The Occurrence of Apiose in *Lemna* (Duckweed) and other Angiosperms

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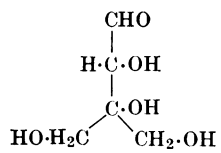
1. The branched-chain pentose apiose reacts with the benzidine–trichloroacetic acid reagent on paper chromatograms to give a yellow spot with an intense white fluorescence in ultraviolet light; on chromatograms developed with butanol–acetic acid–water this spot lies between fucose and rhamnose. 2. Examination of paper chromatograms of hydrolysates of whole plant material has shown the presence of a substance with these properties in a wide variety of species. 3. Among the plants examined two members of the Lemnaceae (*Lemna minor* and *Wolffia arrhiza*) were found to be especially rich sources, comparable with *Posidonia australis* (Bell, Hardwick, Isherwood & Cahn, 1954). 4. Measurements of the apiose content of fractions derived from *Lemna* have shown that the sugar is present at a concentration of about 4% in the holocellulose, and part of this is retained in the α-cellulose left by extraction with 24% (w/v) potassium hydroxide containing borate.

Apiose (I) is the best-known branched-chain sugar. It has long been known to occur in parsley (*Apium petroselinum* L.) as a flavone glycoside; Hudson (1949) has reviewed the chemistry of the glycoside and the sugar. It was first shown by Bell *et al.* (1954) to be a constituent of a polysaccharide, in this case from *Posidonia australis*. Bell (1962) has referred to reports of its presence in a few members of the Euphorbiaceae, Leguminosae and Compositae.



(I)

As depicted (I) the sugar has only one asymmetric centre (C-2), which has the D-configuration, and hence it is called D-apiose. When a furanose ring is formed, e.g. in the glycoside, two other centres of asymmetry appear (C-1 and C-3); this has been discussed fully by Bell *et al.* (1954) and Bell (1962). Williams & Jones (1964) have described a method of synthesis of the sugar that should make it possible to determine the configuration at C-3 in the naturally occurring form of the glycoside, which is at present unknown.

Apiose was examined in this Laboratory in 1959 because of its possible identity with unknown sugars in hydrolysates of soil polysaccharides. Dr J. S. D. Bacon found that when paper chromatograms were developed with butan-1-ol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948) and sprayed with benzidine–trichloroacetic acid (Bacon & Edelman, 1951) the sugar appeared as a yellow spot lying between fucose and rhamnose and giving an intense white fluorescence in ultraviolet light. It was eluted from charcoal columns by aqueous ethanol somewhat more readily than rhamnose.

In 1961 a substance with similar properties was found in hydrolysates of aqueous extracts of leaves of *Nerium oleander*, and Bacon (1963) observed it in small amounts in hydrolysates of leaf tissue from several species.

It was therefore decided to examine a representative selection from a collection of dried materials, representing a great variety of plants, made by Mr A. H. Knight of the Plant Physiology Department of this Institute. These included representatives of many families in the British flora: numbers 14–17, 19–23, 25, 26, 30–41, 43–52, 54–57, 59–65, 68, 69, 71–76, 78–86, 89–93, 96–100, 102, 104–110, 113–115, 117, 119, 120, 122, 127, 129–131, 133–135 and 137–139 in the classification of Clapham, Tutin & Warburg (1957). In addition about 20 dried specimens of garden and hot-house plants were examined, and also fresh material from many of the families referred to above.

A small quantity (25–35mg. of dry matter) of each sample was hydrolysed with 0·4N-sulphuric acid (0·3ml.) at 100° for 1·5hr. and neutralized with barium carbonate, and about 5μl. applied to a

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paper chromatogram, which was run and sprayed as described above, and examined under ultraviolet light. This usually showed the presence of oligosaccharide, galactose, glucose, arabinose, fucose and rhamnose; occasionally some xylose was also present. Apiose would be detectable if a minimum of 0·15 μg. was present. Of 175 plant samples examined many showed no apiose when first tested and 31 showed only traces. Of the others 51 had moderate amounts (at least 1 μg.), and 17 were good sources (perhaps 10μg./spot, i.e. apiose content 2% of dry matter). No pattern could be discerned in the distribution of the species giving a negative test. A random sample of these (one in every ten) was therefore taken and re-examined; all now showed traces of apiose, making it appear very likely that all the samples would show some evidence of the presence of the sugar if examined more carefully. Among the richest sources were *Lemna minor*, *Wolffia arrhiza* and three species of *Zostera*.

