199. *Quantitative Analysis of Mixtures of Sugars by the Method of Partition Chromatography. Part II. The Separation and Determination of Methylated Aldoses.*

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(*a*) Methylated derivatives of reducing sugars can be separated by the method of paper partition chromatography, and this method has been used for the identification of the sugar derivatives produced on hydrolysis of methylated polysaccharides. (*b*) The quantitative determination of methylated aldoses on the micro-scale has been achieved by use of alkaline iodine, and a method has been elaborated for quantitative determination following chromatographic separation. (*c*) As examples of the utility of these methods results obtained with the hydrolysis products from methylated derivatives of maize starch, waxy-maize starch, glycogen, and araban are described.

In Part I (Flood, Hirst, and Jones, *J.*, 1948, 1679) chromatographic separations of reducing sugars and their subsequent determination by use of Somogyi's copper reagent were described.

An extension of this work to the methylated sugars has now shown that the partition method of chromatography using filter-paper is exceedingly sensitive in that it allows of the separation of closely related methylated sugar derivatives. For example, 2 : 4 : 6-trimethyl glucose can be identified in the presence of 2 : 3 : 6-trimethyl glucose, and 2 : 3-dimethyl xylose can be readily separated from 2 : 4-dimethyl xylose (Brown, Hirst, Hough, Jones, and Wadman, *Nature*, 1948, **161**, 720).

This method of separation provides a semimicro-procedure for the rapid identification of the methylated sugars liberated by hydrolysis of methylated polysaccharides. 2 : 3 : 4 : 6-Tetramethyl D-glucose is used as a standard reference substance and the ratio between the distance the sugar derivative travels to the distance through which tetramethyl D-glucose has moved gives a constant (R_G) characteristic of the sugar (see Table I). When a mixture of sugars is separated in this way the R_G values give indications of the identity of the components, but it must be remembered that the formation of a single spot on the paper chromatogram is not a complete proof of the homogeneity of the sugar since a number of the methylated sugars possess similar R_G values.

In order to test the usefulness of the new method, an examination was made of the products of hydrolysis of the methylated derivatives of maize starch, waxy maize starch, glycogen, sugar-beet araban, and peanut araban. The presence of all of the methylated sugars previously isolated by fractional distillation of the mixed glycosides was readily established and indications were obtained of the presence of traces of other partly methylated sugars. The latter originated in all probability from slight under methylation of the product submitted to hydrolysis, but the method is so sensitive that products due to the very slight demethylation of methylated sugars by methanolic hydrogen chloride would be detectable despite the minute proportions in which they occur.

These results, although highly useful, are qualitative only, but if a suitable reagent could be found for the determination of methylated derivatives of reducing sugars it would be possible to separate chromatographically and determine quantitatively the sugars produced on hydrolysis of small amounts of methylated polysaccharides. It was soon found, in agreement with other workers, that copper reagents are not entirely satisfactory for the determination of the methylated sugars. Sobotka (*J. Biol. Chem.*, 1926, **69**, 297) and Jeanloz (*Helv. Chim. Acta*, 1946, **29**, 57) have demonstrated, however, that alkaline iodine can be used for the determination of methylated aldoses, and we have adapted this method for the micro-determination of reducing methylated aldoses. The procedure for the separation and extraction of the methylated sugars is identical with that previously described for the estimation of the non-substituted reducing sugars (Part I, *loc. cit.*). In contrast with the non-substituted sugars, the methylated derivatives show a wide spatial separation on the chromatogram and comparatively large quantities of the sugars can, therefore, be separated, the process of determination being thereby simplified. The sugar extracted from the filter paper is determined by oxidation with 0·1N-iodine solution, introduced from an " Agla " micrometer syringe, in the presence of a sodium carbonate–sodium bicarbonate buffer (pH 10·6). Blank experiments have shown that 0·5—3·0 mg. of methylated sugar can be determined with an accuracy of $\pm 8\%$ and that the recovery of the methylated sugars from the paper chromatogram is of the order of 96%. As examples of the utility of this procedure we record the results obtained with (*a*) a synthetic mixture of 2 : 3 : 4 : 6-tetramethyl D-glucose, 2 : 4 : 6-trimethyl D-glucose, and 2 : 4-dimethyl D-galactose, and (*b*) a mixture of 2 : 3 : 4-trimethyl D-xylose, 2 : 4-dimethyl D-xylose, 2 : 4-dimethyl D-galactose, and L-rhamnose (see Experimental section).

