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BIOSYNTHESIS AND METABOLISM OF ALLITOL AND D-ALLULOSE IN *ITEA* PLANTS; INCORPORATION OF $^{14}CO_2$

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Abstract—The rapid incorporation of $^{14}CO_2$ into allitol and D-allulose has indicated that these two carbohydrates, not hitherto found in any genus other than *Itea*, play an important role in the metabolism of these plants. The percentage ^{14}C incorporation into allitol of both leaves and stems increases markedly with duration of photosynthesis and decreases during metabolism in the dark.

ALDITOLS are of widespread occurrence in the plant kingdom but studies of their biochemical functions in plants are very limited in contrast to the considerable attention paid to their role in animal metabolism.[1] Mannitol formation is associated with photosynthetic activity in the brown algae and Black[2] has demonstrated that the mannitol content of fronds shows a wide seasonal variation, reaching a maximum about midsummer.

Reserves of polyols built up during periods of anabolic activity, can be used subsequently for the supply of energy or conversion to other substances. Plums[3, 4] and pears[5] ripening on the tree accumulate D-glucitol as well as sucrose and reducing monosaccharides. Fruits detached from the tree and stored lose D-glucitol rapidly, but the level of sucrose and reducing monosaccharides is maintained until the concentration of D-glucitol has fallen considerably.

Plant tissues can make selective use of exogenous polyols supplied to them. Thus, D-glucitol, but not D-mannitol or galactitol, is converted to starch in detached leaves of *Rosaceae*; the leaves of *Adonis vernalis* and of *Oleaceae* use galactitol and D-mannitol respectively for starch synthesis.[6] D-Glucitol was converted to cellulose in wheat shoots after splitting of the carbon chain[7, 8] while Barsha and Hibbert[9] have demonstrated that the bacterium, *Acetobacter xylinum*, will produce a polysaccharide, identical with plant cellulose, from a number of carbohydrates including mannitol.

Anderson *et al.*[10, 11] have studied the biosynthesis and metabolism of D-glucitol in plum leaves and have shown that despite the large amounts present in the leaves, D-glucitol is rapidly equilibrated with the primary products of photosynthesis (15 per cent was newly synthesized from ^{14}C-labelled precursors in 3 hr). Results of experiments involving metabolism of specifically labelled glucoses indicated that conversion of D-glucose to D-glucitol

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occurs without rupture of the carbon chain. The suggested role of D-glucitol in the metabolism of the plum was that it may act as an intermediate in the conversion of D-glucose, D-fructose and L-sorbose in analogy with the interconversion of D-*threo*- and L-*threo*-pentulose via xylitol in guinea pig liver, a metabolic function of an alditol which has been conclusively demonstrated.[12]

Plant allitols are usually assumed to originate from monosaccharides by reduction, and that re-oxidation to monosaccharides is the first step in their metabolism. The simultaneous occurrence of certain pairs of polyols and ketose sugars (e.g. D-*glycero*-D-*gala*heptitol and D-*manno*heptulose in pears, D-mannitol and D-fructose in the brown algae in addition to D-glucitol and D-fructose in the *Rosaceae*) has suggested that their interconversions may occur widely in plants.[13, 14] Recent reports of the occurrence of allitol and D-*ribo*hexulose (D-allulose) in *Itea* plants[15, 16] have prompted the present investigation of their biosynthesis and metabolism.

INCORPORATION OF $^{14}CO_2$ INTO LEAVES OF *I. ILICIFOLIA*

(*a*) *After Photosynthesis for One Hour*

Several leafy spurs of *I. ilicifolia*, previously kept in the dark for 15 hr. were allowed to photosynthesize in an atmosphere containing 0·5% v/v $^{14}CO_2$ for 1 hr. This was followed by photosynthesis for further periods of 0–47 hr in a normal atmosphere (the flush period) with the exception of one of the samples which was placed in the dark for 23 hr. The distribution of ^{14}C between the alcohol-soluble and -insoluble portions of the leaves and of the stems was determined (Table 1). Crystalline allitol and di-*O*-isopropylidene-D-allulose were isolated from the leaf extracts and the specific activities determined. Crystalline allitol was also isolated from the stem extracts and assayed. Variations in allitol content of *Itea* leaves and stems during photosynthesis and dark periods has been discussed previously.[17]

TABLE 1. INCORPORATION OF ^{14}C INTO LEAFY SPURS OF *I. ilicifolia* BY PHOTOSYNTHESIS IN $^{14}CO_2$ FOR 1 HR FOLLOWED BY FURTHER PERIODS IN $^{12}CO_2$