This procedure does not distinguish between apiose in glycosides and that in polysaccharides, and a positive test might be given by substances other than apiose, although in each case where attempts have been made to isolate and identify the sugar these have been successful (see below). Nevertheless, these findings were sufficient to show that apiose must be very widely distributed in the plant kingdom; they were briefly reported by Duff & Knight (1963).

The original examination of aqueous extracts of *N. oleander* leaves had shown that some of the apiose was present in material soluble in 80% (v/v) ethanol. On the other hand in *Zostera nana* and *Lemna minor* the greater part of the apiose was present in fractions insoluble in 80% (v/v) ethanol. Analysis of acid hydrolysates of the whole dried material by quantitative paper chromatography showed the presence of about 4–5% of apiose in *Zostera* and 4–8% in *Lemna*. In view of the eminent suitability of the latter as experimental material for studies of plant physiology and biochemistry (Hillman, 1961), and its potential use in the elucidation of the pathways of apiose synthesis, a systematic investigation was made of the distribution of the sugar in polysaccharide fractions prepared from the dried plant.

Distribution of apiose in Lemna *polysaccharides*

Apiose was measured in hydrolysates of the various fractions by quantitative paper chromatography (Duff & Eastwood, 1950). A comparison of hydrolysis at 100° for 1·5hr. in 0·4N-sulphuric acid with 4hr. in N-sulphuric acid showed no significant difference, and the latter method was therefore applied as a routine.

Table 1. *Fractionation of* Lemna minor

Air-dried whole plants were milled and dried to constant weight at 80°. The fractionation procedure is described in the text. The weights of fractions are expressed as though the whole of each insoluble residue was carried through to the next stage; in fact, samples were withdrawn for analysis as described under Chromatography. —, not measured.

Fraction	Wt. of fraction (g.)	Wt. of fraction % of original material	Apiose content of fraction (%)
Original material	4·10	[100]	—
Ether-soluble material	0·10*	3	—
Ethanol-soluble material	0·30*	7	—
Residue from ether and alcohol extractions	3·81	93	—
Holocellulose	2·49	61	4·0
Hemicellulose soluble in 4% KOH	0·83*	20	1·2
Residue from 4% KOH extraction	1·35	33	10·0
Hemicellulose soluble in 24% KOH	0·08*	2	11·5
Residue from 24% KOH extraction	0·94	23	4·3
Material soluble in 24% KOH–borate	0·11*	3	12·2
Residue from 24% KOH–borate extraction	0·73*	18	2·4
*Total material recovered...	2·15	52	

The milled dried whole plant material was first extracted with ether and with ethanol, and a chlorite holocellulose preparation made from the residue. The holocellulose was then extracted successively with 4% (w/v) potassium hydroxide and 24% (w/v) potassium hydroxide at room temperature (22°) in an atmosphere of nitrogen. Analyses of the materials from one such fractionation are given in Table 1. These and the results of other fractionations show that the greater part of the apiose is present in the fraction extracted by 24% potassium hydroxide, but that an appreciable amount still remains in the residue of so-called 'α-cellulose'. Extraction of the latter with potassium hydroxide containing borate (Jones, Wise & Jappe, 1956) removed a small fraction rich in apiose, but some apiose was still left in the residue.

The results suggest that properties other than their apiose content determine the order in which polysaccharides are extracted in this procedure. That some of the apiose is extremely difficult to separate from the α-cellulose fraction does not necessarily mean that it is here combined with glucose residues, because the hydrolysate of the α-cellulose contained mannose and xylose, as well as glucose.

