Studies on Fructosans. Part V. Short-chain Fructosans from* Lolium perenne.

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A mixture of short-chain fructosans has been isolated from mid-season perennial rye-grass (*Lolium perenne*). On fractionation of the methylated material a product was obtained which, on hydrolysis, yielded 1 : 3 : 4 : 6-tetra- (13·2%) and 1 : 3 : 4-tri-*O*-methyl-D-fructose (76·2%) along with 2 : 3 : 4 : 6-tetra- (5·3%) and 2 : 4 : 6-tri-*O*-methylglucose (4·1%). A di-*O*-methylfructose (1·2%) was also isolated. The findings indicate the presence of short, straight-chain polysaccharides made up of 2 : 6-linked fructofuranose residues, terminated in some cases by a glucopyranose residue linked as in sucrose.

The levans have been studied extensively (see Part IV * and references contained therein; Aspinall and Telfer, *Chem. and Ind.*, 1952, 1244; Bell and Palmer, *J.*, 1952, 3763), and, while Schlubach and Holzer (*Annalen*, 1953, **578**, 207) have isolated a fructosan from *Lolium perenne* which contained no glucose residues (cf. Laidlaw and Reid, *J.*, 1951, 1830) it seems probable that these polysaccharides occur in the plant as chains of 2 : 6-linked fructofuranose residues some of which, at least, are terminated by a glucopyranose residue linked as in sucrose. This view is in agreement with the theory that fructosans are built up in the plant from sucrose by enzymic transfructosidation. Similar conclusions have been drawn from the results of the present investigation, which had as its object the isolation and investigation of the short-chain " intermediate " levan fractions.

The rye-grass used was oven-dried, milled material prepared from grass cut in May, 1949, at the Jealott's Hill Agricultural Research Station. Extraction with 80% aqueous ethanol gave a mixture of short-chain fructosans. Hydrolysis of the purified material and analysis of the product by paper chromatography (Hirst and Jones, *J.*, 1949, 1659; Duff and Eastwood, *Nature*, 1950, **165**, 848) showed the presence of glucose (8·5%) and fructose (91·5%).

Methylation of the fructosan mixture yielded a product which was fractionated by dissolution in chloroform–light petroleum to give a soluble (64·4%) and an insoluble fraction (35·6%). The fully methylated insoluble fraction (OMe, 47·1%) on hydrolysis yielded 1 : 3 : 4 : 6-tetra-*O*-methyl-D-fructose (13·2%), 1 : 3 : 4-tri-*O*-methyl-D-fructose (76·2%), 2 : 3 : 4 : 6-tetra-*O*-methylglucose (5·3%), an aldose sugar believed to be probably 2 : 4 : 6-tri-*O*-methylglucose (4·1%), and a di-*O*-methylfructose (1·2%). The tetra- and tri-*O*-methylfructoses were identified as crystalline derivatives. The properties of the aldose—its R_G value, the colour given by aniline oxalate on the chromatogram, the failure to yield a methylfuranoside with cold methanolic hydrogen chloride, and the negative Weerman test given by the derived amide—agreed with those of 2 : 4 : 6-tri-*O*-methylglucose, though we could not, at the time when the experiments were done, prove

* Part IV, Aspinall, Hirst, Percival, and Telfer, *J.*, 1953, 337.

conclusively that this material was, in fact, a derivative of D-glucose. It is to be regretted that no crystalline derivatives of the fully methylated aldose fraction could be obtained, but from paper chromatography and hypoiodite oxidation experiments there would appear to be no doubt of the identity of this fraction. It seems likely that the small amount of di-*O*-methylfructose isolated was an artifact, probably arising from undermethylation.

The proportion of tetra-*O*-methylfructose obtained indicates an average chain length of 7—8 units, while the tetra-*O*-methylglucose content suggests that only one chain in three is terminated by a non-reducing glucose residue, and this accords with the reducing properties of the fructosan and the liberation of formaldehyde on periodate oxidation. It is possible, however, that some degradation of the polysaccharide, with hydrolysis of the sucrose linkage, took place during the preparation of the methylated levan (cf. Part IV, *loc. cit.*).

