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METABOLISM OF ^{14}C-LABELLED D-GLUCOSE, D-FRUCTOSE AND ALLITOL BY *ITEA* PLANTS

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Abstract—The metabolism of D-[1-^{14}C]glucose, D-[6-^{14}C]glucose, D-[1-^{14}C]fructose and D-[6-^{14}C]fructose by leafy spurs of *Itea* plants results in rapid incorporation of label into allitol and D-allulose. The patterns of labelling found in the allitol and D-allulose are discussed, a direct interconversion from D-glucose and D-fructose being indicated. Allitol has been found to be an active metabolite in *Itea* plants.

INTRODUCTION

ALDITOLS are of widespread occurrence in the plant kingdom where they are usually assumed to originate from aldoses by reduction and to be metabolized by a reversal of this process, namely reoxidation to ketoses. The simultaneous occurrence of generically related pairs of alditols and ketoses (e.g. D-*glycero*-D-*gala*heptitol and D-mannoheptulose in pears, D-mannitol and D-fructose in the brown algae, D-glucitol and D-fructose in the Rosaceae) has suggested that their interconversion may occur widely in plants.[1–4] However, studies of the biochemical function of alditols in plants are limited, in contrast to the considerable attention paid to their role in animal metabolism. Hence the discovery of the co-existence of allitol and D-*ribo*hexulose (D-allulose) in *Itea* plants[5,6] prompted an investigation of their biosynthesis and metabolism.

Experiments involving the photosynthetic assimilation of $^{14}CO_2$ by *Itea* plants[7,8] have shown that both allitol and D-allulose play important biosynthetic roles. Thus incorporation of ^{14}C into leaf allitol was rapid and at equilibrium (24 hr) accounted for over 60% of the total ^{14}C in the leaf extract but both the percentage of allitol in the leaf and its ^{14}C content decreased rapidly in the dark suggesting that this polylol acts in a reserve capacity by metabolism during the hours of darkness. Incorporation of ^{14}C into leaf D-allulose was also rapid and the results supported a previous conclusion that the hexulose is converted into

[1] N. L. EDSON, *Rept. Austral. New Zealand Assoc. Adv. Sci.* **29**, 281 (1953).
[2] C. S. HUDSON, *Adv. Carbohyd. Chem.* **1**, 1 (1945).
[3] O. TOUSTER and D. R. D. SHAW, *Physiol Rev.* **42**, 181 (1962).
[4] D. H. LEWIS and D. C. SMITH, *New Phytol.* **66**, 143 (1967).
[5] V. PLOUVIER, *Compt. Rend.* **249**, 2828 (1959).
[6] L. HOUGH and B. E. STACEY, *Phytochem.* **2**, 315 (1963).
[7] L. HOUGH and B. E. STACEY, *Phytochem.* **5**, 215 (1966).
[8] L. HOUGH and B. E. STACEY, *Phytochem.* **5**, 171 (1966).

allitol in the light while the reverse process is operative in the dark. To obtain more detailed information on these biosynthetic processes ^{14}C-labelled carbohydrates were fed to *Itea* plants and the location of label in the isolated allitol and D-allulose investigated.

RESULTS

The radioactive substrates (Table 1) were fed as aqueous solutions to leafy spurs of *Itea ilicifolia* through the cut ends of the stems and allowed to metabolize for various periods of time. At the end of each experiment the leaves and stems were separately extracted with alcohol and the ^{14}C activity in each alcoholic extract determined. Crystalline allitol was isolated directly from each of the alcoholic extracts while D-allulose was obtained as the crystalline di-*O*-isopropylidine derivative,[6] recoveries being improved by the use of preparative TLC with the iodine vapour technique[9] for location. The allulose derivative was usually slightly contaminated with iodine, which was removed by vacuum sublimation, the method proving a superior method to recrystallization from light petroleum used previously.[6,8]

TABLE 1.

