The Biosynthesis of L-Rhamnose of Plum-Leaf Polysaccharides

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1. The utilization of D-[1-¹⁴C]- and D-[6-¹⁴C]-glucose in the biosynthesis of L-rhamnose units of plum-leaf polysaccharides has been studied. 2. After the precursors had been metabolized in the leaves, polysaccharide fractions were prepared therefrom and the constituent L-rhamnose was isolated and purified. 3. Both the specific activity and the distribution of ¹⁴C along the carbon chain of L-rhamnose from two polysaccharide fractions from each experiment were determined. 4. The results indicated a close affinity between L-rhamnose and pectin, and show that biosynthesis of the 6-deoxyhexose from D-glucose occurs in the main without scission or inversion of the carbon chain. 5. A degradation scheme for L-rhamnose via L-rhamnitol was described which gives the labelling at C-1, C-2 + C-3 + C-4, C-5 and C-6 on a 0.3 millimole scale.

L-Rhamnose is a minor constituent of plum-leaf polysaccharides, being found both in the polysaccharide fraction extracted from the leaves with water and in the fraction extracted with hot alkali (Andrews, & Hough 1958a). Present knowledge of the structure of pectic polysaccharides (Aspinall & Canas-Rodriguez, 1958; Andrews, Hough, Powell & Wood, 1959) suggests that this rhamnose exists in polysaccharides of which D-galactose, L-arabinose and D-galacturonic acid are the major monosaccharide constituents. In accord with this possibility, rhamnose, galactose and arabinose of plum-leaf polysaccharide fractions all attained similar specific activities after the incorporation and metabolism of ¹⁴CO₂ by the leaves (Andrews & Hough, 1958b).

Labelling in plum-leaf polysaccharides after the incorporation of D-[1-¹⁴C]- and D-[6-¹⁴C]-glucose gave information on the biosynthetic pathways of the major constituent monosaccharides and showed that the metabolic activity of pectin and xylan was much less than that of starch but greater than that of cellulose (Andrews, Hough & Picken, 1965a). Examination of L-rhamnose from the polysaccharide fractions obtained in these experiments indicated the presence of sufficient ¹⁴C to justify its further study, with a view to determining the mode of biosynthesis. The present paper records our observations in this connexion, and includes a degradation scheme for L-rhamnose applicable to

the determination of ¹⁴C-labelling in different carbon atoms on a 0.3 millimole scale.

MATERIALS AND METHODS

Radioactivity measurements

The ¹⁴C-labelled compounds were burnt in a stream of O₂ and the ¹⁴CO₂ so produced was converted into Ba¹⁴CO₃ disks of infinite thickness for counting. The ¹⁴CO₂ liberated in degradation experiments was likewise converted into Ba¹⁴CO₃ for counting. Radioactivity was determined either with a thin end-window Geiger-Müller tube or in a windowless flow-counter. Further details are given by Anderson, Andrews & Hough (1962).

Procedure for incorporation experiments

The labelled monosaccharide was fed to five non-fruiting spurs, each bearing seven to nine leaves, cut from plum trees (*Prunus domestica* var. Giant Prune). D-[1-¹⁴C]Glucose (3.8 mg.; 100 μC) and D-[6-¹⁴C]glucose (1.2 mg.; 50 μC) (purchased from The Radiochemical Centre, Amersham, Bucks.) were each dissolved in water (1.5 ml.), sufficient to provide 0.3 ml. of solution for each spur, which imbibed the solution through the cut end of the stem. Metabolism of the labelled compounds proceeded for 5 hr., during which time the cut ends of the stems were immersed in water, and the leaves received an illumination of about 400 ft.-candles. Further details are given by Andrews *et al.* (1965a).

