127. *Analysis of Mixtures of 2 : 3 : 4 : 6-Tetramethyl Glucose with 2 : 3 : 6-Trimethyl and Dimethyl Glucoses by Partition on a Silica–Water Column : A Small-scale Method for Investigating the Structures of Glucopolysaccharides.*

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By using a method of partition between organic solvents and water held in a column of silica gel, amounts of 50—200 mg. of 2 : 3 : 4 : 6-tetramethyl glucose can be quantitatively separated from 2 : 3 : 6-trimethyl glucose present in 1—200 molecular proportions. Separation of dimethyl glucoses from the tri- and tetra-methyl sugars can also be effected. The method is thus suitable for the investigation of relatively small amounts of the appropriate polysaccharides. The results of analyses of specimens of methylated glycogens and whole starch show that, with respect to end-group assay, the accuracy of the method is comparable with the distillation procedure. Isolation and estimation of the dimethyl glucose fractions are greatly facilitated. Clear-cut separation of sugars is obtained without the complication of mixed fractions.

At present the structure of oligo- and poly-saccharides is best investigated by analysis of the hydrolysis or methanolysis products of the methylated substances. Haworth, Hirst, and others have developed the methods of Purdie and of Irvine to produce the gravimetric method of " end-group assay " based on the fractional distillation of the methylated constituent radicals in the form of their methylglycosides (Haworth and Machemer, J., 1932, 2270; Hirst and Young, J., 1938, 1247; Peat and Averill, J., 1938, 1244; Peat and Whetstone, J., 1940, 276). Such analyses have often revealed the existence of " unit-chains " of monosaccharide radicals, each terminating in a characteristic " end-group." Furthermore, evidence has been obtained for the presence of certain radicals apparently concerned in glycosidic links between adjacent unit chains in such a manner that the whole assemblage or macromolecule possesses, in Haworth's term, a " laminated " structure.

Provided sufficient material be available, end-group assay is accurate with respect to the determination of actual terminal radicals, *e.g.*, tetramethyl methylhexosides terminating unbranched unit chains of hexose radicals. The method suffers from two disadvantages. First, by fractional distillation it is impossible quantitatively to obtain absolute separation of a higher from a lower methylated glycoside *; mixed fractions can, however, be handled with reasonable accuracy, as Hirst and Young (*loc. cit.*) have shown in the case of 2 : 3 : 4 : 6-tetramethyl and 2 : 3 : 6-trimethyl methylglucosides (see also Bacon, Baldwin, and Bell, *Biochem. J.*, 1944, **38**, 198). The second and more serious drawback is the tendency of partly methylated sugars, during the preparation of their methylglycosides, to undergo autocondensation and demethylation to an extent at present unpredictable. This introduces uncertainty into the assay of non-terminal radicals. Such is certainly the case with polysaccharides of the amylose, amylopectin, and glycogen types, where estimation of the dimethyl glucose radicals, which occur in only small proportion, affords important evidence in favour of a laminated aggregation of unit chains (cf. Freudenberg and Boppel, *Ber.*, 1940, **73**, 609).

We desired to estimate not only the end-groups, but also the radicals which yield dimethyl glucoses, in polysaccharides of the above-mentioned types. To avoid the complicating feature of autocondensation we considered the problem of separating the free methylated sugars. Macdonald (*J. Amer. Chem. Soc.*, 1935, **57**, 771) has shown that the partition coefficients of 2 : 3 : 4 : 6-tetramethyl and 2 : 3 : 6-trimethyl glucoses differ by a factor of 100, thus allowing quantitative separation of the two sugars by customary partition procedure. The manipulations involved, however, are unsuitable for the small quantities we desired to estimate, although for amounts of the order of grams, simple partition methods are practicable for assaying 2 : 3 : 4 : 6-tetramethyl glucose, and have in fact been used to estimate unit-chain lengths (Bell, *Biochem. J.*, 1935, **29**, 2031; 1936, **30**, 1612; Hassid and Dore, *J. Amer. Chem. Soc.*, 1937, **59**, 1503). By partitioning between chloroform and water held in the form of a rigid column by means of silica gel, we have achieved, on a small scale, absolute separation of

* See, however, Levi, Hawkins, and Hibbert, *J. Amer. Chem. Soc.*, 1942, **64**, 1957.

