904. *The Biosynthesis of Polysaccharides. Part II.*[1] *Incorporation of $^{14}CO_2$ into Plum-leaf Polysaccharides during Photosynthesis.*

By P. Andrews and L. Hough.

The rate of incorporation of $^{14}CO_2$ into plum leaves during 48 hours' photosynthesis and the distribution of the ^{14}C between the methanol-soluble and -insoluble portions of the leaves, were determined. At the end of this period the polysaccharides in the insoluble portion were fractionated and the specific activities of their main monosaccharide constituents estimated. ^{14}C-Labelled D-glucose, D-galactose, L-rhamnose, L-arabinose, D-xylose, and D-galacturonic acid were obtained crystalline or as crystalline derivatives. The extent of ^{14}C incorporation into these monosaccharides varied widely from one to another, and the specific activities of each monosaccharide, except D-galactose, differed significantly in different polysaccharide fractions.

In Part I,[1] the polysaccharide constituents of Victoria plum leaves were shown to consist of appreciable quantities of D-galactose, L-arabinose, D-xylose, L-rhamnose, and D-galacturonic acid, in addition to D-glucose. In a tracer experiment, Hough and Pridham[2] observed the incorporation of $^{14}CO_2$ into these units during photosynthesis, suggesting the existence of a dynamic equilibrium between the polysaccharides and their monosaccharide precursors. Since the majority of plant polysaccharides contain one or more of these monosaccharides, which may well be preformed in the leaves for their subsequent inclusion into polysaccharides in other parts of the plant, it was of interest to examine further the assimilation of $^{14}CO_2$ by plum leaves, particularly the appearance of ^{14}C in the constituent polysaccharides.

Mature leaves from Victoria plum trees were kept in the dark for 24 hr. to deplete their polysaccharide content and then allowed to photosynthesise in the presence of $^{14}CO_2$. The leaves were placed initially in a closed system[3] containing 5% of $^{14}CO_2$, only traces of which were recovered after 6 and 8 hours' photosynthesis by the leaves under the illumination provided (about 400 foot-candles). Incorporation and metabolism of $^{14}CO_2$ was allowed to proceed for periods varying from 1 to 48 hr., but in those experiments of more than 8 hr. duration the leaves were removed from the closed system at that time and allowed to continue photosynthesis in the open. Considerable differences have been observed in the photosynthetic activities of apple leaves;[4,5] therefore in an attempt to minimise the effects of such variations in our experiments, about twenty plum leaves were selected each time and the conditions for photosynthesis were standardised as far as possible. However, some variation in the total weights of the leaves was unavoidable.

The leaf-blades were broken up and immediately dropped into hot ethanol to halt their metabolism, then the pieces were exhaustively extracted with methanol and ether. The extracts and the insoluble leaf residues from each experiment were assayed in order to determine the overall distribution of ^{14}C in the leaves after various intervals. At all

[1] Part I, Andrews and Hough, preceding paper.
[2] Hough and Pridham, *Nature*, 1956, **177**, 1039.
[3] Livingston and Medes, *J. Gen. Physiol.*, 1947, **31**, 75.
[4] Heinicke, Agric. Expt. Station, Cornell, Bull. No. 577, 1933.
[5] Asselbergs, *Plant Physiol.*, 1957, **32**, 326.

times the extracts contained more of the original $^{14}CO_2$ activity than did the insoluble leaf residue (Table 1). The activity in the extracts was the first to reach a maximum, which,

TABLE 1. *^{14}C in leaf fractions, as percentage of ^{14}C supplied in $^{14}CO_2$.*

Period of photosynthesis (hr.)	1	1·5	3	6	15·5	24	48
Activity in leaf extract	18·4	24·5	53·8	58·4	56·3	52·3	49·0
Activity in insoluble leaf residue ...	6·2	9·5	25·0	39·9	40·7	38·7	32·3
Total activity in leaves	24·6	34·0	78·8	98·3	97·0	91·0	81·3

from a plot of the figures, seemed to occur after about 5 hours' photosynthesis. A less marked maximum in the activity in the leaf residues was attained after 8—10 hr. After reaching their maxima both curves showed a gradual and parallel decline. Over 98% of the initial $^{14}CO_2$ activity was accounted for in the two leaf fractions after photosynthesis for 6 hr., and the following loss of activity may well have been due to the translocation of labelled compounds into the stems.[6]

