Studies on Fructosans. Part VI. The Degradation of Fructosans in Aqueous Solution.*

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[Reprint Order No. 5855.]

It has been shown that the degradation of fructosans in hot aqueous solution involves the formation of small quantities of acidic materials, which cause the fructosans to undergo slow hydrolysis. The oligosaccharides, formed from the partial breakdown of the fructosan from perennial rye-grass, have been examined. The isolation of sucrose provides further evidence that in the grass levans the chains of fructofuranose residues are terminated by a non-reducing glucopyranose unit.

It has been shown in Parts II, III, and IV of this series (Arni and Percival, *J.*, 1951, 1822; Laidlaw and Reid, *J.*, 1951, 1830; Aspinall, Hirst, Percival, and Telfer, *J.*, 1953, 337) that fructosans are degraded in aqueous solution at 100° and that after several hours the products consisted of fructose, glucose, and a series of oligosaccharides, amongst which sucrose was suspected. The present investigation was undertaken to study such degradations in greater detail and to obtain further information about the role of glucose in fructosan molecules. The isolation of 2 : 3 : 4 : 6-tetra-*O*-methyl-D-glucose from the hydrolysis of methylated inulin (Hirst, McGilvray, and Percival, *J.*, 1950, 1297) and of the methylated levan from cocksfoot grass (*Dactylis glomerata*) (Part IV, *loc. cit.*) showed that terminal non-reducing glucopyranose residues are present in these fructosans. Although, in the former case, evidence was also obtained for the presence of 2 : 4 : 6-tri-*O*-methyl-D-glucose in the hydrolysate of the methylated polysaccharide, it seems probable that this sugar arose from incomplete methylation as it has been shown by periodate oxidation (Aspinall and Telfer, *Chem. and Ind.*, 1953, 490) that no 1 : 3-linked glucose residues are present in inulin. Biogenetic evidence from the investigations of Bacon and Edelman (*Biochem. J.*, 1951, **48**, 114; **49**, 446, 529) and of Dedonder (*Compt. rend.*, 1950, **230**, 549, 997; 1951, **231**, 790; **232**, 1134, 1142) indicates that fructosans are built up in the plant from sucrose by enzymic transfructosidation, and thus provides further support for the view that terminal glucopyranose residues are present in fructosan molecules linked to the penultimate fructofuranose residues as in sucrose.

The degradation of two fructosans has been studied. Inulin was extracted from dahlia tubers with hot water and purified by deposition from aqueous solution on cooling. The levan from perennial rye-grass (*Lolium perenne*) was isolated from the sample of grass used by Laidlaw and Reid (*loc. cit.*) in their investigations. Both fructosans gave fructose and small quantities of glucose (2·8% and 2·0%, respectively) on hydrolysis. The degradation in aqueous solution at 100° was followed by observing changes in pH and optical rotation, and by periodic chromatographic examination of the products. The same general pattern of breakdown was observed with both fructosans; a gradual fall in pH was accompanied by a change in optical rotation with the formation at first of oligosaccharides and finally of fructose and glucose, complete breakdown occurring after about 24 hours. In addition, chromatographic evidence showed the presence of small quantities of difructose anhydrides. These substances were formed more readily from the hydrolysis of inulin. A parallel experiment showed that sucrose undergoes similar breakdown in aqueous solution at 100°, no sucrose being present after 30 hours. Typical data for the degradation of inulin are recorded in the Table.

The fall in pH together with chromatographic indication of the presence of acidic breakdown products showed that degradation of the fructosans was, at least in the later stages, an acid hydrolysis. When inulin was heated in phosphate buffer (pH 6·8) no oligosaccharides or monosaccharides were formed, although the dark coloration of the

* A preliminary account of some of the following results has appeared elsewhere (*Chem. and Ind.*, 1952, 1244). Part V, *J.*, 1954, 2364.

solution showed that some decomposition had occurred. It was also shown that the presence of atmospheric oxygen is an important factor in initiating the degradation; when inulin was heated in aqueous solution through which nitrogen was bubbled the breakdown occurred much more slowly, and after 60 hours considerable quantities of oligosaccharides were still present.

