Phytochemistry, 1967, Vol. 6, pp. 525 to 533. Pergamon Press Ltd. Printed in England

THE INCORPORATION OF ^{14}C-LABELLED D-GLUCURONATE AND D-GALACTOSE INTO SEGMENTS OF THE ROOT-TIP OF CORN

R. M. ROBERTS*

Botany School, South Parks Road, Oxford, England

(*Received* 2 *September* 1966)

Abstract—D-glucuronate-^{14}C was incorporated into uronic acids and pentose sugars combined in the cell wall polysaccharides of maize roots, but not into other ethanol-insoluble components of the cells. Advantage of this was taken to study the patterns of synthesis of these compounds along the first centimetre of the root-tip. Segments of root were incubated with glucuronate labelled either uniformly or at carbon atom 6. The formation of pentosan involved the loss of carbon 6 as CO_2, but small amounts of CO_2 were also released from elsewhere in the carbon skeleton. Uronic acid synthesis was highest in the first two millimetres, but was always low in relation to the incorporation into xylose and arabinose. The synthesis of pentose showed a high peak in the fourth segment, in which cells were at an early stage of elongation, but fell away markedly in segments older than this. D-[1-^{14}C] galactose, supplied to root segments, was also incorporated efficiently into the wall, largely as galactose units, and could be used as a specific precursor of this particular cell wall sugar. In relation to the amount of polysaccharide present, incorporation into galactose was highest in the fifth and sixth millimetres, thus contrasting with the patterns for both pentose and uronic acid synthesis.

INTRODUCTION

GLUCURONIC acid, when supplied to plant tissues, is efficiently incorporated into the pentose sugars and uronic acids combined in the cell wall polysaccharides.[1, 2] This conversion most probably involves an initial phosphorylation at carbon atom 1 and a conversion of the ester to UDP-glucuronic acid. This compound can act as a precursor of the related nucleoside diphosphates of galacturonic acid (by epimerization) and xylose and arabinose (by a decarboxylation at carbon atom 6 and a subsequent epimerization of UDP-xylose to UDP-arabinose).[3] The sugar nucleotides are then thought to act as glycosyl donors to growing polysaccharide chains.[4]

D-galactose, when supplied externally to plant tissues, is also considered to enter the nucleotide pool and participate directly in cell wall synthesis. In the ripening strawberry, a tissue rich in pectin, the sugar is converted efficiently to galacturonic acid units.[1] In maize root-tips (R. M. Roberts, unpublished results) it acted as an excellent precursor of galactose units of the cell wall, whilst only a relatively small diversion of label occurred into glucose, which is the predominant hexose sugar. Pentose and uronic acid were only slightly labelled. Like glucuronic acid, galactose probably becomes phosphorylated at C-1 and the sugar phosphate converted to UDP-galactose by a specific pyrophosphorylase.[5]

* Present address: Department of Biology, State University of New York at Buffalo, N.Y. 14214.

1 F. A. LOEWUS, R. JANG and C. G. SEEGMILLER, *J. Biol. Chem.* **232**, 533 (1958).
2 W. G. SLATER and H. BEEVERS, *Plant Physiol.* **33**, 146 (1958).
3 W. Z. HASSID, *Biochem. Soc. Symp.* **21**, 63 (1962).
4 J. B. PRIDHAM and W. Z. HASSID, *Biochem. J.* **100**, 21P (1966).
5 W. Z. HASSID, E. F. NEUFELD and D. S. FEINGOLD, *Proc. Natl Acad. Sci. U.S.* **45**, 905 (1959).

