721. *Structural Studies on Inulin from* Inula helenium *and on Levans from* Dactylis glomerata *and* Lolium italicum.

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Chromatographic analyses are reported of the hydrolysates of methylated inulin from elecampane (*Inula helenium*) and of methylated levans from two grasses, leafy cocksfoot (*Dactylis glomerata*) and Italian rye (*Lolium italicum*).

The structure of this inulin is similar to that already described for dahlia ("Blue Danube"). Quantitative analyses of grass levan structures are fully reported for the first time.

It has been noted that gravimetric chromatographic analyses may be complicated by the adventitious formation of difructose dianhydrides and similar bodies.

In a previous communication (Bell and Palmer, *J.*, 1949, 2522) we described an effective partition chromatographic method for the quantitative separation of pure tetra-, tri-, and di-methylated fructoses. Separating the hydrolysis products of methylated inulins and grass levans, we encountered an unexpected complication. This difficulty can be circumvented; our experience of such analyses forms the subject of this report.

Analyses of the mixed methylated sugars derived hydrolytically from trimethyl inulin or trimethyl levan requires the separation of relatively small amounts (less than 10%) of 1 : 3 : 4 : 6-tetramethyl D-fructose from the appropriate predominating product, 3 : 4 : 6- or 1 : 3 : 4-trimethyl D-fructose. Again, the latter sugars may have to be separated from small quantities of dimethyl fructoses which have originated either as branch-points or from incomplete methylation. The presence of D-glucose radicals in inulin (cf. Palmer, *Biochem. J.*, 1951, **48**, 389) and in grass levans (Palmer, *loc. cit.*; Laidlaw and Reid, *J.*, 1951, 1830), albeit in small amounts, adds a further analytical problem.

Despite the mild hydrolytic treatment used we find that with methylated inulin, where the predominating radicals are linked 2 : 1′, a non-reducing fructose derivative is present among the hydrolysis products and during the chromatography "loads" the tetramethyl fructose fraction. The latter (Bell and Palmer, *loc. cit.*; Laidlaw and Reid, *loc. cit.*; Arni and Percival *J.*, 1951, 1822) was assayed colorimetrically by strongly acid resorcinol, a

method which does not distinguish between free fructose and such compounds as the non-reducing difructose dianhydrides. This contaminant may well be a hexamethyl difructose dianhydride (see below). However, 1 : 3 : 4 : 6-tetramethyl fructose can be determined, when mixed with non-reducing material, by the reduction of alkaline 3 : 5-dinitrosalicylate (Bell, Manners, and Palmer, *J.*, 1952, 3760) so that an accurate end-group assay can be made. We obtained an impure specimen of the suspected dianhydride, which can arise in appreciable amounts during hydrolysis of methylated inulin and also possibly when 3 : 4 : 6-trimethyl fructose itself is similarly treated. The material had $[\alpha]_D$ +60° in water, thus resembling 3 : 4 : 6 : 3′ : 4′ : 6′-hexamethyl difructofuranose 2 : 1′-2′ : 1-dianhydride (cf. McDonald, *Adv. Carbohydrate Chem.*, 1946, **2**, 253; McDonald and Turcotte, *J. Res. Nat. Bur. Stand.*, 1947, **38**, 423; Wolfrom, Binkley, Shilling, and Hilton, *J. Amer. Chem. Soc.*, 1951, **73**, 3553).

Methylated grass levans, on the other hand, from their structures might not be expected to yield difructose derivatives of the known 2 : 1′-2′ : 1 types. Nevertheless, in one experiment, we found a tetramethyl fraction contaminated with two additional, essentially non-reducing, ketose derivatives. We therefore emphasise the necessity for rigorous characterisation of chromatographic fractions, especially with ketoses (cf. Bott, Hirst, and Smith, *J.*, 1930, 665; Haworth, Hirst, Jones, and Woodward, *J.*, 1938, 1575). One of us has found evidence (unpublished) that chromatographic fractions of trimethyl fructose can be "loaded" by material arising from 3 : 4-dimethyl fructose. The experience of Bourne, Fantes, and Peat (*J.*, 1949, 1109) with glucose derivatives should also be noted.