Fractionation of the leaf polysaccharides and isolation therefrom of ¹⁴C-labelled L-rhamnose

Metabolism of the labelled compounds was terminated by breaking up the leaves and immediately plunging the

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laminae into boiling ethanol. Subsequently the pieces were extracted exhaustively with methanol, then with ether, and dried [yield about 15% (w/w) of fresh leaves]. Polysaccharide fractions isolated from the dry material (about 6g.) by extraction successively with water (7×200 ml.) at 80° for 10 min. and then $2.5N$ -NaOH (7×200 ml.) at 80° for 10 min. were partially purified by ethanol precipitation, then hydrolysed with N -H₂SO₄ at 100° for 8 hr. An intermediate fraction, isolated by extraction with N -NaOH at room temperature, was not used for isolation of rhamnose. The monosaccharide mixtures resulting from hydrolysis of the two polysaccharide fractions were fractionated on large-sheet paper chromatograms (Whatman no. 3MM paper) with butan-1-ol-pyridine-water (10:3:3, by vol.) as developing solvent, and the monosaccharide bands located by spraying guide strips with *p*-anisidine hydrochloride reagent (Hough, Jones & Wadman, 1950). L-Rhamnose was eluted from the appropriate areas of the paper with water, and the solutions were then passed through columns of Amberlite IR-120 (H⁺ form) and IRA-400 (HCO₃⁻ form) ion-exchange resins, evaporated to dryness under reduced pressure and the residues weighed. Further details of the procedures involved in isolating purified monosaccharides from plum-leaf polysaccharides are given by Andrews *et al.* (1965a).

The samples of ¹⁴C-labelled L-rhamnose prepared from each group of leaves were chromatographically pure when examined on paper sheets with either the butan-1-ol-pyridine-water mixture or ethyl acetate-acetic acid-water (9:2:2, by vol.) as developing solvent and *p*-anisidine hydrochloride or ammoniacal AgNO₃ as spray reagent. Crystalline L-rhamnose monohydrate (130 mg.) was added to each sample and the monosaccharide recrystallized from an ethanol-acetone mixture (yield approx. 110 mg.). The recrystallized material was assayed for ¹⁴C.

Degradation of L-rhamnose

L-Rhamnitol (II) from L-rhamnose (I). To a solution of L-rhamnose (I) monohydrate (100 mg.) in water (5 ml.) was added KBH₄ (40 mg.), which dissolved readily when the solution was stirred. After 2 hr. at room temperature, the solution was brought to about pH 6 with *n*-acetic acid and then passed through a column (20 cm. $\times$ 1.5 cm.) of Amberlite IR-120 (H⁺ form) ion-exchange resin for removal of cations. The resultant solution was evaporated to dryness under reduced pressure, and boric acid subsequently removed as the volatile methyl ester by alternate additions of methanol (5×20 ml.) and evaporation. The residue was then dissolved in water to give a neutral solution, from which anions and any unchanged rhamnose were removed by passage through a column of Amberlite IRA-400 (OH⁻ form) ion-exchange resin. Evaporation of the column effluent gave syrupy L-rhamnitol (II), which was crystallized from a methanol-acetone mixture, and had m.p. and mixed m.p. 122–123°. The yield from L-rhamnose (I) was 80%.

Degradation of L-rhamnitol (II). Periodate oxidation of L-rhamnitol (0.5 m-mole) in 0.2 M-sodium phosphate buffer, pH 5.9 (25 ml.), was carried out in a reaction vessel through which passed a stream of N₂ (CO₂-free), with the effluent gas passing through a solution of KMnO₄ (300 mg.) in *N*-H₂SO₄ (20 ml.) to trap the acetaldehyde from C-5 + C-6 of L-rhamnitol. Sodium metaperiodate (special grade 'for glycerol determinations'; Hopkin and Williams Ltd.,

Chadwell Heath, Essex) (450 mg.; 2.1 m-moles) was added to the rhamnitol solution and oxidation allowed to proceed for 2 hr. at room temperature. Excess of periodate was then destroyed by the addition of KI (300 mg.), followed by the addition of saturated NaAsO₂ until precipitation of I₂ ceased. The solution was decanted and decolorized by the addition of more NaAsO₂, then returned to the reaction vessel and warmed at 60° while the flow of N₂ was maintained for a further 2 hr. to complete the transfer of acetaldehyde into the acidified permanganate solution, where it was oxidized to acetic acid. The solution remaining in the reaction vessel contained formaldehyde from C-1 of L-rhamnitol and formic acid from C-2 + C-3 + C-4. These products were isolated and oxidized to CO₂ as described by Andrews *et al.* (1965a).

