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THE BIOSYNTHESIS OF POLYSACCHARIDES*

4. PLUM LEAF CELLULOSE

P. ANDREWS,† L. HOUGH and J. M. PICKEN‡

Department of Organic Chemistry, The University, Bristol

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Abstract—Detached plum tree spurs were allowed to take up and metabolize $^{14}CO_2$, D-glucose-1-^{14}C, D-glucose-6-^{14}C and D-glucuronic acid-6-^{14}C for various periods of time. Incorporation of ^{14}C from $^{14}CO_2$ into cellulose continued for at least 40 hr after the supply of isotope to the leaves had ceased. The labelling patterns in D-glucosyl units constituting the cellulose after incorporation of labelled glucoses suggest that units for cellulose synthesis originate from two distinct sources within the leaf, and a structural basis for the location of these sources is proposed. The labelling pattern in D-glucosyl units of cellulose following incorporation of labelled D-glucuronic acid indicates that $^{14}CO_2$ arising from decarboxylation of the glucuronate is re-incorporated into hexose within the leaf. The results further suggest that transfer of labelled precursor of high specific activity to the pool providing for cellulose synthesis occurs for only a brief period following the incorporation of labelled compounds by the leaves, so that the precursor's labelling pattern at this time is effectively trapped by its mixing with precursor already in the pool.

INTRODUCTION

A STUDY of the incorporation of ^{14}C from $^{14}CO_2$, D-glucose-1-^{14}C and D-glucose-6-^{14}C into the polysaccharides of plum leaves[1,2] has indicated the existence in the leaves of specific interrelationships between the polysaccharides and precursor pools, rather than a situation in which polysaccharide synthesis draws on a common supply of precursor monosaccharides. These previous investigations were concerned mainly with starch, pectin and hemicellulose polysaccharides, although the labelling in D-glucosyl units of cellulose following incorporation of the labelled glucoses was also determined.[2] To obtain more information on cellulose biosynthesis in the leaves, cellulose-containing leaf fractions from the $^{14}CO_2$ incorporation experiments, from further experiments involving incorporation of labelled glucose, and from experiments involving the incorporation of D-glucuronic acid-6-^{14}C by plum leaves[3] have now been examined.

RESULTS AND DISCUSSION

In experiments involving the incorporation of $^{14}CO_2$, plum leaves were allowed to photosynthesize initially in the presence of $^{14}CO_2$, and then in those experiments of more than 6 hr duration in a normal atmosphere, for total periods ranging from 1·5 to 48 hr. (cf.[1]). Cellulose was isolated from the leaves, hydrolysed, the resultant D-glucose was purified, and

* For part 3 see Ref. 2.

† Present address: National Institute for Research in Dairying, Shinfield, Reading, England.

‡ Present address: Dept. of Chemistry, Ohio State University, Columbus, Ohio, U.S.A.

[1] P. ANDREWS and L. HOUGH, *J. Chem. Soc.* 4483 (1958).

[2] P. ANDREWS, L. HOUGH and J. M. PICKEN, *Biochem. J.* 94, 75 (1965).

[3] J. D. ANDERSON, P. ANDREWS and L. HOUGH, *Biochem. J.* 84, 140 (1962).

its specific activity determined.[2] From these results and a knowledge of the specific activity of the $^{14}CO_2$ supplied to the leaves, the proportion of carbon in the cellulose which originated from the $^{14}CO_2$ was calculated. The extent of incorporation of ^{14}C into cellulose was proportional to the period of photosynthesis up to about 24 hr, after which it increased even further but at a slower rate (Fig. 1), although the intake of $^{14}CO_2$ by the leaves had ceased after 6 hr (cf. 1). These results contrast with those obtained for the constituent monosaccharides (D-glucose, D-galactose, L-rhamnose, L-arabinose, D-xylose) of the water-soluble and alkali-soluble leaf polysaccharides, whose specific activities reached maximum values after about 15 hr photosynthesis and then declined.[4]