The presence of D-glucose derivatives in the hydrolysates of both methylated inulins and levans was confirmed. These appeared mainly as mixtures of all the trimethyl compounds unsubstituted at position 5, the 2 : 4 : 6-isomer predominating, and paper chromatography showed traces of 2 : 3 : 4 : 6-tetramethyl glucose. Although the glucose was not all in the fully methylated form we are prepared to allow that it originated from terminal non-reducing radicals linked as in sucrose (Hirst, McGilvray, and Percival, *J.*, 1950, 1297; Bacon and Edelman, *Biochem. J.*, 1951, **48**, 114; Edelman and Bacon, *Biochem. J.*, 1951, **49**, 446, 529; Dedonder, *Compt. rend.*, 1950, **230**, 549, 994; 1951, **231**, 790, 1134; **232**, 1134, 1442; *Bull. Soc. Chim. biol.*, 1952, **34**, 144, 157, 171; Laidlaw and Reid, *loc. cit.*). The failure of fructosans to reduce, *e.g.*, alkaline 3 : 5-dinitrosalicylate, supports this contention (Bell, Manners, and Palmer, *loc. cit.*).

Inula inulin liberated on hydrolysis 2·9 moles of D-glucose per 100 hexose radicals (conveniently termed 2·9 moles %). If the molecule is terminated by the glucose moiety of a sucrose radical the molecular weight of the polysaccharide would be about 5600 and the chain length 35 radicals. Hydrolysis of the trimethyl inulin gave 1 : 3 : 4 : 6-tetramethyl fructose (1 mol.), 3 : 4 : 6-trimethyl fructose (32·7 mols.), dimethyl fructose (1·5 mols.), and trimethyl glucose (0·54 mol.). Traces of tetramethyl and dimethyl glucoses were observed by paper chromatography. Assuming that the glucose was poorly recovered by the imperfect technique employed (it was roughly equivalent to 50% of glucose in the original inulin) we calculate a chain length of about 36 radicals, corresponding to a molecular weight of 5800. This is the chain-length found for dahlia ("Blue Danube") inulin by Hirst, McGilvray, and Percival (*loc. cit.*) although these authors found chromatographically nearly twice as much glucose in their unmethylated material. Through the friendly co-operation of Professor Hirst and the late Dr. Percival we were able to assay the glucose in two samples, termed "1*a*" and "2*a*" in their paper. Determined by D-glucose oxidase, hydrolysates of these samples contained D-glucose corresponding to 3·0 and 2·0 moles % respectively.

The grass levans, unlike inulin, do not crystallise from water on freezing. They require a relatively high concentration of ethanol (80%) for complete precipitation, and from a comparison of the molecular weights with the methylation data we consider that the "mean" structure consists of a singly branched molecule. The levans as commonly isolated, are clearly not homogeneous; Dr. A. G. Ogston of Oxford University, from sedimentation diffusion data, found for Italian rye-grass levan a molecular weight of about 5500 before dialysis, and 8700 after dialysis for some time. Leafy cocksfoot levan had a molecular weight of the same order (5500). Palmer (*loc. cit.*) found that these two levans, precipitated

in 80% ethanol, contained closely similar amounts of D-glucose radicals (2·7 moles %) corresponding to molecular weights of 6000 and chain lengths (if unbranched) of 37 radicals. She showed, however, that neither was homogeneous since fractions having lower contents of D-glucose radicals were obtained by precipitation at lower alcohol concentrations.

