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2. THE METABOLISM OF ^{14}C-LABELLED D-GLUCOSE, D-GLUCURONIC ACID AND D-GLUCITOL (SORBITOL) BY PLUM LEAVES*

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Experiments involving the photosynthetic assimilation of [^{14}C]carbon dioxide by plum leaves (*Prunus domestica*, var. Victoria) have indicated that D-glucitol (I) (sorbitol), the acylic polyol of these leaves, plays an important role in their metabolism (Anderson, Andrews & Hough, 1961). With leaves previously kept in the dark for 24 hr. a higher proportion of the incorporated ^{14}C was utilized for sorbitol synthesis than for starch synthesis. Further, under natural conditions the concentration of glucitol in the leaves did not alter during the course of a day.

The close structural relationship between D-glucose and D-glucitol suggests their ready interconversion when they occur together in Nature. To investigate this possibility, D-[1-^{14}C]- and D-[6-^{14}C]-glucose were metabolized by plum leaves and the resultant labelling in D-glucitol was determined. D-[1-^{14}C]Glucitol was also fed to plum leaves in an attempt to detect the main pathways of glucitol metabolism. In addition, the labelling in D-glucitol after metabolism of D-[6-^{14}C]glucuronic acid by plum leaves was examined.

Two chemical procedures have been developed for the degradation of D-[^{14}C]glucitol to determine the labelling at some of the individual carbon atoms. They are outlined in Schemes 1 and 2.

MATERIALS AND METHODS

Radioactivity measurements

The ^{14}C-labelled compounds were burnt in a stream of oxygen and the $^{14}CO_2$ so produced converted into $Ba^{14}CO_3$ disks of infinite thickness for counting as described by Anderson *et al.* (1961). To ensure complete conversion into $^{14}CO_2$, potassium gluconate samples were mixed with twice their weight of sodium dichromate before combustion (Grant, 1945). The $^{14}CO_2$ liberated in degradation experiments was likewise converted into $Ba^{14}CO_3$ for counting. Rough estimates of ^{14}C in solutions were sometimes obtained by counting the thin films of residue (not more than 0·5 mg.) obtained by evaporating portions (usually 5–100 μl.) to dryness on nickel planchets.

Radioactivity was determined either in a counter with a thin end-window Geiger tube, or in a windowless flow-counter (Type SC-16G; Tracerlab, Waltham, Mass., U.S.A.). In both cases the apparatus was calibrated for counting $Ba^{14}CO_3$ disks by determining the count rate of 'infinitely thick' $Ba^{14}CO_3$ disks prepared from compounds of known specific activity, and for counting thin films with weighed portions (2–12 μl.) of an aqueous solution containing known amounts of D-[6-^{14}C]glucose (specific activity, 42 μc/mg.). The performance of the counters was frequently checked with a disk of poly([^{14}C]methyl methacrylate) as a standard reference source.

^{14}C-*labelled compounds*

The ^{14}C-labelled D-glucose and potassium D-glucuronate of high specific activity (Table 2) for incorporation experiments were purchased from The Radiochemical Centre, Amersham, Bucks. Low-specific-activity D-[^{14}C]glucoses (about 1 μc/m-mole) were prepared by carrier dilution and recrystallization of samples generously supplied by Dr J. R. Catch of The Radiochemical Centre.

D-[^{14}C]*Glucitol for degradation experiments.* The D-[1-^{14}C]-, D-[2-^{14}C]- and D-[6-^{14}C]-glucitol were prepared from the corresponding labelled glucoses by reduction with borohydride (tetrahydroborate) (Abdel-Akher, Hamilton & Smith, 1951). The glucose (250 mg.; specific activity about 1 μc/m-mole) was dissolved in water (25 ml.) together with a large excess (250 mg.) of potassium tetrahydroborate. After 8 hr. at room temperature, acetic acid (1 ml.) was added to destroy excess of tetrahydroborate, and cations were removed from the solution by passage through a column (2 cm. × 30 cm.) of Amberlite IR-120 (H^+ form) ion-exchange resin. Thorough washing of the column gave a solution (300 ml.) which was evaporated to a small volume under reduced pressure; then boric acid was removed by evaporation to dryness (repeated 8–10 times) in the presence of methanol until the product no longer gave an acid reaction. In some preparations, inactive D-glucitol (750 mg.) was added at this stage. Then the product was dissolved in water (20 ml.) and the solution heated at 100° for 4 hr. with Bio-Deminrolit mixed-bed ion-exchange resin (The Permutit Co. Ltd., London, W. 4) to remove any unchanged glucose and other impurities (Anderson *et al.* 1961). The resin was filtered off and the solution evaporated to dryness, yielding a colourless solid which was dried over phosphorus pentoxide. Estimations of activity indicated radiochemical yields of about 90% in these preparations. The method was evaluated more critically in the preparation of high-specific-activity D-[^{14}C]glucitol.