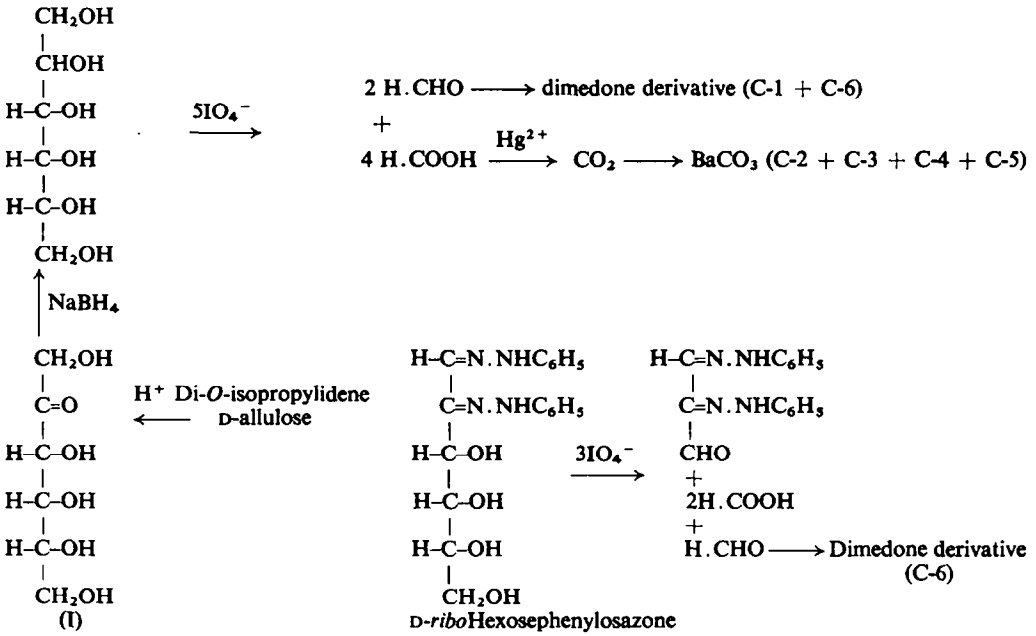
Experiment No.	Compound supplied: Name	wt (mg)	activity (μCi)	Duration of experiment (hr)
1	D-[1-^{14}C]Glucose	4·32	100	4
2	D-[6-^{14}C]Glucose	8·24	100	4
3	D-[1-^{14}C]Fructose	1·80	11·6	4
4	D-[1-^{14}C]Fructose	1·80	11·6	24
5	D-[1-^{14}C]Fructose	1·80	11·6	24 (+24 in dark)
6	D-[6-^{14}C]Fructose	2·94	10	4
7	D-[6-^{14}C]Fructose	2·94	10	24
8	D-[6-^{14}C]Fructose	2·94	10	24 (+24 in dark)
9	[U-^{14}C]Allitol	7·85	1·81	24 (+24 in dark)

Degradation of ^{14}C-Labelled Compounds

Numerous methods have been put forward for the determination of the distribution of ^{14}C along the carbon chain of a monosaccharide:[10] many involve a combination of microbiological and chemical methods and will often give the location of ^{14}C at each individual carbon atom.[11] Brice and Perlin[12] used a chemical method involving oxidation with lead tetraacetate for the determination of ^{14}C-activity of each of the carbon atoms of the fructose molecule. However, in the present investigation a simpler method was adopted, account being taken of the fact that since the ^{14}C-substrates were labelled in the terminal carbon atoms only, interest was focussed mainly on determining the ^{14}C in C-1, (C-2 + C-3 + C-4 + C-5) and C-6 of allulose and allitol.

[9] G. C. BARRETT, *Nature, Lond.* **194**, 1171 (1962).
[10] J. K. N. JONES and R. T. STOODLEY, in *Methods in Carbohydrate Chemistry* (edited by R. L. WHISTLER and M. L. WOLFROM), Vol. 2, p. 489, Academic Press, New York (1963); and references therein.
[11] M. GIBBS, P. K. KINDEL and M. BUSSE, in *Methods of Carbohydrate Chemistry* (edited by R. L. WHISTLER and M. L. WOLFROM), Vol. 2, p. 496, Academic Press, New York (1963).
[12] C. BRICE and A. S. PERLIN, *Can. J. Biochem. Physiol.* **35**, 7 (1957).

