248. *The Quantitative Separation of Methylated Sugars.*

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Two methods for the quantitative separation of fully methylated sugars from their partly methylated derivatives are described. The first is a standardised procedure for the quantitative chromatographic separation of methylated sugar glycosides on activated alumina by means of which quantities as small as 245 mg. of tetramethyl methylglucoside can be separated from a large excess of 2 : 3 : 6-trimethyl methyl-*d*-glucoside. In the second method the fully methylated sugar glycoside, which may weigh as little as 36 mg., can be recovered from admixture with a large excess of admixed partly methylated sugar glycosides by preferential extraction of the fully methylated derivative with light petroleum from water using two continuous extraction apparatuses, one fitted above the other.

Detailed knowledge of structure in the carbohydrate group has been largely dependent upon the possibility of separating and identifying the various components of the mixture of methylated sugars obtained on hydrolysis of the methyl ethers of the materials under investigation. The quantitative aspect of this problem assumed special importance in the development of the chemical end group method applied by Haworth and Machemer (*J.*, 1932, 2270) in their determination of the chain length of cellulose. This involved the quantitative separation, by fractional distillation, of a very small amount of tetramethyl glucose from a larger amount of 2 : 3 : 6-trimethyl glucose, similar problems being met with in studies on starch and glycogen. Even in this comparatively simple case complications are encountered in the course of the fractional distillation of the mixture of methylated methylglucosides, and special methods had to be adopted in order to obtain accurate results (see Hirst and Young, *J.*, 1938, 1247; Averill and Peat, *ibid.*, p. 1244; Peat and Whetstone, *J.*, 1940, 276). In the course of attempts to overcome the difficulties inherent in the method of separation by distillation other procedures have been elaborated, including, for example, (*a*) phosphorylation of the partly methylated glycosides followed by extraction of the fully methylated glycoside from aqueous solution by organic solvents (Hess and Neumann, *Ber.*, 1937, **70**, 710; Leckzyik, *ibid.*, 1938, **71**, 829; Hess, Grigorescu, Steurer, and Forham, *Ber.*, 1940, **73**, 505), and (*b*) separation of the sugars on silica (Bell, *J.*, 1944, 473), or chromatographic separation of the glycosides on alumina (Jones, *J.*, 1944, 333).

An outline of the method of separation of methylated sugar glycosides on alumina has already been given (Jones, *loc. cit.*). We have now standardised this method and find it so sensitive that even the α- and β-methylglucosides of tetramethyl glucopyranose may be separated partially one from another. The recovery of the sugars by this procedure amounts to 93% of the fully methylated sugar, so that a small correction is necessary in order to obtain the exact amount of end-group present.

A further method, which we have developed quantitatively on the semi-micro scale, depends upon the fact that fully methylated sugars are much more readily extracted from water with light petroleum (b. p. 38—40°) than are partly methylated sugars. Tetramethyl methylglucoside has a partition coefficient of about 10 between chloroform and water whilst the partition coefficient found for 2 : 3 : 6-trimethyl methylglucoside is about 2 between chloroform and water (cf. the work of Macdonald, *J. Amer. Chem. Soc.*, 1935, **57**, 771, who determined the coefficient for the corresponding free sugars). We have found that the partition coefficient of fully methylated glucose between light petroleum (b. p. 38—40°) and water is about 0·1 whilst the correspond-

ing figure for trimethyl methylglucoside is $<0{\cdot}01$. The ratio between these last two figures is such that we were able to develop a method for the separation of the sugar derivatives by a continuous extraction process.