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* Part 1: Anderson, Andrews & Hough (1961).

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High-specific-activity D-[1-^{14}C]*glucitol for incorporation experiments.* D-[1-^{14}C]Glucose (3·8 mg.; 100 μc) was reduced with potassium tetrahydroborate (10 mg.) in water (2 ml.), the solution was brought to pH 6 with N-acetic acid, and boric acid removed as described above. After treatment with Bio-Deminrolit resin the product (3·2 mg.) contained 84 μc (estimated on 'infinitely thin' samples), and examination on paper chromatograms with butan-1-ol–ethanol–water (40:11:19, by vol.) or butan-2-one–acetic acid–saturated aq. boric acid (9:1:1, by vol.) (Rees & Reynolds, 1958) as mobile phase, and silver nitrate–sodium hydroxide (Trevelyan, Procter & Harrison, 1950) or *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) as spray reagents, indicated that glucitol was the sole constituent. Only this one radioactive spot was detected on the chromatograms by scanning with a thin end-window Geiger counter, or by radioautography (Ilford 'Industrial G' X-ray film). The product was dried and stored over phosphorus pentoxide.

D-[6-^{14}C]*Glucuronic acid.* Hough & Pridham (1955) found that sodium D-glucuronate was toxic to plum leaves; accordingly the free acid was prepared for the incorporation experiment. A solution (5 ml.) of potassium D-[6-^{14}C]-glucuronate (18·4 mg.; 100 μc) was passed through a column (1 cm. × 30 cm.) of Amberlite IR-120 (H^+ form) ion-exchange resin, and the column thoroughly eluted with water (200 ml.). Activity measurements (with 'infinitely thin' samples) indicated the presence of about 100 μc in this effluent. The solution was distilled *in vacuo* until the volume was about 0·5 ml.; then this solution was transferred, together with washings of the distilling flask (3 ml.), to a 5 ml. beaker, and evaporation continued over conc. H_2SO_4 in a desiccator to a final volume of about 1 ml. Finally the solution was made up to 1·5 ml., the volume being measured by weighing.

Incorporation experiments

Non-fruiting spurs, bearing 7–9 leaves, were cut from plum (*Prunus domestica*, var. Victoria) trees at about 10.0 a.m. on days during August and kept with the cut ends of the stems in water until required (1–2 hr. after cutting). Five spurs were used in each of the experiments with labelled glucose and glucuronic acid, and two in each [^{14}C]glucitol experiment. Illumination, provided by a battery of tungsten lamps shining through a layer of running water for cooling (Folkes, Willis & Yemm, 1952; Andrews & Hough, 1958), was about 400 ft.-candles at the level of the leaves. To promote transpiration and the uptake of solution through the stems, a gentle current of air was maintained over the leaves during experiments.

Labelled compounds were dissolved in sufficient water to provide 0·3 ml. of solution for each spur, which imbibed the solution, contained in a small glass tube, through the cut end of the stem in about 10 min. Two further 0·3 ml. portions of water were taken up by each spur from the same vessels, then water was supplied freely from larger containers. Radioactivity was detected in the leaves with a thin end-window counter soon after uptake of the labelled glucoses.

In the experiment with D-[6-^{14}C]glucuronic acid, immediately after uptake of the solution of labelled compound and a further 0·3 ml. of water (time taken, 20 min.), the leaves were enclosed in a desiccator (volume, 4 l.) through which was passed a stream of CO_2-free air. The air issuing from the desiccator was bubbled through N-NaOH to trap CO_2 expired by the leaves.

To terminate experiments, the petioles and mid-ribs were removed and the leaf laminae placed in boiling ethanol for a few minutes to inactivate enzymes, then extracted exhaustively with methanol in a Soxhlet apparatus. The alcoholic extracts from each experiment were evaporated to dryness *in vacuo*, and the residues dried and stored over phosphorus pentoxide. The alcohol-insoluble residues were dried similarly, weighed, and their ^{14}C content determined by combustion to $^{14}CO_2$ and assay as $Ba^{14}CO_3$. The carbon content of this material was 43·3 %.

Estimation of total ^{14}C *and* ^{14}C *in* D-*glucitol of plum-leaf ethanol-soluble fractions.* The low specific activity of ^{14}C in these fractions, and the consequent high self-absorption of samples large enough to give satisfactory counting-rates, precluded the estimation of ^{14}C in this case by counting thin films. Therefore the following isotopic dilution method was used, which also facilitated the estimation of ^{14}C in D-glucitol.