Duration of photosynthesis (hr)	Total ^{14}C in spur (μc)	Distribution of ^{14}C between alcohol-soluble and -insoluble fractions (as % of total ^{14}C in spur)			
		Leaf extract	Leaf residue	Stem extract	Stem residue
1	187	60	40	0·25	0·32
2	97	59	41	0·23	0·24
6	240	63	36	0·60	0·24
8	136	61	38	0·76	0·28
24	163	59	37	3·2	0·74
48	119	52	43	3·6	1·1
1 (+ 23 in dark)	126	76	21	2·9	0·41

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The relative ^{14}C-activities of alcoholic leaf extracts and corresponding leaf residues showed only slight variation throughout the 48 hr of experiment (Table 1), showing little net transfer of radioactivity from monosaccharide components to the polysaccharide fractions. These observations are in contrast to the results obtained with plum leaves,[18] where, in the early stages, the proportion of ^{14}C in the leaf extracts greatly exceeded that in the insoluble residue (1 hr—75 : 25) but the latter quickly increased so that after 6 hr photosynthesis in $^{14}CO_2$ about 40 per cent of the total activity assimilated by the leaves then resided in this fraction; thereafter, when the leaves were allowed to photosynthesize in a normal atmosphere, there was little significant change in the relative activities. *Itea* leaves contain much less starch than plum leaves and consequently the above difference in behaviour might well be due to the high rate of starch synthesis in plum leaves. On the other hand, there is net synthesis of allitol during photosynthesis of *Itea* leaves [17] and the high proportion of ^{14}C incorporated into this polyol could account for the insignificant changes in the relative ^{14}C-activities of the extract and insoluble residues.

Similar experiments with sugar beet leaves involving photosynthesis in $^{14}CO_2$ for 60 sec plus additional periods of 0–47 min in a normal atmosphere, have shown that ^{14}C assimilated by the leaf continued to reach the polysaccharides for about 3 min after the initial 1 min period and that little, if any, change in distribution occurred during the remainder of the experiment.[19] However, more than 80 per cent of the assimilated radioactivity was still in the sucrose and other simple compounds[20] and Mortimer and Wylam[19] suggested that all the radioactive percursors for polysaccharide formation must have been replaced by non-radioactive substance formed after the initial 1 min.

In the hour subsequent to $^{14}CO_2$ assimilation, there was an unexpected preliminary decrease in the ^{14}C-activities of the alcoholic stem extracts and alcohol-insoluble residues of *Itea* spurs. This was followed by an expected increase with duration of photosynthesis indicating translocation of labelled compounds from the leaves into the stems[21] followed by macromolecular synthesis from these compounds (Table 1). The greater incorporation of ^{14}C into the stem residue compared with the alcohol soluble stem material after the first hour of photosynthesis was also unexpected and remains to be explained.

Incorporation of ^{14}C into leaf allitol was rapid, accounting for 21 per cent of the total ^{14}C-activity in the leaf extract after the initial hour of photosynthesis and increasing rapidly to over 50 per cent after 8 hr; this was followed by a more gradual rise to an equilibrium value of 61–62 per cent after 24 hr (Table 2). This behaviour is similar to that observed when $^{14}CO_2$ was incorporated into D-glucitol of plum leaves[10] but in contrast to the biosynthesis of allitol, no net synthesis of D-glucitol occurred during photosynthesis, indicating a rapid turnover of the D-glucitol pool in equilibrium with the primary products of photosynthesis. The allitol content of *Itea* leaves increased markedly during photosynthesis[17] whereas the percentage of allitol in the leaf and its ^{14}C content decreases rapidly in the dark (after 23 hr in the dark, only 4·6 per cent of the activity in the leaf extract resided in the allitol); hence this polyol acts as a reserve which is metabolized during the hours of darkness. Incorporation of ^{14}C into stem allitol was also quite rapid, accounting for nearly 10 per cent of the total activity in the alcoholic stem extract after the initial hour of photosynthesis and increasing to 44 per cent after 48 hr (Table 3). The specific activity of this allitol was, however, considerably less

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TABLE 2. INCORPORATION OF ^{14}C INTO ALLITOL ISOLATED FROM LEAVES OF *I. ilicifolia* AFTER PHOTOSYNTHESIS IN $^{14}CO_2$ FOR 1 HR FOLLOWED BY FURTHER PERIODS IN $^{12}CO_2$

Duration of photosynthesis (hr)	Yield of allitol* (mg)	Specific activity of allitol (μc per milli-atom-equiv. of C)	Total ^{14}C in allitol (μc)	Total ^{14}C in allitol (as % of total ^{14}C in alcohol-soluble leaf fraction)
1	42	16·5	24	21
2	92	5·62	18	31
6	61	23·6	49	33
8	104	12·5	44	53
24	119	14·6	59	61
48	173	6·55	39	62
1 (+ 23 in dark)	10	13·0	4·4	4·6

* Representing 96 per cent of total in leaves.

