339. *The Carbohydrates of Grass. Isolation of a Polysaccharide of the Levan Type.*

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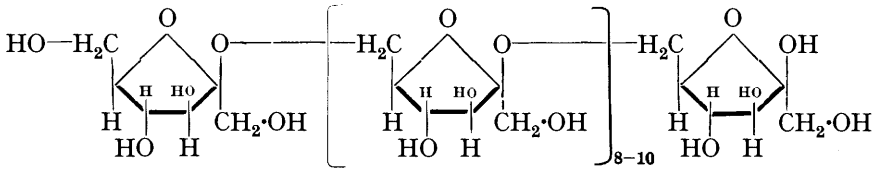
Under a scheme promoted by Imperial Chemical Industries, Ltd., we have been able to examine the carbohydrate content of rough-stalked meadow grass (*Poa trivialis*) grown at the Agricultural Research Station, Jealott's Hill, Bracknell. Other constituents of the same specimen of grass have been reported on by Dr. A. C. Chibnall and his collaborators (*Biochem. J.*, 1933, **27**, 1879; 1934, **28**, 326), to whom we are indebted for providing an aqueous extract of the dried grass from which protein and insoluble polysaccharides were absent.

Our detailed examination has revealed the presence *inter alia* of a water-soluble poly-

saccharide from which only fructose is liberated on hydrolysis. From its properties, including those of its acetyl and methyl derivatives, we have been able to relate this polysaccharide to levan, the analogue of inulin, and previously obtained only by the action of micro-organisms (*e.g.*, *B. mesentericus*) on sugars containing fructose (Harrison, Tarr, and Hibbert, *Canadian J. Research*, 1930, **3**, 449; Hibbert, Tipson, and Brauns, *ibid.*, 1931, **4**, 221). Simultaneously with this work on the identification of the soluble polysaccharide from grass we have investigated the constitution and chain-length of levan (this vol., p. 676). Its molecular structure is represented by the following formula :



Whilst the points of union of contiguous fructose residues in inulin are at the positions 1 and 2, the linking of the units in levan are through the hydroxyl groups at positions 2 and 6 in the fructo-furanose residues. Hydrolysis of methylated levan and of the methylated water-soluble polysaccharide from grass gave the same crystalline 1 : 3 : 4-trimethyl fructose. We have shown that the chain-length of levan is represented by 10—12 fructo-furanose units linked as above, and that the grass polysaccharide is identical in all its physical and chemical properties. We have not, however, been able to determine the chain-length of the grass polysaccharide, but from its general behaviour we judge that this is not very different if at all from that of levan. It is evident that this compound represents an important means of storage of carbohydrate material in meadow grass.

Of the reducing sugars present in the aqueous extract of the grass, glucose and fructose predominated and occurred in approximately equal amounts. The former was isolated as the penta-acetate (crystalline) and as the osazone. The syrupy mixture of these sugars gave phenylglucosazone in the cold, a circumstance which furnishes abundant confirmatory evidence of the presence of fructose. It does not seem to be generally known that fructose yields an osazone without heating in the presence of phenylhydrazine. In one specimen of the extract, galactose was detected and crystalline mucic acid isolated from it.

It becomes clear that in plant life reserve carbohydrate may be stored as chains of fructo-furanose units in two distinct materials : (a) as the inulin group of products, and (b) as the levan type, each differing in their mode of linking. No example is yet known of the occurrence of fructo-pyranose units in a natural polysaccharide. On the other hand, in natural products, glucose units appear to be exclusively pyranose in type. The present work affords another example of this statement in that the only disaccharide which could be identified with any certainty in the grass extract was gentiobiose, which was isolated and examined crystallographically as the octamethyl derivative.

Experimental.

