14. *Constitution of the Starch synthesised* in vitro *by the Agency of Potato Phosphorylase.*

By W. N. Haworth, R. L. Heath, and S. Peat.

The constitution of the granular form of the starch synthesised *in vitro* by Hanes has been investigated. The methylated derivative of the polysaccharide yields on hydrolysis 2 : 3 : 6-trimethyl glucose and tetramethyl glucose and the proportion of the latter (1·5%) corresponds to a minimum chain-length of 80—90 glucose units.

It is concluded that the polysaccharide is a starch in that it is composed solely of glucose residues which are mutually united by 1 : 4-α-glucosidic linkages. The average length of its basal or unit chain, measured by the number of glucose residues, is, however, three to four times that which is found for potato or cereal starches. It is suggested that this polysaccharide, representing 85% of the total synthetic product prepared outside the intact cell by the action of a phosphorylase on glucose 1-phosphate, is related to amyloamylose. The constitution of the remaining 15% of the synthetic polysaccharide has not yet been determined.

The remarkable synthesis of a starch by the action of plant phosphorylase on glucose 1-phosphate has been described by Hanes (*Proc. Roy. Soc.*, 1940, *B*, **128**, 421; **129**, 174). In many respects the synthetic product is indistinguishable from natural starch, although some points of difference are observable. For instance, Hanes emphasises the sparing solubility of the synthetic product in hot water and the rapidity with which it retrogrades from solution. Secondly, the colour given with iodine is more brilliantly blue than the colour given by natural starch. The third, and most characteristic difference, lies in the behaviour towards β-amylase. In the case of natural starches, degradation by β-amylase ceases when some 60% of the starch substance has been converted into maltose. Synthetic starch, on the other hand, is completely converted into maltose by β-amylase and in this respect resembles the amyloamylose of Samec.

Interest obviously attaches to the further examination of the relationship between natural starch and synthetic starch and it has been possible for us, by the courtesy of Dr. Hanes, to investigate the chemical constitution and the chain-length of the synthetic starch.

During the digestion of glucose 1-phosphate with phosphorylase, the synthetic starch is deposited from solution in grains. A sample (45 g.) of this granular form was supplied to us by Dr. Hanes. It must be emphasised, however, that the granular form represents only 85% of the total starch synthesised; the remainder is not deposited but remains in solution and may be obtained therefrom by appropriate treatment. This investigation is not concerned with the soluble form of synthetic starch, which may very well exhibit a difference in chain-length from the grain form.

It was found that the behaviour of the synthetic starch towards methylating agents was entirely analogous to that of natural potato starch. By repeated treatment of the granular synthetic starch with methyl sulphate and aqueous alkali it was possible to prepare a methylated starch which contained, after purification, 44·5% of methoxyl. The methylated starch was a white powder, very sparingly soluble in cold water and insoluble in hot. It showed $[\alpha]_D^{20°}$ + 203° in chloroform solution and in this respect is not significantly different from methylated potato starch ($[\alpha]_D$ + 208°; Haworth, Hirst, and Webb, J., 1928, 2689), methylated amylose ($[\alpha]_D$ + 207°) or methylated amylopectin ($[\alpha]_D$ + 207°; Hirst, Plant, and Wilkinson, J., 1932, 2380). Hanes (*loc. cit.*) has already reported that the synthetic starch shows rotations in water and in aqueous alkali which are almost identical with those shown by potato starch.

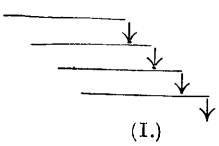
The methylated starch was separated into fractions by its precipitation from chloroform solution with light petroleum. The properties of these fractions indicated the homogeneity of the polysaccharide in a chemical sense. The viscosities of the fractions in *m*-cresol solution showed, however, variations indicating the lack of uniformity of molecular size which is a common feature of high polymers.