The unsubstituted pentose and hexose sugars can also be determined by alkaline hypoiodite under carefully controlled conditions of alkalinity (Hawthorne, *Nature*, 1947, **160**, 714), but in agreement with Myrbäck (*Svensk Kem. Tids.*, 1940, **52**, 293) we find that this reagent is unsatisfactory for the determination of D-mannose. L-Rhamnose, D-lyxose, and D-talose are also incompletely oxidised under these conditions. Nevertheless, D-ribose, which has the same configuration of hydroxyl groups on C_2 and C_3 as these sugars, may be determined satisfactorily. Mannose, rhamnose, lyxose, and talose can be satisfactorily determined, however, if a control determination is carried out simultaneously with the determination of the sugar, and the appropriate correction made.

As test cases for the application of the quantitative procedure to methylated polysaccharides we selected methylated waxy-maize starch and methylated rabbit-liver glycogen, using samples which had already been investigated by the standard method of methanolysis followed by fractional distillation. In both cases no less than five methylated derivatives of glucose were found chromatographically after hydrolysis. The separated sugars were determined by

oxidation with alkaline hypoiodite. The hydrolysis products from methylated waxy-maize starch had the following percentage composition: 2 : 3 : 4 : 6-tetramethyl glucose (4·2%), 2 : 3 : 6-trimethyl glucose (80%), 2 : 3-dimethyl glucose (3%), a dimethyl glucose (11%) (not previously observed; identity uncertain), and a monomethyl sugar (2%). This sample of methylated waxy-maize starch had been examined previously by Dr. T. G. Halsall by the standard procedure and the yield of tetramethyl D-glucose was 4·4%. The yield by the present method is 4·6% calculated on the weight of polysaccharide hydrolysed, and is, therefore, in good agreement with the earlier value. This applies also to the methylated glycogen, the hydrolysis products from which contained 2 : 3 : 4 : 6-tetramethyl glucose (8·5%, equivalent to 9·2% on the weight of polysaccharides), 2 : 3 : 6-trimethyl glucose (70%), 2 : 3-dimethyl glucose (9%), a dimethyl glucose (10·6%) (not previously observed; identity uncertain), and a monomethyl derivative (2·5%). This methylated glycogen, when examined by the method of methanolysis and fractional distillation had given 9% of tetramethyl glucose. A comparison of the yields of the other sugars, both from waxy-maize starch and from glycogen is not possible since reliable methods of estimation have not hitherto been available. It is of interest to note that Freudenberg and Boppel (*Ber.*, 1940, **73**, 609) found two kinds of dimethyl glucose amongst the hydrolysis products of a sample of methylated starch. The structural significance of this is, however, uncertain since some of the dimethyl glucose and probably all of the monomethyl glucose may be ascribable to the incomplete state of methylation of the polysaccharides (OMe, 43% instead of 45·6%). The new method would detect also any products resulting from the demethylation of methylated sugars by the hydrochloric acid used for the hydrolysis. In the present instances, however, these latter effects are of negligible importance.

Experimental.

(1) *Qualitative.—The chromatographic separation of methylated derivatives of sugars.* The apparatus and solvents used were similar to those described previously (Partridge, *Nature*, 1946, **158**, 270; *Biochem. J.*, 1948, **42**, 238; Flood, Hirst, and Jones, *loc. cit.*). With the aid of a capillary tube a small spot of solution containing the sugars under investigation is placed on the starting line of the paper (15 × 50 cm.). Alongside this spot and at a distance of about 3 cm. is placed a small spot of a 10% aqueous solution of tetramethyl D-glucose. The experiment is allowed to run until the solvent [the top layer of a mixture of *n*-butanol (40%), ethanol (10%), water (49%), and ammonia (1%)] is approximately 40 cm. from the starting line on the paper. The paper then is removed, dried, and sprayed with an ammoniacal solution of silver nitrate (10%); on heating, the positions of the separated sugars are indicated by brown spots. The R_G values of the separated sugars are calculated from the ratio between

Table I.