Isolation of a Polysaccharide present in an Aqueous Extract of Dried Rough-stalked Meadow Grass (Poa trivialis).—An aqueous extract (23 l. from which proteins had been removed) of dried rough-stalked meadow grass (dry weight, 2780 g.) was concentrated under diminished pressure at 20° to 2200 c.c. Alcohol (4500 c.c.) was added, and the precipitate separated on the centrifuge. The solid material, which contained only traces of reducing sugars, was dissolved in water and an excess of neutral lead acetate was added to the solution, which at this stage was slightly acid to litmus (acidification, if necessary, with acetic acid). After removal of the small precipitate ammonia was added until the solution was strongly alkaline. The copious precipitate of the lead–sugar complex was collected on the centrifuge, dissolved in 30% aqueous acetic acid, the solution centrifuged, and the lead–sugar complex reprecipitated with ammonia and washed with dilute aqueous ammonia. It was then suspended in water at 60°, and the lead removed as lead carbonate by bubbling carbon dioxide through the mixture. After filtration, the solution was evaporated under diminished pressure at 40° to a thin syrup, which was poured into alcohol. The precipitated polysaccharide was purified by solution in water, followed by reprecipitation

by alcohol. It was a non-reducing white powder, soluble in water but insoluble in organic solvents (yield, 20 g.).

Further purification was effected by formation of the acetyl derivative (which can be readily purified) and subsequent de-acetylation. The polysaccharide then showed $[\alpha]_D^{20°}$ — 41° in water (*c*, 1·0). It was non-reducing, soluble in water, insoluble in organic solvents, and gave no colour with iodine [Found (for air-dried material containing 8% of moisture): C, 40·5; H, 6·7. These figures are equivalent to C, 44·1; H, 6·3 for moisture-free material. $C_6H_{10}O_5$ requires C, 44·4; H, 6·2%].

The hydrolysis of the polysaccharide in *N*/10-hydrochloric acid at 20° was followed polarimetrically. The rotation changed smoothly from $[\alpha]_D$ — 40° to — 96° in the course of 3 hours. When the final figure is calculated on the basis of concentration of sugar after hydrolysis, the value is — 87°, in close agreement with the value for fructose ($[\alpha]_D$ — 91°). Evidence for the presence of fructose was obtained by the formation at room temperature of glucosazone, m. p. 205°. No mannose phenylhydrazone could be detected and mannose was presumably absent. Before hydrolysis the aqueous solution of the polysaccharide was non-reducing and gave no blue colour when tested by Bredereck's specific colorimetric method for fructose. After hydrolysis, a colorimetric estimation by Bredereck's method indicated that a quantitative conversion of the polysaccharide into fructose had taken place. It appeared, therefore, that no sugar other than fructose is liberated during hydrolysis.

Acetylation of Polysaccharide.—The substance (1 g.) was dissolved in water (2 c.c.), and pyridine (25 c.c.) mixed slowly with the solution. Acetic anhydride (25 c.c.) was then gradually added, the temperature not being allowed to exceed 35°. After 2 days at room temperature the clear solution was slowly poured into water (400 c.c.) and the white precipitate was washed repeatedly with water until free from acid. The crude acetate (1·4 g.) was soluble in chloroform, sparingly soluble in acetone, and slightly soluble in hot alcohol. Purification of the acetate was effected by extraction with warm alcohol, followed by solution of the undissolved material in chloroform and reprecipitation by addition of light petroleum. The polysaccharide acetate so obtained was a non-reducing white powder indistinguishable in its properties from levan acetate. When heated, it softened at 110° and melted gradually over a wide range of temperature (about 100°). $[\alpha]_D^{22°}$ + 23° in chloroform (*c*, 1·0) (Found: C, 49·9; H, 5·7; CH_3·CO, 44·2. Calc. for $C_{12}H_{16}O_8$: C, 50·0; H, 5·6; CH_3·CO, 44·8%).

The rotation of the polysaccharide acetate varied somewhat according to the mode of preparation. Similar observations were recorded in control experiments on the acetylation of levan. The details of these will be published later (compare inulin acetate, Haworth and Streight, *Helv. Chim. Acta*, **1932**, **15**, 609).