D-Allulose (I) obtained by the acid hydrolysis of its di-*O*-isopropylidine derivative, was reduced to the polyol with $NaBH_4$. The polyol was then oxidized under buffered condition at pH 7 with sodium metaperiodate, resulting in the formation of formaldehyde from the two terminal carbon atoms and formic acid from the intermediate carbon atoms (Scheme 1). The formaldehyde was collected as its dimedone derivative[13] and assayed for activity by scintillation counting to give the specific activity of the terminal carbons (C-1 + C-6). The formic acid was oxidized to carbon dioxide with mercuric acetate,[14] the liberated carbon dioxide trapped in saturated barium hydroxide and the insoluble barium carbonate assayed to obtain the specific activity of the intermediate carbon atoms ($C_2 \rightarrow C_5$). To determine the ^{14}C in C-6 of allulose, the latter was converted to the phenylosazone which on subsequent oxidation with sodium periodate yielded formaldehyde, assayed as the dimedone derivative. The latter was isolated by a modification of the procedure of Jones *et al.*[13] After isolating the precipitated mesoxaldehyde 1,2-*bis*-phenylhydrazone by filtration, the filtrate containing formic acid and formaldehyde was treated with saturated barium hydroxide to remove the excess periodate and iodate and the resulting solution treated directly with dimedone. The resulting pale yellow methylene *bis*-methone derivative obtained was purified by treating with charcoal during recrystallization. The specific activity of C-1 was then obtained by subtraction, [(C-1 + C-6) − C-6].

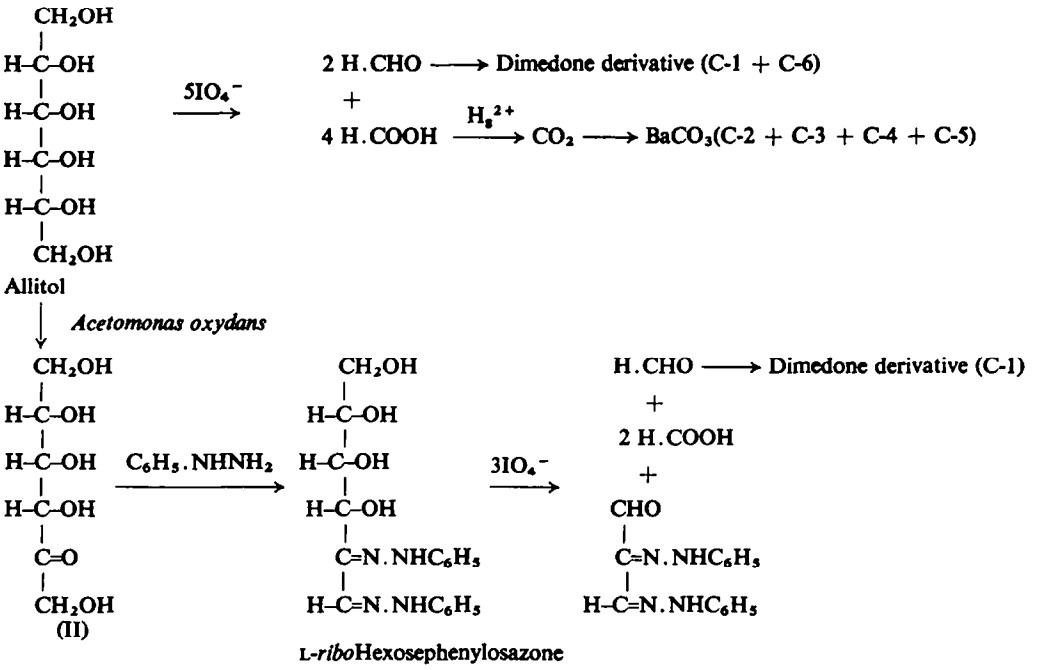


SCHEME 1. DEGRADATION OF D-ALLULOSE.

The degradation of ^{14}C-allitol presented a special problem: direct oxidation with sodium metaperiodate would give the ^{14}C in (C-1 + C-6) and (C-2 + C-3 + C-4 + C-5) but since allitol is a completely symmetrical molecule a purely chemical procedure would not

[13] J. K. N. JONES, M. B. PERRY and R. J. STOODLEY, *Can. J. Chem.* **40**, 856 (1962).
[14] H. D. WIEHE and P. B. JACOBS, *Ind. Eng. Chem. Analyt.* **8**, 44 (1936).

differentiate between the two terminal carbon atoms. Recourse was therefore made to specific oxidation by a microbiological method whereby allitol was converted by *Acetomonas oxydans* to L-allulose (II, L-*ribo*hexulose).[15] The latter was converted to the phenylosazone and subsequently degraded with periodate, (Scheme 2) to give formaldehyde which originated from C-1 of the allitol.* The formaldehyde was assayed as its dimedone derivative and the ^{14}C in C-6 of allitol* obtained by subtraction, [(C-1 + C-6) − C-1].



SCHEME 2. DEGRADATION OF ALLITOL.