Substance.	R_G.	Substance.	R_G.
1. Galactose	0·08	32. 4-Methyl rhamnose	0·57
2. Glucose	0·09	33. 3 : 4-Dimethyl mannose	0·58
3. Sorbose	0·11	34. Glucomethylose 3-methyl ether	0·60
4. Mannose	0·12	35. 2-Deoxyrhamnose	0·61
5. Fructose, Tagatose	0·13	36. 2 : 3-Dimethyl arabinose	0·63
6. Arabinose	0·14	37. 2 : 3 : 4-Trimethyl galactose	0·64
7. Xylose	0·15	38. 2 : 4-Dimethyl xylose	0·66
8. 4-Methyl galactose	0·17	39. 3 : 4-Dimethyl fructose	0·66
9. 6-Methyl galactose	0·19	40. 2 : 4 : 6-Trimethyl galactose	0·67
10. Talose, Lyxose	0·19	41. Altromethylose 3-methyl ether	0·68
11. 2-Methyl galactose	0·21	42. 2 : 3 : 6-Trimethyl galactose	0·71
12. Ribose, Fucose	0·21	43. 2 : 3-Dimethyl xylose	0·74
13. Riboketose	0·25	44. 2 : 4 : 6-Trimethyl glucose	0·76
14. 3-Methyl glucose	0·27	45. 3 : 4 : 6-Trimethyl mannose	0·80
15. Xyloketose	0·26	46. 2 : 3 : 6-Trimethyl glucose	0·81
16. 6-Methyl glucose	0·27	47. 2 : 3 : 6-Trimethyl mannose	0·81
17. Quinovose (glucomethylose)	0·28	48. 1 : 3 : 4-Trimethyl fructose	0·84
18. Rhamnose	0·30	49. Cymarose	0·87
19. 3 : 4-Dimethyl galactose	0·32	50. 2 : 3 : 4 : 6-Tetramethyl galactose	0·88
20. 2-Deoxyallose	0·33	51. Oleandrose	0·88
21. 2-Methyl arabinose	0·36	52. 3 : 4-Dimethyl rhamnose	0·88
22. 3 : 6-Anhydroglucose	0·37	53. 1 : 3 : 4 : 5-Tetramethyl fructose	0·90
23. Rhamnoketose	0·37	54. 2 : 3 : 4-Trimethyl xylose	0·94
24. 2-Methyl xylose	0·39	55. 2 : 3 : 5-Trimethyl arabinose	0·95
25. 2 : 4-Dimethyl galactose	0·41	56. 2 : 3 : 4 : 6-Tetramethyl mannose	0·96
26. 4 : 6-Dimethyl glucose	0·46	57. 2 : 3 : 4 : 6-Tetramethyl glucose	1·0
27. 2 : 6-Dimethyl galactose	0·49	58. 2 : 3 : 4-Trimethyl rhamnose	1·01
28. 2-Methyl fucose	0·51	59. Raffinose	No movement
29. 3 : 6-Dimethyl glucose	0·51	60. Sucrose	0·04
30. 2 : 3-Dimethyl mannose	0·54	61. β-Methyl arabopyranoside	0·33
31. 2 : 3-Dimethyl glucose	0·57	62. α-Methyl mannopyranoside	0·30

the distance from the starting line to the centre of the spot and the distance through which the tetramethyl D-glucose has moved. Table I gives the R_G values of a variety of methylated sugars determined by this procedure. The values for a number of non-reducing sugars have also been determined by spraying the chromatogram with a solution of naphtharesorcinol in alcoholic hydrogen chloride (Forsyth, *Nature*, 1948, **161**, 239).