Until recently few quantitative chemical examinations have been made of levans isolated from leaves and stems of grasses by using methylation. Challinor, Haworth, and Hirst (*J.*, 1934, 1560) showed that levan from *Poa trivialis* (rough-stalked meadow grass) yielded crystalline 1 : 3 : 4-trimethyl D-fructose and, assuming that the properties of this material were close to those of bacterial levans on which end-group assays had been done (Challinor, Haworth, and Hirst, *J.*, 1934, 676; Lyne, Peat, and Stacey, *J.*, 1940, 237), suggested a chain-length of about 10—12 fructofuranose radicals. Haworth, Hirst, and Lyne (*Biochem. J.*, 1939, **31**, 786) reported on a specimen of levan isolated from barley leaves by Archbold and Barter (*Biochem. J.*, 1935, **29**, 2689). The latter had noted that the reducing sugar liberated on hydrolysis was not all fructose; this was confirmed by Haworth *et al.* who also showed that the methylated fructosan yielded mostly 1 : 3 : 4-trimethyl D-fructose. Schlubach and Bandmann (*Ann.*, 1939, **540**, 285) attempted the first structural analysis of a graminaceous levan, the so-called " secalin " obtained from the freshly cut stems of rye (*Secale* sp.), obtaining 1 : 3 : 4 : 6-tetramethyl fructose (1 mol.), 1 : 3 : 4-trimethyl fructose (2 mols.), and dimethyl fructose(s) (1 mol.). A similar substance " pyrosin " from freshly cut stems of wheat, examined by Schlubach and Huchting (*Annalen*, 1949, **561**, 173) gave 1 : 3 : 4 : 6-tetramethyl fructose (1·4 mols.), 1 : 3 : 4-trimethyl fructose (3 mols.), and dimethyl fructose (1 mol.). Both polysaccharides showed the presence of small amounts of aldose (unidentified) by the method of Auerbach-Bodländer. Subsequently, a preliminary note on the present work on Italian rye and leafy cocksfoot grasses appeared (Bell and Palmer, *Biochem. J.*, 1949, **45**, XIV), followed by the work of Laidlaw and Reid (*loc. cit.*) on perennial rye grass (*Lolium perenne*).

Leafy cocksfoot levan was examined in two fractions, (*a*) precipitated (100%) in 80% ethanol, and (*b*) precipitated (86%) in 62% ethanol. These fractions are hereafter termed " levan-80 " and " levan-62." The results of methylation experiments, followed by chromatographic analyses of the hydrolysis products are given in Table 1. Trimethyl " levan-80 " was partly (40%) soluble in acetone at room temperature; the soluble and insoluble fractions were examined separately; trimethyl levan-62 was completely insoluble in acetone. No search for glucose derivatives was made during the analyses of levan-80, but levan-62 was investigated as described below. These results demonstrate that this levan is not homogeneous, and also that its structure differs from those of the fructosans examined by Schlubach and his co-workers.

TABLE 1. *Levan from leafy cocksfoot grass.*

	Levan-80		Levan-62
D-Glucose radicals (moles %)	2·9		2·2
Mol. wt., calc. from above	5600		7400
Mol. wt., by ultracentrifuge (Dr. A. G. Ogston)	5500		—
Trimethyl derivatives :			
Yield (%)	75		80
	Acetone-sol. (40%)	Acetone-insol. (60%)	
OMe (%)	42·5	42·5	45·3
D-Fructose derivatives found in hydrolysates :			
1 : 3 : 4 : 6-Me_4 (mols.)	1	1	1 *
1 : 3 : 4-Me_3 (mols.)	11	13	22
? Me_2 (mols.)	2	1·6	0·5
Chain length	14	15—16	23—24
Mol. wt. of chain	2300	2700	3900

* This fraction was initially contaminated. See Experimental section for assay procedure.

The column-chromatogram fractions from levan-62 were examined by paper chromatography for the presence of glucose derivatives. Traces of 2 : 3 : 4 : 6-tetramethyl glucose and a relatively large amount of trimethyl isomers were found, the latter contaminating the dimethyl fructose fraction. This trimethyl glucose was concentrated by appropriate chromatographic treatment, 1·6% of the total sugars present in the hydrolysate

being obtained as a crystalline mixture in which 2 : 4 : 6-trimethyl glucose predominated. Varying smaller amounts of the 2 : 3 : 4-, 2 : 3 : 6-, and 3 : 4 : 6-isomers were also present.