DISCUSSION

Metabolism of D-*Glucose and* D-*Fructose* (Table 1, expts 1–8)

^{14}C-Hexose was rapidly incorporated into leaf allitol accounting in each of the experiments for over 15% of the total ^{14}C activity in the leaf extract after 4 hr; percentage incorporation based on ^{14}C administered varied between 6·3 and 13% (Table 2). In previous experiments using $^{14}CO_2$,[7] allitol activity accounted for 32% of total ^{14}C in leaf extract after 4 hr photosynthesis indicating that carbon dioxide is incorporated more rapidly than either D-glucose or D-fructose into the metabolic pool from which allitol or its precursor is derived. Incorporation of ^{14}C into stem allitol, (Table 3) was somewhat lower accounting for 4–15% of total ^{14}C in the alcohol-soluble stem fraction but once again this was lower than that observed in $^{14}CO_2$ experiments (20%).

The specific activity of D-allulose was generally greater than that of allitol isolated from the same extract except that in the case of the 'dark' experiments the activities of allitol and D-allulose in leaf extracts were in fact equal, indicating a controlled equilibrium between the

* Considered as the D-compound analogous to the naturally occurring D-allulose.

[15] J. G. CARR, R. A. COGGINS, L. HOUGH, B. E. STACEY and G. C. WHITING, *Phytochem.* **7**, 1 (1968).

two compounds. The specific activity of D-allulose isolated from stem (Table 3) was appreciably greater than that of the corresponding leaf sample (Table 2) indicating that the stems were able to metabolize D-glucose and D-fructose in their own right and that the hexulose does not appear in the stems merely by translocation from the leaves. The same comment applies to allitol (except in expt 1).

TABLE 2. INCORPORATION OF ^{14}C INTO ALLITOL AND D-ALLULOSE ISOLATED FROM LEAVES OF *I. ilicifolia*

Experiment No.	Specific activity of allitol (μCi per milli-atom-equivalent of carbon)	Total (μCi)	^{14}C in allitol (as % of total ^{14}C in alcohol-soluble leaf fraction)	Specific activity of D-allulose (μCi per milli-atom-equivalent of carbon)	Total (μCi)	^{14}C in D-allulose (as % of total ^{14}C in alcohol-soluble leaf fraction)
1	1·60	12·8	17	1·68	4·80	6·4
2	1·06	9·35	15	1·33	3·86	6·2
3	0·146	0·75	18	0·173	0·230	5·4
4	0·158	1·23	37	0·178	0·089	2·7
5	0·104	0·26	10·5	0·104	0·170	7·0
6	0·062	0·63	17	0·091	0·28	7·3
7	0·081	0·51	24	0·113	0·097	4·5
8	0·086	0·094	7·2	0·086	0·016	1·2
9	0·133	0·177	16·5	0·139	0·109	10

The pattern of labelling in corresponding samples of allitol and D-allulose showed a similarity suggesting direct interconversion between the two compounds. The major portion of the ^{14}C in both allitol and D-allulose was located at the same terminal positions corresponding to that in the ^{14}C-labelled substrates administered to the spurs, with a small degree of ^{14}C re-distributed at the other terminal carbon atom. This pattern of labelling probably arises from aldolase breakdown of the specifically labelled substrate through the Embden–Meyerhof glycolytic pathway to 1,3-dihydroxypropanone 1-phosphate and D-glyceraldehyde-3-phosphate, followed by isomerization of these compounds by *triose phosphate isomerase* and subsequent re-synthesis of D-glucose and D-fructose.[16]

A considerably smaller proportion of ^{14}C was detected in the non-terminal carbon atoms of allitol and D-allulose isolated from stems compared with the corresponding samples isolated from leaves. The greater randomization of ^{14}C in the intermediate carbon atoms in the leaf samples may be due to the presence of a greater complexity of enzymes in the leaves compared with the stems. Thus minor biosynthetic pathways operative in the leaves may not exist in the stem or perhaps are operative to a less extent.

Randomization of label within the allitol and D-allulose molecules was somewhat greater when [1-^{14}C]-labelled substrates were used compared with when [6-^{14}C]-monosaccharides were incorporated. This would seem to indicate a minor biosynthetic route involving rupture of a hexose unit between C-1 and C-2 to give $^{14}CO_2$ which on subsequent incorporation would give heavier labelling at (C-2 → C-5). Such a redistribution of isotope could possibly be effected by the Hexose Monophosphate Shunt, whereby D-gluconic acid 6-phosphate is oxidatively decarboxylated to D-*erythro*pentulose 5-phosphate.[17]

[16] S. SHIBKO and J. EDELMAN, *Biochem. Biophys. Acta* **25**, 642 (1957).
[17] B. L. HORECKER, P. Z. SMYRNIETIS and J. E. SEEGMILLER, *J. Biol. Chem.* **193**, 383 (1951).

