41. *Polysaccharides. Part XIX. The Molecular Structure of Waxy Maize Starch.*

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On the basis of previous work the hypothesis we have advanced in this series of papers is that the two main constituents of starch represent physical units having a greater or lesser degree of aggregation of the primal chemical unit. The latter we recognise as a continuous chain terminating after a length of 26—30 α-glucopyranose units has been attained. These two main constituents of starch have been variously described in the literature as amylopectin and amylose (Maquenne and Roux, *Compt. rend.*, 1903, **137**, 88) and as α-amylose and β-amylose respectively (A. Meyer, *Ber. deut. bot. Ges.*, 1886, **4**, 337; "Untersuchungen über die Stärkekörner," 1895). In our discussion of this subject we have preferred to use the nomenclature of Maquenne and Roux, and to avoid confusion it should be pointed out that the alternative nomenclature employing the terms α- and β-amylose has no essential connexion with a stereochemical difference of the units composing the starch constituents.

It was obviously desirable to examine the chain length of the chemical molecule of a variety of starch differing as widely as possible from that which we had already examined. In the endosperm of a number of cereals a so-called red-staining starch has been encountered. It occurs in *Oryza sativa* var. *glutinosa*, *Panicum miliaceum* var. *canditum glutinosum*, *Sorghum vulgare glutinosum*, and in another variety of grass, *Coix lachryma-jobi*. The most readily available source of a starch of this character is, however, waxy maize, of which we have been able to obtain a supply through the courtesy of Professor R. A. Brink, Department of Genetics, Wisconsin University, to whom we express our thanks (compare R. A. Brink and F. A. Abegg, *Genetics*, 1926, **11**, 163). This variety of starch is characterised by giving a red-violet coloration with iodine, not unlike the red-brown colour given by glycogen but differing very markedly from the deep blue colour given by potato starch or ordinary maize starch. It has already been suggested that the red and blue colours given by different starches or their analogues are due to differences in the degree of dispersion of the iodine particles and that any agency such as alcohol, potassium iodide, or heat which has the effect of bringing the iodine into true solution tends to change the colour in the direction of blue to red. For this reason it is suggested by Brink that the ultimate particles of waxy maize starch may be smaller than those of the non-waxy type, thus permitting of a greater surface on which the iodine is distributed. Beyond surmise, little can be gained from our present knowledge of colour reactions. Certainly it is the case that in waxy maize starch the amylose (β-amylose) portion greatly preponderates. It forms viscous solutions with water rather than pastes, and differs materially in general properties from starches in which a high proportion of amylopectin (α-amylose) is present.

From waxy maize starch we have prepared the acetate by two methods involving respectively pyridine and a mixture of sulphur dioxide and chlorine as catalytic agents. The starch regenerated from the acetate retained the same properties as the original starch and, moreover, the acetates prepared by the two methods were indistinguishable. From the two acetates we have also prepared the corresponding fully methylated derivatives. These have closely similar properties, but differ somewhat from each other in their specific viscosities in *m*-cresol. Methylated waxy maize starch resembled in all respects the methylated amylose fraction of potato starch described in two preceding papers (Haworth, Hirst, and Webb, J., 1928, 2581; Hirst, Plant, and Wilkinson, J., 1932, 2375). The methylated starches showed no variation in optical rotation from the previous specimens and the magnitude of the rotation was in agreement with the additive rule of Freudenberg (*Ber.*, 1933, **66**, 177) for continuous units of methylated α-glucopyranose.

The gravimetric assay of the end group isolated by hydrolysis of methylated waxy maize starch revealed the presence of an average of 4·5% of tetramethyl glucose, which corresponds to a chain length of 26—30 α-glucopyranose units. This value, which was found with both specimens of waxy maize starch referred to above, irrespective of their

viscosity, is similar to that which we have already determined for methylated amylose and methylated amylopectin from potato starch, and it is interesting to record that two starches of such different origins give results corresponding to the same value. The apparent molecular weight determined by the viscosity method, using Staudinger's factor, gives values many times greater than that found by the gravimetric assay, and this is also the case when the sedimentation method with the ultra-centrifuge (Svedberg, *Ber.*, **1934, 67**, *A*, 117) is applied. On the basis of our theory of the constitution of starch it is clear that this specimen also was an amylose which owing to molecular aggregation is still much more complex than the limiting chemical molecule.

Experimental.

Preparation of Waxy Maize Starch.—Clean waxy maize grains were steeped in dilute sulphurous acid (*d* 1·007) for 24 hours at 40°. The softened grains were washed with water and pounded to a pulp under water. The pulp was enclosed in fine muslin and kneaded under water until all the starch had passed into the water. The aqueous suspension was poured through a fine sieve to remove any large particles and the starch was allowed to settle. After decantation of the supernatant liquid the crude starch was made into a flowing paste with water and poured slowly into *N*/10-aqueous sodium hydroxide. The mixture was stirred at intervals during 4 hours, the starch allowed to settle, washed with water, and again allowed to settle. The washing with water was repeated several times until all traces of alkali were removed. The starch was digested at 15° in succession with 95% alcohol, absolute alcohol, and ether. The starch thus prepared was a white impalpable powder which contained 6·8% of moisture. After the starch had been kept in the air for some time, the moisture content rose to 20%.