The permanganate solution was subjected to steam-distillation in a Markham (1942) apparatus until the distillate was no longer acidic. The distillate was passed through a column of Amberlite IR-120 (Na⁺ form) ion-exchange resin to convert the acetic acid into the sodium salt, and then evaporated to dryness. The residual sodium acetate was dried over silica gel and weighed (yield from L-rhamnitol, 90–95%).

Degradation of sodium acetate. To the dry sodium acetate were added NaN₃ (1.2 mol.prop.) and 98% (w/w) H₂SO₄ (0.2 ml.), while a stream of N₂ (CO₂-free) was passed through the reaction vessel at a rate of about 1.3 l./hr. The effluent gas was led through a trap containing 30% (w/v) H₂O₂ (3 ml.) in *N*-H₂SO₄ (25 ml.), then through a trap containing saturated Ba(OH)₂ solution (25 ml.) heated at 70° to assist the precipitation in granular form of BaCO₃ derived from C-5 of L-rhamnitol. Degradation of the sodium acetate was effected by heating the reaction vessel for 1 hr. at 60° and then the precipitated BaCO₃ in the second trap was collected for ¹⁴C assay. The contents of the reaction vessel were diluted to 40 ml. with water and made alkaline to phenolphthalein with $2N$ -NaOH, when the solution was evaporated to a small volume with the receiver-adaptor of the distillation apparatus dipping into $4N$ -HCl (10 ml.). The HCl solution was evaporated to dryness and the residue extracted with hot ethanol (10 ml.). Evaporation of the ethanol extract to dryness gave flakes of methylamine hydrochloride, derived from C-6 of L-rhamnitol, which were assayed for ¹⁴C.

RESULTS AND DISCUSSION

Degradation of L-rhamnitol. Attempts to isolate a crystalline salt of L-rhammonic acid, prepared by oxidation of L-rhamnose, for use in a degradation scheme similar to that described by Bernstein (1953) and Eisenberg (1954) for gluconic acid, and applied by us (Andrews *et al.* 1965a) to other aldonic acids, were unsuccessful. Various modifications of the hypiodite oxidation procedure of Moore & Link (1940) gave poor yields of the potassium, barium and strontium salts of rhammonic acid, and the products were either poorly crystalline or failed to crystallize at all. Instead, degradation was effected by the route shown in Scheme 1, whereby L-rhamnose (I) was first reduced with borohydride to give L-rhamnitol (II) and then this was oxidized with

Scheme 1. Degradation scheme for L-rhamnose (I) via L-rhamnitol (II). Experimental details are given in the text.

periodate to give formaldehyde from C-1, formic acid from C-2 + C-3 + C-4 and acetaldehyde from C-5 + C-6. Permanganate oxidation of the latter gave acetic acid, which was degraded by the Schmidt reaction to give carbon dioxide from C-5 of the original rhamnose and methylamine from C-6. The procedure for the Schmidt reaction described in the Materials and Methods section gave consistent yields of about 70% of methylamine and carbon dioxide, and when applied to a sample of sodium [$1\text{-}^{14}\text{C}$]acetate it gave $^{14}\text{CO}_2$ with the expected specific activity and unlabelled methylamine. Degradation of 100mg. of ^{14}C -labelled L-rhamnose monohydrate by Scheme 1 gave yields of $^{14}\text{CO}_2$ from the various carbon atoms that were sufficient for the preparation of $\text{Ba}^{14}\text{CO}_3$ disks (2cm. diam.; minimum wt. 40mg.) for ^{14}C assay under conditions of 'infinite thickness'.