Preliminary chromatographic examination of the products of partial degradation of the levan from perennial rye-grass showed that an optimum yield of oligosaccharides, travelling on the chromatogram at the same rate as or slower than sucrose could be obtained

The degradation of inulin in aqueous solution.

	Time (hr.)	pH	$[\alpha]_D$	Paper chromatography * U	O	D + T	F	G	DFA
Inulin in 3% soln. at 100°	0	6·28	−40·3°	3	—	—	—	—	—
	4	5·14	−39·3	3	2	1	—	—	—
	8	4·40	−45·9	3	2	2	1	—	—
	12	3·93	−60·9	3	2	2	3	1	—
	16·5	3·70	−79·1	—	1	2	3	1	1
	25	3·35	−84·2	—	—	1	3	1	2
Inulin in 3% soln. at 100° in N_2	10	6·11	−44·6	3	1	—	—	—	—
	25	5·36	−47·7	3	2	1	1	—	—
	42	4·88	−53·6	3	2	2	2	—	—
	52	4·66	−67·1	2	2	2	3	1	1
	75	4·24	−78·5	—	1	1	3	1	2
Inulin in phosphate buffer (pH 6·80)	0	6·80	−38·5	3	—	—	—	—	—
	16	6·76	−39·2	3	—	—	—	—	—
	32	6·48	−40·0	3	—	—	—	—	—

* Numbers denote relative intensity, 3 denoting the greatest. U, unchanged fructosan; O, higher oligosaccharides; D + T, di- and tri-saccharides; F, fructose; G, glucose; DFA, difructose anhydrides.

if the reaction was stopped after 6 hours. The oligosaccharides, which, from their rate of movement on the chromatogram, appeared to be di-, tri-, and tetra-succharides, were separated chromatographically, eluted from the paper, and hydrolysed. The amounts of fructose and glucose thus obtained suggested that the " spots " on the chromatogram contained mixtures of oligosaccharides, each spot containing a sugar giving only fructose on hydrolysis, in addition to a sugar giving both fructose and glucose. Further investigation showed that each spot could be at least partially resolved into two components. The levan was heated at 100° in aqueous solution for 6 hours and the hydrolysis products were fractionated successively on charcoal–Celite (Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677) by elution with water and aqueous ethanol, and by partition chromatography on cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511). In this way several oligosaccharide-containing fractions were obtained. In some cases, these fractions required further separation by partition on filter sheets before the individual components were isolated.

From these extensive fractionations four sugars of particular interest were isolated: (i) a reducing disaccharide, which gave only fructose on both acid and enzymic hydrolysis; (ii) a reducing trisaccharide, which gave only fructose on acid hydrolysis, and fructose and the afore-mentioned disaccharide on incubation with yeast invertase; (iii) sucrose (identified by its physical constants and by conversion into the octa-acetate); and (iv) a non-reducing trisaccharide, which on hydrolysis gave fructose and glucose in the ratio of 2 : 1, and on partial hydrolysis gave fructose and sucrose together with traces of glucose and the reducing disaccharide. In view of their derivation from a fructosan of known general structure, it is highly probable that the reducing di- and tri-saccharides contain D-fructofuranose residues linked through positions $C_{(2)}$ and $C_{(6)}$, and experiments to establish the mode of linkage are at present in progress. Again, it is probable that the non-reducing tris accharide is identical with the trisaccharide, kestose, synthesised from sucrose by yeast invertase and shown by Albon, Bell, Blanchard, Gross, and Rundell (*J.*, 1953, 24) to be *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl (6→2)-β-D-fructofuranoside.

Evidence was also obtained for the presence in very small yield of a fructosylglucose. It is doubtful, however, if this sugar is of structural significance and it is likely that the sugar arose either as a reversion product or more probably by epimerisation from the reducing disaccharide during prolonged contact with the pyridine-containing chromatographic solvent. In a control experiment, fructose and glucose were heated together in aqueous solution but no evidence could be obtained for the formation of reversion products.