It cannot be decided on the present evidence whether the tri-*O*-methylglucose found was derived from a 1 : 3-linked glucose unit or whether it was an artifact produced by undermethylation. While previous workers (Hirst, McGilvray, and Percival, Part I, *J.*, 1950, 1297; Bell and Palmer, *loc. cit.*) have reported the isolation of small but significant amounts of 2 : 4 : 6-tri-*O*-methyl-D-glucose from among the products of hydrolysis of methylated inulins, it has been demonstrated by periodate oxidation (Aspinall and Telfer, *Chem. and Ind.*, 1953, 490) that 1 : 3-linked glucose residues were absent from the inulin of " Crimson Flag " dahlia tubers. It was concluded that the tri-*O*-methylglucose isolated in previous experiments must have arisen from undermethylation. Similar considerations may well apply in the levan series. In the present investigation, however, a relatively large proportion of tri-*O*-methylglucose was found, and the quantity of this material, estimated visually on the paper chromatogram after hydrolysis of the methylated fructosan, did not appear to diminish on repeated methylation. It is thus possible that, in this case, 1 : 3-linked glucose may be present. The further question, whether such units, if present, constitute an intrinsic part of the fructosan molecule or whether they are derived from an associated short-chain glucosan, remains unsettled. If the former alternative is correct, these glucose residues may occupy any intermediate position in the fructosan chains, or they may be present as reducing end-groups (cf. the production of a reducing disaccharide, 6-*O*-β-D-fructofuranosyl-D-glucose, by transfructosylation, Whelan and Jones, *Biochem. J.*, 1953, **54**, xxxiv). A structure containing end-groups of this type would give glucose on hydrolysis with invertase, and the reducing glucose unit would be disrupted by periodate oxidation. It is unfortunate that lack of material prevented further examination of the product by these methods.

Hydrolysis of a fully methylated soluble fraction of the fructosan mixture (OMe, 44·4%; $[\alpha]_D^{18}$ +46°) gave a product which furnished very high proportions of tetra- and tri-*O*-methylglucoses. It is likely that considerable degradation of this fraction had taken place during the processes of methylation, etc.

The original fructosan consumed 1·1 mols. of sodium metaperiodate per $C_6H_{10}O_5$ residue, a result in agreement with the conception of a molecule composed of fructofuranose units linked through the 2 : 6-positions. Oxidation with potassium periodate (Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27) yielded approximately one mol. of formic acid per seven $C_6H_{10}O_5$ residues. While these results cannot be correlated directly with the methylation data, they may be interpreted as supporting the general conclusions drawn above.

Further evidence of the presence of sucrose residues was afforded by the weakly positive Raybin test given with diazouracil (*J. Amer. Chem. Soc.*, 1933, **55**, 2603; 1937, **59**, 1402).

These results indicate that rye-grass contains a series of levans of low molecular weight. On the basis of their mobilities on the paper chromatogram and from the proportions of tetra-*O*-methylfructose isolated on hydrolysis of the methylated sub-fractions it appears that the fraction studied consisted of a mixture of materials with chain-lengths of *ca.* 5—10 units. It seems likely that the chains are terminated by glucopyranose units, linked as in sucrose, and this is in agreement with the theories advanced by Dedonder (*Compt. rend.*, 1950, **230**, 549, 997; 1951, **231**, 790; **232**, 1134, 1442) and by Bacon and Edelman (*Biochem. J.*, 1951, **48**, 114; **49**, 446, 529; see also MacLeod, *J. Inst. Brew.*, 1953, **59**, 462). The lower members of the rye-grass " fructosan series " have not been investigated as yet.

Experimental

Evaporations were conducted under diminished pressure. Unless otherwise stated, fractions from the cellulose column were evaporated to dryness; solutions of the residues in water were digested with charcoal and filtered hot, then evaporated to dryness; these residues were exhaustively extracted with boiling acetone, and the extracts were evaporated to dryness.

Preparation of the Polysaccharide.—Mid-season perennial rye-grass (oven-dried; 200 g.; cut May 26th, 1949) was extracted with two successive portions of boiling aqueous ethanol (80%; 500 c.c.) for 4 hr. with continuous stirring. The extracts were evaporated to dryness and water (250 c.c.) was added. The mixture was heated to 95°, 0·4N-cadmium sulphate and saturated aqueous barium hydroxide (50 c.c. of each) were added, and the temperature was maintained at 95° for 3 min. (cf. Laidlaw and Reid, *J. Sci. Food Agric.*, 1952, **3**, 19). After cooling and filtration the filtrate was de-ionised by resins (Amberlite IR-4B and IR-100), and the final solution was evaporated to give a syrup.

The combined syrups from 2 kg. of grass were dissolved in water (400 c.c.) and fractionated on a carbon column (120 g. of charcoal + 120 g. of " Filter-Cel ") (Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677). After removal of the monosaccharides with water, a further fraction (A; 28·5 g.) was obtained by elution with 40% aqueous ethanol. Fraction A was dissolved in water (200 c.c.), and ethanol (1800 c.c.) was added, to yield a precipitate which was purified by two further precipitations as above. After a further precipitation from 95% ethanol the product was finally obtained as a white powder (6·5 g.) which had $[\alpha]_D^{16}$ −30° (*c*, 1·2 in H_2O) (Found: N, 0·1%). The naphtharesorcinol test for uronic acid was negative. The substance quickly reduced boiling Fehling's solution. The test for sucrose with diazouracil (Raybin, *loc. cit.*) gave a positive result.