Galactose, xylose, arabinose, and the uronic acids comprise a major part of the sugars which make up the pectins and hemicelluloses of the cell walls of a large number of plant species. Differences in composition, however, are known to exist between walls in different parts of the plant. Furthermore, the relative amounts of the various sugars are known to change during the development of cells within a single organ such as a root-tip. Such changes have been recognized by chemical analysis of the cell wall polysaccharides in thin segments of tissue cut at increasing distances from the meristematic regions near the tip, where all cells of the primary root originate.[6] The present work describes the incorporation of ^{14}C from glucuronate and galactose into 1-mm segments cut from the first centimetre of the maize root. It indicates on a macro-scale how the patterns of synthesis of different component sugars of the wall vary in relation to each other and to the stage of development of the cells, if it is assumed that the metabolic activities of the individual segments are similar to those of the corresponding regions in the intact root.

RESULTS

(a) *Growth of cells in the root.* Changes in the dimension of cells were followed on 6 μ, longitudinal sections cut from whole root-tips in an attempt to relate the observations made on incorporation of sugars and uronic acids into the cell wall to the growth of cells along the root. Most meristematic activity occurs in the first millimetre, but this segment also contains the tapering root-cap which is usually about 300 μ in length. Radial expansion of cells of all tissues in the main root axis was completed by about 2 mm from the tip, but elongation did not become rapid until about half-way along the third millimetre. Growth then continued at an almost linear rate until 7 mm, after which there was no further expansion. During this phase of rapid elongation, cortical cells lengthened approximately twenty-fold, and pith cells almost thirty-fold. The latter reached a final length of about 300 μ. These results will be presented in detail elsewhere. Segments beyond the seventh contain cells which are not growing.

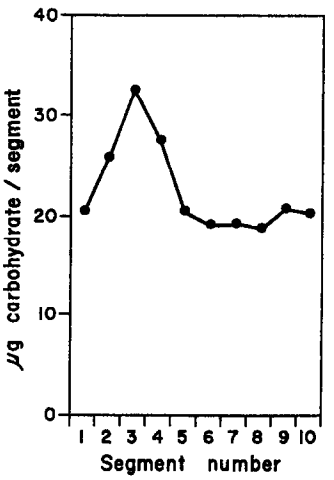

FIG. 1. INSOLUBLE CARBOHYDRATE CONTENT OF MILLIMETRE SEGMENTS OF MAIZE ROOT-TIP.

W. A. JENSEN and M. ASHTON, *Plant Physiol.* 35, 315 (1960).

(b) *Changes in carbohydrate content of segments along the root.* Changes in ethanol insoluble carbohydrate content which occur over the first 10 mm were determined in 1-mm segments by dissolving the polysaccharides in 72% w/v H_2SO_4. Small aliquots of this digest were taken and carbohydrates determined by sulphonated α-naphthol (Fig. 1). In terms of glucose equivalents, there is an increase over the first three segments to give a peak. At this stage, the cells are fully extended radially, but extended only a little longitudinally, so that the number of cells and amount of cell wall material is at a maximum. Subsequently, as the cells continue to elongate, the amount of carbohydrate falls. After segment 8, there is a slight increase in insoluble polysaccharide, possibly related to a thickening of the wall.

(c) *The incorporation of label from* D-*glucuronate into root segments.* Glucuronic acid, labelled uniformly and specifically at carbon atom 6 was supplied separately in each experiment to millimetre segments cut from the same batch of seedlings. Samples of about 130 seg-

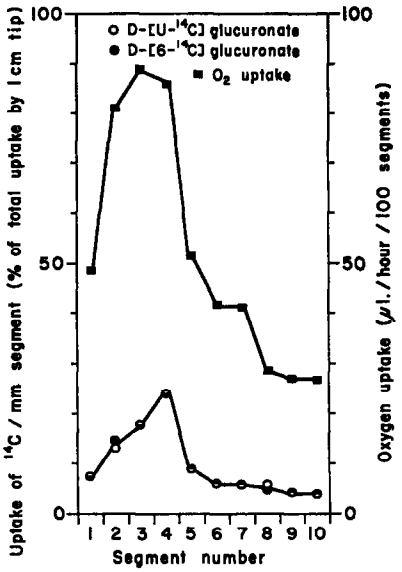

FIG. 2. UPTAKE OF D-GLUCURONATE BY SEGMENTS OF ROOT IN RELATION TO RATES OF OXYGEN UPTAKE.