The isolation of sucrose from the partial hydrolysis of the levan from perennial ryegrass provides conclusive evidence that glucose residues are present in this polysaccharide as an integral part of the molecule. Schlubach and Holzer (*Annalen*, 1953, **578**, 207) claim to have isolated a fructosan from *Lolium perenne* devoid of glucose residues, and it is, indeed, possible that some fructosan molecules may contain no glucose. The present investigation, however, stresses the need for the utmost caution in handling these extremely labile polysaccharides lest inadvertent scission of the fructosan chain results in loss of the glucose-containing moiety. The evidence from both structural investigations and studies of enzymic transfructosidation shows that the majority, at least, of fructosan molecules, both of the inulin and of the levan type, contain terminal glucose residues linked as in sucrose.

Experimental

Paper partition chromatography was carried out on Whatman No. 1 filter paper with the solvent systems: (*A*) butan-1-ol–benzene–pyridine–water (**5 : 1 : 3 : 3**; v/v; top layer); (*B*) butan-1-ol–ethanol–water (**4 : 1 : 5**; v/v; top layer); and (*C*) ethyl acetate–acetic acid–water (**3 : 1 : 3**; v/v; top layer). Sprays of aqueous aniline oxalate and naphtharesorcinol in hydrochloric acid were used to detect aldoses and ketoses, respectively.

Isolation of the Polysaccharides.—(a) *Inulin.* Dahlia tubers (variety "Crimson Flag") were extracted by Hirst, McGilvray, and Percival's method (*loc. cit.*). The inulin, which separated on cooling from aqueous solution, had $[\alpha]_D^{15}$ −40·3° (*c*, 2·4 in H_2O). Chromatographic examination of the hydrolysate (Hirst and Jones, *J.*, 1949, 1659; Duff and Eastwood, *Nature*, 1950, **165**, 848) in solvent *C* showed the presence of fructose (97·2%) and glucose (2·8%).

(b) *Levan.* The fructosan from *Lolium perenne* was isolated as described by Laidlaw and Reid (*loc. cit.*).

Degradation of Inulin in Aqueous Solution.—Inulin (1·5—2·0 g.) was dissolved in water (50 c.c.), and the solution heated on the water-bath and examined periodically for changes in optical rotation and pH, samples being withdrawn for chromatographic examination in solvent *A*. The results are given in the Table. In some cases, oligosaccharides, which appeared, from their rate of movement, to be di-, tri-, and tetra-saccharides, were eluted from the chromatogram and hydrolysed with aqueous 1% oxalic acid, and the hydrolysates examined chromatographically in solvent *C*. Fructose and glucose were present in each hydrolysate, but visual estimates of the ratio of the sugars suggested that each "discrete spot" on the chromatogram contained two oligosaccharides, one giving fructose and glucose and the other giving only fructose on hydrolysis. The inulin solution was also tested periodically with the ammonium thiocyanate–ferrous ammonium sulphate reagent (Young, Vogt, and Nieuland, *Ind. Eng. Chem. Anal.*, 1936, **8**, 198), but in no case were hydroperoxides present.

Inulin (1·5 g.) was heated in phosphate buffer solution (50 c.c.; pH 6·8) and the reaction was followed as described previously. Although the solution darkened considerably no breakdown products could be detected chromatographically. The degradation of inulin in unbuffered aqueous solution through which nitrogen was bubbled was similar to that in air but much slower (see Table).

Degradation of Levan in Aqueous Solution.—The degradation of *Lolium perenne* levan in aqueous solution was followed in the manner described for inulin, and the reaction pattern was essentially similar. In a typical experiment the following changes were observed: $[\alpha]_D^{16}$ −42·8° (initial value), −36·3° (4 hr.), −48·6° (8 hr.), −72·6° (16 hr.), −79·6° (24 hr., const.); pH 5·01 (initial value), 4·48 (4 hr.), 4·11 (8 hr.), 3·54 (16 hr.), 3·20 (24 hr.). Chromatographic examination of the product in solvent *B*, followed by development with methyl-red–methylene-blue (Conway and Byrne, *Biochem. J.*, 1933, **27**, 419), showed the presence of two acids, one of which travelled at the same rate as lactic acid.

Degradation of Sucrose in Aqueous Solution.—A solution of sucrose (1·7 g.) in water (50 c.c.) was heated on the water-bath. The following changes were observed: $[\alpha]_D^{16}$ +65·3° (initial

value) ⟶ −12·3° (36 hr., const.); pH 5·98 (initial value) ⟶ 3·32 (36 hr.). Chromatographic examination of the solution showed gradual breakdown with the formation of glucose and fructose, and after 30 hr. sucrose could no longer be detected. Two acids were also detected.