The dry residue from each experiment was dissolved in ethanol (0·5 or 1·0 l.) and to each of two 50 ml. portions were added different amounts (e.g. 75 and 350 mg.) of carrier D-glucitol. The two solutions were evaporated to syrups in a stream of air over a boiling-water bath, and traces of ethanol removed by repeating the evaporation after addition of water (5 ml.). The specific activity of ^{14}C in the final syrups was determined on portions converted into $Ba^{14}CO_3$. The remainder was dissolved in water (25 ml.), Bio-Deminrolit mixed-bed ion-exchange resin (10 g.) was added, and the mixture heated at 100° for 8 hr. The resin was filtered off, and the filtrate evaporated to dryness. The residual crude D-[^{14}C]glucitol was converted into the hexa-*O*-acetate by treatment overnight at room temperature with excess of acetic anhydride and a large excess of anhydrous pyridine (Verley & Bölsing, 1901; Anderson *et al.* 1961). One recrystallization from aq. ethanol was sufficient to give a product (m.p. 98–99°) with specific activity unchanged on further recrystallization.

Isolation of D-[^{14}C]*glucitol from plum leaves.* The leaves of five plum-tree spurs (wt. of alcohol-insoluble residue usually 6–7 g.) contained 650–750 mg. of D-glucitol (Anderson *et al.* 1961). For isolation of [^{14}C]glucitol for degradation experiments, suitable portions of the ethanol-soluble material were treated with Bio-Deminrolit resin as above. The crude product was purified by conversion into the crystalline hexa-*O*-acetate, from which D-[^{14}C]glucitol was regenerated by deacetylation. For example, to a solution of the acetate (200 mg.) in dry chloroform (20 ml.) was added a solution of sodium methoxide in methanol (2 %, w/v; 2 ml.). After 2 hr. the precipitated glucitol was isolated by centrifuging and dissolved in water, and the solution was de-ionized with Bio-Deminrolit resin. Evaporation to dryness gave a clear colourless syrup, to which carrier D-glucitol was added as necessary.

Degradations of ^{14}C*-labelled compounds*

L-*Xylose (IV) from* D-*glucitol (I).* A modification of the method of Salomon, Burns & King (1952) was used for the preparation of 2,4-*O*-benzylidene-D-glucitol (II). D-Glucitol (I) (1 g.) in water (0·8 ml.) was vigorously shaken with benzaldehyde (0·55 ml.) and conc. HCl (0·1 ml.) in a sealed glass tube (1 cm. diam.) at 4° for 18 hr., by which time the contents of the tube had partly solidified; then the

tube was opened, cooled in ice, and the acid neutralized by adding M-Na_2CO_3 (2 ml.) with thorough mixing. The crystalline product was filtered off, washed with ice–water, and dried *in vacuo* over phosphorus pentoxide and paraffin-wax chips (yield about 750 mg.). This material was extracted with boiling 1% (w/v) Na_2CO_3 solution (8 ml.), and rapid filtration gave a clear solution which yielded crystalline 2,4-*O*-benzylidene-D-glucitol (II) (300–500 mg. in different preparations), m.p. 172–173° [Vargha (1935) obtained m.p. 172–173°]. In experiments commencing with [^{14}C]glucitol, a further recrystallization was sometimes required to attain constant specific activity.