Little dry matter and negligible amounts of apiose were extracted from the original dried material by ether and ethanol. On treatment with chlorite about 30% of the dry matter was lost, but the apiose content of the resulting holocellulose was not appreciably higher than that of the starting material; about 30% of the sugar must therefore have passed into solution. Very little of the apiose was found in the material precipitable by ethanol after dialysis of the fraction solubilized by chlorite; it is therefore possible that apiose was destroyed, released as the free sugar, or present originally in glycosides insoluble in ether and ethanol.

The stages in the extraction were examined under the electron microscope by Dr E. A. C. Follett, of the Department of Pedology. The fibrous structure of the holocellulose became more apparent on conversion into α-cellulose, and no further change was noticed on extraction with alkaline borate.

O. Kandler & E. Beck (personal communication) have recently obtained evidence of a completely different kind that also suggests that apiose is part of a cell-wall component. During photosynthesis in $^{14}CO_2$ the proportion of radioactivity incorporated into apiose and xylose rose while that in glucose fell. During a subsequent dark period the ratio of ^{14}C in apiose to that in glucose rose, and even higher ratios were obtained by supplying ^{14}C-labelled glucose over several days. Extracts made with aqueous ethanol contained only traces of radioactive apiose.

Identification of apiose

Although readily detected by the benzidine–trichloroacetic spray on paper chromatograms, apiose did not prove very easy to identify by a characteristic derivative. It did not itself crystallize, and the di-*O*-isopropylidene derivative, which is preferable to the phenylosazone because it preserves the structure of the sugar and gives a very characteristic infrared-absorption spectrum (D.M.S. no. 12900), is sufficiently volatile to be lost during its separation from the other products of condensation with acetone. This may explain why Dr E. T. Dewar (cf. Bell, 1962), who had evidence from paper chromatography for its presence in *Zostera marina* (personal communication), was unable to confirm it by isolation of this derivative. *Zostera* is one of the richest sources examined in the present work; Williams & Jones (1964) have published independent confirmation of this observation.

EXPERIMENTAL

Materials

Plant specimens. These were dried at 80° in air and milled to pass through a 1 mm. sieve. About 100 different families were represented in the 176 plants examined.

Di-O-isopropylidene apiose. An authentic specimen was obtained from Dr D. J. Bell. The material was also prepared in bulk from *Posidonia australis* fibre by the method described by Bell (1962). The infrared-absorption spectrum was identical with that of the authentic specimen. The material had $[\alpha]_D^{21°} + 53{\cdot}5 \pm 0{\cdot}5°$ (*c* 1·3 in ethanol) and m.p. 78° (Kofler block) after two recrystallizations from water, not altered on admixture with the authentic sample.

Chromatography

On paper. Qualitative paper chromatography has been described above. Preparative separations of small quantities of sugars were carried out on Whatman 3MM or Seed Test paper. In this case the paper was first washed by descending development with N-acetic acid followed by water, and butan-1-ol saturated with water then used to develop the chromatograms. After location of the sugars, strips were eluted with water chromatographically, a short length of Whatman 3MM paper being stapled to one end of the Seed Test paper for this purpose.

Quantitative paper chromatography. Sugars were estimated after elution with cold water (Laidlaw & Reid, 1950) by the Nelson–Somogyi reagents (Nelson, 1944; Somogyi, 1945), as described by Duff & Eastwood (1950). Glucose, mannose, xylose and apiose were all recovered in 95% or better yields. Results obtained by this method were confirmed by densitometry of chromatograms sprayed with the alkaline silver nitrate reagent of Trevelyan, Procter & Harrison (1950). Each chromatogram carried a standard apiose spot prepared by hydrolysis of di-*O*-isopropylidene-apiose with 2% (w/v) oxalic acid at 100° for 1 hr., and the intensities were compared in a reflectance densitometer (Joyce, Loebl and Co. Ltd., Gateshead, Co. Durham).