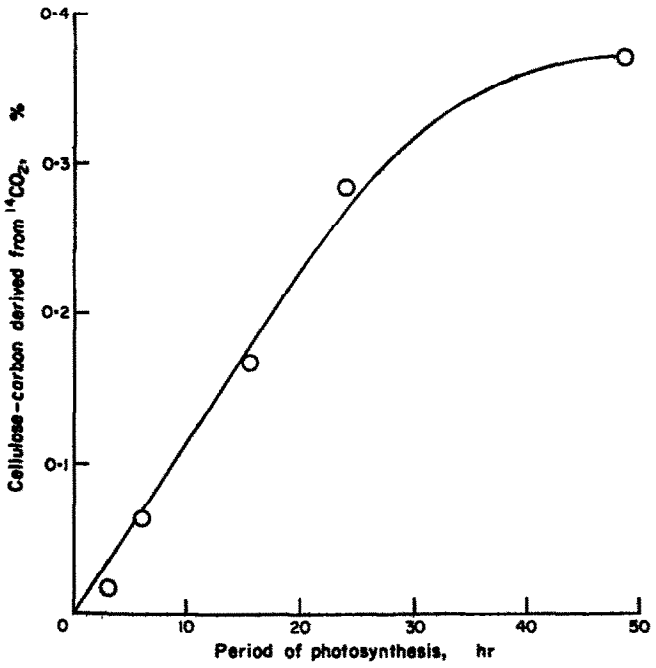

FIG. 1. INCORPORATION OF ^{14}C INTO PLUM LEAF CELLULOSE FOLLOWING PHOTOSYNTHESIS BY THE LEAVES IN $^{14}CO_2$.

The results may reflect differences both in the turnover rates of precursor pools for synthesis of cellulose and of the other polysaccharides, and in the metabolic activity of the polysaccharides themselves. The experiments involved the assimilation of large amounts[1] of carbon dioxide by the leaves during several hours, so that the precursor pools were presumably heavily labelled. If the pool for cellulose synthesis is large but is drawn on only slowly, and replenished with material which for a while is still quite heavily labelled because of the amount of isotope in other metabolic pools in the leaf, net incorporation of isotope into cellulose could well continue steadily for a long period, especially if simultaneous breakdown of the cellulose does not occur. On the other hand, if the precursor pools for other polysaccharides are smaller, and they are drawn on more rapidly, then the diminishing specific activity of material with which they are replenished will soon affect labelling in newly syn-

[4] P. ANDREWS. Unpublished work.

thesized polysaccharide. If the degradation of the polysaccharides also occurs, the contrast with cellulose will be further enhanced. Although our results are insufficient to show whether or not the ^{14}C was irreversibly incorporated into cellulose, this is clearly a possibility. Margerie and Lenvel[5] adduced evidence against the biologically inert nature of cellulose in the root tips of germinating wheat, by showing a continual exchange of radioactivity between this cellulose and alcohol-soluble material of the plant, but a difference in metabolic activity may exist between cellulose in a rapidly growing tissue such as root tip and that in mature plum leaves.

The distribution of ^{14}C in D-glucose obtained by hydrolysis of cellulose labelled in the 3 hr photosynthesis period was determined by a method[2] which gave the specific activity of each terminal carbon atom, and a mean value for the other four carbon atoms. The percentage distribution of ^{14}C along the carbon chain was calculated from these values. The results (Table 1) showed no marked asymmetry of labelling as between the terminal carbon atoms and the other carbon atoms of the glucose units.

D-Glucose-1-^{14}C, D-glucose-6-^{14}C and D-glucuronic acid-6-^{14}C were incorporated into the leaves of plum tree spurs through the cut ends of the stems, and subsequently metabolized by the leaves.[2,3] D-Glucose was isolated as before from the leaf cellulose, and the distribution of ^{14}C along the carbon chain determined. The results are shown in Table 1.

