592. *The Enzymic Synthesis and Degradation of Starch. Part IX. Methylation and End-group Assay of Some Synthetic Polysaccharides.*

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Three of the synthetic polysaccharides mentioned in Part VIII have been submitted to methylation and end-group assay, potato amylose (B.V., 1·30) and potato amylopectin (B.V., 0·19) being used as standards. The average chain lengths thus determined were: amylose, 204; amylopectin, 21; polysaccharide Ia, 150; polysaccharide IV, 19; and polysaccharide V, 17 (all ±5%).

In the preceding paper (Part VIII) it was shown that mixtures of the P- and the Q-enzyme of the potato, containing different proportions of these enzymes, synthesise from glucose-1 phosphate a range of polysaccharides of the starch class. Comparison of the polysaccharides with regard to iodine stains, β-amylolysis limits, activating powers in synthesis by P-enzyme, and complex formation with thymol indicated that in the extreme cases the products were amylose (highest proportion of P-enzyme relative to Q-enzyme) and amylopectin (lowest proportion of P-enzyme relative to Q-enzyme). We now report some results obtained by methylation and end-group assay of the synthetic polysaccharides which confirm this conclusion.

For these studies three of the synthetic polysaccharides were selected, namely, polysaccharide Ia (amylose type) and polysaccharides IV and V (amylopectin type). Samples of amylose (B.V., 1·30) and amylopectin (B.V., 0·19), obtained from potato starch by selective precipitation with thymol (Bourne, Donnison, Haworth, and Peat, *J.*, 1948, 1687), were used as standards of reference.

The five polysaccharides were methylated at −70° in liquid-ammonia suspension with sodium and methyl iodide, as described by Freudenberg and Boppel (*Ber.*, 1938, **71**, 2505), until the methoxyl content of the product exceeded 44·4%.

Hydrolysates of the polysaccharide ethers were analysed qualitatively by paper partition chromatography (Hirst, Hough, and Jones, *J.*, 1949, 928), the staining reagent being aniline hydrogen phthalate (Partridge, *Nature*, 1949, **164**, 443). The hydrolysates of the methyl ethers of potato amylopectin (OMe, 44·6%) and polysaccharide IV (OMe, 45·1%) each gave four spots (see Plate). Three of these spots had R_F values identical with those of 2 : 3-dimethyl-, 2 : 3 : 6-trimethyl-, and 2 : 3 : 4 : 6-tetramethyl D-glucose, used as standards on the same chromatogram. The fourth component in each case appeared to be present only in traces and had an R_F value slightly smaller than that of 2 : 3-dimethyl glucose. It will be recalled that Hirst, Hough, and Jones (*loc. cit.*) in an examination of a hydrolysate of methylated waxy maize starch (OMe, 43·2%) found, in addition to 2 : 3-dimethyl-, 2 : 3 : 6-trimethyl-, and 2 : 3 : 4 : 6-tetramethyl D-glucose, a second dimethyl glucose (R_G, 0·51 compared with 0·58 for the 2 : 3-isomer) and also a monomethyl glucose. They believed that the presence of the last two sugars could be attributed wholly or in part to the incomplete state of methylation of the polysaccharide. In support of this, we have found that a hydrolysate of incompletely methylated polysaccharide IV (OMe, 42·9%) afforded spots of glucose and a monomethyl glucose, in addition to those shown by the remethylated polysaccharide (OMe, 45·1%); furthermore the unidentified dimethyl glucose component gave a far more pronounced spot in the case of the partly methylated polysaccharide.

Chromatographic analysis of hydrolysates of the methyl ethers of potato amylose (OMe, 45·1%) and polysaccharide Ia (OMe, 44·4%) revealed only 2 : 3 : 6-trimethyl D-glucose. Under the same conditions a reference solution containing 2 : 3-dimethyl-, 2 : 3 : 6-trimethyl-, and 2 : 3 : 4 : 6-tetramethyl D-glucose (molecular proportions, 1 : 200 : 1) gave only one spot, that of the trimethyl sugar.

Two methods were employed for end-group assay of the methylated polysaccharides. The

Paper partition chromatography of methylated polysaccharides.

A, *Mixture containing* 2 : 3-*dimethyl,* 2 : 3 : 6-*trimethyl, and* 2 : 3 : 4 : 6-*tetramethyl glucose* (*molecular proportions,* 1 : 20 : 1).
B, *Hydrolysate of methylated synthetic polysaccharide IV.*
C, *Hydrolysate of methylated potato amylopectin.*

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first was the quantitative method of paper partition chromatography developed by Flood, Hirst, and Jones (*J.*, 1948, 1679) and later improved by Hirst, Hough, and Jones (*loc. cit.*). The second was Bell's method of partition on a silica gel (*J.*, 1944, 473), as modified by Bourne, Fantes, and Peat (*loc. cit.*). Each method was tested against known mixtures of methyl glucoses.

