400. *Studies on Fructosans. Part III.* A Fructosan from* Lolium perenne.

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A fructosan from early perennial rye-grass (*Lolium perenne*), on hydrolysis, gave D-fructose (98%) and D-glucose (2%). Acetylation, methylation, and hydrolysis yielded 1 : 3 : 4 : 6-tetramethyl D-fructose (4%), 1 : 3 : 4-trimethyl D-fructose (93%), and 3 : 4-dimethyl D-fructose (2·8%), along with small amounts of aldose derivatives and a trace of fructose. It is concluded that the methylated fructosan has a unit chain length of *ca.* 25—30 residues.

Challinor, Haworth, and Hirst (*J.*, 1934, 1560) in their investigations on the fructosan from rough-stalked meadow grass (*Poa trivialis*) showed this polysaccharide to be of the levan type. Methylation and hydrolysis yielded 1 : 3 : 4-trimethyl D-fructofuranose together with a small quantity of more fully methylated material. Similar results were obtained for the fructosan from barley leaves (Haworth, Hirst, and Lyne, *Biochem. J.*, 1937, **31**, 786) and for phlein, isolated from the roots of timothy-grass (*Phleum pratense*) (Schlubach and Sinh, *Annalen*, 1940, **544**, 101). In the present investigation a similar product from early perennial rye-grass (*Lolium perenne*) has been studied.

The rye-grass used was oven-dried milled material prepared from grass cut in May, 1949, at the Jealott's Hill Agricultural Research Station. After preliminary extractions with ether

* Part II, Arni and Percival, preceding paper.

and ethanol, the fructosan was extracted from the grass with water. Hydrolysis of the purified material and analysis of the product by paper chromatography (Hirst and Jones, *J.*, 1949, 1659; Laidlaw and Reid, *Nature*, 1950, **166**, 476) showed the presence of fructose (98%) and glucose (2%).

Simultaneous deactylation and methylation of the fructosan acetate, followed by treatment with silver oxide and methyl iodide, fractional precipitation, methylation with thallous ethoxide and again with the Purdie reagents, gave a methylated fructosan (OMe, 44·4%). This was hydrolysed and the mixture of sugars separated on a cellulose column (Hough, Jones, and Wadman, *J.*, 1949, 2511). Three fractions were obtained, corresponding to those previously observed on the paper chromatogram to be tetramethyl fructofuranose, a trimethyl fructose, and a dimethyl fructose.

Because of its volatility, the tetramethyl fructofuranose in fraction I was estimated by a colorimetric method (Bell and Palmer, *J.*, 1949, 2522; Arni and Percival, Part II, preceding paper). The results indicated the presence of *ca.* 4% of tetramethyl fructose in the hydrolysate. The identity of the sugar was confirmed by conversion into the crystalline tetramethyl D-fructofuronamide (Avery, Haworth, and Hirst, *J.*, 1927 2313). Hypoiodite oxidation showed the presence of *ca.* 9% of tetramethyl aldose in I.

Fraction II (93%) crystallised almost completely and on recrystallisation showed m. p. 74°, not depressed on admixture with an authentic specimen of 1 : 3 : 4-trimethyl D-fructose. Oxidation with alkaline hypoiodite indicated that only very small amounts of aldose derivatives were present in this fraction.

Fraction III was a syrup (*ca.* 3%), $[\alpha]_D$ −50°. Oxidation with sodium metaperiodate gave formaldehyde (1·78 moles/mole). The yield of formaldehyde, taken in conjunction with the specific rotation, makes it highly probable that the main component of fraction III is 3 : 4-dimethyl D-fructose. Oxidation of III with alkaline hypoiodite showed it to contain *ca.* 9% of dimethyl aldose.

In their studies on Italian rye- and leafy cocksfoot-fructosans, Bell and Palmer (*Biochem. J.*, 1949, **45**, xiv) calculated a chain length of 14 units from the yield of tetramethyl fructofuranose, which is about half the present estimate. They also reported the presence of an appreciable amount of dimethyl fructoses.