The modified constant $k_m = 1{\cdot}6 \times 10^{-4}$ for the Staudinger equation was suggested by Carter and Record (J., 1939, 670) to conform with osmotic pressure values determined for natural starch and with the laminated or branched structure based upon the unit chain length of 24—30 glucose residues. A synthetic starch having a unit chain-length of 100 or more glucose residues would require a different constant. If the original constant used in the Staudinger equation for cellulose chains be applied in this case, namely, $k_m = 10 \times 10^{-4}$, then the average molecular size of methylated synthetic starch, calculated from viscosity values, is 80—160 glucose residues; but this can only be a very speculative approximation, although it is not in disagreement with the value determined by the end-group method. Furthermore, Hanes (private communication) has observed that synthetic starch has a copper-reducing value consistent with its existence as a chain molecule of 100 glucose residues.

The chief product of hydrolysis of the methylated synthetic starch was 2 : 3 : 6-trimethyl glucose, some 40% of which was isolated as the crystalline 2 : 3 : 6-trimethyl β-methylglucoside. The end-group was identified in the hydrolysate by the isolation of tetramethyl methylglucoside, which was characterised as the crystalline tetramethyl glucopyranose. The quantitative estimate of the unit chain-length was made on the basis of the refractive indices and specific rotations of the fractions obtained by the distillation of the glucoside mixture from methylated synthetic starch. The proportion of tetramethyl methylglucoside found was 1·5% and this is a maximum value. The actual proportion of end-group may be less than 1·5%, but it cannot be more. The conclusion is reached that the unit chain-length of synthetic starch cannot be less than 80—90 glucose residues. From these facts it is evident that the synthetic starch is constituted on the same structural plan as natural starch, namely, that it is composed of chains of glucose residues mutually united by 1 : 4-α-glucosidic linkages. The difference between natural and synthetic starch is manifested in the number of glucose residues composing the unit chain-length.

As a first approximation, the laminated structure represented by (I) has been postulated for natural starch and other chain polysaccharides.

In the diagram (I), the horizontal lines represent the unit chain of monosaccharide residues (24—30 glucose residues in the case of natural starch) and the arrow heads indicate the linking of the reducing terminal units of the chains (cf. Haworth, Hirst, and Oliver, J., 1934, 1917; Haworth, Hirst, and Isherwood, J., 1937, 577; Haworth, *Chem. and Ind.*, 1939, **58**, 917). The union of the repeating units [represented in (I) by the points of contact of the arrow heads] has been shown (Bawn, Hirst, and Young, *Trans. Faraday Soc.*, 1940, **36**, 880) to be effected by primary valencies and recently Barker, Hirst, and Young (*Nature*, 1941, **147**, 296) have adduced evidence to show that this glycosidic union involves the primary alcohol groups at C_6 of one of the glucose residues, as was first portrayed in the formulation originally given in 1937 by Haworth, Hirst, and Isherwood (*loc. cit.*). It seems necessary to emphasise this structural formulation inasmuch as some workers in this field have erroneously assumed that the unit chain-length we have postulated is also identical with the molecular size of starch; whereas these are repeating structural units composing the much larger molecule.



(I.)

The reason for the difference in unit chain-length of natural and synthetic starch is of great interest. The suggestion might be made that the organised enzyme complex in the plant cell first assembles glucose residues in chains of 24—30 units and then completes the mutual linkage of these chains. When the enzyme system is disorganised by extraction and is functioning outside the directive influence of the intact cell, it would appear that the union of glucose residues is not limited to the formation of basal units of 24—30 residues.

It may be of course that the difference in activity of the enzyme in the starch cell and outside it is one of degree only and that basal chains much longer than are represented by 24—30 glucose units are synthesised *in vivo*. It is recognised that the unit length of chain determined by end-group assay is for the whole natural starch and that a very limited portion may well have longer chains. It is well known that starch may be separated into two constituents (amylose and amylopectin) which have widely different physical properties, but the structural difference between these constituents is by no means clear. Some vagueness attaches to the definition of the terms " amylose " and " amylopectin " and the properties of the constituents of starch appear to depend very much on the method used for their separation.