Italian rye grass levan was also examined, but less exhaustively. By methylation through the acetate, an 80% yield of trimethyl levan (OMe, 45·8%) was obtained. Our results are summarised in Table 2.

TABLE 2. *Levan from Italian rye grass.*

D-Glucose radicals (moles %)	Mol. wt., calc. from glucose content	Mol. wt., from ultra-centrifuge (Dr. Ogston)	D-Fructose derivatives found in hydrolysate of trimethyl levan: 1 : 3 : 4 : 6-Me_4 (mols.)	1 : 3 : 4-Me_3 (mols.)	Me_2 (mols.)	Chain length	Mol. wt. of chain
2·8	5800	5500	1	11	1	13	2100

Comparison with the levan from *Lolium perenne* examined by Laidlaw and Reid (*loc. cit.*) is of interest. Here the chain length was 25—30 radicals; 2 moles % of D-glucose radicals were present; and chromatographic examination of a boiled aqueous solution showed the presence of sucrose.

We have carried out control experiments in which chromatographically pure specimens of the appropriate sugars were subjected to the exact hydrolytic treatment and chromatography used in fructosan investigations. With 1 : 3 : 4-trimethyl fructose there was no interference with the quantitative recovery of pure 1 : 3 : 4 : 6-tetramethyl fructose. On the other hand, when 3 : 4 : 6-trimethyl fructose was a component of the mixture there was slight "loading" of the tetramethyl fraction by a non-reducing fructose derivative. This indicates that condensation of free fructose derived radicals may occur in dilute acid solution.

EXPERIMENTAL

Unless otherwise stated, the following standard procedures were employed throughout: (*a*) Solvents were evaporated under reduced pressure below 40°. (*b*) Polarimetric observations were made in aqueous solutions in 2-dm. tubes. (*c*) Paper chromatograms were made with Whatman No. 1 paper and *n*-butanol–water. Ketoses and aldoses were detected by the resorcinol and aniline phthalate sprays respectively. As temperature-controlled tanks were not available, all chromatograms were run in the presence of authentic specimens of the appropriate sugars. (*d*) Column chromatography and the characterisation of methylated fructoses were done according to Bell and Palmer (*loc. cit.*). (*e*) The glucose-radical content of the fructosans was determined by Palmer's method (*loc. cit.*).

Methylations.—The following general procedure was used. One part of polysaccharide was dissolved in 3 parts (v/w) of 30% (w/v) sodium hydroxide solution. With vigorous stirring at 40°, 30 parts (v/w) of methyl sulphate and 60 parts (v/w) of 30% sodium hydroxide solution were added in one-tenth portions at 10-minutes' intervals. Dioxan was added from time to time to promote solution of the methylated product which tended to separate at an early stage. Finally 30—40 parts of hot water were introduced and the whole heated at 100°, with stirring, for an hour. The methylated fructosans separated and were collected by filtration, washed with boiling water, and treated as required.

Hydrolysis of Methylated Fructosans.—One part of polysaccharide was dissolved in 5 parts (v/v) of boiling ethanol, and 7 parts (v/v) of 0·05N-sulphuric acid were added slowly. After 4 hours' heating at 95—100° under reflux the ethanol was removed by distillation at ordinary pressure. After addition of water to replace the ethanol which had distilled, heating was continued until the optical rotation was constant. The solution was brought to pH 7 with barium carbonate and after filtration concentrated to about 50 ml. for examination by paper chromatography (Bell and Palmer, *loc. cit.*).

Quantitative Analysis.—Fructose (ketose) was determined by the method of Cole, Hanes, Jackson, and Loughman (cf. Bell and Palmer, *loc. cit.*). Reducing sugars were determined by reduction of alkaline 3 : 5-dinitrosalicylate (Bell, Manners, and Palmer, *loc. cit.*).

Isolation of the Polysaccharides.—(*a*) *Inulin.* Fresh roots of elecampane harvested in October 1947 were treated according to Onslow ("Plant Biochemistry," 1929, Cambridge Univ. Press, p. 60). The crude inulin, recrystallised three times from water (pH 8—8·5) by freezing the solution to −20° and allowing it to thaw slowly, had $[\alpha]_D$ −41·0° and contained 2·9 moles % of D-glucose radicals.