2 : 3 : 4 : 6-tetramethyl and 2 : 3 : 6-trimethyl glucoses. The procedure is analogous to the method of partition chromatography used by Gordon, Martin, and Synge for the separation of acetamido-acids (*Biochem. J.*, **1941, 35, 1388**; **1943, 37, 79**), and applied by Elsden (*Biochem. J.*, in the press) to the separation of short-chain fatty acids (cf. Smith, *Biochem. J.*, **1942, 36**, xxii).

We have further achieved the separation of trimethyl from dimethyl glucoses by partitioning between the silica–water column and a mixture of chloroform with one-tenth of its volume of *n*-butanol. This mixed solvent extracts from water nearly 12 times as much 2 : 3 : 6-trimethyl glucose as does chloroform alone. In both separations the individual sugars are recovered in high yield and analytically pure within the limitations of the methods employed.

The operations are simple and reliable, provided the precautions detailed below be adopted. Using artificial mixtures of known composition, we have been able to assay 2 : 3 : 4 : 6-tetramethyl glucose in amounts ranging from 50 to 200 mg. in the presence of 1—200 molecules of 2 : 3 : 6-trimethyl glucose. The method has further been successfully tested on methylated derivatives of cellobiose, glycogen, and whole starch. The actual materials had all been previously analysed on a large scale by the distillation method. Results are shown in the following table. Roman numerals indicate: (I) results obtained by the partition method, (II) results obtained by the fractional distillation procedure.

Substance.	Methylated cellobiose.	Methylated glycogen (horse muscle).	Methylated glycogen (*Ascaris*).	Methylated whole starch (rice).	
Weight analysed (g.)	0·1600	1·124	0·9824	3·120	1·539
Number of radicals, to each "end-group":					
"Trimethyl" (I)	1·00	8·95	12·4	21·2	22·6
(II)	1·00	8	12·7	28·1 *	
"Dimethyl" (I)	—	2·05	1·7	4·5	4·5
(II)	—	1·9	0·8	*	
Total number of radicals in average unit-chain: (I)	2	12	15	26—28	
(II)	2	12	13—14	29	

* The non-terminal radicals were estimated together as one fraction.

Experimental.

Obligatory Precautions.—(*a*) Apparatus must be free from grease; stop-cocks may be lubricated with graphite. (*b*) Organic solvents must be distilled from all-glass apparatus; chloroform is thoroughly washed with water before distillation and may conveniently be stored in a large separating funnel, under a layer of water. (*c*) Care must be taken to free substances for analysis from all traces of organic contaminants of low volatility, such as acetone condensation products. (*d*) To guard against autocondensation of sugars, all chloroform solutions must be evaporated in the presence of small amounts of barium carbonate (cf. Purdie and Irvine, J., 1905, **87**, 1022).

Preparation of the Silica.—The instructions of Gordon, Martin, and Synge (*loc. cit.*, 1943) must be strictly followed, but addition of indicator to the silica is omitted. Prolongation of the period stated for "ageing," or delay in completing the washing of the aged material, enhances the absorptive properties of the silica and thus hinders rapid partition.

Preparation of the Silica–Water Column.—One part of dry silica is ground (draught cupboard) in a mortar, and one-half of its weight of water stirred into the powder with further grinding to ensure efficient mixing. A glass tube, of dimensions appropriate to the operation (see below), is loosely plugged at one end by cotton-wool resting on a removable, perforated, porcelain disc. The moist silica is made into a slurry with chloroform and poured into the tube. The gel rapidly packs into a column on top of the plug. The supernatant chloroform is allowed to drain through the gel and the column is then degreased by running through two "column-lengths" of chloroform, one after the other. The apparatus is then ready for use.

