidic components typical of the growing cells are not present (Fig. 13).

The hydrolyses show that these polysaccharides are rich in glucose and xylose and poor in arabinose residues. It is probable therefore that polysaccharides other than pectins are liberated from the cell walls and that the pectins lose a portion of their arabinose residues during or after cell necrosis. The loss of the strongly acidic components from pectin added to filtered medium shows that a polygalacturonase is present, possibly released by necrotic cells.

Since sucrose was not readily available in as high a specific activity as glucose and since it was taken up slowly and has been found to have complex physiological effects in bean tissue cultures (Jeffs & Northcote, 1966) glucose is preferable for metabolic studies on pectin. Likewise mesoinositol, though a precursor of pectin in sycamore and other tissues (Loewus, Kelly & Neufeld, 1962; Albersheim, 1963; Roberts & Loewus, 1966), is well known as a growth hormone in tissue cultures (Steward, Shantz, Pollard, Mapes & Mitra, 1961), and consequently its use leads to complications in interpretation.

From the results of our work, glucose is clearly a precursor of both the galacturonan and the neutral sugar portions of the pectin, but not of the methyl groups. Arabinose is a precursor of the neutral arabinan-galactan and the weakly acidic pectinic acid only, and not of the strongly acidic components. This supports the chemical studies of Stoddart *et al.* (1967), who found evidence for the presence of only traces of neutral sugars in strongly acidic pectinic acid. The incorporation of glucose into pectin accounted for only a small part of the total fixation of glucose, most of which was initially present in methanol-soluble fractions of the cells (low-molecular-weight materials) and in residual material (cellulose, hemicellulose and starch). At no time was the lipid of the tissue labelled (Fig. 5).

Only a fairly weakly acidic fraction (zone B) was labelled (Fig. 8a) in the pectin extracted from stationary-phase cultures exposed to radioactive glucose for 1 hr. Hydrolysis with nitric acid or sulphuric acid released neutral sugars from the extracted pectin, but no radioactivity was associated with them; likewise no radioactivity was associated with the neutral region after transelimination degradation of the whole pectin. No evidence was found for the presence of nucleic acids in the extract and although a small amount of ninhydrin-positive material was present it moved away from the carbohydrate zones on electrophoresis and was unlabelled. The radioactive material was resistant to chemical degradation, but treatment with pectinase gave rise to radioactive galacturonic acid. Thus the properties of this radioactive fraction showed that it was a partially methylated galacturonan.

With continued incorporation of radioactive glucose, the pectin of the cells became labelled in both its neutral sugar and its uronic acid constituents. However, even after 156 hr. exposure to radioactive glucose the methyl ester groups of the pectin were not labelled.

The radioactivity of the pectin found in stationary-phase culture after a 3 hr. chase in non-radioactive medium was located in the weakly acidic region (zone C). Similar material was found in exponential-phase cultures exposed to radioactive glucose for 1 hr. It seems likely therefore that the radioactivity initially found in the partially methylated pectinic acid (zone B) can be transferred to weakly acidic pectinic acid (zone C). No great accumulation of radioactivity in the partially methylated pectinic acid (zone B) was ever observed and, as the weakly acidic pectinic acid peak (zone C) gradually broadened, it became impossible to distinguish the two fractions. We suggest that the partially methylated pectinic acid is rapidly turned over and that the absolute amount of it present at any time is small. It is possible that the partially methylated galacturonan could also give rise to the strongly acidic pectinic acid (zone A) by demethylation.

In continuous-incorporation studies the neutral arabinan-galactan becomes labelled, but displays a time-course of labelling very different from that of the pectinic acids, in that it very rapidly becomes saturated. In pulse experiments the neutral fraction becomes labelled after some hours of chasing, but finally loses its label completely (Fig. 9). This suggests that it is turning over rapidly, relative to the weakly acidic pectinic acid (zone C) and the strongly acidic pectinic acid (zone A). In continuous-incorporation experiments of short duration with radioactive arabinose the neutral arabinan-galactan was the only component significantly labelled at first, whereas longer times of

exposure led to labelling of the weakly acidic pectinic acid (Fig. 11). It therefore seemed likely that there was a rapid transfer of radioactive carbohydrate from arabinan-galactan to the weakly acidic pectinic acid.

The pulse-labelling experiments with radioactive arabinose showed that there was such a redistribution of radioactivity during the period of chasing with unlabelled medium, with little or no change in the specific activity of the whole pectin. The simplest explanation of this redistribution of labelled material is that radioactive material passed from the neutral arabinan-galactan fraction to the weakly acidic pectinic acid fraction. This latter fraction is known to contain high-molecular-weight portions made up of arabinose and galactose.