(*b*) *Levans.* Pure strains of dried grasses were extracted by Bacon's method (Palmer, *loc. cit.*). Levans from leafy cocksfoot and from Italian rye-grass had $[\alpha]_D$ −40·5° and −41·0° respectively.

Methylated Inulin : Chromatographic Analysis.—10 G. of inulin yielded 10 g. (80%) of trimethyl derivative, $[\alpha]_D^{20}$ −52·8° (in chloroform) (Found : OMe, 45·1%). 7·47 G. were hydrolysed and analysed on the partition column, with two extra intermediary elutions; each eluted fraction was examined by paper chromatography (Table 3). Quantitative examinations of each fraction was made by both the ketose and dinitrosalicylate methods. Each fraction was characterised as fully as possible (cf. Bell and Palmer, *loc. cit.*). The results are summarised in Table 4.

TABLE 3. *Analysis of hydrolysate of trimethyl inulin from* Inula helenium.

	Column chromatography :		Paper chromatography :			
			Fructose spots :		Glucose spots :	
Serial no. of fraction	Eluting solvent and no. of column-lengths passed	Wt. of material eluted (g.)	main R_F	trace R_F	main R_F	trace R_F
1	Toluene–0·33% EtOH (16)	0·5	0·78 [a]	—	—	0·79 [b]
2	Chloroform (8)	6·8	0·67 [c]	—	—	?0·76 [b] ?0·64
3	Chloroform–5% Bu^nOH	0·13	—	0·67 [c]	0·60	—
4	Chloroform–10% Bu^nOH	0·12	0·44	0·67 [c]	—	0·60 0·46
5	Methanol	0·20	0·44	—	—	0·37

[a] Same R_F as 1 : 3 : 4 : 6-tetramethyl fructose. [b] Same R_F as 2 : 3 : 4 : 6-tetramethyl glucose. [c] Same R_F as 3 : 4 : 6-trimethyl fructose.

TABLE 4. *Examination of the fractions from Table* 3.

Serial no. of fraction	Composition of fraction	Analytical constants and remarks
1	0·23 g. of 1 : 3 : 4 : 6-tetramethyl fructose 0·27 g. of non-reducing material, ? hexamethyl difructose dianhydride	Impossible to characterise
2	6·80 g. of 3 : 4 : 6-trimethyl fructose	Virtually pure; $[\alpha]_D^{18}$ +30·3°; n_D^{20} 1·4658; OMe, 41·6%; oxidation by IO_4^- (pH 7·5) gave 1·0 mol. of CH_2O
3	0·01 g. of trimethyl fructose 0·12 g. of trimethyl glucose	Examined separately (see below), $[\alpha]_D$ −70°
4	0·12 g. of dimethyl fructose	Combined fractions, essentially dimethyl fructose; $[\alpha]_D^{18}$ −43·4°; n_D^{20} 1·4852; OMe, 26·9%; oxidation by IO_4^- (pH 7·5) gave 1·5 mols. of CH_2O
5	0·20 g. of dimethyl fructose	

Recovery of sugars was 7·8 g. (96%) present in the following molar proportions : tetramethyl fructose, 1·0; trimethyl fructose, 32·7; trimethyl glucose, 0·54; dimethyl fructose 1·5. Examination of fraction 3 (0·13 g.) showed that it contained 8·2% of trimethyl fructose. The amount of aldose was therefore 0·12 g. From the specific rotation, the $[\alpha]_D$ of the glucose fraction must have been of the order of +74°, typical for a trimethyl D-glucopyranose.

Paper chromatography (ethyl methyl ketone–water and *n*-butanol–water systems) showed 2 : 4 : 6-trimethyl glucose to be the main component, admixed with small amounts of all the other pyranose isomers.