Sugimori and Suhadolomik,[18] studied the biosynthesis of psicofuranine (6-amino-9-D-allulofuranosyl purine), a nucleotide isolated from the cultures of *Streptomyces hydroscopicus*, and suggested that the allulose moiety arises directly from D-glucose or a

TABLE 3. INCORPORATION OF ^{14}C INTO ALLITOL AND D-ALLULOSE ISOLATED FROM STEMS OF *I. ilicifolia*

Experiment No.	Specific activity of allitol (μCi per milli-atom-equivalent of carbon)	Total (μCi)	^{14}C in allitol (as % of total ^{14}C in alcohol-soluble stem fraction)	Specific activity of D-allulose (μCi per milli-atom-equivalent of carbon)	Total (μCi)	^{14}C in D-allulose (as % of total ^{14}C in alcohol-soluble stem fraction)
1	0·91	0·69	3·8	2·54	1·05	5·8
2	1·16	1·45	7·8	3·75	1·64	8·8
3	0·31	0·36	7·8	0·690	0·147	3·3
4	0·66	0·50	13·5	0·823	0·141	4·3
5	0·37	0·48	14	0·500	0·169	4·9
6	0·137	0·434	15	0·475	0·56	19
7	0·369	0·298	13	0·677	0·16	6·7
8	0·450	0·395	18	*	*	*
9	0·159	0·0314	11	0·243	0·0224	7·7

* Sample lost.

nucleotide hexose intermediate, whilst Gibbons and Simpson[19] from their studies on the metabolism of D-allose by *Aerobacter aerogenes* have postulated the presence of a 3-epimerase which could interconvert D-fructose 6-phosphate and D-allulose 6-phosphate. The second type of process might well be operative in *Itea* plants.

TABLE 4. DEGRADATION OF ^{14}C-LABELLED ALLITOL AND D-ALLULOSE ISOLATED FROM LEAVES OF *I. ilicifolia*

Experiment No.	Location of ^{14}C (as % of ^{14}C in original allitol or D-allulose) Allitol C-1	Allitol C-2 + C-3 + C-4 + C-5	Allitol C-6	D-Allulose C-1	D-Allulose C-2 + C-3 + C-4 + C-5	D-Allulose C-6
1	45	24	28	47	19	30
2	28	17	53	28	16	53
3	59	18	22	56	16	27
4	53	17	28	56	21	22
5	61	26	17	51	27	24
6	27	12	64	28	13	53
7	27	18	56	20	19	55
8	28	13	60	28	17	54

If a direct interconversion between D-fructose and D-allulose takes place via the phosphate derivatives one would expect a very small proportion of ^{14}C to be present in the intermediate carbon atoms of D-allulose with a major proportion occurring in the terminal carbon atoms, the predominant position corresponding to the position of the labelling in the original ^{14}C-fructose incorporated. However, the present degradation studies have indicated that an appreciable proportion of ^{14}C is associated with the intermediate carbon

[18] T. SUGIMORI and R. J. SUHADOLMIK, *J. Am. Chem. Soc.* **87**, 1136 (1965).
[19] L. N. GIBBONS and F. J. SIMPSON, *Can. J. Microbiol.* **10**, 829 (1964).

atoms. The scrambling in the case of D-allulose may be due to the breakdown of fructose 1,6-diphosphate to give two 3-carbon fragments and in turn the recombination of these fragments to yield D-allulose. It is known that ketoses are formed as a result of combination between two smaller fragments under the influence of enzymes such as *aldolase*, *transaldolase* and *transketolase*. In all such cases the products possess the D-*threo* configuration of hydroxyls at the carbon 3 and 4. An exception to the reverse of this process has, however, been reported; D-tagatose 1,6-diphosphate in which the hydroxyl at carbon 3 and 4 possess the L-*erythro* configuration is split by muscle aldolase to yield two 3-carbon fragments.[20] It is feasable therefore that *Itea* plants contain an enzyme capable of effecting combination of the triose units to yield D-allulose possessing D-*erythro* configuration at carbon 3 and 4. To account for the ^{14}C associated with the intermediate carbon atoms of D-allulose, one could speculate that the scrambling of ^{14}C between C-1 and C-3 of 1,3-dihydroxypropanone phosphate could be effected either via the dephosphorylated compound or via the diphosphate derivative, both of these compounds being symmetrical.