There is apparently a close similarity between the synthetic starch we have examined and the amylo-

amylose fraction of natural starch separated by the method of Samec, which involves the dispersion of potato starch in water by autoclaving at 120° and the submission of the solution to electro-dialysis. This amyloamylose is reported to be completely hydrolysed by β-amylase (Samec and Waldschmidt-Leitz, *Z. physiol. Chem.*, 1931, **203**, 16; Freeman and Hopkins, *Biochem. J.*, 1936, **30**, 446). More recently it has been reported that an amylose (about 5%) extracted with water at 60—70° from the intact grain of maize or potato starch also resembles Hanes's synthetic starch in being completely degraded to maltose by β-amylose (Meyer *et al.*, *Helv. Chim. Acta*, 1940, **23**, 865; 1941, **24**, 378). The latter workers have attempted a very rough end-group estimation of an incompletely methylated amylose from potato starch and we have used their approximate figures in a calculation of the unit length, which appears to be of the order of 300 glucose residues. The molecular weight determined by osmotic pressure measurement is of the same order.

Since this work was completed a publication has appeared by Hassid and McCready (*J. Amer. Chem. Soc.*, 1941, **63**, 2171), who have been unable to detect the presence of tetramethyl glucose in their hydrolysed specimen of methylated synthetic starch. It would appear that the quantity of the methylated polysaccharide employed for the determination (5 g.) may have been insufficient to ensure the assay of the exiguous amount (1·5%) of end-group which we, using 32 g. of initial material, have estimated.

Experimental.

The sample of synthetic starch provided by Dr. C. S. Hanes weighed 45·1 g. and contained moisture, 6·7%, and total nitrogen, 0·03%.

Methylation.—The starch was methylated in three batches.

Batch I (10 g.), dissolved in 1% sodium hydroxide solution, was treated at 50° with methyl sulphate (180 c.c.) and 30% sodium hydroxide solution (450 c.c.), added simultaneously. After the addition of one-half of the reagents, methylated starch began to separate and in order to maintain it in solution, dioxan was added. At the end of the methylation the mixture was heated on a water-bath for ½ hour and the insoluble methylated product was collected on a cloth filter. In subsequent methylations, the solvent (dioxan or acetone) was added at the beginning of the methylation. After 15 methylations, the product showed OMe, 43·8%.

Batch II (17 g.) was methylated in a similar manner except that the solvent used throughout was acetone instead of dioxan. After 12 methylations the methoxyl content was 43·5%.

Batch III (18 g.) yielded, after 12 methylations, a product containing OMe, 43·8%.

Fractional Precipitation of the Methylated Starch.—The three batches of methylated starch were now combined and dissolved in chloroform (750 c.c.). The solution was dried with anhydrous magnesium sulphate, filtered, and evaporated to small bulk. The graduated addition of light petroleum to the chloroform solution effected precipitation in fractions, the properties of which are tabulated :

Fraction.	Weight, g.	Ash, %.	OMe, % (corr. for ash).	$[\alpha]_D^{20°}$ in $CHCl_3$.	$\eta_{sp.}/c$ in *m*-cresol (c, 0·4%).	Apparent mol. wt. ($k_m = 1 \times 10^{-3}$).
A	2	34·0	—	—	—	—
B	6	1·0	42·5	+190°	0·912	18,200
C	6	0·8	44·6	+203	0·840	16,800
D	7	0·2	44·9	+203	1·537	30,700
E	6·5	0·2	45·5	+203	1·225	24,500
F	9·5	0·15	44·7	+202	0·985	19,700

The bulk of the ash (sodium sulphate) is contained in the small first fraction and if this fraction is excluded it is seen that the yield of purified methylated product is 35 g. from 45 g. of starch.

Methanolysis of the Combined Fractions.—The fractions were combined as follows : B (4·96 g.), C (5·47 g.), D (6·76 g.), E (6·49 g.) and F (8·41 g.). The total weight of the mixture is thus 32·09 g. and its mean methoxyl content is 44·5%.