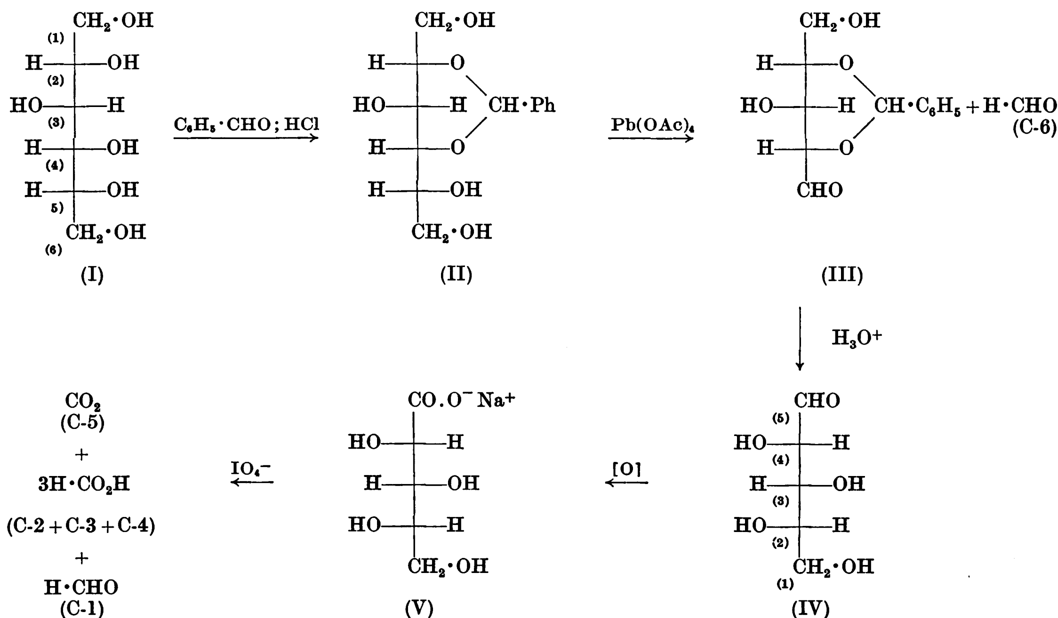
Lead tetra-acetate was prepared by the method of McClenahan & Hockett (1938), and standardized as a 5% (w/v) solution in acetic acid (Hockett & McClenahan, 1939). A portion of this solution, containing 1 mol.prop. of lead tetra-acetate, was added to 2,4-*O*-benzylidene-D-glucitol (500 mg.) and the solution heated at 50° while a slow stream of nitrogen was bubbled through it. After about 15 min. the solution gave a negative starch–iodide reaction, whereupon it was evaporated to dryness under reduced pressure. 2,4-*O*-Benzylidene-L-xylose (III) was extracted from the residue with ethyl acetate (50 ml.) three times. Evaporation of the extract gave a syrup which was dissolved in 50 ml. of acetic acid (15%, v/v), and the solution was heated at 100° for 3 hr. to hydrolyse the benzylidene group. After hydrolysis, the solution was concentrated to a syrup which was dissolved in water (2 ml.), and the solution was passed through a column (1 cm. × 15 cm.) of a mixture of equal amounts of Amberlite IR-120 (H^+ form) and IR-400 (HCO_3^- form) resins. Elution of the column with water (200 ml.) and evaporation of the effluent gave a syrupy product, from which crystalline L-xylose (IV), m.p. 140–142°, was obtained by crystallization from acetone–methanol (the yield in different experiments was 40–65%, based on the benzylidene–xylose). Further recrystallization effected no change in specific activity in experiments with labelled compounds.

No attempt was made to recover formaldehyde from C-6 of D-glucitol. The ^{14}C at this position was calculated from difference in specific activities of (II) and (IV).

Degradation of L-*xylose* (*IV*). The procedure for oxidation of L-xylose (IV) to L-xylonic acid (V) was modified from that of Moore & Link (1940). L-Xylose (75 mg.), dissolved in methanol (2 ml.) plus a few drops of water, was added to a solution of $BaI_2,2H_2O$ (280 mg.) and iodine (280 mg.) in methanol (6 ml.) contained in a 50 ml. centrifuge tube. The solution was stirred and heated at 40° while 5% (w/v) KOH in methanol (6 ml.) was added dropwise during 15 min. The reaction mixture was then cooled and centrifuged, and the sediment, consisting mainly of barium xylonate and barium carbonate, was washed twice with methanol, dried over phosphorus pentoxide and weighed. This material was extracted with water (3 ml.) three times, and the undissolved residue dried and weighed. The yield of barium L-xylonate, calculated by difference, was usually about 85% (based on L-xylose).

Removal of barium from the xylonic acid solution was necessary to obtain satisfactory degradation by periodate. This was effected by addition of Na_2SO_4 to the solution until precipitation of $BaSO_4$ was complete, and clarifying the solution by centrifuging.

Periodate oxidation of sodium xylonate was carried out by the method of Bernstein (1953), with some important modifications of experimental detail. The solution (about 9 ml.) of sodium L-xylonate (about 0·4 m-mole), prepared as above, and an equal volume of M-sodium phosphate buffer, pH 5·9, were flushed with N_2 (CO_2-free), in a darkened



Scheme 1. Degradation of D-glucitol (I) via 2,4-*O*-benzylidene-D-glucitol (II) and L-xylose (IV) (the numbering of carbon atoms refers to their original positions in D-glucitol). See text for experimental details.

aeration tube, then sodium metaperiodate (Hopkin and Williams Ltd.; special grade 'for glycerol determinations') (428 mg.; 2·0 m-moles) was added. The effluent aeration gas containing CO_2 derived from C-5 of D-glucitol was passed through $Ba(OH)_2$ solution (previously saturated at 20°), and heated at 80° to encourage the formation of a granular precipitate of $BaCO_3$; the yield was about 95% from sodium xylonate.