Barger's method (Barger, *J.*, 1904, 286; Caesar, Gruenhut, and Cushing, *J. Amer. Chem. Soc.*, 1947, **69**, 617) was used in an attempt to estimate the molecular weights of the acetylated and methylated fructosan. With the methylated material, no satisfactory results could be obtained; with the acetylated product, at a concentration of *ca.* 1%, the observed molecular weight corresponded to a molecule of *ca.* 20—30 $C_6H_{10}O_5$ units, although on increasing the concentration of the solution it appeared that all the droplets tended to increase in size, a phenomenon previously noted by Barger for compounds of high molecular weight. Bell and Palmer (*loc. cit.*) have also reported the molecular weight of perennial rye-grass levan to be of the order of 5000.

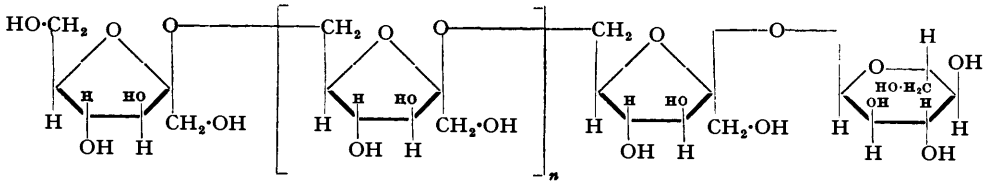
Oxidation of the polysaccharide with potassium metaperiodate (Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27) yielded 1 mole of formic acid per 20 $C_6H_{10}O_5$ residues. Oxidation with sodium metaperiodate showed that *ca.* 1·02 moles of periodate were used up for every $C_6H_{10}O_5$ residue, in accordance with the conception of a molecule composed of fructofuranose residues linked through the 2 : 6-positions.

When the crude fructosan was heated with water for several days at 100° and the products were examined on the chromatogram, spots were obtained corresponding to sucrose, glucose, and fructose. Hydrolysis of the first material gave glucose and fructose. The possibility exists, therefore, that the chain is terminated, not by a reducing fructose units, but by fructose and glucose linked as in sucrose as recently proposed for inulin (Hirst, McGilvray, and Percival, Part I, *J.*, 1950, 1297). This would account for the non-reducing properties of the fructosan; furthermore, in a polysaccharide of this type, oxidation with periodate should yield only one mole of formic acid from each chain (from the glucopyranose residue). The chain length determined by this method would then be *ca.* 20 units, in reasonable agreement with that deduced from methylation data.

It cannot be decided on the present evidence whether branching of the chain occurs, or whether the dimethyl derivative isolated is an artifact due to incomplete methylation. Similarly, no significance can be attached at present to the small amount of fructose isolated from the products of hydrolysis of the methylated polysaccharide.

The small quantities of aldose derivatives present in the hydrolysate precluded any detailed structural investigations. It was evident, however, that a relatively large proportion of these

appeared in the fully methylated fraction. A polysaccharide of such a structure as in the annexed formula would yield, after methylation and hydrolysis, tetramethyl fructofuranose and tetramethyl glucopyranose in equal amounts, whereas the experimental figures for tetramethyl



aldose are much lower. This divergence may be caused by hydrolysis of the labile sucrose linkages during the processes involved in the preparation of the methylated fructosan. Nevertheless, this explanation is only tentative and further work will be necessary before any detailed structure can be established.

Experimental.

Evaporations were conducted under diminished pressure. Temperatures recorded are bath-temperatures. Fractions from the cellulose column were evaporated to dryness, dissolved in water, digested with charcoal, and filtered hot; the aqueous solution was then evaporated to dryness and exhaustively extracted with boiling acetone, and the extracts were evaporated to dryness.

Preparation of the Polysaccharide.—Early perennial rye grass (oven-dried; 450 g.; cut, May 3rd, 1949) was exhaustively extracted in a Soxhlet apparatus for 48 hours with ether. The solvent was then changed to alcohol containing 20% of water, and the extraction continued for a further 60 hours. The residue was removed, dried in air, and extracted with water (5 l.) at room temperature with continuous shaking for 5 hours. The grass was removed by filtration and washed with water, and the extract and washings were combined and evaporated to 2 l. The solution was heated to 95°, cadmium sulphate solution (100 c.c.; 10%) and sodium hydroxide (50 c.c.; 0·5N.) were added, and the whole was kept at 95° for 3 minutes. After cooling, the protein complex was removed by filtration through " Filter Cel " (cf. Doak, *N.Z. J. Sci. Tech.*, 1939, **21**, 90B). The clear filtrate was deionised on columns of Amberlite resins (IR100 and IR4B), and the last traces of protein were removed by shaking the solution with chloroform and butanol (Sevag, Lackmann, and Smollens, *J. Biol. Chem.*, 1938, **124**, 425), six treatments being required. Evaporation of the neutral solution to 200 c.c. and digestion with charcoal gave a clear red solution from which the polysaccharide was precipitated by ethanol (4 l.). The brown product was separated by filtration, redissolved in water (1500 c.c.), digested with charcoal at 40° for 30 minutes, refiltered, and evaporated to 180 c.c. The fructosan was precipitated by pouring the solution into methanol containing 25% of light petroleum (b. p. 60—80°), whereupon it separated as a highly hygroscopic cream-coloured solid. The polysaccharide was dissolved in water (60 c.c.) and freeze-dried to yield a crisp brownish solid (20 g.).