TABLE 2. *Relative distribution of ^{14}C between leaf fractions.*

Period of photosynthesis (hr.)	1	1·5	3	6	15·5	24	48
Leaf extracts (%)	75	72	68	59·5	58	57·5	60
Insoluble leaf residue (%)	25	28	32	40·5	42	42·5	40

In the early stages the proportion of ^{14}C in the leaf extracts greatly exceeded that in the insoluble residue (Table 2), but that in the latter quickly increased, so that after 6 hr., when a steady state was reached, about 40% of the total activity assimilated by the leaves then resided in this fraction. Thereafter the proportion rose very slowly to 42·5% after 24 hr. and gradually diminished to 40% again during the next 24 hr. These results are consistent with a rapid initial incorporation of the ^{14}C into sugar phosphates and other compounds of low molecular weight, and their subsequent use in macromolecular synthesis. The rate of appearance of ^{14}C in the leaf residue may have been accelerated especially in the early stages by factors consequent on the pre-starvation of the leaves, which would have depleted their total carbohydrate content, and the high initial carbon dioxide concentration. However, since the total activity in the leaf residues never exceeded that in the extracts, in fact it decreased slowly after reaching a maximum, the macromolecular substances as a whole are clearly not accumulated as end-products of the leaf metabolism. Their constituent units are probably further redistributed by participation in the biological processes in the leaf.

From a knowledge of the specific activities involved, values were calculated for the percentage of the carbon in the insoluble leaf residues which had originated from $^{14}CO_2$. In order to compare these values one with another, allowance was now made for the variations in the quantity of leaves used in different experiments by adjusting each value to accord with a weight of 3 g. for the insoluble leaf residue. The values so obtained (Table 4) lay on a curve which rose rapidly to a maximum value of about 3·4% after 9 hours' photosynthesis and then declined slowly.

To obtain information on the extent of ^{14}C incorporation into the monosaccharide constituents of the various leaf polysaccharides after photosynthesis for 48 hr., the appropriate insoluble leaf residue was extracted [1] successively with hot water and cold and hot sodium hydroxide solution. The three polysaccharide fractions so obtained were hydrolysed separately, and the components of the resultant monosaccharide mixtures isolated by paper chromatography. Further purification of those sugars obtained in quantities (>2 mg.) considered sufficient for accurate assay was effected with ion-exchange resins, then each monosaccharide was diluted with the appropriate inactive monosaccharide, crystallised, and assayed. The results were confirmed by the preparation and assay of various derivatives of the sugars (Table 5). In order to relate these figures with those in Table 4, the corresponding values for the percentage of sugar-carbon

[6] Nelson and Gorham, *Canad. J. Bot.*, 1957, **35**, 340, 704.

originating from the $^{14}CO_2$ were calculated, again on the basis of 3 g. of insoluble leaf residue (Table 3).

TABLE 3. *Sugar-carbon (%) originating from* $^{14}CO_2$ *after 48 hours' photosynthesis.*

	Water-soluble polysaccharides	Polysaccharides soluble in cold N-NaOH	Polysaccharides soluble in hot 2·5N-NaOH
D-Glucose	—	21·6	18·5
D-Galactose	1·66	—	1·72
L-Rhamnose	0·94	—	1·12
L-Arabinose	1·58	0·90	0·74
D-Xylose	0·21	0·30	0·49
D-Galacturonic acid	0·08	—	—

Whilst the incorporation of isotopic carbon into the D-glucose of plum-leaf polysaccharides greatly predominated, significant activity was determined in D-galactose, L-rhamnose, L-arabinose, D-xylose, and D-galacturonic acid. Hough and Pridham's observations [2] are therefore confirmed, in that all these monosaccharides have acquired different specific activities. However, fractionation of the leaf polysaccharides has shown that the specific activities of the various component monosaccharides differed from one fraction to another, thus revealing a hitherto unsuspected complexity (cf. Perlin [7]). The incorporation of ^{14}C into leaf starches during photosynthesis of the leaves in $^{14}CO_2$ has been studied,[8] but similar aspects of the biological synthesis of other leaf polysaccharides have received little attention. Ginsburg and Hassid [9] isolated uniformly ^{14}C-labelled xylose and arabinose from canna-leaf polysaccharides, and Cowie and Krotkov [10] similarly obtained these labelled pentoses, together with ^{14}C-labelled galactose, from wheat seedlings.