Incorporation of D-glucose-1-^{14}C resulted in cellulose-glucose in which about 75 per cent of the ^{14}C was retained at C-1, the remainder being mainly at C-6. An analogous situation followed incorporation of D-glucose-6-^{14}C, with retention of 75 per cent of the label at C-6 and transfer of 22 per cent to C-1. Similar redistributions of isotope from labelled D-glucose, occurring in both directions, have been observed in tracer experiments on cellulose synthesis by cotton bolls,[6] barley seedlings,[7] wheat seedlings[8] and wheat plants.[9–12] They are generally attributed to the rapid equilibration of hexose phosphate with the Embden–Meyerhof glycolytic pathway, involving its fission to 1,3-dihydroxypropan-2-one 1-phosphate and D-glyceraldehyde 3-phosphate, isomerization of these compounds catalysed by D-glyceraldehyde 3-phosphate ketol-isomerase, and reconstitution of hexose phosphate. In most experiments, including our own, only 12–23 per cent of the label in cellulose-glucose was located at the terminal carbon atom opposite to the one originally labelled, despite the variety of experimental conditions employed. In no case was isotope evenly apportioned between C-1 and C-6 by the redistribution. Apparently the isomerization of triose phosphate occurs slowly compared with the rate of hexose phosphate resynthesis, or else only 24–46 per cent of the labelled hexose phosphate molecules utilized for cellulose synthesis in these experiments had been through the Embden–Meyerhof pathway.

If the first of these explanations of the observed labelling patterns is correct, a greater retention of isotope at its original position might be expected in cases of rapid cellulose synthesis, such as occurs in growing cotton bolls,[6] than in comparatively slow cellulose synthesis, as presumably occurs in mature plum leaves. Similarly, less randomization might be expected in wheat seedlings[8] than in more mature wheat plants.[9–12] However, allowing for differences in experimental conditions, this does not seem to be the case.

[5] C. Margerie and C. P. Lenvel, *Biochim. Biophys. Acta* **47**, 275 (1961).
[6] F. Shafizadeh and M. L. Wolfrom, *J. Am. Chem. Soc.* **77**, 5182 (1955).
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[10] A. C. Neish, *Can. J. Biochem. Physiol.* **33**, 658 (1955).
[11] H. A. Altermatt and A. C. Neish, *Can. J. Biochem. Physiol.* **34**, 405 (1956).
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TABLE 1. SPECIFIC ACTIVITY AND DISTRIBUTION OF ^{14}C IN D-GLUCOSE ISOLATED FROM PLUM LEAF CELLULOSE AFTER INCORPORATION OF ^{14}C-LABELLED PRECURSORS

Precursor			Period of metabolism (hr)	Specific activity of glucose carbon $\times 10^3$ (μc/m-atom)	Location of ^{14}C*		
Name	Wt. (mg)	Activity (μc)			C-1	C-2+C-3+C-4+C-5	C-6
$^{14}CO_2$	367	93	3	5·5	17·3	65·5	17·2
D-Glucose-1-^{14}C†	3·8	100	5	7·2	77·8	1·8	20·4
D-Glucose-1-^{14}C†	3·8	100	20	9·4	71·8	6·6	21·6
D-Glucose-6-^{14}C†	1·2	50	5	3·4	23·1	1·9	75·0
D-Glucose-6-^{14}C†	1·2	50	20	6·5	21·5	6·0	72·5
D-Glucuronic acid-6-^{14}C	15·8	100	6	5·4	29·5	41·0	29·5

* Radioactivity at each location is expressed as a percentage of the total radioactivity found in the degradation products. Possible uncertainties in such figures are discussed elsewhere.[2]

† See Table 3 in earlier paper.[2]

We suggest that the similarity in labelling patterns observed in so many experiments can be better explained if the precursor pool for cellulose synthesis is supplied from two distinct sources. Some of the glucose incorporated into cellulose has clearly been through the Embden–Meyerhof pathway, so this is one source of supply. Assuming that the glucose units from this source have equal distribution of ^{14}C between C–1 and C–6, the cellulose must also contain glucose units whose labelling pattern approximates to that of the ^{14}C-glucose fed to the leaves. The supply from both sources of labelled glucose of high specific activity to the precursor pool may well last for only a short time compared with the experimental periods allowed for isotope incorporation into cellulose, since labelled glucose entering the plants probably soon passes out of the translocation system, and isotope circulating in the Embden–Meyerhof pathway also could be quickly dispersed. The labelling patterns in the short bursts of high specific activity glucose from the two sources then are effectively trapped as they enter the precursor pool for cellulose synthesis, particularly if the pool is relatively large, as