Acetylation.—The impure fructosan (19 g.) was dissolved in water (200 c.c.), pyridine (300 c.c.) added, and the azeotrope removed at 40° (Pacsu and Mullan, *J. Amer. Chem. Soc.*, 1941, **63**, 1487). After several additions of pyridine and evaporation the solution was concentrated to 350 c.c., and acetic anhydride (350 c.c.) added with stirring during 6 hours at room temperature, the reaction vessel being cooled in a water-bath. Next morning the acetate was precipitated by pouring the mixture into water (10 l.). The cream-coloured solid was removed by filtration and thoroughly washed with water. The last traces of pyridine were removed over concentrated sulphuric acid in a vacuum-desiccator. The acetate was dissolved in chloroform, and the solution dried (Na_2SO_4) and evaporated to 200 c.c. The product was precipitated by pouring the solution into light petroleum (b. p. 40—60°; 2·5 l.), and after filtration was washed with light petroleum and dried in a high vacuum over calcium chloride and paraffin wax. The product (25 g.) had $[\alpha]_D^{18}$ +19° (*c* 1·7 in chloroform) and $\eta_{sp.}^{20}/c'$ 1·95 (chloroform) where *c'* is the concn. in g.-mols. of $C_{12}H_{16}O_8$ per l. (Found: Ac, 44·4. Calc. for $C_{12}H_{16}O_8$: Ac, 44·6%).

Deacetylation.—The acetyl compound (2 g.) in chloroform (10 c.c.) was cooled in a freezing mixture, and a solution of sodium (0·1 g.) in absolute methanol (5 c.c.) added (cf. Zemplen and Pacsu, *Ber.*, 1929, **62**, 1613). The mixture was shaken for 4 hours, and ice-water (5 c.c.) added, followed by acetic acid (2 c.c.; 10%) and finally water (13 c.c.). The regenerated fructosan was precipitated from the aqueous layer by methanol–light petroleum as before. The product (0·73 g.) showed $[\alpha]_D^{18}$ −46° (*c*, 0·7 in water).

Hydrolysis.—The regenerated fructosan (0·145 g.) was heated with oxalic acid (20 c.c.; 0·1N) at 70°. $[\alpha]_D^{19}$ were −58° (15 mins.); −83° (45 mins., constant). Neutralisation, filtration, and evaporation gave a reducing syrup (0·1 g.) which was shown by investigation on the paper chromatogram (*loc. cit.*) to contain fructose, 98%, and glucose, 2%.

Periodate Oxidation.—The fructosan (0·2541 g.) was dissolved in water (35 c.c.), and sodium metaperiodate (15 c.c.; 0·3M.) added. The periodate uptake, determined by the arsenite method, was constant after 1 day at a value of 1·02 moles of periodate per $C_6H_{10}O_5$ residue.

Oxidation with potassium metaperiodate (*loc. cit.*) gave the following results (expressed as the number of $C_6H_{10}O_5$ residues per mole of formic acid liberated): 43 (18 hours); 23 (144 hours); 21 (240 hours); 20 (350 hours).

Methylation.—The acetate (15 g.) was dissolved in acetone (300 c.c.) and methylated 3 times with methyl sulphate and sodium hydroxide at room temperature in an atmosphere of nitrogen. The product was then methylated twice with silver oxide and methyl iodide. Dissolution of the methylated fructosan in chloroform followed by fractional precipitation with light petroleum (b. p. 40—60°) gave the following fractions.