TABLE 5. DEGRADATION OF ^{14}C-LABELLED ALLITOL AND D-ALLULOSE ISOLATED FROM STEMS OF *I. ilicifolia*

Experiment No.	Location of ^{14}C (as % of ^{14}C in original allitol or D-allulose) Allitol C-1	Allitol C-2 + C-3 + C-4 + C-5	Allitol C-6	D-allulose C-1	D-allulose C-2 + C-3 + C-4 + C-5	D-allulose C-6
1	48	22	27	61	14	24
2	21	8	68	23	7	69
3	65	19	22	54	24	20
6	22	10	70	32	6	60

Metabolism of Allitol (expt 9)

After metabolism of 48 hr (24 hr in the dark), 60% ^{14}C administered was located in the leaf extract while only 16·5% of this resided in the allitol component (Table 2). Thus the transport of allitol to the leaves and its subsequent metabolism are quite efficient processes and contrast with the results obtained for D-glucitol incorporation into plum leaves when over 80% of the leaf extract activity was accounted for in the D-glucitol pool after metabolism for 5 hr.[21]

In a recent investigation of the metabolism of galactitol in *Euonymus japonica*,[22] vacuum infiltration of the leaves with ^{14}C-galactitol followed by metabolism in the dark for 24 hr resulted in over 40% of the ^{14}C administered being located in the galactitol pool; this contrasts with only 11·5% of the ^{14}C-administered being found in the combined leaf and stem allitol in the present experiment (Tables 2 and 3) and emphasizes the role of allitol as an active metabolite in *Itea* leaves.

The specific activities of allitol and D-allulose isolated from the leaves were found to be equal indicating a direct interconversion between allitol and D-allulose. Thus, these results serve to confirm the conclusions reached from the degradation experiments which showed a similar pattern of labelling in allitol and D-allulose. Furthermore, taking into consideration

[20] T. C. TUNG, K. H. LING, W. L. BYRNE and H. A. LARDY, *Biochem. Biophys. Acta* **14**, 488 (1954).
[21] J. D. ANDERSON, P. ANDREWS and L. HOUGH, *Biochem. J.* **84**, 140 (1962).
[22] C. A. BLISS, N. W. HAMON and T. P. LUKASZWESKI, *Phytochem.* **11**, 1695 (1972).

the results of $^{14}CO_2$ incorporation into *Itea* leaves,[6,7] this direct interconversion appears to favour the synthesis of allitol in the light while conversion of the hexitol to D-allulose is favoured in the dark.

It has generally been considered that most alditols are formed by reduction of an aldose and that the first step in the metabolism of these compounds is oxidation to a ketose.[3,4] On this basis, it might be expected that allose would be the precursor of allitol in *Itea* plants and so its detection was attempted. Allose does not appear to have been found in plants although it has been isolated in extracts prepared from *Ochromonas malhemesis*.[23] In order to detect the presence of D-allose in *Itea* extracts by paper chromatography, it was necessary first to remove glucose and fructose, since the R_fs of glucose, fructose and allose are very close. After isolating the major portion of allitol, the aqueous syrup containing mainly allulose, glucose, fructose and sucrose was fermented with commercial baker's yeast, (*Saccharomyces cerrivasae*), for 24 hr. The concentrated solution was examined by PC, when the presence of only allulose was observed, no spot corresponding to allose being detected.

What might be described as a converse situation occurs in E. *japonica*, regarding the biosynthesis and metabolism of galactitol; while galactose has been shown to be the precursor of galactitol the absence of the corresponding ketose, tagatose (*lyxo*hexulose) has been demonstrated[24] and Bliss *et al.*[22] have presented evidence that the initial product of galactitol metabolism is glucose. It therefore appears that no general mode of alditol biosynthesis and metabolism in plants can be formulated.