The high specific activity of D-glucose is consistent with the important rôle of starch in leaf metabolism, as was suggested by the observations of Vittorio, Krotkov, and Reed,[8] who showed that, during photosynthesis, ^{14}C from $^{14}CO_2$ appeared in the starch fraction of tobacco leaves, previously stored in the dark, more rapidly than it did in the free glucose, fructose, and sucrose. In contrast, the very small amount of ^{14}C now found in D-galacturonic acid suggests that pectic acid is not at all an active metabolite in mature plum leaves. Both D-galactose and L-arabinose attained considerably higher specific activities than did D-galacturonic acid. Therefore if L-arabinose arises from D-galactose by oxidative decarboxylation *via* D-galacturonic acid, as has been suggested by isotopic tracer studies,[11] the intermediary hexuronic acid seems to be decarboxylated, or otherwise metabolised, without being incorporated to any great extent into the pectic acid.

EXPERIMENTAL

Determination of ^{14}C *Activities.*—Activities were determined in a Geiger counter with a thin mica end-window. For estimation of the activities of crystalline ^{14}C-labelled compounds and insoluble leaf residues, samples (usually 6—12 mg.) were burnt in a stream of oxygen, the $^{14}CO_2$ produced was passed into hot barium hydroxide solution (saturated at 20°), and the precipitated $Ba^{14}CO_3$ collected in layers (*ca.* 20 mg./cm.^{2}, equivalent to infinite thickness for counting), which were washed with water and dried at 120° before being used for activity measurements. Sufficient counts were taken to give a standard error of count of $\pm 3\%$ or better. The counting equipment was calibrated in terms of $Ba^{14}CO_3$ by determining the count-rate of infinitely thick layers of $Ba^{14}CO_3$ prepared from poly([^{14}C]methyl methacrylate) of known specific activity (supplied by the Radiochemical Centre, Amersham). A disc of this polymer was used as a standard reference source.

The ^{14}C activities of solutions (*e.g.*, alcohol extracts of leaves) were determined by infinite

[7] Perlin, *Canad. J. Chem.*, 1958, **36**, 810.

[8] Vittorio, Krotkov, and Reed, *Science*, 1954, **119**, 906; Gibbs, *Plant Physiol.*, 1951, **26**, 549; Gibbs and Kandler, *Proc. Nat. Acad. Sci. U.S.A.*, 1957, **43**, 446.

[9] Ginsburg and Hassid, *J. Biol. Chem.*, 1956, **223**, 277.

[10] Cowie and Krotkov, *Canad. J. Bot.*, 1957, **35**, 1.

[11] Neish, *Canad. J. Biochem. Physiol.*, 1955, **33**, 658; Seegmiller, Axelrod, and McCready, *J. Biol. Chem.*, 1955, **217**, 765; Seegmiller, Jang, and Mann, *Arch. Biochem. Biophys.*, 1956, **61**, 422.

thinness counting, for which the samples were prepared by pipetting aliquot parts (usually 5—20 μl.) on to nickel planchettes and evaporating the solvent at 120°. Sample weights were kept below *ca.* 0·2 mg. whenever possible, having regard also to the counting-rates. The variations in the results indicated an overall statistical and sample-preparation error of ±5% in most cases, but up to ±10% on the least active samples. The counting equipment was calibrated for this method of measurement with weighed aliquot parts (2—12 μl.) from a solution (1 c.c.) containing [6-^{14}C]glucose (50 μc; 1·2 mg.).

Apparatus for Photosynthetic Experiments.—Steady illumination was provided in an apparatus similar to that described by Folkes, Willis, and Yemm.[12] It consisted of an iron frame supporting, at the top, twelve 150 w tungsten-filament lamps mounted between a reflecting surface above and a glass-bottomed water tank below. The tank was fitted with a constant-level device, and the water passing through it conducted away heat from the electric lamps. Fans maintained a circulation of air through the apparatus, and the photosynthetic chamber was placed on a wooden stage which could be raised and lowered beneath the water tank. To adapt the apparatus for rearing plants in the laboratory, an automatic switch was put in circuit with the lamps to vary the illumination periods.