Chromatography on charcoal. This was used for the isolation of apiose, and also to make a more positive identification of the sugar during the survey of plant samples. For the latter purpose 1 g. of dried material was hydrolysed with 25 ml. of 0·4 N-H_2SO_4 for 2 hr. at 100°, the filtrate neutralized with $BaCO_3$ and applied to a column packed with a mixture of 20 g. of Ultrasorb SC 120/240 (British Carbo-Norit Union, Grays, Essex) and 20 g. of Celite no. 535 (Johns–Manville; L. Light and Co. Ltd., Colnbrook, Bucks.). Elution with a gradient of aqueous ethanol (0–20%, v/v) removed first the bulk of the pentose and hexose. The apiose was accompanied by other sugars, particularly fucose and rhamnose, but the loading of the paper chromatogram could now be increased sufficiently to reveal it unequivocally.

Partition chromatography on Celite columns. This was carried out essentially as described by Lemieux, Bishop & Pelletier (1956).

Isolation of apiose from Lemna

A sample of *Lemna* (2·07 g.; moisture content, 7·2%), a mixture of cultures grown on tap and pond water, was hydrolysed with 25 ml. of 0·4 N-H_2SO_4 for 1·5 hr. at 100°. After filtration through sintered glass the residue was treated a second time with N-H_2SO_4; paper chromatograms showed only a very small amount of apiose in this second hydrolysate. The combined hydrolysates were neutralized with $BaCO_3$, and the filtrate and washings evaporated *in vacuo* and applied to a Celite column (80 g.;

40 cm. × 2·9 cm.). Development was carried out with redistilled butan-1-ol–water and the effluent collected in 5–7 ml. fractions. Fractions containing apiose with a small amount of impurities (one of low mobility and one with $R_{\text{Tetramethylglucose}}$ 0·42) were passed through a British Drug Houses Ltd. (Poole, Dorset) activated charcoal (decolorizing powder)–Celite column (20–20 g.) and eluted successively with 50 ml. portions of water, 15%, 20%, 20% and 25% (v/v) ethanol. The 20% ethanol eluate gave, after evaporation and drying over P_2O_5 *in vacuo*, 20 mg. of chromatographically pure syrupy apiose.

The remaining apiose-containing fractions were transferred to another Celite column (80 g.) and developed with a 4:1 (v/v) mixture of butan-1-ol and light petroleum (b.p. 80–100°) saturated with water. By this means a further 52 mg. of chromatographically pure apiose was obtained. Another fraction contained only apiose and xylose; quantitative paper chromatography indicated the presence of about 9 mg. of apiose in this mixture, making a total yield of 80 mg. of apiose (about 4% of the oven-dried plant).

A second isolation was carried out with the same sample of *Lemna* (2·00 g.; moisture content, 7·2%). In this case the neutralized hydrolysate was concentrated to small bulk, applied in a band 20 cm. long to Whatman Seed Test paper (1·6 mm. thick) and developed with butanol–water. The apiose so obtained was further purified by elution from a small Celite column (6 g.) with butan-1-ol–water–light petroleum as solvent. A total of 62 mg. of chromatographically pure syrupy apiose was obtained.

Identification of apiose

As the di-O-isopropylidene derivative. This was carried out by a small-scale modification of the procedure of Bell (1962). A sample (34 mg.) of the suspected apiose from *Lemna* was evaporated in the presence of 150 mg. of Celite and dried by twice evaporating from the flask small portions of a 1:1 (v/v) mixture of ethanol and benzene, and finally leaving it over P_2O_5. The material was treated with 1·5 ml. of a solution made by dissolving 1 ml. of conc. H_2SO_4 (A.R.) in 19 ml. of acetone that had been dried over $CaCl_2$ and redistilled. After 5·5 hr. of shaking at room temperature (22°), anhydrous Na_2CO_3 was added until a wet indicator paper showed the pH to be 6·5–7·0. After standing a further 30 min. the mixture was filtered through paper by gravity and the residue washed with dry ether. A small drop of N-NaOH was added to the filtrate and it was evaporated at 30° under slight suction. Before the solution reached dryness a small sample was developed on a paper chromatogram for 5 hr.; this showed that most of the apiose was now present as a di-isopropylidene derivative (R_F about 0·9). The whole of the ether was then removed and the residue carefully distilled under reduced pressure with a bath temperature rising from 50° to 90° over a period of 20 min. The distillate, which was crystalline, weighed 13·7 mg. after cautious drying over P_2O_5 at a pressure of 15 mm. Hg for 10 min. The infrared-absorption spectrum was identical with that of an authentic specimen, and $[\alpha]_D^{25°}$ was $+54 \pm 1°$ (*c* 0·55 in ethanol).