Testing the Rate of Partition of the Column.—Different batches of silica show variations in absorptive properties. We therefore carry out an empirical test on each batch. Three columns, each of 2·5 g. of silica, are prepared in tubes of 10 mm. diameter; degreasing is not necessary. One ml. each of chloroform solutions (1 mg./ml.) of 2 : 3 : 4 : 6-tetramethyl and 2 : 3 : 6-trimethyl glucoses are carefully pipetted on to the surface of each column and the liquid allowed to sink in. One column-length of chloroform is then allowed to pass through each column. One tube is now ready for examination. The second tube is then treated with four column-lengths of chloroform, and the third tube held in reserve. The first and the second column are now expressed from the tubes by placing a glass-rod against the base-plug and smoothly drawing the tube back, over the plug, so as to leave the extruded column lying on a glass or porcelain plate. The gel is then dried at 110° and allowed to cool. Fine drops of 2% alcoholic α-naphthol are then applied at regular intervals along the length of the dried columns, followed by similar "spotting" with concentrated sulphuric acid. A deep mauve coloration (Mölisch reaction) indicates the areas occupied by the sugars, obvious threads of cellulose being disregarded. Both columns will show a pronounced band at the top (trimethyl sugar), and the first should show a second band (tetramethyl sugar) near the base, with little or no "tailing." If the silica is "good" (low absorption), the second column will show only the trimethyl band, for all the tetramethyl glucose will have been eluted. Should the second column still retain tetramethyl sugar, the reserve column should be treated with eight column lengths and examined as before. If, in its turn, this column still retains tetramethyl glucose, the batch of silica must be rejected. As the relative rates of movement on the column of the two sugars differ so greatly, complete separation is easily accomplished.

Procedure for Hydrolysis of Methylated Glucopolysaccharides.—The following procedure has given satisfactory results with derivatives of polysaccharides linked between positions 1 and 4 in both α- and β-orientations. Hydrolysis must be complete; if it is not, oligosaccharides will appear in the "tetramethyl fraction." The substance, dissolved in a mixture of 5 parts of glacial acetic acid with 10 parts of 5% (w/v) hydrochloric acid, is heated for 5 hours on the boiling water-bath. After cooling a slight excess of saturated lead acetate is added, and the precipitate of lead chloride filtered off and washed with a little ice-water. Hydrogen sulphide is passed through the filtrate and washings to precipitate lead ions, lead sulphide is filtered off, and the colourless solution, with frequent additions of water, is distilled in a vacuum below 50° to remove acetic acid. The solution must not be evaporated to dryness, as condensation between sugar molecules is catalysed by traces of acid. Chloride ions are removed from the acetic acid-free solution by addition of silver carbonate, followed by filtration and precipitation of colloidal silver from the filtrate by hydrogen sulphide. After

filtration through kieselguhr, the solution may be either evaporated to dryness in a vacuum, or directly used for analysis as described in the following section.

Analysis of the Hydrolysate.—The following sequence of operations covers a wide range of possibilities.

(I) An amount of the hydrolysate containing 100—200 mg. of tetramethyl glucose dissolved in 10—15 parts of water is filtered through charcoal into a graduated separating-funnel, and the final concentration brought to approximately 5% by water washings. The solution is shaken nine times with its own volume of chloroform, and the latter evaporated, without dehydration, at ordinary pressure. The extract contains all the tetramethyl glucose, and, in addition, about 10% of the trimethyl sugar of the hydrolysate. If the amount of the latter sugar extracted is suspected to exceed 300 mg., a second partition, similar to the first, must be carried out, in order not to overload the column.

(II) The sugars, extracted in (I), are dissolved in chloroform and quantitatively transferred by pipette to a column prepared from 25 g. of silica in a tube of 40 mm. diameter. When the solution has completely entered the column, the tetramethyl glucose is eluted by passage of the requisite number of column-lengths of chloroform. This number is determined by the routine test on the batch of silica used, with the addition of two column-lengths to provide a factor of safety. A good silica thus requires 5 + 2 = 7 column-lengths, whereas poor material requires 9 + 2 = 11. The eluate is evaporated, and dried for an hour by means of the water pump over sulphuric acid and solid sodium hydroxide.