ments of each class (millimetre segments 1–10) were incubated in 0·5 µc of the labelled glucuronates supplied at a concentration of 1 µmole/3 ml in 0·02 M phthalate buffer (pH 5·5) at 25° for 6 hr. Measurements of the losses of isotope from the medium corresponded closely with the recovery of ^{14}C from segments of any group at the end of the incubation. Results are presented to show the uptake of each segment as a percentage of the uptake by the whole 1-cm root-tip (Fig. 2). Uptake by the fourth segment (i.e. that cut from the 3–4-mm region of the root) was highest. About 34 per cent of the ^{14}C provided in the medium was taken in by the tissue; this constituted about 25 per cent of the uptake of the whole tip. The third segment, however, which had the highest respiration rate and carbohydrate content showed only 17·5 per cent of the total ^{14}C uptake. Segments 5 and beyond exhibited a progressively lower utilization of glucuronate. Tissue from segment 10, for instance, took up only about 6 per cent of the radioactive glucuronate provided.

The radioactivity in the ethanol was very low (Fig. 3); of the total isotope taken up by the ten segments, only 6·1 per cent was present in this fraction from the C-6 labelled compound.

The corresponding figure for the uniformly labelled glucuronate was 9·7 per cent. Younger regions accumulated most of this soluble isotope. Although by paper chromatography in solvent A, glucuronate was shown to be a major soluble radioactive compound, several other unidentified materials were present in each segment running near to the origin. In tissues supplied with D-[U-^{14}C] glucuronate, several faster moving components with mobilities similar to those of pentose sugars were observed, but no final identification was made. No distinctive differences were observed between segments of increasing age.

Carbon atom 6 contributed most extensively to the respired CO_2 in the fourth segment where there was a sharp maximum, and 21·8 per cent of the ^{14}C from the C-6 labelled glucuronate taken up by the whole tip was released as CO_2 by this segment. However, small amounts of isotope from other carbon atoms also contributed to the CO_2 from all segments, since when uniformly labelled glucuronate was supplied, more ^{14}C was released than expected for a single decarboxylation at carbon atom 6.

The incorporation of label from carbon atom 6 of glucuronate into the insoluble fraction

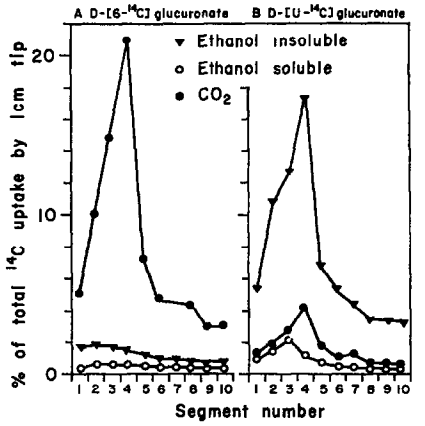

FIG. 3. INCORPORATION OF ^{14}C FROM D-GLUCURONATE INTO ROOT SEGMENTS AND RESPIRED CO_2.

appeared exclusively in uronic acid residues in all segments. Following acid hydrolysis, with 2 N HCl, two main radioactive regions were revealed on chromatograms developed in solvent A which corresponded closely in mobility with galacturonic and glucuronic acids. A further, unidentified, radioactive area was detected slightly ahead of glucuronic acid and this may have been a methyl substituted uronic acid or an unidentified aldobiuronic acid. Radioactivity at the origin also revealed that hydrolysis was not completed in 6 hr. Labelled glucuronic and galacturonic acids can also be separated in solvent B and have been identified in hydrolysates from intact roots that had been incubated in the labelled glucuronates. Incorporation of label from D-[6-^{14}C] glucuronate indicated, therefore, the synthesis of cell wall uronic acids in the root.