EXPERIMENTAL

Radioactivity measurements. 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. ^{14}C-$BaCO_3$ was assayed by suspension counting using 'Gel' phosphor N.E. 221 (Nuclear Enterprises Ltd.).[25]

Incorporation of ^{14}C-labelled carbohydrates into leaf spurs of Itea ilicifolia. (a) D-[1-^{14}C]- *and* D-[6-^{14}C]-*glucose.* Three non-flowering spurs each bearing 10–12 leaves were kept in the dark for 16 hr prior to use. The labelled glucose was dissolved in H_2O (0·3 ml) which was distributed between 3 glass cups (2·5 × 0·6 cm dia.) held in clamps (*ca.* 0·13 ml in each, including washings). Each spur was placed in a cup with its cut end immersed in glucose solution and placed under the light battery. A gentle current of warm air was maintained over the spurs to assist transpiration and the uptake of solution through the stems. The glucose solution was inbibed in 20–30 min and was flushed with two similar vol. of H_2O; water was then supplied freely from larger containers. After a total photosynthetic period of 4 hr, the leaves were detached from the stems and each placed in boiling EtOH for *ca.* 5 min to deactivate enzymes. The residues were then exhaustively extracted with MeOH. The combined alcoholic extracts were made up to a known volume (leaves 250 ml, stems 100 ml) and assayed for ^{14}C-activity. (b) D-[1-^{14}C]- *and* D-[6-^{14}C]-*fructose.* A procedure similar to the above was employed but with the following modification. ^{14}C-Labelled D-fructose was dissolved in H_2O and aliquots (0·1 ml) delivered to each of several cups. A leafy spur was placed in each cup and allowed to photosynthesize for different periods of time, (4–24 hr) one of the spurs being placed in the dark for 24 hr after being kept under illumination for 24 hr subsequent to feeding. The source of illumination used for photosynthesis consisted of six 40-W fluorescent lamps 62 cm in length and gave an intensity of illumination of 5400–6500 lx at the leaf surface. (c) [U-^{14}C]-*allitol.* The ^{14}C-labelled allitol (obtained from experiments involving the photosynthesis of *Itea* leaves in an atmosphere containing $^{14}CO_2$) was fed in the form of an aqueous solution (0·1 ml) to one *Itea ilicifolia* spur bearing 9 leaves which was allowed to photosynthesise for 24 hr and then kept in the dark for a further period of 24 hr before the leaves and stems were extracted with MeOH.

[23] H. KAUSS, *Z. pflanzenphysiol.* **53**(1), 58 (1965).
[24] J. SCHRADIE, Ph.D. Thesis, University of Southern California, University Microfilms Inc., Ann Arbor (1966).
[25] H. J. CLULEY, *Chem. Analyst.* **87**, 170 (1962).

Isolation of allitol and D-allulose from leaf and stem extracts. A portion of the alcoholic extract (usually 96% of total) was concentrated to a syrup and shaken with water for 15 min. After centrifugation the supernatant was passed slowly through a pad of activated charcoal-Hyflosupercell to give an almost colourless solution which on concentration yielded crystalline allitol. On adding 70% aq. EtOH, a further amount of allitol crystallized. The allitol was removed by filtration washed with EtOH followed by Et_2O and finally dried. The allitol was recrystallized from aq. EtOH, m.p. 149–150° (lit.[5,26] 149; 150–151°). The mother liquor was concentrated to a dry syrup and shaken with Me_2CO (9 ml), $CuSO_4$ (1 g) and conc. H_2SO_4 (0·02 ml) for 48 hr. After centrifugation the solution was neutralized by shaking with K_2CO_3 (0·3 g), centrifuged and evaporated to a syrup; TLC revealed the presence of seven other compounds in addition to the di-*O*-isopropylidine D-allulose. The pure allulose derivative was isolated by preparative TLC using $CHCl_3$–Et_2O (3:1). The band corresponding to di-*O*-isopropylidine D-allulose (located by the iodine vapour technique[9]) was extracted with Me_2CO and on concentration yielded a yellowish syrup which on trituration with light petrol. (40–60) gave a slight brown crystalline product: vacuum sublimation under diminished pressure using a cold finger apparatus yielded colourless crystals of di-*O*-isopropylidine D-allulose, m.p. 57–58° (lit. 57–58·5°).[26]