TABLE 3. INCORPORATION OF ^{14}C INTO ALLITOL ISOLATED FROM STEMS OF *I. ilicifolia* AFTER PHOTOSYNTHESIS IN $^{14}CO_2$ FOR 1 HR FOLLOWED BY FURTHER PERIODS IN $^{12}CO_2$

Duration of photosynthesis (hr)	Yield of allitol* (mg)	Specific activity of allitol (μc per milli-atom-equiv. of C)	Total ^{14}C in allitol (μc)	Total ^{14}C in allitol (as % of total ^{14}C in alcohol-soluble stem fraction)
1	10	0·121	0·044	10
2	22	0·054	0·044	20
6	15	0·53	0·29	20
8	15	0·61	0·33	32
24	15	3·20	1·77	34
48	16·5	3·17	1·92	44
1 (+ 23 in dark)	5·4	1·44	0·285	8

* Representing 90 per cent of total in stems.

than that in the leaves. As in the case of leaf allitol, metabolism in the dark led to a lower yield and lower total ^{14}C-activity of stem allitol.

Rapid incorporation of ^{14}C into leaf D-allulose was observed, giving specific activities comparable with but greater than those of allitol during the first 2 hr of photosynthesis, while the reverse was the case after 6 hr photosynthesis (Table 4). This effect could be due to the conversion of ^{14}C-D-allulose into ^{14}C-allitol, with consequent relative increase in the specific activity of allitol. The percentage of ^{14}C-activity in the hexulose (calculated on the basis of the crude di-*O*-isopropylidene derivative isolated)† decreased with duration of photosynthesis, whereas, in the dark, the ^{14}C-activity increased considerably and actually exceeded the proportion of ^{14}C then incorporated into the allitol. These results support the previous conclusion[17] that the conversion of D-allulose to allitol occurs in the light and the reverse process is operative in the dark.

† The true allulose content of the leaves may well be 100 per cent or more higher than the values calculated from the yields of the di-isopropylidene derivative owing to losses in isolation [17]

TABLE 4. INCORPORATION OF ^{14}C INTO D-ALLULOSE ISOLATED FROM LEAVES OF *I. ilicifolia* AFTER PHOTOSYNTHESIS IN $^{14}CO_2$ FOR 1 HR FOLLOWED BY FURTHER PERIODS IN $^{12}CO_2$

Duration of photosynthesis (hr)	Yield of di-*O*-isopropylidene D-allulose (mg)	Specific activity (μc per milli-atom-equiv. of allulose carbon)	Total ^{14}C in D-allulose (μc)	Total ^{14}C in D-allulose (as % of total ^{14}C in alcohol-soluble leaf fraction)
1	17	18·6	7·6	6·8
2	5·6	7·5	1·0	1·8
6	1·6	19·5	0·75	0·5
1 (+23 in dark)	18·7	16·2	7·3	7·6

No attempt was made to estimate the ^{14}C present in compounds other than allitol and D-allulose. Such a study concerned with sucrose, glucose and fructose would no doubt yield interesting information, especially since the two monosaccharides occur only as minor components of *Itea* leaves in contrast with other leaf species.[16]

(*b*) *After Photosynthesis for Fifteen Minutes*

The primary aim of this set of experiments was to determine if any significant transfer of ^{14}C occurred between the alcohol-soluble and -insoluble fractions of the leaves during the first hour of photosynthesis (cf. p. 217).

The leafy spurs, collected August 1963 and previously kept in the dark for 15 hr, were allowed to photosynthesize in an atmosphere of $^{14}CO_2$ for 15 min, and then for further periods of 0–105 min in a normal atmosphere.