Levan from Leafy Cocksfoot.—For the methylation experiments two distinct preparations were used : (*a*) " levan-80 " obtained by complete precipitation in ethanol at 80% concentration, and (*b*) " levan-62 " where 86% of the levan was precipitated in ethanol at 62% concentration. The glucose-radical contents were 2·9 moles % for " levan-80 " and 2·2% for " levan-62." Both samples were methylated twice. Trimethyl " levan-80 " (75% yield) had OMe, 42·5% (not raised by repeated treatment with Purdie's reagents); " trimethyl levan-62 " (80% yield) had OMe, 45·3%.

Trimethyl " Levan-80."—Only 40% of this methylated levan was soluble in acetone at room temperature, unlike the material from *Poa trivialis* (Challinor, Haworth, and Hirst, *loc. cit.*) Both soluble and insoluble portions had OMe, 42·5%. Analysis of the hydrolysis products from both fractions gave similar results. No search was made for glucose derivatives. Results are in Table 5.

The amounts of fractions marked * were insufficient for accurate determination of $[\alpha]_D$. n_D^{20} and R_F values of the single spots given on paper chromatograms, compared with authentic 1 : 3 : 4 : 6-tetramethyl fructose, indicated that both fractions 1 were composed essentially of the latter sugar.

Fractions 2 and 3, in each case, were homogeneous with respect to fructose derivatives, on paper chromatograms. Fractions 2 were crystalline and were essentially 1 : 3 : 4-trimethyl D-fructose. Fractions 3 must, from their $[\alpha]_D$, have been composed of dimethyl fructoses unsubstituted on position 6.

TABLE 5. *Analysis and characterisation of the hydrolysis products of the acetone-soluble and acetone-insoluble portions of trimethyl "levan-80."*

Fractions were eluted by the following solvents: 1, toluene–0·33% ethanol; 2, chloroform–5% *n*-butanol; 3, methanol.

Acetone-soluble portion (2·87 g. hydrolysed)

No. of fraction	Eluted (g.)	R_F	n_D^{20}	OMe (%)	$[\alpha]_D^{18}$	Molar ratio
1	0·20	0·8	1·4509	*	*	1
2	2·14	0·6	1·4653	40·0	−57·2°	11
3	0·33	0·4	1·4820	28·0	−60·4	2

Recovery: 88% of theoretical fructose content

Acetone-insoluble portion (1·18 g. hydrolysed)

No. of fraction	Eluted (g.)	R_F	n_D^{20}	OMe (%)	$[\alpha]_D^{18}$	Molar ratio
1	0·076	0·8	1·4509	*	*	1
2	0·96	0·6	1·4658	42·0	−56·0°	13
3	0·11	0·4	—	28·8	−60·6	1·6

Recovery: 89% theoretical fructose content

Trimethyl "Levan-62."—This was completely insoluble in acetone at room temperature. After hydrolysis and analysis on a silica column each fraction eluted was examined by paper chromatography. Faint traces of tetramethyl and dimethyl glucose were detected in the appropriate fractions, but the bulk of the glucose appeared as trimethyl derivatives and was concentrated in the dimethyl fructose fraction. The first "ketose" fraction, *i.e.*, that expected to contain 1 : 3 : 4 : 6-tetramethyl fructose as sole component, contained two additional substances strained by the acid resorcinol spray. The amount of tetramethyl fructose in the mixture, determined by dinitrosalicylate, was about one-third of the total material eluted (see Table 6).

TABLE 6. *Hydrolysis products of trimethyl "levan-62."*

11·60 G. of trimethyl levan were hydrolysed. Eluting solvents were as in Table 5.

No. of fraction	Eluted (g.)	R_F: Fructose derivative	R_F: Glucose derivative	OMe (%)	$[\alpha]_D^{18}$	n_D^{20}	
1	0·80	0·75 0·82 [a] 0·89	(Trace 0·81) [b]	52·5	+14·7° [c]	1·4511 [d]	0·54 g. of reducing Me_4 fructose was chief component
2	11·08	0·60	(Traces, 0·81, 0·6)	41·3	−56·1	1·4662	Almost pure crystalline 1 : 3 : 4-Me_3 fructose
3	0·30	(Trace 0·6) 0·4	0·6 (Trace 0·4)	—	—	—	A complex mixture (see below)

[a] R_F of 1 : 3 : 4 : 6-tetramethyl fructose. [b] R_F of 2 : 3 : 4 : 6-tetramethyl glucose. [c] $[\alpha]_D$ of 1 : 3 : 4 : 6-tetramethyl fructose, +30°. [d] n_D^{20} of 1 : 3 : 4 : 6-tetramethyl fructose, 1·4506.