Examination on the Paper Chromatogram.—In benzene–pyridine–butanol–water (1 : 3 : 5 : 3) the product remained as a spot on the starting-line, while stachyose travelled *ca.* 10 cm. In *iso*amyl alcohol–pyridine–water (7 : 5 : 7), after prolonged running, the spot moved a very short distance below the starting-line and gave a tail several cm. long, with indications of several discrete spots.

Invertase Hydrolysis.—A concentrated spot of the fructosan was placed on the starting-line of a paper chromatogram and sprayed with 4% (v/v) invertase concentrate (B.D.H.) solution. The paper was suspended over water in an air-oven at 50° for 20 min., and then developed with ethyl acetate–acetic acid–water (6 : 1 : 6). The results indicated the presence of a large amount of apparently unchanged fructosan at the starting-line, a considerable amount of fructose and a small amount of glucose.

Hydrolysis.—The fructosan was hydrolysed at 100° with 3% aqueous oxalic acid (25 c.c.) for 1 hr. (rotation constant). The product on examination on the chromatogram was shown to contain fructose, glucose, and a trace of arabinose. Estimation by the periodate method (Hirst and Jones, *loc. cit.*) gave glucose 8·5% and fructose 91·5%, while the colorimetric Somogyi procedure (Duff and Eastwood, *loc. cit.*) gave glucose 8·4% and fructose 91·6%.

Periodate Oxidation Experiments.—Formic acid. The fructosan was oxidised with potassium periodate (Brown, Halsall, Hirst, and Jones, *loc. cit.*), and the formic acid liberated was determined by titration with 0·01N-sodium hydroxide, *viz.*: 8·9 (24 hr.), 7·6 (48 hr.), 6·9 (96 hr.), 6·7 (144 hr., constant) $C_6H_{10}O_5$ residues per mole of formic acid liberated.

Periodate uptake and formaldehyde production. To the fructosan (*ca.* 0·3 g.) in water (35 c.c.), sodium metaperiodate (15 c.c.; 0·3M) was added. The periodate uptake, determined in 5-c.c. portions by the arsenite method, was 1·05 (30 min.), 1·12 (1 hr., constant) moles of periodate consumed per $C_6H_{10}O_5$ residue.

After one day, 5 c.c. of the solution were withdrawn and the periodate and iodate destroyed by the addition of 1·2M-sodium arsenite (2 c.c.). Aqueous phenylhydrazine hydrochloride (1%; 2 c.c.) and potassium ferricyanide (5%; 1 c.c.) were added, and the solution was acidified with hydrochloric acid, the port-wine colour characteristic of formaldehyde being developed. Cocksfoot-grass levan, on similar treatment, gave no colour (cf. Part IV, *loc. cit.*).

Methylation.—The fructosan (4·6 g.) was methylated under nitrogen at room temperature with methyl sulphate and sodium hydroxide (8 times), then three times with methyl iodide and silver oxide. After each methylation, a small portion of the product was removed and hydrolysed, and the sugars were examined on the chromatogram. After the first few methylations, no appreciable change in the amount of tri-*O*-methylglucose present could be detected. The product was dissolved in chloroform (75 c.c.), and the solution dried (Na_2SO_4) and poured into light petroleum (b. p. 60—80°; 750 c.c.), to give a precipitate X (1·44 g.). Evaporation of the filtrate yielded a syrup Y (2·61 g.).

Precipitate X.—Hydrolysis of X and fractionation of the sugars. Fraction X {OMe, 47·1%; $[\alpha]_D^{20}$ −60° (*c*, 0·86 in $CHCl_3$); properties unchanged on further methylation} (*ca.* 1 g.) was heated with methanol (100 c.c.) and water (33 c.c.) containing oxalic acid (1·35 g.) at 80° for 24 hr. The solution was filtered, water (300 c.c.) was added, and the methanol–water mixture was removed by evaporation to small volume. The aqueous solution (150 c.c.) was heated at 80° for 5 hr. Neutralisation, evaporation to small volume, extraction of the residue with acetone, and evaporation of the extracts almost to dryness yielded a thin syrup which was fractionated on a column of cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511) with benzene–ethanol–water (169 : 47 : 15) (Andrews, Hough, and Jones, *J.*, 1952, 2746) as solvent, to give fractions (1)—(7).

In investigations of the fractions by paper chromatography two solvent systems were used. These were benzene–ethanol–water (as above) (A) and butanol–ethanol–water–ammonia (40 : 10 : 49 : 1) (B). Solvent A had previously been shown to separate 1 : 3 : 4 : 6-tetra-*O*-methylfructose, 2 : 3 : 4 : 6-tetra-*O*-methylglucose, 1 : 3 : 4-tri-*O*-methylfructose, and 2 : 3 : 4- and 2 : 4 : 6-tri-*O*-methylglucoses (R_G values *ca.* 1·14, 1·00, 0·66, 0·40, and 0·32 respectively in this solvent). All chromatograms were run in the presence of authentic specimens of the appropriate sugars.