Degradation of ^{14}C-labelled allitol. (a) *Determination of ^{14}C in* (C-1 + C-6) *and* (C-2 → C-5). The ^{14}C-labelled allitol (*ca.* 0·2 mmol) was dissolved in H_2O (5 ml) and 0·2 M phosphate buffer, pH 8 (8 ml) added followed by 0·2 M sodium metaperiodate (10 ml). After 8–9 hr, excess of 0·5 M barium acetate was added and, after standing for 30 min the insoluble salts removed by filtration. The filtrate was acidified with M HOAc and dimedone (1%; 25 ml) added. The precipitated methylene *bis*-methone (C-1 + C-6) was collected after 20–24 hr and after two recrystallizations from aq. EtOH (70–80% yield) was assayed for ^{14}C activity. The filtrate containing barium formate was acidified with M H_2SO_4 and was heated under reflux for 15–20 min in a stream of N_2 to displace any CO_2. After cooling a solution (20 ml) of 0·3 M mercuric acetate containing 3% HOAc was added and the solution again refluxed, the liberated CO_2 being trapped in $Ba(OH)_2$ and $BaCO_3$ subsequently assayed for ^{14}C activity. (b) *Determination of ^{14}C in* C-1. An aqueous solution of allitol (0·5% w/v) and yeast extract (1% w/v) was innoculated with *Acetomonas oxydans* (CR 49)[15] and incubated at 25° for 3 weeks. The mixture was then centrifuged and the supernatant deionized by passing through Amberlite (IR 120 H^+ and IRA 400 acetate $^-$ ion exchange resins). The neutral solution was concentrated and was converted to L-*ribo*hexosephenylosazone by treating with phenylhydrazine. The resultant osazone was further degraded with sodium metaperiodate, and the specific activity of C-6 of the L-allulose (C-1 of the corresponding allitol) was determined.

Degradation of ^{14}C-labelled D-allulose. (a) *Determination of ^{14}C in* (C-1 + C-6) *and* (C-2 → C-5). Di-*O*-Isopropylidine D-allulose (*ca.* 0·1 mmol) was hydrolysed by heating with 0·005 M H_2SO_4 (1 ml) at 100° for 40 min. After neutralizing with NaOH, $NaBH_4$ (25 mg) was added and the solution left at room temp. overnight. Excess borohydride was destroyed by acidifying with H_2SO_4 and the resultant hexitol oxidized with 0·2 M sodium metaperiodate (5 ml) in phosphate buffer. The specific activities of the formaldehyde (C-1 + C-6) and formic acid (C-2 → C-5) were determined by conversion into methylene *bis*-methone and barium carbonate respectively. (b) *Determination of ^{14}C in* C-6. Di-*O*-isopropylidine-D-allulose (*ca.* 64 mg, 0·25 mmol) was hydrolysed by heating with 10% HOAc (0·5 ml) at 100° for 40–45 min. H_2O (1·7 ml) was added to the cooled solution containing D-allulose which was then converted to the phenylosazone by heating with redistilled phenylhydrazine (0·1 ml) and a trace of sodium bisulphite (to inhibit formation of tarry impurities) at 65–70° in N_2 for *ca.* 4 hr during which time a yellow precipitate gradually formed. The precipitate was removed by filtration and washed successively with dil. HOAc and H_2O. The phenylosazone was recrystallized from aq. EtOH and assayed for activity. The phenylosazone (*ca.* 0·05 mmol) was degraded by dissolving in 50% EtOH (10 ml) and adding 0·2 M sodium metaperiodate (2 ml). After 30 min the precipitate of mesoxaldehyde 1,2-*bis*phenylhydrazone was removed by filtration. The filtrate was treated with excess $Ba(OH)_2$ and the insoluble salts were removed by filtration. The filtrate was made acid with HOAc and dimedone solution (10–15 ml) added. The derivative was collected after 24 hr, recrystallized from aq. EtOH and the specific activity determined.

Attempted detection of allose in Itea *Extracts.* A procedure similar to that adopted by Somogyi[27] was used for fermenting the sugar solution. A 20% (w/v) suspension of the washed bakers' yeast was centrifuged and the supernatant solution discarded. The tube was drained for a few sec and the moisture adhering to the walls of the tube removed by using strips of filter paper. The sugar solution (1%; 15 ml) to be fermented was introduced and the contents well stirred for 3–4 min and allowed to stand in a thermostat kept at 35–40° for a period of 24 hr. The tube was occasionally inverted to avoid the settling of the bakers' yeast. After standing for 24 hr the tube was centrifuged and the clear solution filtered, concentrated and finally examined by PC. D-Glucose, D-fructose, D-allose, D-allulose, allitol and D-xylose were subjected to the process of fermentation by the above method. Only D-glucose and D-fructose were fermented, the others being

[26] M. Steiger and T. Reichstein, *Helv. Chim. Acta* **19**, 184 (1936).
[27] M. Somogyi, *J. Biol. Chem.* **119**, 741 (1937).

unaffected. After isolating the major proportion of allitol from the *Itea* extracts, the aqueous solution containing mainly D-allulose was fermented by the above method. Examination of the concentrated syrup by PC revealed the presence of allulose but no spot corresponding to allose could be detected.

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