The examination of a methylated polysaccharide by this procedure is conveniently carried out with approximately 50 mg. of material which is hydrolysed with 4% methanolic hydrogen chloride (1 ml.) in a sealed tube immersed in a boiling water-bath. After hydrolysis the tube and its contents are cooled, and the tube opened cautiously. An aqueous solution of hydrochloric acid (4%; 5 ml.) is then added and the methyl glycosides are hydrolysed at 100° for 3 hours. The solution is then neutralised with silver carbonate, filtered, and the silver is removed by passage of hydrogen sulphide. After filtration, acidic materials and uronic acid derivatives are removed by the use of Amberlite Resin, IR4B, and the neutral solution evaporated to a thin syrup which is examined on the paper chromatogram. The R_G values of the separated components give an indication of identity, and further evidence is obtained by comparison on another chromatogram with an artificial mixture of the suspected sugars. Table II gives the results of the application of this procedure to some methylated polysaccharides which have previously been examined by the method of methanolysis followed by fractional distillation of the methylated methyl glycosides.

TABLE II.

Methylated polysaccharide.	R_G *Values of the separated sugars.*
Maize starch Waxy-maize starch Glycogen	1·00(57)*; 0·805(46)*; 0·57(31)*; 0·51†; 0·26†
Sugar-beet araban Peanut araban	0·95(55)*; 0·635(36)*; 0·37(21)*; 0·27‡; 0·11‡

The numbers in parentheses are those of the sugar concerned as listed in Table I.
* Previously observed by fractional distillation method.
† Not previously observed; identity uncertain.
‡ In small amount; not previously observed; identity uncertain.

TABLE III.

Substance.	Amount weighed out (mg.).	Found by titration (mg.).	Recovery, %.
L-Arabinose	1·03	1·03	100
,,	2·06	2·06	100
,,	3·09	3·07	99·5
D-Galactose	0·565	0·56	99
,,	1·13	1·13	100
,,	2·26	2·22	98
,,	2·82	2·78	98·5
D-Ribose	0·565	0·56	99
,,	1·13	1·13	100
,,	2·22	2·26	101·5
,,	2·78	2·82	101·5
D-Xylose	1·60	1·58	98·5
,,	3·20	3·16	98·5
D-Lyxose	1·18	1·06	90
D-Mannose	0·51	0·48	94
,,	1·02	0·92	90
,,	2·04	1·64	80·5
L-Rhamnose	0·97	0·87	89·5
,,	1·94	1·64	84·5
,,	2·91	2·11	72·5
D-Talose	1·26	1·17	93
2 : 4-Dimethyl D-xylose	0·475	0·49	103
,, ,,	0·95	0·935	98·5
,, ,,	1·225	1·29	105
2 : 4 : 6-Trimethyl D-glucose	0·40	0·415	104
,, ,,	0·80	0·75	94
,, ,,	1·20	1·22	101·5
,, ,,	1·60	1·58	99
2 : 3 : 6-Trimethyl D-glucose	0·555	0·53	96
,, ,,	1·105	1·065	97
,, ,,	2·21	2·20	99·5
2 : 3 : 4 : 6-Tetramethyl D-glucose	1·36	1·37	100·5
,, ,,	2·04	2·00	98
,, ,,	2·38	2·52	106

(2) *Quantitative.—Micro-determination of the aldoses and their methylated derivatives using sodium hypoiodite.* A 0·1N-solution of iodine (1 ml.) is added from an " Agla " micrometer syringe to the sugar solution (5 ml.) (which should not contain more than 2·5 mg. of sugar; otherwise oxidation will be incomplete) contained in a boiling tube (22 × 3 cm.) fitted with a B24 ground-glass stopper. A solution (2 ml.) containing 0·2M-sodium hydrogen carbonate and 0·2M-sodium carbonate (pH 10·6) is then pipetted

into the solution, and the tube stoppered. In order to prevent loss of iodine due to evaporation, the stopper is moistened with a little potassium iodide solution (10%). A control determination is carried out on 5 ml. of water contained in a similar boiling tube. After 2—2½ hours the stopper is washed, and the reaction mixture diluted to 25 ml. and then acidified by carefully running 2N-sulphuric acid down the side of the tube and avoiding unduly vigorous evolution of carbon dioxide. After acidification the tube is stoppered pending titration of the liberated iodine with 0·01N-sodium thiosulphate solution.