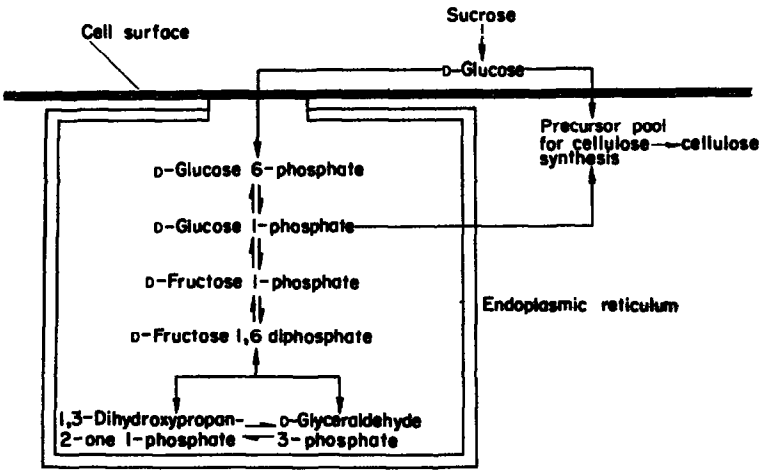

FIG. 2

suggested above, and its turnover is slow. Any isotope subsequently added to the pool is present only in glucose with a lower specific activity, and coming possibly from the Embden–Meyerhof pathway. As a result, cellulose synthesis draws on a pool of glucose which changes little in its labelling pattern over a long period.

Figure 2 illustrates diagrammatically the spatial separation envisaged between the two sources of precursor, the precursor pool, and the synthetic site itself. Work on cell fractionation[13] indicates that the glycolytic pathway enzymes are located in the cytoplasm or soluble fraction of the cell, and are not associated with any of the particulate components. Therefore the suggestion is that the endoplasmic reticulum of the cell forms a partition between the enzymes of the Embden–Meyerhof pathway and the precursor pool which, together with the synthetic site, seems likely to be at or near the cell surface.

The metabolism of D-glucuronic acid-6-^{14}C by plum leaves resulted in cellulose-glucose bearing a labelling pattern (Table 1) which differed from that resulting from D-glucose-6-^{14}C incorporation and from $^{14}CO_2$ incorporation. The terminal carbon atoms were equally labelled as in the $^{14}CO_2$ experiments, but a lower proportion of the activity in the molecule

[13] H. HOLTER, *Advanc. Enzymol.* 13, 1 (1952).

was located at C-2 to C-5. The results are similar to those of Neish[12] on the incorporation of the same labelled compound by wheat plants, in that considerable labelling appears in the middle carbon atoms, but differ in that Neish found a greater concentration of isotope at C-1 than at C-6. The decarboxylation of D-glucuronic acid by plant tissues is well known,[11,14] and is a major route for its metabolism by plum leaves.[3] Some of the $^{14}CO_2$ arising in this way from C-6 of the glucuronic acid is evidently reutilized by the leaves for hexose synthesis, since the labelling pattern characteristic of this mode of synthesis, namely an initial high concentration of isotope at C-3 and C-4 of the hexose (cf.[15,16]), is trapped in sorbitol of plum leaves following incorporation and metabolism of D-glucuronic acid-6-^{14}C by the leaves.[3] The labelling at C-2 to C-5 of cellulose-glucose following incorporation of D-glucuronic acid-6-^{14}C presumably arose at least in part in a similar way. Equal labelling at C-1 and C-6 of cellulose-glucose following the glucuronic acid incorporation indicates that the precursor pool for cellulose synthesis received isotopically labelled glucose from the Embden–Meyerhof pathway, but no uniquely labelled glucose derived from the glucuronic acid by direct reduction. The likelihood is, therefore, that glucuronic acid is utilized by plum leaves mainly, if not entirely, by pathways which involve its degradation.