As before (Table 1), the relative activities of the alcoholic leaf extracts and corresponding leaf residues showed only slight variation during photosynthesis (Table 5). However, in this set of experiments, a much greater proportion of ^{14}C resided in the leaf extracts. Thus, during the period of photosynthesis in $^{14}CO_2$, the percentage of ^{14}C in the alcohol-solubles of the leaf decreases with time (15 min, 83%; 1 hr, 60%) (Tables 5 and 1) with a corresponding increase of ^{14}C in the alcohol-insoluble leaf residue (15 min, 16%; 1 hr, 40%), but once the $^{14}CO_2$ is replaced by normal carbon dioxide, little transfer is observed. The yield and the percentage of ^{14}C incorporated into the allitol once again increased with duration of photosynthesis (Table 6), showing gradual conversion of radioactive precursor to allitol.

TABLE 5. INCORPORATION OF ^{14}C INTO LEAF SPURS OF *I. ilicifolia* BY PHOTOSYNTHESIS IN $^{14}CO_2$ FOR 15 MIN FOLLOWED BY FURTHER PERIODS IN $^{12}CO_2$

Duration of photosynthesis (min)	Total ^{14}C in spur (μc)	Distribution of ^{14}C between alcohol-soluble and -insoluble fractions (as % of total ^{14}C in spur)			
		Leaf extract	Leaf residue	Stem extract	Stem residue
15	25·8	83	16	1·6	0·048
30	33·3	86	11	2·7	0·052
45	27·1	80	17	3·1	0·067
60	56·4	84	14	2·2	0·053
120	18·6	85	11	4·0	0·052

TABLE 6. INCORPORATION OF ^{14}C INTO LEAFY SPURS OF *I. ilicifolia* BY PHOTOSYNTHESIS IN $^{14}CO_2$ FOR 15 MIN FOLLOWED BY FURTHER PERIODS IN $^{12}CO_2$

Duration of photosynthesis (min)	Yield of allitol (mg)	Specific activity (μc per milli-atom-equiv. of C)	Total ^{14}C in allitol (μc)	Total ^{14}C in allitol (as % of total ^{14}C in alcohol-soluble leaf fraction)
15	40	0·68	0·93	4·4
30	50	2·57	4·4	15
45	62	1·65	3·5	16
60	61	7·0	14·7	31
120	52	2·47	4·4	28

Thus, all the above results agree with those of the previous $^{14}CO_2$ incorporation experiment and serve to corroborate the conclusions that allitol is accumulated by *Itea* leaves during photosynthesis and that when $^{14}CO_2$ is replaced by $^{12}CO_2$, there is no significant transfer of radioactivity from the alcohol-solubles to the alcohol-insoluble leaf residue, presumably because allitol synthesis is the dominant feature in the metabolism of *Itea*.

EXPERIMENTAL

Alcohol-insoluble leaf residues were burnt in a stream of oxygen in a micro-analytical combustion apparatus (F. J. McMurray, Dartford, Kent) and the carbon dioxide produced passed into hot (60–70°) barium hydroxide solution (saturated at room temperature). The granular precipitate of barium carbonate was collected with suction on filter paper (2·8 cm dia.) as uniform discs (2 cm dia.; wt. ~ 20 mg per cm equivalent to infinite thickness for counting).[10, 17] After careful washing with water, the still damp disc and filter paper were mounted on a circular brass plate and finally dried at 120° (20 min). Sufficient material was oxidized to give at least 60 mg of barium carbonate for infinite thickness counting, which was carried out in a windowless flow-counter (Type SC-16G; Tracerlab), coupled with an automatic scaler (Ekco Electronics Ltd., Type N530F). The counting equipment was calibrated with respect to $Ba^{14}CO_3$ by determining the count rate of an "infinitely thick" layer of $Ba^{14}CO_3$ prepared from samples of poly ([^{14}C]methyl methacrylate) of known specific activity; a disc of this polymer was used as a standard reference source.

Radioactive alcoholic extracts and soluble ^{14}C-compounds were assayed in a liquid scintillation counter (liquid measuring head "2022" and a coincidence control unit "2032", I.D.L.) coupled with an automatic scaler (I.D.L.). Liquid scintillators, N.E. 213 and N.E. 220 (Nuclear Enterprises, Ltd.), the latter for aqueous solutions, were employed. The efficiency of counting was determined by the addition of an internal standard, [^{14}C]-*n*-hexadecane to each sample.

INCORPORATION OF $^{14}CO_2$ INTO LEAFY SPURS OF *I. ILICIFOLIA*

Apparatus

The apparatus was similar to that described by Folkes *et al.*[22] Steady illumination was provided by twelve 150 W tungsten-filament lamps mounted between a reflecting surface above and a glass-bottomed water tank below, the whole being held in an iron frame. The

[22] B. F. FOLKES, A. J. WILLIS and E. W. YEMM, *New Phytol.* **51**, 31 (1953).

tank was fitted with a constant level device and a steady flow of cold water served as a heat filter.