End-group assay of polysaccharides of the starch type.

Polysaccharide.	B. V.	Average chain length (±5%).	
		By filter paper method.	By silica gel method.
Potato amylose	1·30	—	204
Potato amylose *	1·21	—	191
Polysaccharide Ia	1·18	—	150
Potato amylose *	0·91	—	101
Potato starch *	0·40	—	26
Potato amylopectin	0·19	21	21
Polysaccharide IV	0·15	19	—
Polysaccharide V	0·12	—	17

* Figures quoted by Bourne, Fantes, and Peat (*J.*, 1949, 1109).

From the results given in the above table, it is seen that the average chain length of polysaccharide Ia (*ca.* 150 units) confirmed the conclusion reached in Part VIII that this polysaccharide was predominantly an amylose. Likewise the average chain lengths (19 and 17, respectively) of polysaccharides IV and V supported the belief that these polysaccharides were similar to potato amylopectin (chain length, 21 units). Furthermore, it can be seen that when the natural and synthetic polysaccharides are arranged together in order of decreasing blue value, then this is also the order of decreasing chain length. In view of these observations there can no longer be any doubt that P-enzyme is responsible for the synthesis of linear amylose chains, and that P- and Q-enzymes together synthesise branched polysaccharides of the amylopectin class.

EXPERIMENTAL.

Methylation of the Polysaccharides.—The method of Freudenberg and Boppel (*loc. cit.*), in which sodium and methyl iodide are added alternately to a suspension of the polysaccharide in liquid ammonia at −70°, was employed, a modified procedure being used for the isolation of the methylated product. After the last addition of methyl iodide had been made, the liquid ammonia was siphoned off at −70° by the application of a small pressure. The last traces of ammonia were removed under anhydrous conditions at 90°/12 mm. The solid residue was suspended in water and dialysed in Cellophane against frequent changes of distilled water. When iodide ions could no longer be detected in the dialysate by the silver nitrate test, the solution of the polysaccharide ether was concentrated at 40°/12 mm. to about 150 c.c and then freeze-dried. The finely-divided feathery product was subsequently dried at 60° over phosphoric oxide in a vacuum.

(a) *Potato amylose.* Amylose (B.V., 1·30; dry wt., 6·680 g.), isolated from potato starch by selective precipitation with thymol (Bourne, Donnison, Haworth, and Peat, *loc. cit.*), was methylated as above, 8 treatments with the methylating agents being given. During dialysis, part of the product separated from solution. This portion was collected in the centrifuge, washed with a small volume of water, and freeze-dried, giving fraction X (7·405 g.) (Found : OMe, 45·1. Calc. for trimethyl amylose : OMe, 45·6%). The supernatant solution afforded fraction Y (0·420 g.) (Found : OMe, 44·0%). The total yield was 93%.

It was fraction X which was subsequently employed as a standard during the examination of the methylated synthetic polysaccharides.

(b) *Potato amylopectin.* Potato amylopectin (B.V., 0·19; dry wt., 10·10 g.), isolated by the thymol method, was treated 8 times with the methylating reagents. The freeze-dried product was dissolved in the minimum volume of chloroform and precipitated with light petroleum (b. p. 100—120°; 4 volumes). The precipitate was washed with light petroleum, ground with ether, and dried. The methylated polysaccharide (7·569 g.) had OMe, 37·4%. Part (6·867 g.) of this material was submitted to 8 more treatments with the methylating reagents and gave a product (6·654 g.; overall yield, 58%) having OMe, 44·6%.

(c) *Synthetic polysaccharide* Ia. Polysaccharide Ia (dry wt., 4·338 g.; see Part VIII) was treated 8 times with the methylating reagents. During dialysis, a large proportion of the polysaccharide ether separated from solution, but in order to conserve material and to obtain a more accurate estimate of the average chain length of polysaccharide Ia the soluble and insoluble fractions were not isolated separately. The product (5·053 g., 93%) had OMe, 44·4%.

(d) *Synthetic polysaccharide* IV. After 8 treatments with the methylating reagents, polysaccharide IV (dry wt., 4·465 g.; see Part VIII) gave a product (4·544 g., 81%) having OMe, 42·9%. Part (0·920 g.) of this material was remethylated (8 treatments) and the final product (0·842 g.; overall yield, 74%) had OMe, 45·1%.

(e) *Synthetic polysaccharide* V. Treated 8 times with the methylating reagents, polysaccharide V (dry wt., 2·943 g.; see Part VIII) afforded a product (3·017 g., 81%) having OMe, 44·5%.