The methylated starch was boiled for 8 hours with dry methyl alcohol (1 l.) containing 1·6% of hydrogen chloride. The clear solution was neutralised with lead carbonate, and the solution evaporated to dryness in the presence of a little lead carbonate. The dry residue was extracted with chloroform, the extract dried, and the solvent evaporated. The mixture of methylglucosides so obtained weighed 39·45 g.

Fractional Distillation of the Products of Methanolysis.—The usual procedure was adopted of distillation and redistillation in a high vacuum from a Widmer flask with a vacuum-jacketed column. The following fractionation was ultimately attained :

Fraction	1	2	3	4	5	6	7
Weight, g.	0·104	0·078	0·113	0·827	0·301	1·70	4·28
$n_D^{20°}$	1·4428	1·4428	1·4433	1·4543	1·4560	1·4560	1·4562
OMe, %	—	60·2	—	52·2	—	53·3	—
Fraction	8	9	10	11	12	13	14
Weight, g.	0·91	13·82	9·66	0·84	1·14	0·474	0·405
$n_D^{20°}$	1·4562	1·4563	1·4572	1·4592	1·4601	1·4638	1·4740
OMe, %	—	—	—	—	—	49·1	43·4

The undistillable residue weighed 0·85 g.

Inspection of refractive indices and methoxyl values indicates that the whole of the tetramethyl αβ-methylglucoside ($n_D^{20°}$ 1·4428; OMe 62·0%) produced is present in fractions 1 to 6; that the main product is 2 : 3 : 6-trimethyl αβ-methylglucoside ($n_D^{20°}$ 1·4560; OMe 52·6%); and that a small proportion of dimethyl methylglucoside (OMe, 41·9%) is also present in the final fractions.

That the tetramethyl methylglucoside is a derivative of glucose is indicated by the observation that for fraction 3 ($[\alpha]_D^{15°}$ + 61·8°) the $n_D/[\alpha]_D$ ratio lies on the $n_D/[\alpha]_D$ curve for mixtures of the α- and the β-forms of tetramethyl methylglucopyranosides (cf. Hirst and Young, J., 1938, 1248). Furthermore, hydrolysis of fraction 3 with aqueous acid yielded crystalline tetramethyl αβ-glucopyranose of m. p. and mixed m. p. 79—84° and $[\alpha]_D^{16°}$ + 82·5° (*c*, 0·4 in water; equilibrium value).

The main product of hydrolysis of the methylated starch is 2 : 3 : 6-trimethyl glucose. Fractions 5 to 10 crystallised on keeping and from each was separated crystalline 2 : 3 : 6-trimethyl β-methylglucoside (m. p. and mixed m. p. 71°). The yield of the crystalline β-isomer from these fractions was 40%. Hydrolysis of the non-crystalline portion yielded crystalline 2 : 3 : 6-trimethyl glucose, m. p. and mixed m. p. 112—115°.

The proportion of end-group, tetramethyl methylglucoside, was assessed on the basis of the n_D values of fractions 1 to 6, the extreme values so calculated being 1·40 g. and 1·54 g. derived from 100 g. of methylated starch. These are corrected values (cf. Averill and Peat, J., 1938, 1244) and correspond to chain lengths of 88 and 80 glucose units respectively.

It must be pointed out that 1·5% represents the maximum amount of end-group that can be present and that therefore the average chain length of synthetic starch cannot be less than 80 glucose units. Thus, fraction 1 showed certain anomalies (*e.g.*, the $n_D/[\alpha]_D$ value did not conform to the curve for tetramethyl αβ-methylglucoside) which led to the suspicion that this fraction was not composed entirely of glucoside but contained an unknown proportion of methyl lævulate or other decomposition product. Nevertheless, in arriving at the value of 1·5%, it has been assumed that the whole of fraction 1 consists of tetramethyl αβ-methylglucoside. The proportion of end-group may therefore be somewhat less than 1·5%.

The amount of dimethyl methylglucoside is not great. The bulk of it is contained in fraction 14 and it is estimated that roughly 1·5 g./100 g. of methylated starch are present.

The A. E. Hills Laboratories,
The University, Edgbaston, Birmingham.

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