Because of the high volatility of tetra-*O*-methylfructofuranose at reduced pressures, solutions containing this material were never evaporated to dryness before the weight of the sugar present had been determined. The estimation was carried out on an aliquot portion in aqueous solution by the resorcinol method (Bell and Palmer, *J.*, 1949, 2522; Arni and Percival, *J.*, 1951, 1822). It seems unlikely, in view of Bell and Palmer's findings (*J.*, 1952, 3763), that any appreciable amounts of contaminating non-reducing materials were present in these fractions.

Fraction (1), on investigation by paper chromatography, was shown to contain only 1 : 3 : 4 : 6-tetra-*O*-methylfructofuranose and estimation by resorcinol indicated the presence of 91·5 mg. of this sugar in (1). The material was identified by conversion into tetra-*O*-methyl D-fructofuronamide (Avery, Haworth, and Hirst, *J.*, 1927, 2313), m. p. and mixed m. p. 101° (Found: OMe, 48·5. Calc. for $C_{10}H_{19}O_6N$: OMe, 49·8%).

Fraction (2) was shown by paper-chromatography to be a mixture of tetra-*O*-methylglucopyranose and tetra-*O*-methylfructofuranose. The tetra-*O*-methylfructose content, estimated as above on a portion of the fraction, was 31·5 mg. The remainder of the fraction was evaporated to dryness. Hypoiodite oxidation (Part I, *loc. cit.*) of the resulting syrup indicated the presence of 25·3 mg. of tetra-*O*-methylaldose in fraction (2).

Fraction (3) (24 mg.) gave one spot on the chromatogram corresponding to tetra-*O*-methylglucose. Hypoiodite oxidation indicated 91% purity. Attempts to prepare crystalline derivatives from this fraction were unsuccessful.

Fraction (4) (651 mg.) crystallised completely. Chromatographic examination indicated the presence of 1 : 3 : 4-tri-*O*-methyl-D-fructose and no aldose. After recrystallisation from carbon tetrachloride–light petroleum, 1 : 3 : 4-tri-*O*-methyl-D-fructose, m. p. and mixed m. p. 74°, $[\alpha]_D^{18}$ −45° (10 min.), −53° (1 hr., constant) (*c*, 1·96 in H_2O), was obtained (Found: OMe, 40·9. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (5) (82·2 mg.) was shown by chromatography to contain tri-*O*-methylfructose and an aldose derivative along with traces of more fully methylated materials. Hypoiodite oxidation indicated the presence of 24·7 mg. of tri-*O*-methylaldohexose. Fraction (5) was separated into its components by partition on Whatman's 3MM paper.

Fraction (6) (13·7 mg.) was shown by chromatography and by hypoiodite oxidation to consist almost entirely of an aldose, and the fraction was combined with the aldose isolated from (5). The combined fraction had the same R_G value as 2 : 4 : 6-tri-*O*-methylglucose. The syrup (15·6 mg.) in 1% methanolic hydrogen chloride showed $[\alpha]_D^{18}$ +70°, unchanged in 24 hr. The syrup was recovered from the polarimetric solution and converted *via* the lactone into the syrupy amide, which gave a negative Weerman test.

Fraction (7) (10·8 mg.) had an R_G value corresponding to that of 3 : 4-di-*O*-methylfructose and was not further investigated.

Syrup Y.—Further methylation of the syrup Y with silver oxide and methyl iodide gave a product which was dissolved in chloroform (20 c.c.). Light petroleum (750 c.c.) was added and the precipitate (0·45 g.) which formed was removed by filtration. This material presumably consisted of fructosans of a molecular size intermediate between those of precipitate X and the soluble fraction, but was not further investigated. Evaporation of the filtrate yielded a syrup (1·93 g.) which was dissolved in ether and added to excess of light petroleum. The black precipitate was filtered off and the filtrate was evaporated to a syrup (1·42 g.) (Found: OMe,

44·4%). Further methylation did not increase the methoxyl content. The product showed $[\alpha]_D^{18}$ +46° (*c*, 0·35 in $CHCl_3$).

The syrup was hydrolysed under the conditions used for precipitate X, and a portion (0·39 g.) was fractionated on a column of cellulose. The amounts of the component sugars were estimated as detailed above and were: tetra-*O*-methylglucose, 59·4 mg.; tetra-*O*-methylfructose, 63·8 mg.; tri-*O*-methylglucose, 64·4 mg.; tri-*O*-methylfructose, 73·8 mg. No di-*O*-methyl sugars could be detected.

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