Large-scale Degradation of Levan and Separation of Oligosaccharides.—A solution of the levan (50 g.) in water (1·4 l.) was heated on the water-bath for 6·5 hr. {$[\alpha]_D$ −41·1° ⟶ −48·6° (6 hr.); pH 4·40 ⟶ 4·08 (6 hr.)}. The cooled solution was neutralised with barium carbonate, and the clear filtrate was concentrated; chromatographic examination of the syrup showed the presence of fructose and a series of oligosaccharides.

A solution of the syrup in water (500 c.c.) was poured on to charcoal–Celite (38·5 × 4·6 cm.) (Whistler and Durso, *loc. cit.*). The aqueous eluate, however, contained oligosaccharides in addition to monosaccharides and appropriate portions were, therefore, combined to give three fractions. Fraction *A* (10·2 g.) was shown chromatographically to contain only fructose and glucose and was not examined further. Fraction *B* (23·0 g.) contained fructose, glucose, sucrose, and a sugar having $R_{sucrose}$ 1·18. Fraction *C* (3·2 g.) contained fructose, sucrose, and sugars having $R_{sucrose}$ 1·18 and 0·71. Elution of the column with 50% aqueous ethanol gave fraction *D* (14·1 g.), which contained a sugar having $R_{sucrose}$ 0·71 and slower-moving oligosaccharides. Fraction *B* was further separated on charcoal–Celite (62 × 4·5 cm.), elution with water giving fraction *B*(i) (17·2 g.) containing fructose and glucose (trace), and elution with 5% aqueous ethanol giving fraction *B*(ii) (4·42 g.) containing a sugar having $R_{sucrose}$ 1·18 and *B*(iii) (0·546 g.) containing sucrose and sugars having $R_{sucrose}$ 1·18 and 0·85. Fraction *C* was separated on cellulose (70 × 3 cm.), solvent *A* being used, to give fractions containing fructose, glucose, and substances travelling on the chromatogram faster than fructose (probably difructose anhydrides), four oligosaccharide-containing fractions (1—4) and a fraction *C*(i) (0·914 g.) containing sucrose and the sugar of $R_{sucrose}$ 1·18. Fractions *B*(iii) and *C*(i) were combined and separated on cellulose (100 × 1·7 cm.) to give fractions containing glucose, fructose, and (probably) difructose anhydrides, and two further oligosaccharide-containing fractions (5 and 6). In some fractions traces of an unidentified substance were present; this travelled on the chromatogram considerably faster than fructose and gave a blue coloration with naphtharesorcinol and hydrochloric acid.

Examination of Oligosaccharide-containing Fractions.—Fraction 1. The syrup (0·442 g.), which reduced Fehling's solution and ammoniacal silver nitrate, had $[\alpha]_D^{17}$ −20·8° (*c*, 4·0 in H_2O) and $R_{sucrose}$ 1·18 in solvent *A*. Both mild acid hydrolysis and incubation with yeast invertase (B.D.H. "Invertase Concentrate") gave only fructose. The rate of movement on the chromatogram indicated that the sugar was a disaccharide, and its derivation from a fructosan of known general structure suggests that the sugar was 6-*O*-β-D-fructofuranosyl-D-fructofuranose (referred to as fructobiose).

Fraction 2. Chromatographic examination of the syrup (0·242 g.) showed the presence of four sugars, probably (*a*) fructobiose, (*b*) sucrose, (*c*) fructosylglucose ($R_{sucrose}$ 0·85), (*d*) fructotriose. Separation of small quantities of the sugars was effected on the chromatogram by using solvent *A*, (*a*), (*c*), and (*d*), but not (*b*), reducing ammoniacal silver nitrate. On mild acid hydrolysis or incubation with yeast invertase (*a*) and (*d*) gave only fructose, while (*b*) and (*c*) gave glucose and fructose.