When the uniformly labelled compound was supplied, xylose and arabinose, as well as uronic acids were found to be radioactive in each segment. However, because the label was predominantly in the pentose sugars, incorporated radioactivity from D-[U-^{14}C] glucuronate was assumed to represent pentosan synthesis.

The incorporation of label from D-[6-^{14}C] glucuronate was most marked in segments 1–4 (Fig. 3A), but was always small compared to the incorporation from D-[U-^{14}C] glucuronate

(Fig. 3B). As predicted, the pattern of ^{14}C incorporation into pentose along the root closely paralleled the release of carbon atom 6 as CO_2. However, the amount of polysaccharide and therefore cell wall material, varied considerably in different regions (Fig. 1); and when the incorporation was calculated on the basis of unit carbohydrate and hence unit cell wall material present (if the starch content is ignored), the uronic acids were synthesized most extensively in walls of the first millimetre (Fig. 4). Incorporation was also high in segment 2, but declined in older regions. The labelling of pentose, on the other hand, was very high in segment 4, but considerably lower in segments older and younger than this, even those that were considered to be elongating (5 and 6). There is no adequate explanation of the slight depression in incorporation in segment 3 compared with adjacent regions. Clearly, however there was a characteristic region of high synthesis of pentosan in cell walls before elongation was completed, and the pattern of uronic acid synthesis was quite distinct from this.

(d) *The incorporation of ^{14}C from* D-[1-^{14}C] *galactose into root segments.* 1·0 μc of the

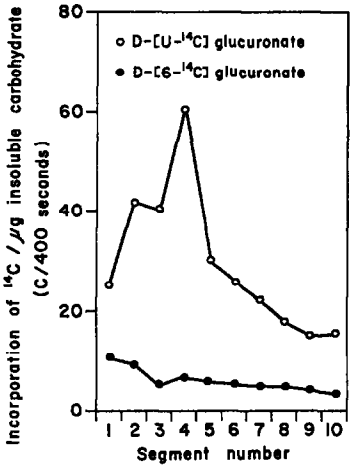

FIG. 4. INCORPORATION OF ^{14}C FROM D-GLUCURONATE INTO ETHANOL-INSOLUBLE FRACTIONS OF ROOT SEGMENTS IN RELATION TO THE AMOUNT OF INSOLUBLE CARBOHYDRATE PRESENT.

labelled galactose was supplied to each group of segments at a concentration of 0·3 μmole/3 ml water at 25° for 3 hr. Only 75 segments were employed in each flask to ensure that uptake was not completed before the end of the incubation period. Uptake by the third and fifth segments was most rapid; they took up about 65 per cent of the total isotope available. These segments each contributed about 15 per cent towards the total uptake by the whole root (Fig. 5). Uptake was low in the youngest and oldest regions of the root and highest in elongating segments.

The ethanol-soluble radioactive pool was very complex and, although galactose was present in each segment, it did not appear to accumulate in cells, but was quickly transformed into other compounds. The soluble fraction from all segments comprised about 38 per cent of the total radioactivity (Fig. 5). Labelled CO_2 was given off from all regions of the root in small amounts; about 7·7 per cent of the total radioactivity was released. $^{14}CO_2$ release was most extensive from the younger segments, particularly the third, which also showed the highest rates of uptake and respiration.