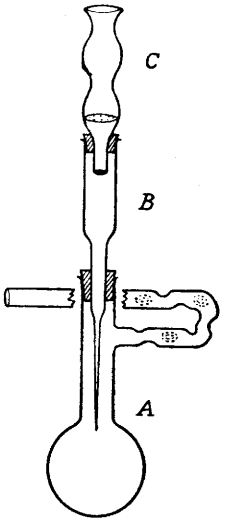
(III) The tetramethyl glucose from (II), dissolved in light petroleum–dry ether (3 : 1), is pipetted into the evaporation apparatus (see below). The residual sugar is dried to constant weight at the vacuum of the water pump.

(IV) The aqueous phase from (I) is shaken with an equal volume of chloroform–butanol (9 : 1). This extracts approximately 15% of the trimethyl sugar present. The process is repeated until not more than 500 mg. of the latter remain in the aqueous phase, which is then evaporated to dryness under reduced pressure. The residual sugars are dissolved in the chloroform–butanol mixture, and pipetted on to the same column as before. The trimethyl glucose is eluted by chloroform–butanol, the number of column-lengths required being the same as that employed in (II).

(V) The chloroform–butanol solutions from (IV) are united and evaporated to dryness, water being added to assist the removal of the butanol, under reduced pressure. The residual trimethyl glucose is dissolved in ether–acetone (2 : 1), the solution made up to a definite volume, and an aliquot, corresponding to about 1 g. of sugar, concentrated in the evaporation apparatus. The sample is dried to constant weight in a high vacuum.

(VI) The column is expressed from the tube, and the dimethyl sugar (and any traces of lower homologues) extracted by washing the gel five times with 100-ml. lots of acetone. The resulting solution is evaporated to dryness, and the residue, dissolved in warm, dry ethyl acetate, transferred to the evaporation apparatus and finally dried to constant weight in a high vacuum.

The Evaporation Apparatus (see Fig.).—This consists of three parts. *A* is a small flask of about 5 ml. bulb capacity. A side tube inserted in the neck is bent upwards and backwards though an angle of 180° and is " dimpled " at intervals to provide baffles. *B* is an adapter with the narrow limb drawn into a capillary which reaches just into the bulb of *A*. *C* is a small sintered-glass filter (G4). On the filter disc is packed a 2-mm. layer of barium carbonate covered by a further 2-mm. layer of charcoal. These absorbents remove traces of impurities from the sugar solutions. After being weighed, *A* is attached to *B* and *C* and warmed in a small air-bath at 70—80°. Gentle suction is applied to the side limb of *A* by a water pump (manometer about 730 mm. Hg), and the solution to be evaporated is pipetted, in small portions, through *C*. Excessive spurting in *A* must be regulated by the length and bore of the capillary of *B* and by choosing a suitable degree of vacuum. When the solution and necessary washings have been evaporated to a syrup, which may crystallise spontaneously, the inside of the adapter and the outside of the capillary are washed with a few drops of dry acetone. Solvents remaining in *A* are removed by applying full vacuum for 5 minutes. *A* and its contents are then dried to constant weight in a vacuum desiccator over sulphuric acid and solid sodium hydroxide.



Typical Recovery Experiments upon Artificial Mixtures.—(i) A mixture of 183 mg. of tetramethyl and 114 mg. of 2 : 3 : 6-trimethyl glucose was dissolved in chloroform and analysed on a column of " good " silica. The chloroform eluate yielded 171 mg. of tetramethyl glucose (93%), and the chloroform–butanol eluate yielded 107 mg. (92%) of the trimethyl sugar. Both sugars were recovered in analytically pure condition.

(ii) 147 Mg. of tetramethyl glucose and 102 mg. of trimethyl glucose were analysed on a column of " poor " silica; 138 mg. (93%) and 94 mg. (92%) were the respective amounts of the two sugars recovered analytically pure.

(iii) A mixture composed of 58·6 mg. (1 mol.) of tetramethyl and 11,220 mg. (200 mols.) was subjected to the complete processes of hydrolytic and partition treatments before being analysed on the column. The two sugars were recovered in respective amounts of 50·0 mg. (86%) and 10,550 mg. (94%), *i.e.*, 1 : 224 mols.

(iv) Experiments with dimethyl glucoses gave recoveries of the same high order.