Fraction 3. The chromatographically pure syrup (0·393 g.) had $R_{sucrose}$ 0·71 in solvent *A*. The sugar had $[\alpha]_D^{16}$ −21·1° (*c*, 3·18 in H_2O) and reduced Fehling's solution and ammoniacal silver nitrate. Mild acid hydrolysis gave only fructose, and incubation with yeast invertase gave fructose and fructobiose. It seems probable that the sugar was 6-*O*-β-D-fructofuranosyl-6-*O*-β-D-fructofuranosyl-β-D-fructofuranose (referred to as fructotriose).

Fraction 4. Chromatographic examination showed the presence of two trisaccharides and separation on filter sheets with solvent *A* gave fractions (*e*) (450 mg.), having $R_{sucrose}$ 0·71 (identical with fructotriose), and (*f*) (40 mg.), having $R_{sucrose}$ 0·60. Fraction (*f*) and $[\alpha]_D^{18}$ +24° (*c*, 0·5 in H_2O) and was non-reducing towards Fehling's solution and ammoniacal silver nitrate. Incubation with yeast invertase gave glucose and fructose, and quantitative estimation (Hirst and Jones, *loc. cit.*) of the acid hydrolysate showed glucose and fructose to be present in the ratio 1 : 2. A portion of the syrup (10 mg.) was dissolved in water (5 c.c.), and the solution was heated at 100° with Amberlite resin IR-100 (0·5 g.). After 30 min. chromatographic examination showed fructose and sucrose, together with traces of glucose and fructobiose. Hydrolysis to fructose and glucose was complete after 1 hr. It is probable that the sugar was *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl (6→2)-β-D-fructofuranoside (kestose) (Albon, Bell, Blanchard, Gross, and Rundell, *loc. cit.*). Albon *et al.* record $[\alpha]_D^{20}$ +27·3° for the crystalline sugar.

Fraction 5. The syrup (0·553 g.) contained sucrose and sugars having $R_{sucrose}$ 1·18 and 0·85. The sugars were separated on filter sheets, solvent *A* being used, to give fractions 5(*a*) (228 mg.), which travelled on the chromatogram at the same rate as fructobiose, 5(*b*) (287 mg.), which travelled on the chromatogram at the same rate as sucrose, and 5(*c*) (31 mg.). Fraction 5(*b*) crystallised from aqueous ethanol and had m. p. (and mixed m. p. with sucrose) 184—185° (Found: C, 42·2; H, 6·4. Calc. for $C_{12}H_{22}O_{11}$: C, 42·1; H, 6·4%). A sample was hydrolysed with aqueous 1% oxalic acid {$[\alpha]_D$ +59° ⟶ −24° (1 hr., const.)}, and quantitative estimation (Hirst and Jones, *loc. cit.*) of the hydrolysate showed glucose and fructose to be present in the ratio 1 : 1·05. The identity of the sugar was confirmed by an *X*-ray powder photograph (by courtesy of Dr. C. A. Beevers), which was identical with that of sucrose, and by conversion into sucrose octa-acetate, m. p. and mixed m. p. 72—73°, γ_D^{20} (fused crystals) 1·4602, $[\alpha]_D^{17}$ +60° (*c*, 1·1 in $CHCl_3$) (Found: C, 49·5; H, 5·8; Ac, 50·3. Calc. for $C_{28}H_{38}O_{19}$: C, 49·5; H, 5·6; Ac, 50·7%).

Fraction 6. The syrup (31 mg.) was combined with fraction 5(*c*), both fractions containing a sugar having $R_{sucrose}$ 0·85 in solvent *A* together with traces of other sugars. The sugar reduced Fehling's solution and ammoniacal silver nitrate, and gave glucose and fructose on treatment with yeast invertase. Quantitative estimation of the acid hydrolysate showed glucose and fructose to be present in the ratio of 1 : 1·27. Reduction of alkaline hypoiodite (55% of theoretical for a disaccharide) showed that the major component of the syrup was a fructosylglucose.

The authors thank Professor E. L. Hirst, F.R.S., for his interest and advice, the Department of Scientific and Industrial Research for the award of a maintenance allowance (to R. G. J. T.), and Imperial Chemical Industries Limited, Central Agricultural Control, for the supplies of *Lolium perenne* used in these investigations.

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF EDINBURGH. [*Received, November 8th*, 1954.]