Paper chromatography showed that there was still some of the di-*O*-isopropylidene derivative remaining in the residue; a further period of distillation left a residue of about 5 mg., which contained a substance with R_F about 0·75, which was probably a mono-isopropylidene derivative. The total yield of crystalline di-isopropylidene derivative was 23 mg.

As the phenylosazone (*cf.* Bell. et al. 1954). Apiose from *Lemna* was also identified by treatment with phenylhydrazine in a mixture of sodium acetate buffer, pH 4·5, and ethanol. Thus 24·2 mg. yielded 26·3 mg. of crystalline osazone (Found: C, 61·4; H, 6·3; N, 16·7. Calc. for $C_{17}H_{20}N_4O_3$: C, 62·2; H, 6·1; N, 17·1%). Its infrared-absorption spectrum was identical with that of an authentic specimen. The m.p. of the osazone was not sharp: 148–154° for the authentic specimen, 148–153° for that from *Lemna*, and 149–157° for the mixture.

Fractionation of Lemna *polysaccharides*

Whole plants, milled and air-dried, were first extracted in a Soxhlet apparatus with diethyl ether. The residue was dried overnight in a vacuum oven at 40° and extracted with ethanol until the extract was colourless (15 hr.). The residue was again dried in the vacuum oven and then converted into a holocellulose preparation by the method of Wise (1945): about 2 g. was treated with a mixture of 100 ml. of water, 10 ml. of acetic acid and 10 g. of sodium chlorite at 60° for 30 min., and left at 30° with occasional shaking until it became colourless (4·5 hr.). The residue was filtered off on sintered glass and washed with ice–water, acetone and diethyl ether, and dried in a vacuum oven. The filtrate was treated with $Na_2S_2O_3$ until it no longer gave a colour with starch–iodide paper, dialysed against water until free from sulphate, and evaporated to dryness.

The holocellulose was extracted with 100 ml. of 4% KOH, previously heated to 85° and then cooled to room temperature, added while a stream of nitrogen was passed through the flask. Extraction was continued for 24 hr., while nitrogen was bubbled through. The residue was recovered and washed with 25 ml. of 4% KOH on the centrifuge, then with water followed by acetone and ether on a filter. The 4% KOH extracts were poured into 10 ml. of acetic acid and then, no precipitate appearing, into 4 vol. of ethanol, the precipitate being collected and washed like the residue.

The residue was then extracted in the same way with 24% KOH, for 24 hr. at room temperature. Again no precipitate was formed when the extract was made acid.

The ethanol supernatant was in each case evaporated to dryness, dialysed against running tap water and evaporated to dryness.

In one experiment a further extraction of the final residue was made with 100 ml. of 24% KOH containing 4 g. of boric acid (Jones & Painter, 1957).

Hydrolysates were made of samples from each of the fractions and examined qualitatively by paper chromatography. Quantitative estimations were made of apiose (Table 1), xylose, glucose and mannose. The presence of mannose in the final residues was confirmed by isolation by thick-paper chromatography and conversion into the phenylhydrazone; 0·25 g. of residue yielded 10 mg. of mannose phenylhydrazone, m.p. 198–199°, unchanged by admixture with an authentic sample, which had an identical infrared-absorption spectrum.

The final residues, which are sometimes referred to as α-cellulose preparations, contained appreciable amounts of sugars other than glucose. Thus hydrolysis with N-H_2SO_4 for 4 hr. at 100° of the residue from extraction with 24%

KOH containing borate yielded about 4% of mannose and 2·5% of xylose; the apiose content is reported in Table 1.

The high value for the apiose content of the residue from extraction with 4% KOH (Table 1) is probably incorrect. In two other fractionations this material had an apiose content only a little higher than the holocellulose from which it was prepared.

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