After 90 min., KI (300 mg.) was added, the reaction mixture adjusted to pH 2 with 2N-H_2SO_4, and saturated $NaAsO_2$ was added until precipitation of I_2 ceased. The solution was decanted and decolorized with more $NaAsO_2$ solution. If the precipitate of I_2 were not removed but redissolved by further addition of $NaAsO_2$, free I_2 appeared later during steam-distillation of formic acid at pH 2. The solution was made alkaline to phenolphthalein with N-NaOH and distilled at atmospheric pressure, the distillate being collected in a receiver cooled in ice. Several evaporations almost to dryness, with intermediate additions of water, gave a distillate (about 200 ml.) containing formaldehyde originating from C-1 of D-glucitol. The formaldehyde was oxidized to formic acid with hypoiodite, and the formic acid to CO_2 with mercuric acetate, as described by Bernstein (1953); a Markham (1942) apparatus was used for steam-distillation, and the CO_2 was collected as $BaCO_3$ for ^{14}C assay (the yield was about 70% from L-xylose).

The solution remaining after distillation of formaldehyde contained formic acid originating from (C-2 + C-3 + C-4) of D-glucitol. After addition of 2N-H_2SO_4 to adjust the pH to 2, the formic acid was isolated by steam-distillation and oxidized to CO_2 (trapped as $BaCO_3$; the yield was about 75% from L-xylose) with mercuric acetate.

Degradation of D-glucitol (I) via D-glucose (VI). D-[^{14}C]Glucitol (100 mg.; containing not less than 1 μc) in water (25 ml.) was oxidized to D-glucose (VI) with $FeSO_4,7H_2O$ (30 mg.) and hydrogen peroxide (30%, v/v; 0·15 ml.) [Fenton's reagent (Fenton & Jackson, 1899)] at 0°. The solution was stored at 2° for 9 hr., then unlabelled D-glucose (750 mg.) was added as carrier, excess of H_2O_2 was decomposed by addition of a few drops of blood, and the solution was de-ionized by passage through a column (2 cm. × 30 cm.) of equal parts of Amberlite IR-120 (H^+ form) and IR-45 (OH^- form) resins. Evaporation of the solution gave a syrup (850 mg.) which was dissolved in methanol and used for preparing potassium D-[^{14}C]gluconate (VII) by hypoiodite oxidation (Moore & Link, 1940). One recrystallization from aq. methanol was sufficient to give a product of constant specific activity. The radiochemical yield from [^{14}C]glucitol was about 2%.

The degradation of potassium gluconate (VII) by periodate oxidation, and conversion into CO_2, were carried out as described for sodium xylonate.

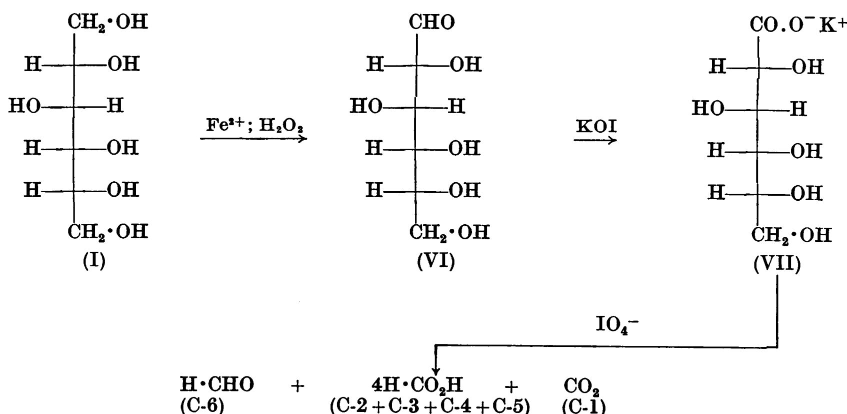
RESULTS AND DISCUSSION

Degradations of ^{14}C-labelled compounds

Methods for the degradation of D-[^{14}C]glucitol (I) with the aim of determining the isotope content at individual carbon atoms have not hitherto been described, although Muntz & Carroll (1960) reported the oxidation of D-glucitol to D-fructose by a rat-liver enzyme preparation, and degradation of this ketose with lead tetra-acetate (Brice & Perlin, 1957) could give the labelling at all carbon atoms. However, two simpler chemical degradations (Schemes 1 and 2) were developed for D-glucitol and they gave satisfactory results when tested with specifically labelled compounds (Table 1). The second of these methods (Scheme 2) was the more rapid, but had the disadvantage that the yield of D-glucose (VI) from D-glucitol (I) was very low (about 2%).

Incorporation experiments

The overall distribution and recovery of ^{14}C in the leaf laminae after metabolism by plum leaves of the labelled compounds listed in Table 2 was determined in an attempt to assess the relative importance of these compounds as intermediates in the leaf metabolism. To estimate the total ^{14}C in the ethanol-soluble fractions, and the ^{14}C in D-glucitol, an isotopic dilution method was used which obviated the determination of the carbon or



Scheme 2. Degradation of D-glucitol (I) via D-glucose (VI). See text for experimental details.