After hydrolysis of the alcohol-insoluble fraction in dilute sulphuric acid, galactose was

the main radioactive compound in all regions, but glucose also contained high amounts of label (Fig. 6). Xylose, arabinose and possibly uronic acids were faintly radioactive, but there were only traces of isotope in amino acids. The latter could be detected only by long exposure

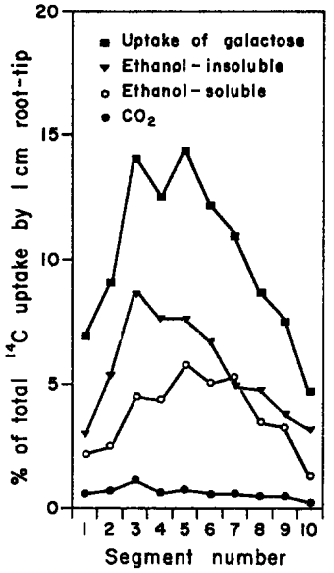

FIG. 5. INCORPORATION OF LABEL FROM D-[1-^{14}C] GALACTOSE INTO ROOT SEGMENTS AND RESPIRED CO_2.

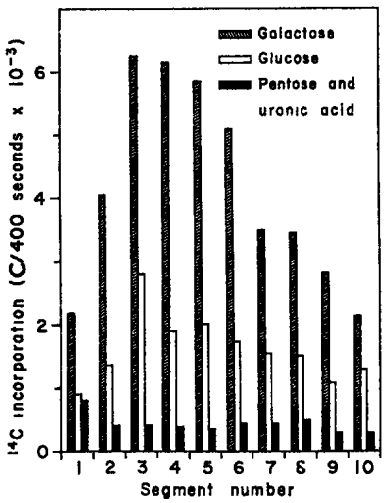

FIG. 6. ^{14}C INCORPORATION INTO SUGARS RELEASED BY HYDROLYSIS OF THE CELL WALL OF ROOT SEGMENTS THAT HAD BEEN SUPPLIED WITH D-[1-^{14}C] GALACTOSE.

of the chromatograms to X-ray film. When the paper strips were automatically scanned for radioactivity, just the labelled sugars could be detected. The relatively high activity in galactose and glucose allowed an accurate assessment of incorporated label in these sugars to be made. The incorporation into galactose was highest in the third segment and markedly

lower than this in the two younger regions. In older segments a slower decline in incorporation was evident. When results are expressed to show the amount of radioactivity in galactose per unit insoluble carbohydrate material in each segment, incorporation was highest in the fifth and sixth millimetre, and considerably lower in very young and fully extended tissues (Fig. 7).

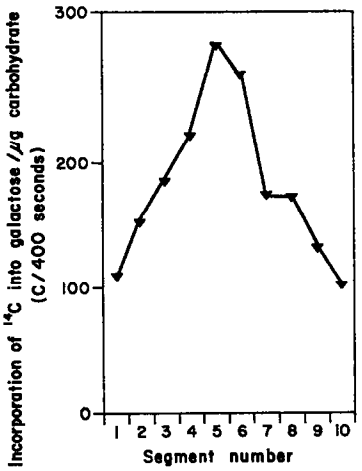

FIG. 7. RADIOACTIVITY IN CELL WALL GALACTOSE IN RELATION TO THE AMOUNT OF INSOLUBLE CARBOHYDRATE PER SEGMENT.

DISCUSSION

D-galactose and D-glucuronic acid, which are known precursors of cell wall polysaccharide units in several plant tissues that have been tested, have been supplied to small segments cut from the initial centimetre of the primary root of 3-day-old maize seedlings. Because meristematic activity is confined to a relatively discrete region near the tip, these segments constitute a series of cell preparations of increasing developmental age. In addition, all growth in the root occurs within the first 7 mm from the tip, so that the metabolic processes concerned with cell wall extension are also localized within the 1-cm region.

The utilization of glucuronate by all segments appeared similar to that observed in other tissues. The incorporation of carbon atom 6 into the cell wall occurred exclusively as uronic acids. The formation of pentose involved a loss of this carbon atom as CO_2, and there was a close similarity, therefore, between the patterns for labelled CO_2 production and the synthesis of cell wall pentose units along the root. CO_2, however, was released in small amounts from other parts of the carbon skeleton and not exclusively from carbon 6. This aspect of glucuronate metabolism will not be discussed here.