Analysis of the Hydrolysates of the Methylated Polysaccharides by Paper Partition Chromatography.—(a) *Standard method.* The hydrolysates were prepared and analysed by a method which was essentially that used by Flood, Hirst, and Jones (*loc. cit.*) and later by Hirst, Hough, and Jones (*loc. cit.*). The polysaccharide ether (*ca.* 60 mg.) was treated with 3·6% methanolic hydrogen chloride (1·2 c.c.) in a sealed tube at 100° for 9 hours and then with 4% hydrochloric acid (6·0 c.c.) at 100° for 5 hours. The resulting solution of methyl glucoses was freed from ions and concentrated to a thin syrup, which was redissolved in water (1·0 c.c.).

Spots of the hydrolysate, containing in all some 3·5 mg. of mixed methyl glucoses, were placed along a line drawn 10 cm. from the upper edge of a sheet (57 × 23 cm.) of filter paper (Whatman No. 1). An additional spot was placed 3 cm. from each end of the main group. The sheet was irrigated in a closed tank with the top layer of a mixture of *n*-butanol (40%), ethanol (10%), water (49%), and ammonia (1%) until the solvent boundary had moved 40—45 cm. from the starting line. The sheet was removed and dried, and the terminal reference strips were stained with aniline hydrogen phthalate (Partridge, *loc. cit.*). By matching these chromatograms with the main sheet, it was possible to cut the latter into strips of suitable width (3—6 cm.) to ensure that each of the sugar components was isolated on one strip. In addition, blank strips of equal area were cut from the sugar-free regions of the paper. The corresponding strips from two such sheets of filter paper were eluted together with water (10 c.c.) for 1 hour and analysed by the method of Hirst, Hough, and Jones (*loc. cit.*). For the larger quantities of methyl glucoses thus obtained the analysis was conducted with double volumes of all reagents. Since the method of estimation had been shown to be stoicheiometric for methyl glucoses, the volumes of 0·01N-sodium thiosulphate equivalent to the respective sugar components were proportional to the number of molecules of these components present in the extracts.

(b) *Analysis of a known mixture of methyl glucoses.* A solution, containing 2 : 3-dimethyl glucose (3·46 mg./c.c.), 2 : 3 : 6-trimethyl glucose (64·8 mg./c.c.), and 2 : 3 : 4 : 6-tetramethyl glucose (4·29 mg./c.c.), was analysed by the above method. The molecular proportions of the three sugars, expressed on a percentage basis, were found to be, respectively, 5·48, 89·0, and 5·39 (average of 3 determinations), compared with the theoretical figures, 5·09, 89·3 and 5·57.

(c) *End-group assay of methylated potato amylopectin.* Four components were revealed when a hydrolysate of methylated potato amylopectin (OMe, 44·6%) was examined by paper partition chromatography. Three of them had R_F values identical with those of 2 : 3-dimethyl glucose, 2 : 3 : 6-trimethyl glucose, and 2 : 3 : 4 : 6-tetramethyl glucose, used as standards on the same chromatogram. The fourth and slowest-moving component appeared to be another dimethyl glucose. Quantitative results obtained in five independent determinations are given in the following table.

Probable derivative of glucose.	Molecular composition (%) indicated by assay number : I.	II.	III.	IV.	V.	Mean.
Unknown dimethyl	2·25	7·10	0·46	1·28	1·10	1·32
2 : 3-Dimethyl	3·80		3·52	5·01	6·58	4·90
2 : 3 : 6-Trimethyl	88·1	88·9	90·8	90·6	86·5	89·0
2 : 3 : 4 : 6-Tetramethyl	5·81	4·00	5·23	3·25	5·88	4·83

The mean value for the molecular proportion of tetramethyl glucose (4·83%) corresponded to an average chain length of 20·7 glucose units.

(d) *End-group assay of methylated synthetic polysaccharide* IV. Four components were revealed when a hydrolysate of the methyl ether of polysaccharide IV (OMe, 45·1%) was examined by paper partition chromatography. The R_F values of three of these components were identical with those of 2 : 3-dimethyl glucose, 2 : 3 : 6-trimethyl glucose, and 2 : 3 : 4 : 6-tetramethyl glucose, used as standards on the same chromatogram. The slowest-moving component appeared to be a second dimethyl glucose. The chromatogram could not be distinguished visually from that obtained in the case of methylated potato amylopectin (see Plate). Quantitative results obtained in five independent determinations are shown in the following table.

Probable derivative of glucose.	Molecular composition (%) indicated by assay number : I.	II.	III.	IV.	V.	Mean.
Unknown dimethyl	1·25	1·07	1·82	2·51	0·54	1·44
2 : 3-Dimethyl	4·78	6·67	5·32	5·28	5·09	5·43
2 : 3 : 6-Trimethyl	88·0	88·1	86·1	87·7	88·9	87·8
2 : 3 : 4 : 6-Tetramethyl	5·88	4·01	6·58	4·49	5·42	5·27

The mean value for the molecular proportion of tetramethyl glucose corresponded to an average chain length of 19·0 glucose units.