In carrying out the separation two continuous extractors are fitted, one above the other. In the top apparatus is placed the mixture of glycosides dissolved in water or sodium hydroxide solution, whilst the bottom apparatus initially contains water only. The apparatus is connected to a reservoir flask containing boiling light petroleum (b. p. 38—40°). The vapours are condensed and drop through the top solution and extract a mixture of sugars, mainly the fully methylated α- and β-glycosides; the extracts are then washed by passing through the water in the lower apparatus and finally return to the reservoir. This extraction is interrupted at intervals, solvent evaporated, the residual syrup weighed, and the refractive index determined. When a refractive index, combined with optical rotatory determination, shows the extract to be free from the fully methylated derivative (Hirst and Young, *loc. cit.*), the previous extracts are combined and dissolved in water, and the whole process is repeated in a similar apparatus. Eventually, following this technique, fractions consisting of fully methylated glycosides free from partly methylated sugars are obtained. In trial experiments the recovery of fully methylated sugar glycosides was of the order of 98—102%. By the use of this method the quantitative separation of the methylglucosides of 2 : 3 : 4 : 6-tetramethyl *d*-glucose and 2 : 3 : 6-trimethyl *d*-glucose (1 : 1 mixtures), and of 2 : 3 : 4 : 6-tetramethyl *d*-galactose and 2 : 3 : 6-trimethyl *d*-glucose (1 : 1 mixtures) has been achieved. This method has also been applied successfully to the quantitative separation of one part of tetramethyl methylglucoside (36 mg.) from 24 parts of partly methylated methylglucosides (865 mg.), and can be used with reasonable accuracy for still lower concentrations of the fully methylated sugar. These methods can therefore be applied to the determination of the size of the repeating unit of methylated polysaccharides. Since the partition method is applicable also to the quantitative separation of fully methylated pentose sugars from partially methylated pentose and hexose sugars, it can be used for the quantitative determination of the sugars formed on methanolysis of the complex methyl ethers of gums and mucilages; as an example of its utility the quantitative separation of the methyl-glycosides produced on hydrolysis of methylated peanut araban is described. The results obtained are in full agreement with those obtained by using the distillation procedure (Hirst and Jones, in the press).

Experimental difficulties may arise if the methylated polysaccharide is not rigorously purified before methanolysis, since any impurities present in it which are soluble in light petroleum will contaminate the fully methylated sugar finally obtained. In the event of a coloured and impure fraction of fully methylated sugar being isolated it may be purified by distillation in an apparaus of the type described by Ellis (*Chem. and Ind.*, 1934, 77). The loss during the final distillation is less than 1 mg. on quantities of the order of 40 to 100 mg. of tetramethyl methyl-*d*-glucoside.

Experimental.

As a result of many experiments with various solvents and weights of alumina the following technique was found to be the most satisfactory for the separation of tetramethyl α- and β-methyl-*d*-glucoside from a mixture of 2 : 3 : 4 : 6-tetramethyl methyl-*d*-glucoside and 2 : 3 : 6-trimethyl methyl-*d*-glucoside.