The presumed 3 : 4 : 6 : 3′ : 4′ : 6′-hexamethyl difructose 2 : 1′-2′ : 1-dianhydride found in hydrolysates of inulin has the same R_F value as tetramethyl fructose. The component of R_F 0·75 might be tetramethyl ethylfructoside (*cf.* Haworth and Learner, *J.*, 1928, 619). The component of R_F 0·89 might be 1 : 3 : 4-trimethyl ethylfructoside (cf. Arni, Thesis, Univ. Edinburgh, 1951), or a dimer of that sugar as suspected by Lyne, Peat, and Stacey (*J.*, 1940, 237).

Recovery of sugars was 12·18 g. (95·5%) present in the following molar proportions: tetra-, 1·0, tri- 22·0, and di-methyl fructose, 0·6, corresponding to a chain length of *ca.* 24 radicals.

Fraction 3 (0·3 g.) was partitioned on a silica column through which were passed eight column-lengths of chloroform–10% (v/v) *n*-butanol. The eluate contained 0·1 g. of a syrup, $[\alpha]_D^{18}$ +44°, composed of a relatively high concentration of trimethyl glucoses (paper chromatography). Extraction of the silica by methanol gave 0·2 g. of a syrup, $[\alpha]_D^{18}$ −7·5°, n_D^{20} 1·4838 [OMe, 26·9%; IO_4^- oxidation (pH 7·5) yielded 0·48 mol. of formaldehyde]. This fraction appeared to be a mixture of dimethyl fructoses. Glucose derivatives were absent (paper chromatography).

Investigation of the Glucose-containing Fractions from Trimethyl "Levan-62."—A sample of trimethyl levan was hydrolysed as before and the resulting sugars transferred to a silica column

and fractionally eluted as described for methylated inulin (Table 3). The chromatographic scheme and the results of paper chromatography are shown in Table 7.

TABLE 7. *Qualitative chromatographic examination of the hydrolysate of trimethyl "levan-62."*

	Column chromatography	Paper chromatography			
		Fructose components detected :		Glucose components detected :	
No. of fraction	Eluting solvent and no. of column-lengths passed	Main	Trace	Main	Trace
1	Toluene–0·33% EtOH (16)	Me_4	—	—	Me_4
2	Chloroform (8)	Me_3	—	—	Me_4
3	Chloroform–5% Bu^{n}OH (8)	—	Me_3 Me_2	Me_3	Me_3
4	Chloroform–10% Bu^{n}OH (8)	Me_2	Me_3	—	Me_3 Me_2
5	Methanol	Me_2	—	—	Me_2

In this experiment tetramethyl fructose in fraction 1 was unaccompanied by other fructose derivatives. The trimethyl glucose was concentrated in fraction 3.

Fraction 3, which represented 1·6% of the total sugars found in the hydrolysate, rapidly crystallised. The crystal habit differed from that of 1 : 3 : 4-trimethyl fructose, but resembled that of 2 : 4 : 6-trimethyl glucose. Exhaustive comparison, by paper chromatography, made with authentic samples of the 2 : 3 : 6-, the 2 : 3 : 4-, the 2 : 4 : 6-, and the 3 : 4 : 6-isomer showed that fraction 3 consisted mainly of 2 : 4 : 6-trimethyl glucose.

Levan from Italian Rye-grass.—Air-dried levan (10 g.) was methylated through the acetate (cf. Challinor, Haworth, and Hirst, *loc. cit.*). The resulting precipitate, after being washed and dried in a high vacuum, weighed 6·8 g. (80%) (Found : OMe, 45·8%) and had $[\alpha]_D^{18}$ −54·5° in chloroform. No attempts were made to fractionate this levan by means of acetone.