The method is satisfactory for the determination of glucose, galactose, arabinose, xylose, ribose, and their methylated derivatives, but with the rhamnose, lyxose, talose, and mannose theoretical values were not obtained for the unmethylated sugars. For the quantitative determination of these a standard aqueous solution of the particular sugar is analysed simultaneously, its concentration being adjusted so that 5 ml. will contain approximately the same quantity of sugar as is to be analysed. In this way an accurate determination of these four sugars can be achieved, or alternatively, the determination may be carried out by reference to a correction curve calibrated for each individual sugar.

Typical results are shown in Table III. In several instances figures are given for a series of analyses which show how the method responds to varying amounts of the sugar which is being determined.

The Determination of the Aldoses and their Methylated Derivatives after Chromatographic Separation.—In order to obtain measurable quantities of the separated sugars the method of Flood, Hirst, and Jones (*loc. cit.*) is used. The filter-paper strips thus obtained are extracted for ¾ hour with 5 ml. of water contained in a boiling-tube. The sugar solution is then cooled to 20° and analysed by the method described above.

Substance.	Quantity used (mg.).	Found by titration (mg.). Expt. 1.	Expt. 2.	Calc. from reference compound (mg.). Expt. 1.	Expt. 2.	Average recovery, %.
(*a*) *Analysis of a mixture of* D-*galactose and* L-*arabinose using* D-*ribose as the reference sugar* (*for details of method see Part I*) :						
D-Galactose	7·16	1·08	1·24	7·10	7·19	100
L-Arabinose	3·66	0·60	0·685	3·95	3·96	108
D-Ribose	5·24	0·795	0·905	—	—	100 (Ref. substance)
(*b*) *Analysis of a mixture of* 2 : 3 : 4 : 6-*tetramethyl* D-*glucose and* 2 : 4-*dimethyl* D-*galactose using* 2 : 4 : 6-*trimethyl* D-*glucose as the reference sugar :*						
2 : 3 : 4 : 6-Tetramethyl D-glucose	28·93	2·77	3·45	26·8	26·3	92
2 : 4 : 6-Trimethyl D-glucose	23·16	2·43	3·04	—	—	100 (Ref. substance)
2 : 4-Dimethyl D-galactose	12·86	1·24	1·55	12·8	12·8	100
(*c*) *Analysis of a mixture of* 2 : 3 : 4-*trimethyl* D-*xylose*, 2 : 4-*dimethyl* D-*galactose*, and L-*rhamnose using* 2 : 4-*dimethyl* D-*xylose as the reference sugar :*						
2 : 3 : 4-Trimethyl D-xylose	11·63	1·42	1·47	12·4	13·0	109
2 : 4-Dimethyl D-xylose	31·93	3·66	3·60	—	—	(100 Ref. substance)
2 : 4-Dimethyl D-galactose	22·63	2·34	2·18	22·2	21·0	96
L-Rhamnose	16·18	1·75	1·85	15·3	16·4	98

(*d*) *Examination of methylated waxy-maize starch.* The material used had OMe, 43·2%. The methylated polysaccharide (50 mg.) was hydrolysed with 4% methanolic hydrogen chloride (1 c.c.) in a sealed tube at 100°, and the resultant glycosides hydrolysed (7 hours) as described above. The methylated sugars were chromatographically separated and determined by the hypoiodite method.

R_G of sugar derivative.	Derivative of glucose indicated.	Found (mg.).	Composition, %.
1·00	2 : 3 : 4 : 6-Tetramethyl	0·28	4·2
0·81	2 : 3 : 6-Trimethyl	5·37	80·0
0·58	2 : 3-Dimethyl	0·27	4·0
0·51	3 : 6-Dimethyl	0·70	10·4
0·26	Monomethyl	0·09	1·3

(*e*) *Examination of methylated rabbit-liver glycogen.* The methylated polysaccharide (50 mg.) was hydrolysed and portions of the resultant methylated sugars were separated and determined by the method of paper partition chromatography.

R_G of sugar derivative.	Derivative of glucose indicated.	Found (mg.).	Composition, %.
1·00	2 : 3 : 4 : 6-Tetramethyl	0·14	8·7
0·81	2 : 3 : 6-Trimethyl	1·13	69·0
0·58	2 : 3-Dimethyl	0·15	8·9
0·51	3 : 6-Dimethyl	0·18	10·8
0·26	Monomethyl	0·04	2·4

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