Fraction.	Weight (g.).	OMe (%).	$[\alpha]_D^{18}$ (chloroform).
1	4·8	42·7	−57°
2	1·8	40·2	−51°
3	1·2	32·7	—

Fraction 3 was obtained by evaporation of the residual solution.

Fractions 1 and 2 were recombined, and the product (5·6 g.) was dissolved in chloroform and methylated once with thallous ethoxide and methyl iodide. The product (4·8 g.; OMe, 43·7%) was methylated 3 times with the Purdie reagents to give a methylated fructosan (3·8 g.), $[\alpha]_D^{18}$ −57° (*c*, 1·5 in chloroform) (Found : OMe, 44·4. Calc. for $C_9H_{16}O_5$: OMe, 45·6%).

Hydrolysis and Fractionation.—The methylated fructosan (3·4 g.) was heated with methanol (100 c.c.) and water (33 c.c.) containing oxalic acid (1·35 g.) at 80° for 24 hours. A small amount of insoluble material was removed by filtration, water (300 c.c.) added, and the methanol–water azeotrope removed by distillation. The solution was evaporated to *ca.* 150 c.c. (35°/15 mm.) and heated at 80° for 5 hours (constant rotation). Neutralisation, evaporation to small volume (35°/15 mm.), extraction of the residue with boiling acetone, and evaporation of the extracts almost to dryness (30°/15 mm.) yielded a thin syrup, in which examination by the paper chromatogram indicated the presence of tetramethyl fructofuranose, a trimethyl fructose, and a dimethyl fructose. These sugars were separated on a cellulose column (70 × 3 cm.) with light petroleum (b. p. 100—120°)–butanol (7 : 3) saturated with water as eluent (Hough, Jones, and Wadman, *loc. cit.*), to give fractions I, II, and III.

Fraction I contained tetramethyl fructofuranose and tetramethyl glucose only. The solution was evaporated to small volume (35°/15 mm.), and water (300 c.c.) was added. By repeated evaporations and additions of water an aqueous solution of fraction I was finally obtained. An aliquot portion of this was removed and saturated with benzoic acid. The tetramethyl fructofuranose content of this solution was estimated colorimetrically by using resorcinol (Part II, *loc. cit.*). The weight of tetramethyl fructofuranose found in I was 97 mg.

The remainder of the solution containing I was evaporated to dryness (35°/15 mm.). Hypoiodite oxidation of a portion of this syrup (Part I, *loc. cit.*) showed the presence of *ca.* 10 mg. of tetramethyl aldose in I.

Fraction I showed $[\alpha]_D$ +39° in water (*c* 0·5). The syrup (40 mg.) was oxidised according to the method of Avery, Haworth, and Hirst (*loc. cit.*) with nitric acid. Treatment of the product with methanolic ammonia (2 c.c.) at 0° for 48 hours yielded tetramethyl D-fructofuronamide which after several recrystallisations from ether–light petroleum (b. p. 60—80°) had m. p. 97—99°, not depressed on admixture with an authentic specimen.

Fraction II (2·28 g.) crystallised. The whole was spread on a porous tile, and the solid material, on recrystallisation from carbon tetrachloride–light petroleum (b. p. 40—60°) had m. p. 74°, not depressed on admixture with an authentic specimen of 1 : 3 : 4-trimethyl D-fructofuranose. It had $[\alpha]_D^{18}$ −27° (5 mins.); −43° (15 mins.); −55° (1 hour); −57° (19 hours); −58° (68 hours, constant), in water (*c* 1·8) (Found : C, 48·6; H, 8·0; OMe, 40·7. Calc. for $C_9H_{18}O_6$: C, 48·6; H, 8·2; OMe, 41·9%).

Extraction of the tile with boiling acetone and evaporation of the extract gave a small amount of syrup. This had an R_G value on the paper chromatogram corresponding to that of 1 : 3 : 4-trimethyl fructose and gave the characteristic ketose colour with urea oxalate. Periodic acid oxidation of the syrup (Reeves, *J. Amer. Chem. Soc.*, 1941, **63**, 1476) gave formaldehyde, identified by the coloration with phenylhydrazine hydrochloride–potassium ferricyanide–hydrochloric acid. Hypoiodite oxidation indicated the presence of a very small amount of aldose derivative.