Methylation of Polysaccharide.—The polysaccharide acetate was simultaneously de-acetylated and methylated with methyl sulphate and sodium hydroxide in the presence of acetone at 50°, the usual procedure being followed. The methylated derivative separated as an insoluble powder when the solution was heated at 80°, after removal of the acetone by distillation. The solid was collected, washed with hot dilute sodium hydroxide, and re-methylated. After three methylations the product was washed with small quantities of boiling water until almost ash-free. The *trimethyl* derivative was thus obtained as a grey powder (yield, 75% of the theoretical), soluble in chloroform and acetone in the cold, almost insoluble in hot water, but becoming sticky in cold water. $[\alpha]_{5780}^{17°}$ — 60° in chloroform (*c*, 1·0) (Found: C, 52·9; H, 8·1; OMe, 43·3. $C_9H_{16}O_5$ requires C, 52·9; H, 7·9; OMe, 45·6%).

Hydrolysis of Methylated Polysaccharide.—The methylated derivative (3·46 g.) was boiled for 150 minutes with 1·5% methyl-alcoholic hydrogen chloride (300 c.c.) until the rotation had reached the constant value $[\alpha]_{5780}^{20°}$ + 31°. The solution (which was non-reducing and free from furfural) was neutralised with silver carbonate and concentrated in a vacuum to a pale yellow, non-reducing syrup. This was dissolved in ether, leaving an insoluble residue (0·45 g.) which appeared to consist of incompletely hydrolysed material. After removal of the ether the mobile syrup (3·0 g.) which remained was distilled, giving (a) mobile oil (0·4 g.), b. p. 95—100°/0·03 mm. (bath temperature), $n_D^{18°}$ 1·4197; (b) mobile oil (1·5 g.), b. p. about 140°/0·03 mm. (bath temperature), $n_D^{18°}$ 1·4590 (Found: OMe, 49·2. Calc. for $C_{10}H_{20}O_6$: OMe, 52·6%); (c) viscid syrup (0·2 g.), b. p. 190—220°/0·05 mm. (bath temperature), $n_D^{18°}$ 1·4704; (d) undistillable still-residue (0·8 g., probably incompletely hydrolysed material).

Fraction (b) was non-reducing towards Fehling's solution; $[\alpha]_{5780}^{20°}$ + 9° in water (*c*, 2·5). When this substance was heated at 95° with 1·2% hydrochloric acid, the rotation altered in 35 minutes from the above value to the final constant value $[\alpha]_{5780}^{20°}$ — 43° (calc. as trimethyl fructose). The acid was then neutralised with silver carbonate and the solution was evaporated

to dryness under diminished pressure. The product was dissolved in ether to eliminate some mineral impurities and on removal of the solvent 1 : 3 : 4-trimethyl fructose was obtained as a colourless syrup (yield, almost quantitative), which rapidly crystallised after inoculation with a fragment of the authentic material. The crystals formed a solid mass which appeared to be almost free from adhering syrup. $[\alpha]_{5780}^{21°}$ — 49° in water (*c*, 1·0), final equilibrium value after completion of mutarotation. This value is close to that of authentic 1 : 3 : 4-trimethyl fructose prepared from levan ($[\alpha]_{5780}^{18°}$ — 52°, equilibrium value in water) and indicates that there was no serious contamination with other sugars. Further proof of identity was provided by recrystallising the above material from ether. This gave 1 : 3 : 4-trimethyl fructose, m. p. 75° (alone or in admixture with a sample prepared from levan). The properties of this substance were identical with those already recorded for 1 : 3 : 4-trimethyl fructose.

Reducing Sugars in Aqueous Extract of Dried Rough-stalked Meadow Grass.—The aqueous alcoholic solution from which the polysaccharide portion had been removed (see above) strongly reduced Fehling's solution. The sugars were precipitated in the usual way with basic lead acetate, and the lead removed from the insoluble lead complex as the carbonate. Any remaining dissolved lead was removed as sulphide from the aqueous solution of the regenerated sugars. The hydrogen sulphide was removed by aeration, and the solution concentrated under diminished pressure to 1 litre. The total amount of reducing sugars present at this stage was equivalent to 165 g. of glucose (obtained from 2780 g. of grass, dry-weight). Tests were made on this solution and on others obtained by similar procedure with the following results :

(a) *Aldose-ketose ratio.* Estimation of the total reducing sugars by Fehling's solution and of the aldose content by the Willstätter–Schüdel method showed that every 100 g. of reducing sugars (calc. as glucose after titration with Fehling's solution) contained 54 g. of aldose (calc. as glucose).