A solution of 2 : 3 : 4 : 6-tetramethyl α-methyl- (530 mg.) and β-methyl- (515 mg.) *d*-glucoside and 2 : 3 : 6-trimethyl methyl-*d*-glucoside (1·02 g., prepared from pure 2 : 3 : 6-trimethyl *d*-glucose) in light petroleum (b. p. 60—80°; washed with sulphuric acid and sodium hydroxide solution and distilled) was passed through a column of alumina (from the British Aluminium Co., Burntisland, Scotland, activated at 360° for 4 hours; 350 g., length 18 ins., cross section 1¼ ins.), and the column developed with chloroform [dried ($CaCl_2$) and distilled]. The eluate was collected in fractions (100 c.c.) each of which was tested for the presence of sugars by the Molisch test after withdrawing a sample (5 c.c.) and removing the solvent by evaporation. After the passage of chloroform (1100 c.c.), fractions of sugar were isolated having the following properties, all rotations being measured in chloroform solution: (1) 50 mg., $n_D^{16°}$ 1·4440, $[\alpha]_D^{16°}$ +75°; (2) 60 mg., $n_D^{16°}$ 1·4436, $[\alpha]_D^{16°}$ +110°; (3) 125 mg., $n_D^{16°}$ 1·4442, $[\alpha]_D^{16°}$ +130°; (4) 90 mg., $n_D^{16°}$ 1·4437, $[\alpha]_D^{16°}$ +83°; (5) 70 mg., $n_D^{16°}$ 1·4436, $[\alpha]_D^{16°}$ +68°; (6) 105 mg., $n_D^{16°}$ 1·4420, $[\alpha]_D^{16°}$ +20°; (7) 440 mg., $n_D^{16°}$ 1·4414 (superfused), $[\alpha]_D^{16°}$ −25°. The syrup from this fraction crystallised; m. p. 37°. Total yield, 940 mg. (92% recovery). The next fractions gave a weak positive Molisch test but left no weighable residue of sugar on evaporation. The trimethyl methyl-*d*-glucoside was eluted with methyl alcohol. Yield, 1·0 g. Since the yield of fully methylated sugar which would be isolated from a partly methylated starch of weight 5 g. (an amount convenient to handle in methylation work) is about 240 mg., tetramethyl methylglucoside (245 mg., $n_D^{16°}$ 1·4440) and trimethyl methylglucoside (382 mg., $n_D^{16°}$ 1·4572) were separated. The sugars, dissolved in light petroleum (b. p. 40—60°, 50 c.c.), were passed through a column of activated alumina (18 ins. × 1¼ ins.). The alumina was then eluted with alcohol-free, dry chloroform and the following fractions of sugars isolated on concentration of 100 c.c. portions of the chloroform eluate: Fraction A (28 mg.), $n_D^{16°}$ 1·4462; Fraction B (49 mg.), $n_D^{16°}$ 1·4460; Fraction C (51 mg.), $n_D^{16°}$ 1·4449; Fraction D (24 mg.), $n_D^{16°}$ 1·4440; Fraction E (54 mg.), $n_D^{16°}$ 1·4420; Fraction F (20 mg.), $n_D^{16°}$ 1·4415. This last fraction crystallised; m. p. of solid, 37°. Two further

fractions contained a negligible amount of sugar (<1 mg.)—thereafter trimethyl methylglucoside was eluted. This yield of sugars (Fractions A to F, 226 mg.) corresponds to a yield of 93%.

Since the yield of 2 : 3 : 4 : 6-tetramethyl methyl-*d*-glucoside was 92%, a correction is necessary when applying this method to the determination of the repeating unit of methylated starch or glycogen. The technique described above is applied to the separation of approximately equal parts of tetramethyl and trimethyl methyl-*d*-glucosides. To approximate to these conditions when carrying out a determination of the repeating unit of a polysaccharide it is necessary to carry out a preliminary enrichment of the "tetra" fraction present in the hydrolysis products from the methylated polysaccharides. This is carried out by the following general procedure. The methylated polysaccharide is hydrolysed by boiling with methanolic hydrogen chloride. After neutralisation of the solution, with silver carbonate, N-sodium hydroxide (slight excess), or diazomethane, and filtration if necessary, solvent is removed at 760 mm. on the water-bath and finally in a vacuum at 25°, and the residual syrup dissolved in water (*ca.* 50 c.c.) and extracted continuously with redistilled sulphur-free light petroleum in an all-glass apparatus for about 4 hours. This procedure usually removes all the fully methylated sugar from the aqueous solvents along with about an equal weight of trimethyl methylglucoside. To guard against incomplete extraction, the water solution is again extracted with light petroleum (b. p. 38—40°) for about 4 hours, and the extracts are added to the first extract. If the sodium hydroxide neutralisation technique is used, contamination of the fraction rich in fully methylated sugar with methyl lævulate is avoided. These extracts are then combined and concentrated, and the product is weighed and subsequently separated on the column as described above.