During the growth of the plant there is a continuous change in the pattern of the synthesis of the polysaccharides containing galacturonic acid and the neutral polymers. The studies of Jensen & Ashton (1960), for example, have demonstrated a synthesis of galacturonan and of non-cellulose neutral polymers during cell elongation in pea root; and Wilson (1961) found that in auxin-induced pectin synthesis in tobacco-pith cultures there was a marked stimulation of synthesis of arabinan, either as a neutral polymer or as part of the pectic complex. Thornber & Northcote (1961b) found a net synthesis of arabinose and galactose during primary growth, before differentiation, in several tree species. By inducing differentiation in solid callus cultures of *Phaseolus vulgaris*, Jeffs & Northcote (1966) have shown that cytological characteristics of differentiation can be paralleled by chemical characteristics, and in particular that the ratio of xylose to arabinose in the cell wall increases during differentiation. Detailed studies of the pectinic acids and the pectic arabinan of white-mustard seeds before and during germination indicated that there was a similarity between the arabinan and the neutral portion of the pectinic acid, and that there were complex changes in the arabinan during germination (Gould *et al.* 1965; Rees & Richardson, 1966; Rees & Steele, 1966). These changes were related to changes in wall properties and suggested that the arabinan and pectinic acid may have some metabolic relationship to each other. Stoddart *et al.* (1967) have shown that there is a general difference in the composition of the pectic complex between callus and cambial tissues on one hand and mature tissue on the other. Two distinct types of pectinic acid have been identified in sycamore, one of which contains large blocks of neutral sugar polymers. The work reported here indicates that these neutral blocks, which contain arabinose and galactose and which are part of the weakly acidic pectinic acid, are related to the separate neutral pectic arabinan-galactan fraction.

Scheme 1. Metabolic interrelationships between the isolated fractions of the pectic substances of sycamore cells. GalUA, galacturonic acid.

Our results and those of other workers can be most readily explained in terms of a model of the type shown in Scheme 1. Glucose can serve as a source of both acid and neutral precursors, which give rise to a partially methylated galacturonan (the first labelled product) and to the neutral arabinan-galactan. In the formation of the former, small amounts of rhamnose and xylose are incorporated as single residues, and a little xylose is also present in the arabinan-galactan (Stoddart *et al.* 1967). Arabinose can act only as a neutral precursor and hence arabinan-galactan source. The first labelled product can give rise to strongly acidic pectinic acids by partial or entire demethylation and to weakly acidic pectinic acids by receiving neutral blocks or fragments from the arabinan-galactan by transglycosylation. This transfer could occur before or after deposition of the material in the wall. The results on which we base our model have been obtained with a pectin prepared from a single tissue, and the fractions of this pectin have been chemically characterized (Stoddart *et al.* 1967).

The studies of Ordín *et al.* (1955), Albersheim & Bonner (1959) and Albersheim (1963) have shown that pectin in *Avena* coleoptile is synthesized as a methylated galacturonan and that glucose and inositol will serve as uronide precursors. Methionine and formaldehyde serve as methyl group precursors (Jansen *et al.* 1960a; Becker *et al.* 1964), and Becker *et al.* (1964) suggest that N⁵-methyltetra-

hydrofolate is the donor substance. Albersheim (1963) postulates that there is a common methylated galacturonide precursor, derivable from either glucose or *meso*inositol since there is a parallel effect of indol-3-ylacetic acid on glucose, inositol and methyl group incorporation into the pectin. However, particulate preparations have been obtained from mung beans that are capable of pectin (galacturonan) synthesis and UDP-galacturonic acid is the best sugar nucleotide precursor of this pectin (Villemez, Lin & Hassid, 1965; Lin, Elbein & Su, 1966).

Hassid and his co-workers have further found that UDP-methylgalacturonate is not an effective precursor in this system *in vitro* and that *S*-adenosyl-methionine can act as a methyl donor to pectin (Villemez *et al.* 1966; Kauss *et al.* 1967). There is no need to postulate a methylated precursor of pectin, for it is possible that the methylation reaction may take place on the partially completed galacturonan chain and that its progress may be a function of chain extension. Hence our results and those of Albersheim and co-workers do not necessarily conflict with the studies *in vitro* by Hassid and co-workers.

Albersheim *et al.* (1960) and Albersheim & Killias (1963) have applied the method of Gee, Reeve & McCready (1959) to the study of pectin in sections of onion root tip. They have found a highly esterified polycarboxylic acid in the cell plate and middle

lamella, and a certain amount of similar material throughout the primary wall. This is probably pectin and could well correspond to our first labelled product, which perhaps undergoes further changes *in situ* as the wall develops.

The formation of the cell plate at telophase takes place by the fusion of vesicles brought up between microtubules to the central region of the mitotic spindle (Pickett-Heaps & Northcote, 1966a,b). These vesicles are probably derived from the Golgi bodies (Whaley & Mollenhauer, 1963). Northcote & Pickett-Heaps (1966) have shown that a pectic polymer in wheat root-cap cells is secreted in vesicles formed from the Golgi bodies. It is likely therefore that some of the enzymes of pectic metabolism are located within this organelle. The Golgi body also manufactures and transports material in the cell wall during secondary thickening of differentiated tissue such as xylem (Wooding & Northcote, 1964) and phloem (Northcote & Wooding, 1966). Different polysaccharides are used during the formation of the primary and secondary wall and the pectic polysaccharides change during the initial phases of cell growth. Thus the metabolic function of the Golgi bodies changes continuously during the growth and differentiation of the cell.

R. W. S. thanks the Managers of the Brookbank Fund and the Gordon Wigan Fund for grants during the tenure of which this work was carried out. We thank Mr J. Ndebahweje and Mr R. M. Davey for valuable technical assistance.

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