(b) *Qualitative tests for individual sugars.* Glucosazone, m. p. 205°, was formed readily at room temperature and in greatly increased yield at 100°. These observations together with those under (a) indicate that both glucose and fructose were present in quantity. When estimations were carried out by the specific colorimetric method of Bredereck, it was found that the fructose present amounted to one half of the total reducing sugars (the latter being estimated as glucose by Fehling's solution).

No mannose phenylhydrazone was observed and mannose was presumably absent. Negative results were obtained when the cadmium bromide–cadmium xylonate test for xylose was carried out. Qualitative tests for the presence of arabinose (as benzylphenylhydrazone) were negative. The furfural test for pentoses was inapplicable owing to the presence of fructose. It would appear, however, that at most only very small quantities of pentoses could be present. From one solution a trace of mucic acid (test for galactose) was obtained after oxidation with nitric acid, but this was not given by other batches of reducing sugars. In any case the amount of galactose present was negligible.

(c) *Acetylation of mixed sugars.* A neutral aqueous solution (100 c.c.) containing 4% of reducing sugars (calc. as glucose) was evaporated to a syrup under diminished pressure in a stream of carbon dioxide. The last traces of water were removed by simultaneous addition and distillation of glacial acetic acid under diminished pressure. The dry syrup was acetylated with acetic anhydride (200 c.c.) and anhydrous sodium acetate (30 g.). After 2½ hours at 95—100° the mixture was poured into 1500 c.c. of cold water. A viscid syrup (A) separated and after 12 hours at room temperature the supernatant aqueous solution was removed by decantation, neutralised with sodium bicarbonate, and extracted with chloroform. The chloroform extract gave 6·75 g. of neutral brown syrup, which was soluble in water. This syrup was re-acetylated, and the product separated into two fractions; (a) mobile syrup, 2·7 g. (CH_3·CO, 53%), soluble with difficulty in water, and (b) mobile syrup, 3·3 g. (CH_3·CO, 55·5%), readily soluble in water. The syrup (A) was re-acetylated and the product separated into two fractions; (c) viscid syrup, 4·05 g. (CH_3·CO, 51%), and (d) mobile syrup, 1·4 g. (CH_3·CO, 52·2%). Unsuccessful attempts were made to obtain crystalline products from these four syrups by fractional precipitation for a series of organic solvents. In other experiments the syrups were de-acetylated and the regenerated free sugars were examined. No crystalline product except glucose was obtained.

The remaining portions of the four syrups (a), (b), (c), and (d) were then united and joined with another batch of acetylated sugars prepared similarly to (A) (see above). The mixture was extracted with boiling ether and the ether-soluble portion (10 g.) was distilled under diminished pressure, giving (1) 0·8 g., yellow mobile oil, b. p. 190—200°/0·02 mm. (bath temperature), (2) 5·6 g., pale yellow, viscid oil, b. p. 190—215°/0·03 mm. (bath temperature), (3) 2·75 g., pale yellow, viscid oil, b. p. 210—230°/0·03 mm. (bath temperature). Fractions (2) and (3) crystal-

lised on trituration with ether. The syrupy portions were removed by spreading the mixture on porous tile and the solid material was recrystallised from alcohol, giving β-penta-acetyl glucose, m. p. 133°, $[\alpha]_{5780}^{20°}$ + 3° in chloroform (*c*, 1·03). The yields were 1·2 g. from (2) and 1·3 g. from (3). The syrupy portions of (2) and (3), after recovery from the tile, again deposited crystals of β-penta-acetyl glucose. The total quantity of the latter substance in fractions (2) and (3) was at least 4 g.