The photosynthetic chamber[18] was a desiccator (vol. 4 l.) with two inlet tubes, fitted with stopcocks, passing through the centre of the lid. One inlet tube was terminated immediately inside the lid while the other extended to within ~ 1 cm of the bottom of the desiccator, this tube having a wide mouth to facilitate addition of liquid. The source of $^{14}CO_2$, [^{14}C]barium carbonate, was contained in a small beaker placed on the bottom of the desiccator to surround the end of the long inlet tube; the humidity in the desiccator was controlled by the presence of sulphuric acid (30% v/v). The leaf spurs under experiment were placed in small beakers, held in position around the shelf of the desiccator by a metal frame.

Procedure

(*a*) *One hour incorporation.* Eight selected spurs, collected April 1963, each bearing eight to nine leaves, were placed with cut ends in water (within 2 hr of picking), and kept in the dark overnight (15 hr). With the spurs in position in the desiccator, the pressure inside was lowered by ~ 10 cm mercury and sufficient lactic acid (~ 25 ml; 80% v/v) was added to the $Ba^{14}CO_3$ (145 mg; 1790 μc) so that the end of the long inlet tube was immersed by 5–10 mm. When the evolution of $^{14}CO_2$ had ceased, the desiccator was placed under the light battery and the pressure inside increased to atmospheric by admitting air through the long inlet tube, this also serving to effect a uniform distribution of carbon dioxide (~ 0·5% v/v) within the apparatus. The stopcock on the long tube was then left open, loss of gas being prevented by the immersion of the lower end of the tube in lactic acid. The intensity of illumination received by the leaves in the desiccator was about 400 foot-candles.

After 1 hr, the desiccator was removed from under the light battery. One leafy spur was then immediately extracted with boiling ethanol as described below; whilst another spur, with its cut end still under water, was kept for 23 hr in the dark. The remaining six spurs, their cut ends still under water, were replaced under the light battery for different periods of time, 1–47 hr (light intensity received in the open ~ 500 foot candles).

After each period of $^{14}CO_2$ incorporation, the leaves were rapidly detached from the stems, both cut into small pieces and dropped into boiling ethanol (~ 75 ml and 40 ml respectively; 15 min). The residues were then exhaustively extracted with boiling methanol and each combined alcoholic extract made up to a known volume with methanol (leaves, 250 ml; stems, 100 ml), leaf extracts being further diluted with ethanol (1 ml→100 ml) for ^{14}C-assay. The final alcohol-insoluble residues were dried at 60°, to constant weight (alcohol-insoluble leaf residue, C, 44 per cent; alcohol-insoluble stem residue C, 45 per cent). Each alcoholic extract and alcoholic-insoluble residue was then assayed for ^{14}C activity.

Allitol was isolated from aliquots of each leaf and stem extract (240 ml and 90 ml respectively) as described previously[17] and recrystallized from aqueous ethanol while D-allulose was isolated from certain leaf extracts via the di-isopropylidene compound[17] and recrystallized from light petroleum (40–60°). Each sample was assayed for ^{14}C-activity, the total ^{14}C-activity being calculated on the basis of initial yield of compound prior to recrystallization.

(*b*) *Fifteen minute incorporation.* Five leavy spurs (collected August 1963) with approximately ten leaves on each were kept in the dark for 16 hr with their cut ends under water. They were then arranged in the desiccator as used in the previous incorporation experiment with the modification that the desiccator was now fitted with a small fan connected to a 1½ V battery. With the apparatus in a subdued light, lactic acid (25 ml; 80% v/v) was added to the $Ba^{14}CO_3$ (156 mg; 1 mc) and the fan started to assist circulation of $^{14}CO_2$. After

3 min the apparatus was placed under the light battery and after a further 15 min, the spurs were quickly removed from the desiccator. One specimen was immediately extracted with boiling ethanol (leaves and stems separately) while the remaining four spurs were replaced under the light battery with their cut ends under (fresh) water. These spurs were finally extracted with boiling ethanol after total photosynthetic periods of 30, 45, 60 and 120 min respectively. Each residue was then exhaustively extracted with methanol and the distribution of ^{14}C between alcohol-soluble and -insoluble portions of leaves and stems determined (alcohol-insoluble leaf residue, C, 43 per cent; alcohol-insoluble stem residue C, 45 per cent). Finally, allitol was isolated from the leaf alcoholic extracts and assayed for ^{14}C-activity

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