Quantitative Separation of Fully Methylated Sugars from Partly Methylated Sugars by Partition between Water and Light Petroleum (b. p. 38—40°).—The mixture of methylated methylglucosides is dissolved in dilute sodium hydroxide solution (to remove traces of acids and esters) and extracted continuously in an apparatus consisting of two all-glass continuous extraction vessels ("Quickfit" and Quartz/EX8/23) in series. The solution of sugars (40 c.c.) is placed in the top apparatus; the second extractor contains water (75 c.c.) which serves the purpose of washing the light petroleum extracts.

In a trial experiment fully methylated glucose ($n_D^{16°}$ 1·4434; 257 mg.) and trimethyl methylglucoside ($n_D^{16°}$ 1·4572; 223 mg.) were dissolved in water (40 c.c.) and extracted in the apparatus for 2¾ hours. Concentration of the extract gave a syrup (235 mg.; $n_D^{16°}$ 1·4434; $[\alpha]_D^{20°}$ +53°, in water). Further extraction for two hours gave a syrup (20 mg.; $n_D^{16°}$ 1·4430; $[\alpha]_D^{20°}$ +40°, in water). Two further extracts of 2 hours each gave products (9 mg. and 12 mg.) which were trimethyl methylglucoside. Recovery is therefore 255 mg. or 99%.

In a second experiment tetramethyl methylglucoside (126 mg.; $n_D^{16°}$ 1·4437) and trimethyl methylglucoside (129 mg.; $n_D^{16°}$ 1·4572) were dissolved in water (50 c.c.) and extracted as before for 2½ hours. Concentration of the extract left a residual syrup (107 mg.; $n_D^{16°}$ 1·4431; $[\alpha]_D^{20°}$ +38°). Further extraction for 2 hours gave a syrup (23 mg.; $n_D^{16°}$ 1·4423; $[\alpha]_D^{20°}$ +14°). Continuation of the extraction (2 periods of 2 hours) gave on concentration a very small yield of syrup (1—2 mg.). Recovery of fully methylated glucose, 102%.

In the above two experiments the ratio of tetramethyl to trimethyl methylglucoside is about 1 : 1. With most polysaccharides, *e.g.*, starches and celluloses, the proportion of end group is much less than this, and further experiments were carried out to test the accuracy of this method when applied to mixtures containing one part of tetramethyl methylglucoside to 19—34 parts of trimethyl methylglucoside. Fully methylated glucose (140 mg.; $n_D^{16°}$ 1·4438) and trimethyl methylglucoside (2577 mg.; $n_D^{16°}$ 1·4574) were dissolved in water (50 c.c.) and extracted for 5 hours. Concentration of the extract left a syrup (C) (347 mg.; $n_D^{16°}$ 1·4510). Further extraction with light petroleum gave a syrup (93 mg.; $n_D^{16°}$ 1·4550) which contained no fully methylated glucose since on extraction in the same way for 5 hours it yielded trimethyl *d*-methylglucoside (7 mg.) only. Accordingly the extract (C) (347 mg.) was dissolved in water (50 c.c.) and extracted once again for 5 hours. Yield of syrup, 138 mg.; $n_D^{16°}$ 1·4434; $[\alpha]_D^{20°}$ +60° (in water). Further extraction for two hours gave the partly methylated glucose (16 mg.), $n_D^{16°}$ 1·4562. Recovery of fully methylated glucose is therefore 138 mg. (98·5%).

In another experiment a mixture of tetramethyl (53 mg.; $n_D^{16°}$ 1·4438) and 2 : 3 : 6-trimethyl methyl-*d*-glucoside (1715 mg.; $n_D^{16°}$ 1·4574) and 2 : 3-dimethyl methylglucoside (157 mg.; m.p. 79°) were put through this double procedure which resulted in a recovery of 98% of the fully methylated sugar (52 mg.; $n_D^{16°}$ 1·4441; $[\alpha]_D^{20°}$ +80°).