Analysis of Heptamethyl β-Methylcellobioside.—160 Mg. were hydrolysed, yielding 145·5 mg. of mixed sugars (approx. 90% recovery). Separation on the column yielded: (*a*) crystalline 2 : 3 : 4 : 6-tetramethyl glucose, 71 mg. (95%) (Found: OMe, 52·3. Calc.: OMe, 52·5%), $[\alpha]_D$ (water) +82·5°; (*b*) crystalline 2 : 3 : 6-trimethylglucose, 66·0 mg. (94%) (Found: OMe, 41·4. Calc.: OMe, 41·9%), $[\alpha]_D$ (water) +70°. The two sugars were thus recovered in equimolecular proportion.

Simple End-group Assay on Methylated Glycogen (*Horse Muscle*).—The material used (OMe, 44·7%) was the actual sample shown by the distillation method to have a unit chain length of 11—12 radicals (Bell, *Biochem. J.*, 1937, **31**, 1683). 2013 Mg. were hydrolysed, yielding 2000 mg. of mixed sugars (approx. 92% recovery), which were dissolved in water and the volume made up to 50 ml. The solution was divided into two portions for separate analyses on the silica–water column. (*a*) Analysis of 1000 mg. of the mixed sugars yielded 78·8 mg. of crystalline tetramethyl glucose (Found: OMe, 52·1%), $[\alpha]_D$ (water) +83°; after allowance for a 6% loss on column working, this corresponds to an end-group percentage of 8·4, *i.e.*, a unit-chain length of 11—13 radicals. (*b*) 940 Mg. of mixed sugars yielded 77 mg. of crystalline tetramethyl glucose (Found: OMe, 52·1%), $[\alpha]_D$ (water) +80°, again corresponding to a unit-chain length of 11—13 radicals.

Complete Analysis of Glycogen (*Horse Muscle*).—1124 Mg. of the same material as that used in the above experiments yielded, after hydrolysis, 1128 mg. of mixed sugars (recovery, approx. 93%). Analysis on the column gave the following results:

Methylated sugar.	Found, mg.	OMe, %.	$[\alpha]_D$ (water).	Molecular ratio.
Tetramethyl	93	52·1	+80°	1
Trimethyl	783	41·7	+70	8·95
Dimethyl	168	28·2	—	2·05

Unit-chain has average length of 12 radicals.

Complete Analysis of Glycogen (Ascaris lumbricoides).—The material used was the actual sample of methylated glycogen analysed by the distillation method by Baldwin and King (*Biochem., J.*, 1942, **36**, 37), in this laboratory, and found to have a unit-chain length of 13—14 radicals. 982·5 Mg. of material yielded 980·4 mg. of hydrolysis products (recovery, approx. 93%). Analysis on the column gave the following results :

Methylated sugar.	Found, mg.	OMe, %.	$[\alpha]_D$ (water).	Molecular ratio.
Tetramethyl	63	52·5	+83°	1
Trimethyl	748	41·7	+71	12·4
Dimethyl	96	29·8	—	1·7

Unit-chain has average length of 15—16 radicals.

Complete Analysis of Whole Rice Starch.—A commercial sample was methylated directly (Peat and Whetstone, *loc. cit.*) until a methoxyl content of 43·1% was attained. The sample displayed the expected physical properties, and in aqueous solution gave an intense blue with iodine–potassium iodide solution. The analysis was performed in duplicate, with the results shown below.

(i) 3120 Mg. gave 3225 mg. of hydrolysis products (recovery, approx. 95%).
(ii) 1539 Mg. gave 1437 mg. hydrolysis products (recovery, approx. 88%).

	Found, mg.		OMe, %.		$[\alpha]_D$ (water).		Molecular ratio.	
Methylated sugar.	(i).	(ii).	(i).	(ii).	(i).	(ii).	(i).	(ii).
Tetramethyl	121	52	52·3	52·0	+84°	+82°	1	1
Trimethyl	2398	1104	41·6	41·5	+69	+70	21·1	22·6
Dimethyl	481	207	29·2	30·0	—	—	4·5	4·5

Unit-chain length has average length of 26—28 radicals.

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