Label from D-[6-^{14}C] glucuronate, incorporated into the wall, was associated with both glucuronic and galacturonic acids as well as various products of partial acid hydrolysis. Galacturonic acid, in most plant tissues tested, exists predominantly as α 1,4 linked chains with various neutral side groupings attached.[7] These polysaccharides are extractable as pectin. Glucuronic acid and 4-*O*-methyl glucuronic acid, on the other hand, usually occupy terminal positions on the side chains of polysaccharides associated with the hemicellulose fraction of the wall.[8] In these experiments, the two types of acidic polysaccharide were not

[7] G. O. ASPINALL and R. FANSHAWE, *J. Chem. Soc.* 4215 (1961).
[8] T. E. TIMELL, *Advan. Carbohydrate Chem.* **19**, 247 (1964).

distinguished and label was expected to be incorporated into both. Clearly, however, the synthesis of uronic acid was always low in relation to pentose production. Nevertheless, incorporation was relatively high in the youngest regions. It is here that meristematic activity and the formation of new cell walls is localized. The root-cap also occupies a part of the first segment and this tissue is known to produce large amounts of mucilaginous polysaccharide, probably acidic in nature, whose synthesis is probably localized within the Golgi bodies.[9, 10]

In relation to the amount of cell wall material present, the synthesis of pentose units was very high in segment 4. This region corresponds to an early stage of cell wall extension. In segments 5 and 6, in which cells were growing at a comparable rate to that in the fourth, incorporation was much lower. This suggests that the composition of the non-cellulosic polysaccharides is continually changing during an apparently steady phase of growth. The presence of label in both uronic acid, pentose and galactose in the fully grown segments indicates that some thickening of existing wall occurs after elongation is complete.

Label from D-galactose was efficiently incorporated into galactose units of the cell wall polysaccharides. A smaller incorporation occurred into glucose, and only negligible activity was recovered in other ethanol insoluble compounds after acid hydrolysis. Galactose, therefore, like glucuronate, provides a useful tool for tracing the specific synthesis of particular cell wall sugar units. The pattern of incorporation from labelled galactose was very different from that observed using uniformly labelled glucuronate. No marked peak of incorporation was obtained in the fourth millimetre, and galactose units were laid down at the highest rate at a later stage of development than the pentose sugars. Synthesis was highest in cells that were approaching the end of their elongation phase. It may be, therefore, that the incorporation patterns observed are important in relation to the control of the physical properties of the cell wall, particularly extensibility.

However, in experiments of this kind employing segments of root-tips, there are many objections which make interpretations difficult. Each segment, for instance, contains a wide variety of tissues which may be developing independently of one another. No general patterns of cell wall development may exist. Furthermore, segments sliced as thickly as 1 mm contain cells in a relatively broad spectrum of developmental age, so that fine changes in biochemistry may be overlooked. Bearing in mind these difficulties, however, changing patterns of polysaccharide synthesis have undoubtedly been shown to occur in maize root-tips. These patterns have been further studied by means of autoradiography and the results will be published elsewhere.

EXPERIMENTAL

Materials. Potassium D-[U-^{14}C] and D-[6-^{14}C] glucuronate, and D-[1-^{14}C] galactose were obtained from the Radiochemical Centre, Amersham, Bucks.

Grain of *Zea mays* (Orla 266) was soaked for 8 hr in water and allowed to germinate in moist sphagnum moss for 64 hr at 25° in darkness. Segments were cut from the terminal tip of the primary root with a guillotine of razor blades, separated by 1-mm copper spacers. Large quantities could thereby be cut rapidly, and used within 90 min from excision.