Katz, Abraham & Chaikoff (1955) used the Schmidt reaction to degrade radioactive sodium acetate, and reported 90% yields of the products. We were unable to achieve yields much better than 70% in our experiments, although the effects of varying the ratios of reactants, concentration of sulphuric acid and temperature were examined (Table 1). The concentration of sulphuric acid generally used in the Schmidt reaction is 98% (w/w) because higher concentrations may lead to sulphonation, whereas concentrations lower than 96% are ineffective (Wolf, 1946). Since sulphonation of acetic acid cannot take place, 100% (w/w) sulphuric acid was used in its degradation, but the yields of products were not improved. Phares (1951) similarly degraded acetic acid and propionic acid in the presence of 100% (w/w) sulphuric acid, but did not report the yields of products.

Table 1. Effects of varying conditions on yields of products from degradation of sodium acetate by the Schmidt reaction

Further experimental details are given in the text. N.D., Not determined.

Temp.	Molar ratios of reactants		Concn. of H_2SO_4 (% w/w)	Yield (%)	
	H_2SO_4 /sodium acetate	NaN_3 /sodium acetate		CO_2	Methylamine
80°	8.4	1.65	98	44	N.D.
80°	11	2.2	100	65	63
80°	11.6	1.63	98	73	N.D.
60°	8.4	1.12	98	62	61
60°	12.8	1.7	98	70	68

A degradation procedure for L-rhamnose described by Jones, Perry & Stoodley (1963) differs in the main from Scheme 1 in that degradation of acetaldehyde was effected by the iodoform reaction. We found that, for degradation of acetaldehyde, oxidation to acetate followed by the Schmidt reaction gave better and more consistent yields of products than did the iodoform reaction. Jones *et al.* (1962) enumerate the several published degradation schemes for L-rhamnose. Degradation according to our Scheme 1 is probably as simple and as satisfactory in terms of yields as any of them, and gives information on ^{14}C -labelling that is sufficient for many investigations of rhamnose biosynthesis.

^{14}C -incorporation experiments. More than half the ^{14}C supplied to the plum spurs in labelled glucose was found in the leaves after 5hr. of metabolism,

Table 2. *Labelling of L-rhamnose of plum-leaf polysaccharides after the incorporation of ^{14}C -labelled D-glucose*

Degradation was effected by the method outlined in Scheme 1. Experimental details are given in the text. Radioactivity at each location is expressed as a percentage of the total radioactivity found in the degradation products. To facilitate comparison between the two experiments, results for D-[6- ^{14}C]glucose incorporation have been corrected for the use of only 50 μC of ^{14}C in this experiment (compared with 100 μC of D-[1- ^{14}C]glucose), and the distribution of ^{14}C amongst polysaccharides in 8.99 g. of ethanol-insoluble leaf material in the 50 μC experiment as compared with 6.75 g. of ethanol-insoluble material in the 100 μC experiment.

Compound supplied	Polysaccharide fraction	Sp. activity of carbon in L-rhamnose ($\mu\text{C}/\text{mg. atom of C}$)	Location of ^{14}C in L-rhamnose				
			C-1	C-2 + C-3 + C-4	C-5	C-6	
D-[1- ^{14}C]Glucose	Hot-water-soluble	0.022	56	8	2	34	
	Hot-alkali-soluble	0.022	79	3	1	17	
D-[6- ^{14}C]Glucose	Hot-water-soluble	0.044	20	4	3	73	
	Hot-alkali-soluble	0.016	11	13	*	76	

* The sample was lost. No account is taken of any ^{14}C in this position in calculating percentage of ^{14}C among the other five carbon atoms.