Fraction III (63 mg.) was obtained as a syrup. Investigation on the paper chromatogram indicated it to be composed of a dimethyl fructose along with small amounts of tri- and tetra-methyl fructoses. The syrup, $[\alpha]_D^{17}$ −50° in water (*c* 0·6) (12 mg.), was dissolved in water (2 c.c.) and allowed to react with sodium metaperiodate (2 c.c.; 0·3M.) and sodium hydrogen carbonate (2 c.c.; 1N.) for 40 hours whereupon the formaldehyde liberated was estimated as the dimedon complex (Reeves, *loc. cit.*). 29·1 Mg. of complex were obtained, having m. p. 175° not depressed on admixture with an authentic specimen; this corresponds to a yield of 1·78 moles of formaldehyde per mole of dimethyl fructose. An estimation run on xylose simultaneously and under the same conditions gave a yield of formaldehyde corresponding to 1·03 moles/mole.

Treatment of the syrup III (30 mg.) in water (6 c.c.) with recrystallised phenylhydrazine hydrochloride (50 mg.), sodium acetate (30 mg.), and a small amount of sodium hydrogen sulphite at 100° for 1 hour gave, on cooling, a light yellow osazone. When separated at the centrifuge, washed several times with water, and dried in a vacuum-desiccator in the dark over phosphoric oxide, this had m. p. 92—103°. Attempted recrystallisation was unsuccessful.

Oxidation of III with alkaline hypoiodite indicated the presence of *ca.* 6 mg. of dimethyl aldose in this fraction.

Hypoiodite Oxidation of Fructose Derivatives.—(a) 1 : 3 : 4-*Trimethyl* D-*fructose*. A sample of this sugar was recrystallised several times for use in the following investigation. The sugar samples (5·05 mg.) were oxidised with alkaline hypoiodite as above. Water blank titres (c.c.; 0·02N-sodium thiosulphate),

10·837, 10·817. Sample: 10·789, 10·821. Thus the difference between sample and blank is of the order of only 0·02 c.c., and may be neglected.

(b) 1 : 3 : 4 : 6-*Tetramethyl* D-*fructose*. The sample used in this preparation was a synthetic one. The syrup samples (6·10 mg.) were oxidised as above. Water blank titres, 10·870, 10·860, and 10·850 c.c. Sample titres, 10·745, 10·744, and 10·753 c.c. The difference here is of the order of 0·1 c.c. This divergence may be ascribed to impurities in the sample.

Molecular Weight by Barger's Method.—Barger's capillary technique (*loc. cit.*) was used in an attempt to determine the molecular weights of acetylated and methylated fructosan. Droplets of a solution of known concentration were compared with solutions of sucrose octa-acetate (2—12 $\times$ 10^{-3}M.), readings being taken with a travelling microscope. By using a 1% solution of the methylated polysaccharide, the isopiestic condition was found to be at concentrations of sucrose octa-acetate greater than 12 $\times$ 10^{-3}M. This indicates a very low molecular weight and clearly the method is inapplicable here. With 1% solutions of the acetate a molecular weight in the region of 5000 was found ; attempts to increase the accuracy of the estimation by using stronger polysaccharide solutions, however, were abortive.

Autohydrolysis.—(*a*) A portion of the crude fructosan (1 g.) was dissolved in water (300 c.c.) and heated at 100° for 24 hours. Evaporation of the solution and investigation on the paper chromatogram showed the presence of fructose, a small amount of glucose, some apparently unchanged polysaccharide at the starting line, and a small amount of material with a R_G value corresponding to that of sucrose. This material was separated on the paper, extracted, and hydrolysed with oxalic acid (2%; 20 c.c.) at 90° for 1 hour. Neutralisation, filtration, evaporation, and chromatography showed the presence of glucose and fructose. The sugar mixture was redissolved in water and heated at 100° for a further 72 hours. The solution was then evaporated to small volume and poured into excess of ethanol, a precipitate being obtained. This material was removed at the centrifuge and heated with oxalic acid (2%; 10 c.c.) at 100° for 2 hours. The only sugar identified in the hydrolysate was arabinose (paper chromatogram). The ethanolic solution was found to contain glucose and fructose only.

(*b*) A sample (1 g.) of the fructosan purified by acetylation and deacetylation was treated in the same manner. Very similar results were obtained except that here no araban was detected, it presumably having been removed during the acetylation process.

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