D-glucitol content of these fractions. The results (Table 2) were calculated from the experimental values by means of the appropriate simultaneous equations. For comparison, the distribution of ^{14}C in plum leaves resulting from the incorporation of [^{14}C]carbon dioxide is included, although these are not strictly comparable since in this case the weight of labelled compound used was 100 times greater and the leaves had been kept in the dark for 24 hr. before incorporation to deplete the starch content (Anderson *et al.* 1961).

Nearly 80 % of the ^{14}C administered to the leaves was accounted for by assaying the leaf-laminae fractions from those experiments with labelled glucoses, and a similar recovery of isotope from D-[6-^{14}C]glucuronic acid was obtained when the expired [^{14}C]carbon dioxide was taken into account. The 20 % of ^{14}C that was unaccounted for probably remained in the mid-ribs and petioles since activity was detected in the woody stems of the spurs with a thin end-window counter, but it was not measured. However, in the experiments with D-[^{14}C]glucitol a much smaller proportion of administered isotope was detected in the leaves. The low recovery of ^{14}C in these experiments, and the small quantity of ^{14}C found in compounds other than D-glucitol, preclude any conclusions at present about the way in which D-glucitol is metabolized by plum leaves, but do suggest that its transport to the leaves and subsequent metabolism are, by comparison with D-glucose and D-glucuronic acid, relatively inefficient processes. The higher incorporation in the light than in the dark of ^{14}C from D-glucitol into alcohol-insoluble material may be due to equilibration of D-glucitol with D-glucose or other possible intermediates in polysaccharide synthesis under conditions favouring a net synthesis of polysaccharide, especially starch.

The experiments with D-[1-^{14}C]- and D-[6-^{14}C]-glucose demonstrated a rapid synthesis of D-glucitol from D-glucose, since in both cases about 40 % of the isotope accounted for was found in D-glucitol after 5 hr. Hutchinson, Taper & Towers (1959) reported a similar conversion of D-[1-^{14}C]-glucose into D-[^{14}C]glucitol in apple (*Malus*) leaf disks which, like plum leaves, are rich in D-glucitol. The pattern of labelling in D-[^{14}C]glucitol from plum leaves was determined by two degradative schemes (Schemes 1 and 2) with concordant results (Table 3), which confirmed that the hexitol originated largely by direct reduction of D-glucose, since a similar randomization of isotope to that in D-glucitol results. Partial randomization of labelling between terminal carbon atoms and to a lesser

Table 1. *Chemical degradation of specifically labelled* D-[^{14}C]*glucitols*

The location of ^{14}C at numbered carbon atoms is calculated from: specific activity of derived $^{14}CO_2$ × theoretical yield of $^{14}CO_2$. Results are expressed as percentages relative to ^{14}C measured in the corresponding 2,4-*O*-benzylidene-D-[^{14}C]glucitol (II) (Scheme 1) or potassium D-[^{14}C]gluconate (VII) (Scheme 2).

		Location of ^{14}C			
Substance	Degradation method	C-1	(C-2 + C-3 + C-4)	C-5	C-6*
D-[1-^{14}C]Glucitol	Scheme 1	100	1·2	0·9	~0
D-[2-^{14}C]Glucitol	Scheme 1	0·8	99	1·5	~0
D-[6-^{14}C]Glucitol	Scheme 1	←	0·4†	→	99·6
D-[6-^{14}C]Glucitol	Scheme 2	2	0 (C-2 to C-5)		98

* By difference; see text.

† ^{14}C in L-xylose.

Table 2. *Distribution of* ^{14}C *in plum-leaf constituents after incorporation of* ^{14}C*-labelled substrates*

See text for experimental details.

Compound supplied					^{14}C in leaf fractions (as % of total ^{14}C in laminae)		
						Ethanol-soluble material	
Name	Wt. (mg.)	Activity (μc)	Duration of expt. (hr.)	Total ^{14}C in leaf laminae (as % of ^{14}C administered)	Alcohol-insoluble material	D-Glucitol	Other compounds
D-[1-^{14}C]Glucose	3·8	100	5	76	22	42	36
D-[6-^{14}C]Glucose	1·2	50	5	80	23	37	40
D-[6-^{14}C]Glucuronic	15·8	100	6	55*	53	25	22
D-[1-^{14}C]Glucitol	1·6	42	5	17	10	82	8
D-[1-^{14}C]Glucitol	1·6	42	5†	20	4	82	14
$^{14}CO_2$	395	143	6‡	98	59	28	13

* An additional 22 % was recovered as expired $^{14}CO_2$.

† In darkness.

‡ Before photosynthesis in $^{14}CO_2$ the leaves were kept in darkness for 24 hr.

Table 3. *Chemical degradation of* D-[^{14}C]*glucitol from plum leaves*

Results are expressed as in Table 1.