and about a quarter of this was present in the ethanol-insoluble fraction, which contained the polysaccharides (Andrews *et al.* 1965a). The weights of ^{14}C -labelled L-rhamnose isolated from the various polysaccharide fractions were 4–7 mg., less than the amounts of D-glucose, D-galactose or L-arabinose isolated from the same fractions, but similar to the amounts of D-xylose obtained from the water-soluble polysaccharides. The specific activity obtained by L-rhamnose (Table 2) was at least an order of magnitude less than that of D-glucose of starch from the corresponding polysaccharide fractions, and an order of magnitude greater than that of D-glucose from cellulose of the same leaves (Andrews Hough & Picken, 1965b). On the other hand, a relationship between L-rhamnose and leaf pectin is indicated by a marked similarity between the specific activities of L-rhamnose and D-galactose of corresponding fractions, the former generally being about two-thirds of the latter. Further, in the water-soluble polysaccharides, these two monosaccharides attained about twice the specific activity after D-[6- ^{14}C]glucose incorporation than that after D-[1- ^{14}C]glucose incorporation. Though these results suggest a common precursor pool for D-galactose and L-rhamnose, it is clear that ^{14}C is redistributed in the precursors by a pathway of carbohydrate metabolism that leads to a greater utilization of ^{14}C from C-6 than from C-1 before synthesis of deoxyhexose. Investigating L-rhamnose synthesis by a streptococcus, Southard, Hayashi & Barkulis (1959) also found that this monosaccharide attained a higher specific activity when D-[6- ^{14}C]glucose was the precursor than when D-[1- ^{14}C]glucose was the precursor. On the other

hand, Watkin & Neish (1961) and Jones *et al.* (1962) made the opposite observation for L-rhamnose synthesis in buckwheat and *Azotobacter indicum* respectively. Apart from differences in hexose metabolism in these organisms, the contributions of two pathways for the biosynthesis of L-rhamnose, varying from one organism to another, would also account for these observations.

The location of ^{14}C in L-rhamnose isolated from the various plum-leaf polysaccharide fractions is shown in Table 2. For reasons discussed by Andrews *et al.* (1965a) the values for percentage distribution of ^{14}C along the carbon chain are considered to have a possible error of 2 units. The labelling patterns resemble those of other hexoses isolated from the leaf polysaccharides (Andrews *et al.* 1965a). Randomization of label in L-rhamnose from all but the water-soluble polysaccharides from the D-[1- ^{14}C]glucose experiment was markedly less than that in D-glucose from starch, but similar to that in D-galactose from the same fractions and to that in D-glucose from cellulose; in the exceptional case, the labelling in L-rhamnose was even more random than that in D-glucose of starch. On the whole, the observations support the suggestion that the synthesis of structural polysaccharides utilizes glucose from a pool different from that concerned with starch synthesis.

In plum leaves, as in buckwheat (Watkin & Neish, 1961) and in some micro-organisms (cf. Jones *et al.* 1962), the major route for the biosynthesis of L-rhamnose from D-glucose evidently involves neither scission nor inversion of the carbon chain. Presumably in plum leaves the interconversion occurs while glucose, rhamnose and the intermediate

monosaccharides are in the form of UDP derivatives (Barber, 1963), rather than thymidine diphosphate or GDP derivatives as in bacteria (Pazur & Shuey, 1960, 1961; Blumson & Baddiley, 1961; Glaser & Kornfeld, 1961; Markovitz, 1961). Labelling patterns in the rhamnose of water-soluble polysaccharides suggest that a minor but indirect pathway for its biosynthesis is also operative. It could involve, for example, the aldolase-catalysed transfer of a 1,3-dihydroxypropan-2-one unit from D-fructose 6-phosphate to L-pyruvaldehyde and conversion of the resultant 6-deoxy-L-sorbose into L-rhamnulose and thence into L-rhamnose, as proposed by Jones *et al.* (1962). A contribution from such a pathway that varies according to the site of rhamnose synthesis could result in labelling patterns for rhamnose which differ according to the polysaccharide fraction from which it is isolated. A spatial separation of the precursor sources for rhamnose biosynthesis, similar perhaps to the situation for cellulose synthesis discussed by Andrews *et al.* (1965b), might exist in plum leaves.

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