Incubations. Incubations were performed in Warburg flasks containing 3 ml of fluid at 25°. The medium contained penicillin at a concentration of 10 μg/ml. Respiratory CO_2 was collected in 0·2 ml of 20% (w/v) KOH contained in the centre well of the flask. Oxygen

[9] B. E. JUNIPER and R. M. ROBERTS, *J. Roy. Microscop. Soc.* **85**, 63 (1966).
[10] D. H. NORTHCOTE and J. B. PICKETT-HEAPS, *Biochem. J.* **98**, 159 (1966).

uptake was determined manometrically. At the end of the incubation, the segments were filtered on to a sintered disc, washed thoroughly, and ground in 80% (v/v) ethanol in a glass homogenizer. The suspension was centrifuged, and the residue washed several times with ethanol to remove all soluble radioactivity.

Determination of radioactivity. The uptake of radioactivity from the medium was determined by withdrawing 0·1-ml samples and plating these on ½-in. glass coverslips with a drop of ethanol to assist spreading. These were counted directly with an end-window Geiger-Muller counter attached to a 1700 scaler (Isotope Development Ltd., Beenham Grange, Berks.) with a quench unit. Counting efficiency under these conditions was approximately 8·2 per cent. The isotope content of cell wall materials and ethanol extracts was determined by combustion with a potassium dichromate–iodate (2:1) mixture in fuming H_2SO_4–syrupy H_3PO_4 (2:1) containing potassium iodate (1 g/100 ml)[11] in the apparatus described by Stutz and Burris.[12] The CO_2 evolved was trapped in 20% (w/v) KOH and precipitated as $BaCO_3$ by adding an excess of saturated barium chloride solution. The barium carbonate was filtered on standard discs, 2 cm in diameter. These were dried and counted directly. Corrections were made for self-absorption in relation to the weight of $BaCO_3$ precipitated. Alcohol extracts and insoluble residues suspended in acetone were transferred to combustion flasks by pipette, and dried down by evaporation before combustion.

The isotope content of respiratory CO_2 was measured by transferring the KOH in the centre well to 0·05 M sodium carbonate (3 ml) together with two washings with water. Excess saturated barium chloride solution was added, and the barium carbonate collected and counted as described earlier.

Radioactivity on chromatograms was detected qualitatively by placing the paper in contact with Ilford "Ilflex" X-ray film, or quantitatively by passing the chromatogram through an integrated scanner attached to an automatic recorder,[13] the disintegrations being counted using a mica end-window G-M tube.

Hydrolysis of polysaccharide and chromatography of sugars. Hydrolyses were performed in sealed glass tubes in 2 N HCl at 100° for 6 hr, or in 3·6% (v/v) H_2SO_4 for 12 hr after first dissolving the polysaccharides completely in 72% (v/v) acid. Sulphuric acid was neutralized by barium carbonate. Hydrolysates were evaporated to dryness *in vacuo* at room temperature. Sugars and uronic acids were separated by descending chromatography on Whatman No. 1 papers. The solvents used were: (A) benzene–*n*-butanol–pyridine–water (1:5:5:3); and (B) ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Spots were located by spraying with aniline hydrogen phthalate in butanol, followed by heating in an oven at 105°.

Determination of carbohydrate. Polysaccharides made soluble in 72% (v/v) H_2SO_4 were determined by sulphonated α-naphthol.[14]

Acknowledgements—The author is grateful to Mr. J. G. Castle for help in the cutting of segments and in performing some of the combustions. The work was carried out under the tenure of a Christopher Welch Scholarship at Oxford University and constitutes part of the work submitted in a thesis for the degree of D. Phil., 1965.

[11] D. D. Van Slyke and J. Folch, *J. Biol. Chem.* **136**, 509 (1940).
[12] R. E. Stutz and R. H. Burris, *Plant Physiol.* **26**, 226 (1951).
[13] B. C. Loughman and R. B. Martin, *J. Expl Botany* **8**, 272 (1957).
[14] Z. Dische, *Methods in Carbohydrate Chemistry*, Vol. 1, p. 478. Academic Press, New York (1962).