(e) *Examination of methylated synthetic polysaccharide* Ia. Only one component was revealed when a hydrolysate of the methylated polysaccharide Ia was examined by paper partition chromatography. The R_F value of this sugar was identical with that of 2 : 3 : 6-trimethyl glucose, used as a standard on the same chromatogram. Under the same conditions, a hydrolysate of methylated potato amylose and a mixture of 2 : 3-dimethyl glucose, 2 : 3 : 6-trimethyl glucose, and 2 : 3 : 4 : 6-tetramethyl glucose, containing the respective sugars in the molecular proportions 0·50, 99·0, and 0·50%, both gave only one spot. The aniline hydrogen phthalate reagents was apparently not sufficiently sensitive to detect such small quantities of di- or tetra-methyl glucose.

Analysis of Hydrolysates of the Methylated Polysaccharides by Partition Chromatography using a Silica Gel.—(a) *Preparation and testing of the silica.* The silica was prepared by the method of Gordon, Martin, and Synge (*Biochem. J.*, 1943, **37**, 79) with two modifications suggested by Tristram (*Biochem. J.*, 1946, **40**, 721). First, the diluted water glass was cooled to 0° before the addition of 10N-hydrochloric acid, and, secondly, the precipitated silica was "aged" in 0·1N-hydrochloric acid at 0° for 24 hours.

The silica was tested, both qualitatively and quantitatively, with artificial mixtures of 2 : 3 : 6-trimethyl glucose and 2 : 3 : 4 : 6-tetramethyl glucose, as described by Bell (*loc. cit.*). The recoveries of tetramethyl glucose in four such tests are shown in the following table.

Wt. of mixed sugars (mg.).	Mol. propn., " tri " : " tetra."	Recovery of " tetra " (%).	OMe of " tetra " (theory, 52·6%).
123·5	4·7	90·2	52·3
123·6	4·4	92·8	52·5
80·8	1·9	89·5	52·4
64·6	1·3	88·3	52·3
	Mean values :	90·2	52·4

The mean value for the recovery of " tetra " (90·2%) compares with 93% quoted by Bell (*loc. cit.*) and 90·3% quoted by Bourne, Fantes, and Peat (*loc. cit.*). An allowance for this consistently low recovery of tetramethyl glucose was made in all end-group assays, the factor employed being 90 ± 5%.

(b) *End-group assay of the methylated polysaccharides.* The methylated polysaccharides were submitted to end-group assay by the method of Bell (*loc. cit.*), as modified by Bourne, Fantes, and Peat (*loc. cit.*). The methyl ethers of potato amylose and polysaccharide Ia, prepared as above, were resistant to methanolysis and it was necessary to dissolve them in a small volume of chloroform before the methanolic hydrogen chloride was added. The mixture was heated under reflux for 11 hours, the solvents were removed and the resulting syrup was re-treated with methanolic hydrogen chloride in the usual way.

The average chain-length was calculated, as described by Bourne, Fantes, and Peat (*loc. cit.*), from the expression :

$$\text{chain length} = \frac{0{\cdot}9\,B}{E - \dfrac{F(C - E)}{D}}$$

Details of the results obtained with the methyl ethers of various natural and synthetic polysaccharides are given in the following table.

Polysaccharide.	Potato amylose.	Potato amylopectin.	Synthetic Ia.	Synthetic V.
Blue value	1·30	0·19	1·18	0·12
Wt. of methylated polysacc. (*A* mg.)	4428	2459	3986	1846
OMe of methylated polysacc. (%)	45·1	44·6	44·4	44·5
Wt. of glucoside mixture (*B* mg.)	3899	2760	4016	1879
Wt. of first distillate fraction (*C* mg.)	962·3	619·0	558·0	802·9
Wt. of second distillate fraction (*D* mg.)	861·2	808·0	630·4	433·6
Wt. of crystalline " tetra " from first fraction (*E* mg.)	52·6	131·8	35·3	123·0
Wt. of syrupy " tetra " from second fraction (*F* mg.)	33·4	19·0	13·4	13·9
OMe of " tetra " from first fraction (%)	52·3	52·2	51·7	51·7
Chain length	204 ± 10	21 ± 1	150 ± 8	17 ± 1

The " tetra " fraction from polysaccharide V, treated with aniline in methyl alcohol, gave, after several crystallisations from ether, white needles of 2 : 3 : 4 : 6-tetramethyl glucose anilide, m. p. 135°, not depressed on admixture with an authentic specimen prepared by the method of Irvine and Moodie (*J.*, 1908, 95).

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