In a further experiment a mixture of tetramethyl methylglucoside (36 mg.; $n_D^{16°}$ 1·4440), 2 : 3 : 6-trimethyl methylglucoside (865 mg.; $n_D^{16°}$ 1·4582), and 2 : 3-dimethyl methylglucoside (26 mg.; m. p. 79°) was extracted in the same way and gave a recovery of the fully methylated sugar of 100% (36 mg.; $n_D^{16°}$ 1·4441; $[\alpha]_D^{20°}$ +80° in water).

Separation of Tetramethyl Methylgalactoside and 2 : 3 : 6-Trimethyl Methylglucoside.—Heptamethyl methyl-lactoside (199 mg.; m. p. 77—79°) was dissolved in boiling methanolic hydrogen chloride (25 c.c.; 1%) and hydrolysed by boiling the solution for 8 hours. The solution was then neutralised with N-sodium hydroxide and concentrated in a vacuum to a syrup which was dissolved in water (50 c.c.) and extracted continuously with light petroleum (b. p. 38—40°) in the standard apparatus. Continuous extraction of the aqueous solution for 9 hours gave tetramethyl methylgalactoside [105 mg. (96%); $n_D^{16°}$ 1·4492] on concentration of the extracts. Further extraction for 3 hours gave, on removal of the solvent, < 3 mg. of syrup. The syrup (105 mg.) was hydrolysed with boiling N-hydochloric acid, and the syrupy 2 : 3 : 4 : 6-tetramethyl *d*-galactose isolated in the usual manner and identified by conversion into the corresponding crystalline anilide, m. p. and mixed m. p. with an authentic specimen, 190°. The aqueous solutions remaining in the apparatus were then combined and extracted continuously with chloroform. Removal of the solvent left 2 : 3 : 6-trimethyl methylglucoside [102 mg. (98%); $n_D^{16°}$ 1·4574], identified after hydrolysis with boiling N-hydrochloric acid as 2 : 3 : 6-trimethyl *d*-glucose.

Separation of the Hydrolysis Products of Methylated Peanut Araban.—(*a*) Methylated peanut araban (4·4 g.) was boiled with methanolic hydrogen chloride (1%; 100 c.c.) during 7 hours. The cooled solution was neutralised with silver carbonate and filtered. The filtrate on concentration gave a mixture of glycosides (5·2 g.) which was fractionated. A portion of this syrupy product (1·519 g.) was dissolved in water

(50 c.c.) and extracted continuously in a double extractor with purified light petroleum (b. p. 38—40°). The following fractions were obtained: Fraction 1 (0·498 g.), $n_D^{22°}$ 1·4380; Fraction 2 (0·039 g.), n_D^{20} 1·4439; Fraction 3 (0·53 g.), $n_D^{19°}$ 1·4510. Fractions 1 and 2 contained 0·517 g. of trimethyl methyl-*l*-arabinoside corresponding to a yield of 32% (calculated on the weight of glycosides) (Found by the distillation method: 32·2%).

(*b*) A further portion of the methanolysis product (1·31 g.) of methylated peanut araban was dissolved in water (50 c.c.) and extracted continuously in a double extractor as described above. The following fractions were isolated: 2 : 3 : 5-Trimethyl methyl-*l*-arabinoside (0·42 g.), $n_D^{16°}$ 1·4390 (Found: OMe, 59·7. Calc. for $C_9H_{18}O_5$: OMe, 60·2%); 2 : 3-dimethyl methyl-*l*-arabinoside (0·40 g.), $n_D^{16°}$ 1·4530 (Found: OMe, 48·2. Calc. for $C_8H_{16}O_5$; OMe, 48·4%). The residual aqueous solution on concentration gave 2-methyl methyl-*l*-arabinoside (0·46 g.), $n_D^{16°}$ 1·4749 (Found: OMe, 37·2. Calc. for $C_7H_{14}O_5$: OMe, 34·8%). Total recovery of sugars, 98%. These figures indicate that trimethyl, dimethyl, and monomethyl methyl-*l*-arabinoside were present after methanolysis in equimolecular proportions.

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