EXPERIMENTAL

Isotope Incorporation

Labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks. For each experiment non-fruiting spurs, each bearing seven or nine leaves were cut from a plum tree (*Prunus domestica* var. Giant Prune) and kept with the cut ends of the stems in water until required (usually 1–2 hr).

The labelled monosaccharides (Table 1) were dissolved in water (1·5 ml) and portions (0·3 ml) fed from a small glass cup to the cut end of each stem. The solution was imbibed after about 10 min when two further 0·3 ml portions of water were allowed to be taken up by each spur, then water was freely supplied from large containers. Constant illumination of about 400 ft-candles and a gentle current of air was maintained over the leaves during the experiments.

For the incorporation of $^{14}CO_2$, the leaves were kept in the dark for 24 hr. They were then placed in a glass photosynthetic chamber under steady illumination (400 ft-candles) and $^{14}CO_2$ liberated by acidification of $Ba^{14}CO_3$[1]. The leaves incorporated all of the $^{14}CO_2$ after 6–8 hr when, if necessary, the spurs were removed from the chamber and left in the open with their cut ends still in water and under the same illumination.

In each experiment metabolism was terminated after the required interval of time by breaking up the leaves and plunging the laminae into boiling ethanol for 2–3 min and then extracting them in a Soxhlet apparatus for several hours with methanol and then with ether. The insoluble leaf residue was dried at 50°, powdered, dried to constant weight over phosphoric oxide and assayed for ^{14}C.

Radioactivity Measurements

The ^{14}C-labelled compounds were burnt in a stream of oxygen and the $^{14}CO_2$ so produced was converted into $Ba^{14}CO_3$ disks of infinite thickness for counting with a thin-end-window Geiger counter.[2]

[14] W. G. SLATER and H. BEEVERS, *Plant Physiol.* 33, 146 (1958).
[15] M. GIBBS and O. KANDLER, *Proc. Nat. Acad. Sci.* 43, 446 (1957).
[16] J. A. BASSHAM, A. A. BENSON, L. D. KAY, A. Z. HARRIES, A. T. WILSON and M. CALVIN, *J. Am. Chem. Soc.* 76, 1760 (1954).

Isolation of D-*Glucose from Cellulose*

The insoluble leaf residue was extracted exhaustively with 2·5 N-sodium hydroxide at 80° and the insoluble residue (2·5% w/v) heated as a suspension in N-sulphuric acid at 100° until no further radioactive material was released (~8 hr). The insoluble cellulose was then hydrolysed to D-glucose by the method of Monier–Williams,[17] which involves treatment of the material (200 mg) with 1 ml of 72% (w/w) sulphuric acid for a week at room temperature, dilution to 50 ml and then heating at 100° for 8 hr. After de-ionization, evaporation gave crystalline D-glucose, which after recrystallization from methanol/ethanol was assayed for its specific activity.[2]

Degradation of ^{14}C*-Labelled* D-*Glucose*

The aldose (0·48 m-mole) was dissolved in a little water and mixed with a solution of iodine (280 mg) in methanol (8 ml) at 40°. A 5% (w/v) solution of potassium hydroxide in methanol (6 ml) was then added dropwise with stirring. After 15 min, the reaction mixture was cooled, the crystalline potassium D-gluconate filtered off, washed with methanol and ether and dried over silica gel.

A solution of the aldonate was then oxidized with sodium metaperiodate and the products, carbon dioxide from C-1, formic acid from C-2, C-3, C-4 and C-5, and formaldehyde from C-6, separated and each converted to CO_2 and assayed as $BaCO_3$, as described previously.[2]

Acknowledgements—We thank the Agricultural Research Council for the award of a Fellowship (to P. A.) and the Department of Scientific and Industrial Research for a Maintenance Award (to J. M. P.).

[17] G. W. Monier-Williams, *J. Chem. Soc.* 803 (1921).