Labelled compound fed to leaves	Degradation method	Location of ^{14}C in D-[^{14}C]glucitol			
		C-1	(C-2 + C-3 + C-4)	C-5	C-6*
D-[1-^{14}C]Glucose	Scheme 1	71	6	3	20
D-[6-^{14}C]Glucose	Scheme 1	14	5	2	79
D-[6-^{14}C]Glucuronic acid	Scheme 1	12	58	12	18
D-[1-^{14}C]Glucose	Scheme 2	70	9 (C-2 to C-5)		21
D-[6-^{14}C]Glucose	Scheme 2	16	6 (C-2 to C-5)		78

* By difference; see text.

extent into others, a common observation in D-[^{14}C]glucose metabolism, indicates that a competitive but reversible aldolase reaction has occurred involving 25–50% of the hexose molecules (Shibko & Edelman, 1957). A similar randomization of isotope to that of D-glucitol originating from D-[1-^{14}C]glucose was found in the D-[^{14}C]-glucosyl units of the water-soluble polysaccharides of leaves from the same experiment: C-1, 64·6%; C-2–C-5, 13·7%; C-6, 21·7% (P. Andrews, unpublished work).

The pattern of labelling in D-glucitol from leaves which had metabolized D-[6-^{14}C]glucuronic acid differed greatly from that of D-glucitol originating from D-[6-^{14}C]glucose (Table 3). The randomization of ^{14}C throughout the molecule indicated that much of the ^{14}C had entered the synthetic pathway to D-glucitol at a very early stage, and the heavier labelling at (C-2 + C-3 + C-4) than at C-1 and C-5 suggested that ^{14}C was introduced into the pathway by the photosynthetic incorporation of [^{14}C]-carbon dioxide, derived from ^{14}C-6 of D-glucuronic acid by decarboxylation. The decarboxylation of D-glucuronic acid by plant tissues is well known (Altermatt & Neish, 1956; Slater & Beevers, 1958), and was apparently the major route of metabolism on feeding it to plum leaves since the leaves expired a considerable amount of [^{14}C]carbon dioxide in addition to using it for photosynthetic assimilation. Gibbs & Kandler (1957) isolated D-glucose labelled predominantly at C-3 and C-4 from *Chlorella* and from green leaves after short periods of photosynthesis in [^{14}C]carbon dioxide, in general agreement with the process for hexose synthesis proposed by Bassham *et al.* (1954) involving incorporation of carbon dioxide into D-*erythro*-pentulose 1,5-diphosphate. They showed also that non-uniform labelling can still be observed after photosynthesis by *Chlorella* for longer periods, e.g. 3 hr., in the presence of a supply of exogenous glucose. The [^{14}C]carbon dioxide liberated from D-[6-^{14}C]glucuronic acid by plum leaves was, no doubt, rapidly used for synthesis of hexose, some of which was reduced to D-glucitol. Dilution of this labelled glucitol by the large pool of glucitol already present in the leaves could in effect trap non-uniformly labelled molecules and thus prolong the time for which this labelling was detectable.

Gibbs & Kandler (1957) also found that the labelling at C-6 of unevenly labelled photosynthetic D-glucose was either similar to, or less than, that at C-1, but, in our experiment with D-[6-^{14}C]glucuronic acid, there was a significant excess of ^{14}C at C-6 over that at C-1 of D-glucitol. This effect may be attributed to reduction of a small amount of the available D-glucuronic acid to D-glucitol without rupture of the carbon chain; such a reaction could occur with either D-glucose or D-gluconic acid as intermediate. The possibility of the latter pathway is illustrated by the observation (Kessler, Neufeld, Feingold & Hassid, 1961) that mung-bean (*Phaseolus aureus*) seedlings can convert D-glucuronic acid into D-gluconic acid.

The interconversion of D-glucose and D-fructose via D-glucitol in sheep seminal vesicles (Hers, 1958, 1960) suggests that the polyol plays a similar role in plum leaves.

SUMMARY

1. Two chemical degradation schemes for D-[^{14}C]glucitol have been developed. One involves the preparation of 2,4-*O*-benzylidene-D-glucitol followed by oxidation to L-xylose and then L-xylonic acid, and permits the determination of labelling at C-1, (C-2 + C-3 + C-4), C-5 and C-6. The other, which is more rapid, proceeds by direct oxidation of D-glucitol to D-glucose, albeit in poor yield, and then to D-gluconic acid, and gives the labelling at C-1, (C-2 + C-3 + C-4 + C-5) and C-6.

2. When D-[1-^{14}C]- and D-[6-^{14}C]-glucose were metabolized by plum leaves, about 40% of the ^{14}C detected in the leaves after 5 hr. was in D-glucitol. Labelling patterns showed that the conversion of D-glucose into D-glucitol probably occurs without rupture of the carbon chain.

3. D-[6-^{14}C]Glucuronic acid was in the main decarboxylated by plum leaves; some of the [^{14}C]-carbon dioxide was expired and of the remainder,

which was reassimilated during photosynthesis, a considerable portion appeared in D-glucitol after 6 hr.