Analysis of the Hydrolysis Products of Trimethyl Levan from Italian Rye-grass.—The levan (0·94 g.) was hydrolysed and the resulting sugars analysed on the partition column. Careful examination of the three methylated fructose fractions failed to reveal contaminants. The eluted fractions were : (1) 1 : 3 : 4 : 6-tetramethyl fructose (0·08 g., 1 mol.), (2) crystalline 1 : 3 : 4-trimethyl fructose (0·77 g., 11 mols.), and (3) dimethyl fructose (0·06 g., 1 mol.). Recovery of fructose components was 89·5%.

Control Experiments.—These were done to determine whether the hydrolytic procedure was responsible for the production of difructose dianhydrides or similar substances from pure samples of the appropriate monosaccharides. Ternary mixtures of previously chromatographed fructose derivatives were boiled with 0·03N-sulphuric acid for 8 hours and then isolated and chromatographed as were the methylated fructosans. In each case 1 : 3 : 4 : 6-tetramethyl and 3 : 4-dimethyl fructose were used, the trimethyl fructose being the 1 : 3 : 4- or the 3 : 4 : 6-derivative. Results are in Table 8.

TABLE 8.

			Recovered weight			
Expt. no.	Fructose component	Starting wt. (g.)	Ketose method	Gravimetric method	Dinitrosalicylate method	Characterisation of recovered sugars
1	1 : 3 : 4 : 6-Tetramethyl	0·70	70	—	70	OMe, 50·0%; $[\alpha]_D$ −28·0° (*c*, 3·0); n_D^{20} 1·4506; R_F 0·8
	1 : 3 : 4-Trimethyl	0·30	—	0·3	—	OMe, 41·0%; $[\alpha]_D$ −56·0 (*c*, 1·5); n_D^{20} (fused) 1·4661; R_F 0·6. IO_4^- gave 1·0 mol. of CH_2O
	3 : 4-Dimethyl	1·15	—	1·10	—	OMe, 29·2%; $[\alpha]_D$ −58° (*c*, 1·0); n_D^{20} 1·4850; R_F 0·4
2	1 : 3 : 4 : 6-Tetramethyl	0·25	0·29	—	0·25	R_F 0·8. Single spot uncontaminated by trimethyl sugars
	3 : 4 : 6-Trimethyl	1·78	—	1·77 *	—	OMe, 41·6%; $[\alpha]_D$ +28·1° (*c*, 3·0); n_D^{20} 1·4648; R_F 0·6. IO_4^- gave 1·0 mol. of CH_2O
	3 : 4-Dimethyl	0·11	—	0·11	—	OMe, 29·6%; $[\alpha]_D$ −61·5° (*c*, 0·6); n_D^{20} 1·4840; R_F 0·4

* Corrected for 0·4 g. of " difructose dianhydride " contaminating the tetramethyl fraction.

Attempts to Separate the Supposed " Hexamethyl Difructose Anhydride " from Tetramethyl Fructose.—A relatively large amount of the contaminated tetramethyl fructose fraction of inulin

hydrolysates was collected. Chromatography on paper did not indicate possibilities of separation in this way. Attempts to distil away the monomeric sugar at room temperature were unsuccessful as the anhydride proved to be equally volatile. Steam-distillation likewise effected no significant separation between the amounts of reducing and non-reducing components. Finally, by distillation in an apparatus of the type described by Ellis (*Chem. and Ind.*, 1934, 77) when the condenser was cooled by water and not by the usual acetone–solid carbon dioxide, a distillate was obtained which contained only 3% of reducing tetramethyl fructose. This material had $[\alpha]_D$ +62·5° (cf. McDonald, *loc. cit.*, who quotes $[\alpha]_D$ +50° for hexamethyl difructose dianhydride-I) (Found: OMe, 46·6. $C_{13}H_{32}O_{10}$ requires OMe, 45·6%).

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