When crystallisation of β-penta-acetyl glucose in (3) had ceased, the remaining syrup was dissolved in alcohol and on removal of the solvent the clear syrup which remained slowly deposited a fresh crop of crystals. These were recrystallised from alcohol, giving needles, m. p. 192—193°. From solubility, m. p., and b. p., this material may have been an acetylated disaccharide. For confirmatory evidence of the presence of disaccharides in the sugar mixture see the following section.

Methylation of Mixed Sugars.—The solution (300 c.c., containing approx. 4% of reducing sugars calculated as glucose) was concentrated under diminished pressure in a stream of carbon dioxide to 100 c.c. Methylation was then effected in the usual way by methyl sulphate (119 c.c.) and 30% aqueous sodium hydroxide (267 c.c.) in the presence of acetone. The early stages of the methylation were carried out at 35° and considerable reducing power remained even after the addition of half the total quantity of the reagents. The mixture was then kept for 12 hours without further addition of reagents and was then non-reducing. The methylation was completed in the usual way, and the product extracted with chloroform. The syrup so obtained was remethylated with methyl sulphate and afterwards with methyl iodide and silver oxide. The final methylated product (19 g.) was a mobile syrup which consisted of a complex mixture of methylated monosaccharides and disaccharides, the relative proportion of monosaccharide and disaccharide being approximately 7 to 1. After a prolonged series of fractional distillations two fairly homogeneous fractions were isolated: (a) 8 g., colourless mobile oil, b. p. 95—105°/0·05 mm. (bath temperature), $n_D^{16°}$ 1·4444 (Found : OMe, 59·6%). The properties of this material indicated that it was a mixture of fully methylated hexoses (some pentose derivatives may have been present also, but could not be detected by the furfural reaction since fructose was known to be present). On hydrolysis with 6·5% hydrochloric acid a syrup was obtained which crystallised on nucleation with 2 : 3 : 4 : 6-tetramethyl glucopyranose. The solid material was separated on porous tile and was identified as 2 : 3 : 4 : 6-tetramethyl glucopyranose, m. p. 86° alone or in admixture with an authentic specimen (yield, 3 g.). No other crystalline substance was isolated from the syrup after hydrolysis. The anilides formed when the syrup was boiled with aniline in alcoholic solution were syrups.

(b) 1·3 G., non-reducing, viscid, pale yellow oil, b.p. 190—210°/0·03 mm., $n_D^{16°}$ 1·4668 (Found : C, 52·6; H, 8·7; OMe, 52·5. Calc. for $C_{20}H_{38}O_{11}$: C, 52·9; H, 8·4; OMe, 54·6%). This fraction had the composition of a fully methylated disaccharide. When kept, it partly crystallised. Crystallographic examination of the solid material showed that the latter was in all probability heptamethyl methylgentiobioside. Since no detectable hydrolysis took place when the syrup was heated for 30 minutes with *N*/10-hydrochloric acid at 60°, it follows that no appreciable amount of methylated sucrose was present. Hydrolysis occurred when the syrup was heated with 5% hydrochloric acid at 100° for 2 hours; $[\alpha]_D^{20°}$ + 60° (initial value) → + 70° on completion of hydrolysis. The solution was neutralised with barium carbonate and extracted with chloroform. Evaporation of the chloroform left a pale yellow, strongly reducing syrup (0·42 g., $n_D^{23°}$ 1·4587, $[\alpha]_D$ + 75°, equilibrium value in water). This partly crystallised when kept, giving 2 : 3 : 4 : 6-tetramethyl glucopyranose, and evidently consisted mainly of the latter substance.

The aqueous solution was then evaporated to dryness under diminished pressure. The organic material was extracted with boiling chloroform. It was a stiff syrup (0·38 g.) which reduced Fehling's solution strongly; $n_D^{21°}$ 1·4713, $[\alpha]_D^{20°}$ + 75°, equilibrium value in water (*c*, 0·4). It failed to show any sign of crystallisation when inoculated with a fragment of authentic 2 : 3 : 6-trimethyl glucose.

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