4. To a small extent, D-glucuronic acid appeared to be converted into D-glucitol without rupture of the carbon chain.

5. When D-[1-^{14}C]glucitol was fed to plum leaves, only about 20% of the activity was detected in the leaves after 5 hr. and over 80% of this was still present in D-glucitol.

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REFERENCES

Abdel-Akher, M., Hamilton, J. K. & Smith, F. (1951). *J. Amer. chem. Soc.* **73**, 4691.

Altermatt, H. A. & Neish, A. C. (1956). *Canad. J. Biochem. Physiol.* **34**, 405.

Anderson, J. D., Andrews, P. & Hough, L. (1961). *Biochem. J.* **81**, 149.

Andrews, P. & Hough, L. (1958). *J. chem. Soc.* p. 4483.

Bassham, J. A., Benson, A. A., Kay, L. D., Harries, A. Z., Wilson, A. T. & Calvin, M. (1954). *J. Amer. chem. Soc.* **76**, 1760.

Bernstein, I. A. (1953). *J. biol. Chem.* **205**, 309.

Brice, C. & Perlin, A. S. (1957). *Canad. J. Biochem. Physiol.* **35**, 7.

Fenton, H. J. H. & Jackson, H. (1899). *J. chem. Soc.* **75**, 1.

Folkes, B. F., Willis, A. J. & Yemm, E. W. (1952). *New Phytol.* **51**, 317.

Gibbs, M. & Kandler, O. (1957). *Proc. nat. Acad. Sci., Wash.*, **43**, 446.

Grant, J. (1945). In *Pregl's Quantitative Organic Micro-Analysis*, 4th ed., p. 60. London: J. and A. Churchill Ltd.

Hers, H. G. (1958). *Proc. int. Symp. Enzyme Chem., Tokyo-Kyoto*, p. 109. Tokyo: Maruzen Company Ltd.

Hers, H. G. (1960). *Biochim. biophys. Acta*, **37**, 120, 127.

Hockett, R. C. & McClenahan, W. S. (1939). *J. Amer. chem. Soc.* **61**, 1667.

Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.

Hough, L. & Pridham, J. B. (1955). *Arch. Biochem. Biophys.* **59**, 17.

Hutchinson, A., Taper, C. D. & Towers, G. H. N. (1959). *Canad. J. Biochem. Physiol.* **37**, 901.

Kessler, G., Neufeld, E. F., Feingold, D. S. & Hassid, W. Z. (1961). *J. biol. Chem.* **236**, 308.

McClenahan, W. S. & Hockett, R. C. (1938). *J. Amer. chem. Soc.* **60**, 2061.

Markham, R. (1942). *Biochem. J.* **36**, 790.

Moore, S. & Link, K. P. (1940). *J. biol. Chem.* **133**, 293.

Muntz, J. A. & Carroll, R. E. (1960). *J. biol. Chem.* **235**, 1258.

Rees, W. R. & Reynolds, T. (1958). *Nature, Lond.*, **181**, 767.

Salomon, L. L., Burns, J. J. & King, C. G. (1952). *J. Amer. chem. Soc.* **74**, 5161.

Shibko, S. & Edelman, J. (1957). *Biochim. biophys. Acta*, **25**, 642.

Slater, W. G. & Beevers, H. (1958). *Plant Physiol.* **33**, 146.

Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.

Vargha, L. von (1935). *Ber. dtsch. chem. Ges.* **68**, 18.

Verley, A. & Bölsing, F. (1901). *Ber. dtsch. chem. Ges.* **34**, 3354.

Biochem. J. (1962) **84**, 146

Automatic Analysis of Amino Acids by Polarographic Estimation of their Copper Complexes

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Spackman, Stein & Moore (1958) described an automatic recording apparatus for amino acid analysis that used the photometric ninhydrin method to estimate the amino acids. Similar analysers have been described (Simmonds, 1958; Woods & Engle, 1960), and several automatic amino acid analysers of this type are now available commercially.

We have previously determined amino acids as their copper complexes (Blackburn & Robson, 1953; Corfield & Robson, 1953), and have used this analytical method in the construction of an automatic amino acid analyser. During the development of this apparatus, we considered the possibilities of measuring the copper in the amino acid complexes by radiometric, colorimetric and polarographic methods. The radiometric method was rejected because ^{64}Cu has too short a half-life, and the colorimetric method was technically too complicated. The polarographic estimation of copper complexes of amino acids has been used for the quantitative analysis of amino acid mixtures resolved by chromatography on paper (Martin & Mittelman, 1948), on starch columns (Kemula & Witwicki, 1955) and on cation-exchange-resin columns (Blaedel & Todd, 1961), but no compre-