The photosynthetic chamber [3, 8] was a desiccator (vol. 4 l.) with two inlet tubes, both with stopcocks, passing through the centre of the lid. One inlet tube was terminated immediately inside the lid, and the other extended to within *ca.* 1 cm. of the bottom of the desiccator. Small beakers containing water, for holding leaves, were disposed around the shelf of the desiccator and held in place with a metal frame. A small beaker containing $Ba^{14}CO_3$ was placed in the bottom of the desiccator, to surround the end of the long inlet tube; sulphuric acid (30% v/v) was also put in the bottom for humidity control.

Procedure for Photosynthetic Experiments.—For each experiment four spurs, each bearing four or five leaves of length 7—10 cm. were cut from a plum tree (*Prunus domestica* var. Victoria) in early September 1956, and kept for 24 hr. in the dark with the cut ends of the stems in water. With the spurs in position in the desiccator, the pressure inside was reduced through the short inlet tube by *ca.* 6 cm. of Hg, then lactic acid solution (80% v/v) was added through the long inlet tube to the $Ba^{14}CO_3$ in the beaker at the bottom. In each experiment, *ca.* 9 mmoles (1·77 g.) of $Ba^{14}CO_3$ were used, this being enough to give an initial $^{14}CO_2$ concentration in the desiccator of 5%; the specific activity varied from 7 to 23 μc/mmole in different experiments. Sufficient lactic acid (*ca.* 25 c.c.) was used to immerse the lower end of the long inlet tube by 1 cm. When the evolution of $^{14}CO_2$ had ceased, the desiccator was placed under the light battery and the pressure inside adjusted to atmospheric by the admission of air through the long inlet tube. The stopcock on this tube was then left open to the air, loss of $^{14}CO_2$ being prevented by the immersion of the lower end of the tube in lactic acid.

Trials showed that, with the above experimental array, the intensity of illumination received by the leaves in the desiccator was about 400 foot-candles (measured with a Weston Photronic foot-candle meter), and that they incorporated the $^{14}CO_2$ in 6—8 hr. Accordingly at the end of photosynthesis periods of less than 8 hr., or after 8 hr. when longer periods were required, the desiccator was swept out for 10 min. with a rapid stream of carbon dioxide-free air, and any carbon dioxide present was trapped in sodium hydroxide solution. Not more than 2% of the activity supplied was recovered as $^{14}CO_2$ after any of the 6 and 8 hr. periods. In cases where photosynthesis was to be for more than 8 hr. the spurs were removed from the desiccator at that time and left under the light battery with the cut ends of the stems still in water for the remainder of the time. Only very slight activity was detected in this water in any of the experiments. The illumination intensity received in the open was about 500 foot-candles.

Each experiment was concluded by rapidly breaking up the leaves and immediately dropping the leaf-blades into boiling ethanol. Then the pieces were transferred to a Soxhlet apparatus and extracted successively with methanol and ether. The ethanol, methanol, and ether extracts from each experiment were combined; after radioactivity determinations the resultant solutions were evaporated to dryness and the residues retained for further investigation. The insoluble leaf residues were dried at 60°, powdered, and finally dried to constant weight (Found: C, 43·3%) over phosphoric oxide before radioactivity determinations. The results are summarised in Table 4.

Isolation of ^{14}C-Labelled Monosaccharides from Plum-leaf Polysaccharides.—The polysaccharides in the insoluble leaf residue from the 48 hr. experiment were extracted by successive

[12] Folkes, Willis, and Yemm, *New Phytol.*, 1953, **51**, 317.

treatments with hot water, N-sodium hydroxide at 20°, and 2·5N-sodium hydroxide at 80°, the extraction with each solvent being repeated (4—6 times) until no further material was dissolved. The final insoluble residue (217 mg.; 2·4 μc) was retained for further investigation. The aqueous extract was concentrated, and the alkaline extracts neutralised with acetic acid, then the polysaccharides in each extract were precipitated with ethanol. The crude polysaccharide fractions were washed several times with hot methanol, then dissolved in N-sulphuric acid, and the solutions were heated at 100° for 8 hr. In each case some insoluble material formed. The hydrolysates were neutralised with barium carbonate, clarified on the centrifuge, and assayed:

TABLE 4. *Plum-leaf photosynthesis experiments.*

Period of photosynthesis (hr.)	1	1·5	3	6	15·5	24	48
$^{14}CO_2$ supplied to leaves: Quantity (mmole)	8·83	9·05	8·83	8·98	8·94	8·77	9·17
$^{14}CO_2$ supplied to leaves: Specific activity (μc/mmole)	12·7	7·6	9·5	16·0	7·2	15·8	23·0
$^{14}CO_2$ supplied to leaves: Total activity (μc)	112	69	93	143	65	139	211
Activity found in: Leaf extracts (μc)	20·6	16·9	49·9	83·8	36·4	72·6	103·0
Activity found in: Insoluble leaf residue (μc)	7·0	6·6	23·2	57·2	26·4	53·8	68·2
Specific activity of carbon in leaf residue (μc/milli-atom-equiv.)	0·057	0·071	0·225	0·499	0·193	0·494	0·535
Wt. of insoluble leaf residue (g.)	3·35	2·50	2·82	3·12	3·72	2·97	3·40
% of carbon in leaf residue originating from $^{14}CO_2$ *	0·50	0·79	2·22	3·25	3·32	3·09	2·63

* Calc. on the basis of 3 g. of insoluble leaf residue.

the hydrolysates of the water-soluble, cold alkali-soluble, and hot alkali-soluble polysaccharide fractions contained approximately 3·3, 16, and 22 μc, respectively. The solutions were then evaporated and examination of the resultant syrups on the paper chromatogram indicated the presence of monosaccharides, traces of oligosaccharides, some ninhydrin-positive compounds, and some inorganic material. The monosaccharide constituents were: from the water-soluble polysaccharides, mainly galactose and arabinose (ratio *ca.* 1 : 1), with some galacturonic acid, rhamnose, xylose, and glucose, and a trace of 2-*O*-methylxylose; from the N-alkali-soluble polysaccharides, mainly glucose, smaller amounts of arabinose and xylose, and much smaller amounts of galactose, galacturonic acid, and rhamnose; and from the 2·5N-alkali-soluble polysaccharides, galactose, glucose, arabinose, and xylose (ratio *ca.* 1 : 2 : 2 : 1·5), smaller amounts of rhamnose and galacturonic acid, and traces of fucose and 2-*O*-methylxylose.[1] Autoradiographs of the paper chromatograms indicated that glucose was by far the most heavily labelled sugar, but activity was also detected in positions corresponding to galactose, arabinose, and rhamnose after the chromatograms had been left in contact with the emulsion side of *X*-ray films for 6 days.

The sugar mixtures were submitted to chromatography on paper sheets, with butan-1-ol–pyridine–water (10 : 3 : 3 v/v) as solvent. Particular attention was paid to obtaining each monosaccharide free from the others, and for galactose, glucose, and arabinose two separations were usually necessary. Galacturonic acid and small amounts of acidic oligosaccharides remained on the starting-line, and the former was purified by extracting the material from the starting-line with water and submitting it to paper chromatography (Whatman No. 540 paper) in ethyl acetate–acetic acid–water (9 : 2 : 2 v/v) solvent. The separated monosaccharides were eluted from the paper with cold water; the solutions of neutral sugars were then passed through columns of Amberlite IR-120(H) and IRA-400(CO_3) ion-exchange resins and evaporated to dryness. The galacturonic acid solution was passed through IR-120(H) resin only, and evaporated, the last few drops of water being removed *in vacuo* without heating. The sugar syrups were finally dried under reduced pressure over phosphoric oxide and weighed (see Table 5). Insufficient amounts of the minor constituents of the sugar mixtures were obtained for their further investigation. Ninhydrin-positive material could not be detected in any of the sugar syrups; the galactose and arabinose crystallised spontaneously, and the other neutral sugars did so when scratched. Galacturonic acid (10·3 mg.) from the water-soluble polysaccharide fraction contained some inorganic material (detected on the paper chromatogram); the optical rotation of the syrup {$[\alpha]_D$ +49° (*c* 1·03)} indicated that it was *ca.* 90% pure.

Determination of Specific Activities of Isolated Monosaccharides.—The ^{14}C-labelled neutral monosaccharides were recrystallised from the appropriate solvents after sufficient amounts of the corresponding unlabelled sugars had been added to bring the weights to 30—60 mg. (in the case of glucose, *ca.* 200 mg.), and portions of the recrystallised sugars were then burnt and assayed. Crystalline derivatives of the galactose, arabinose, and xylose were then prepared

as follows, and their activities estimated by the same method. Galactose (25—30 mg.) was dissolved in 5N-nitric acid (3 c.c.) and the solution, contained in a 5 c.c. beaker, was heated on the steam-bath until the volume had decreased to *ca.* 0·5 c.c., and then the solution was set aside for 24 hr. to crystallise. The mucic acid so obtained (15—18 mg.) was washed with water, ethanol, and ether, and dried, and had m. p. 208—210°. To a solution of arabinose (25—30 mg.) in water (0·5 c.c.) was added benzhydrazide (35 mg.) in ethanol (0·5 c.c.). The solution was kept at 20° for 24 hr., then evaporated to dryness. Decolorisation (charcoal) and recrystallisation of the residue from methanol afforded arabinose benzoylhydrazone (20—25 mg.), m. p. 206—207°. Xylose (25—30 mg.) and toluene-*p*-sulphonhydrazide (40 mg.) were dissolved in methanol (4 c.c.) and the solution heated under reflux for ½ hr.[13] Then the solution was reduced in volume to *ca.* 1 c.c. and set aside (1—2 days) to crystallise. The xylose toluene-*p*-sulphonylhydrazone obtained (20—25 mg.) had m. p. 148—149°. The ^{14}C-labelled rhamnose (8 mg. remained after the first assay) yielded insufficient of the benzoylhydrazone for accurate ^{14}C determinations by the $Ba^{14}CO_3$ method. The ^{14}C-labelled galacturonic acid sample was dissolved in water and after the addition of sodium calcium D-galacturonate $[NaCa(C_6H_9O_3)_3,6H_2O]$ (*ca.* 25 mg.) the solution was brought to pH 7 by addition of aqueous sodium and calcium hydroxides (molar ratio, 1 : 1). Crystalline ^{14}C-galacturonate (*ca.* 15 mg.) was then obtained by concentration and addition of ethanol.

The results of the assays were converted into the corresponding values for the undiluted radioactive monosaccharides and these are collected in Table 5.

TABLE 5.

		Sugar isolated (mg.)	Counts/min. per C atom of undiluted sugar calc. from assay results for: Diluted sugar	Sugar derivative	Specific activity of undiluted sugar (μc/milli-atom-equiv. of C)
Monosaccharides from water-soluble polysaccharides	D-Galactose	16·0	542	562	0·338
	L-Rhamnose	5·5	310	—	0·190
	L-Arabinose	14·0	521	525	0·320
	D-Xylose	2·6	[a]	70 [b]	0·043
	D-Galacturonic acid	9·3	27 [b]	—	0·017
Monosaccharides from polysaccharides soluble in cold N-NaOH	L-Arabinose	5·4	[a]	298	0·182
	D-Xylose	8·0	106 [b]	98 [b]	0·062
	D-Glucose	41·0	7170 [c]	—	4·40
Monosaccharides from polysaccharides soluble in hot 2·5N-NaOH	D-Galactose	19·7	587	562	0·350
	L-Rhamnose	8·1	374	—	0·227
	L-Arabinose	31·0	249	241	0·149
	D-Xylose	26·5	170	157	0·100
	D-Glucose	45·3	6130 [c]	—	3·76

[a] Diluted sugar not recrystallised and assayed, but used directly for preparation of derivative. [b] Probable error ±10%, calculated from variations in counting rate of $Ba^{14}CO_3$, which in these cases was not more than twice the background count. [c] Unchanged by recrystallisation; derivative not prepared.

We thank the Agricultural Research Council for the award of a Fellowship (to P. A.).

THE UNIVERSITY, BRISTOL. [*Received, July 8th*, 1958.]

[13] Easterby, Hough, and Jones, *J.*, 1951, 3416.