Properties of Waxy Maize Starch.—The granules of waxy maize starch appear under the microscope as small rounded discs with indentations at the circumference. The granules stain with iodine dark blue at the margin and reddish-purple in the interior. Waxy maize starch is not appreciably soluble in cold water, but dissolves readily in warm water (40—50°), giving an opalescent solution. This solution after cooling gives with iodine a reddish-purple colour which very slowly changes to blue. The colour disappears when the solution is heated and on subsequent cooling a deep green colour appears which changes gradually to an intense blue. Waxy maize starch prepared by the above method had no ash content and contained no nitrogen. The phosphorus content was very small (P, 0·0025%) (Found : C, 44·2; H, 6·4. Calc. for $C_6H_{10}O_5$: C, 44·4; H, 6·2%). The starch had no action on Fehling's solution on prolonged boiling, and its iodine number, determined by the method of Bergmann and Machemer, was negligible (0·4—0·6).

Waxy maize starch gave only moderately viscous solutions with warm water. The " pastes " so obtained were much less viscous than potato starch paste prepared in a similar way. For example, the times taken in a viscometer were respectively 28·2 seconds for potato starch paste and 10·8 seconds for waxy maize starch paste. In each case a 1% paste had cooled to 20°. At this temperature the time for water in the same instrument was 9 seconds.

Owing to the opalescence of the solutions determination of the rotation of waxy maize starch was difficult to carry out in water or sodium hydroxide. For this reason, " prepared " starch was used for the polarimetric measurements. " Prepared " starch was obtained by precipitating an aqueous solution of waxy maize starch with alcohol, grinding the precipitate under alcohol, washing it with ether, and drying it in a vacuum. The prepared starch differed from the original waxy maize starch in being immediately soluble in cold water. In all other respects its properties were identical with those recorded above. In particular it was non-reducing and had a negligible iodine number. Its aqueous solution gave a deep red-purple colour with iodine. $[\alpha]_{5780}^{20°}$ + 212° in water (*c*, 0·97); + 153° in 4% aqueous sodium hydroxide (*c*, 1·30).

Experiments carried out under strictly comparable conditions and with the same enzyme preparation showed that the rates of hydrolysis of waxy maize starch and potato starch with barley diastase were almost identical. After 4 hours at 50° both solutions showed reducing properties corresponding to 60% conversion into maltose. When the product was worked up in the usual way, crystalline maltose was obtained.

Attempts to separate waxy maize starch into fractions corresponding to amylose and amylopectin by Ling and Nanji's method were unsuccessful. The starch was made into a paste (1·5%) with water and frozen for 30 minutes in ice–salt. During the subsequent extractions with warm water the whole of the starch dissolved, except a small portion (less than

0·1% of the total) consisting mainly of inorganic material derived from the aluminium pans used during the freezing process. It appears, therefore, that waxy maize starch cannot be separated into fractions corresponding to the amylose and amylopectin portions of potato starch. It consists entirely of the more soluble form corresponding to amylose. The starch extracted by the warm water in the above experiments was precipitated by alcohol from the concentrated extract. It was ground under alcohol and washed with alcohol and then with ether. Its properties were identical with those of " prepared " starch (see above).

Acetylation of Waxy Maize Starch.—(a) A solution of the starch in hot water was poured, with brisk stirring, into an excess of alcohol. The precipitate was ground under alcohol to a fine powder, which was washed with ether and dried in a vacuum. This prepared starch (**5 g.**) was steeped in glacial acetic acid (30 c.c.) through which chlorine had been passed for 90 seconds. Acetic anhydride (50 c.c.) containing sulphur dioxide equivalent to the amount of chlorine was then added and the mixture was stirred for 1 hour at 20° and for 1 hour at 55°. The clear solution was filtered through glass wool and poured into a large volume of water. The white granular precipitate of acetylated waxy maize starch was washed with water until acid-free, then with alcohol and ether, and dried in a vacuum (yield, quantitative). The *acetyl* derivative was a crisp white powder readily soluble in acetone and in chloroform. It gave no colour with iodine and was non-reducing towards Fehling's solution. $[\alpha]_D^{22°}$ + 167° in chloroform (*c*, 0·3) (Found : C, 50·2; H, 5·8; CH_3·CO, 44·6. $C_{12}H_{16}O_8$ requires C, 50·0; H, 5·6; CH_3·CO, 44·6%). After systematic fractionation from chloroform solution by addition of light petroleum the main portion (88%) of the acetate was obtained as a white powder with the rotation and analytical figures given above and having $\eta_{sp.}$ 0·51 in *m*-cresol at 20° (*c*, 0·4%) (corresponding to an apparent molecular weight of 37,000 by Staudinger's formula). (The viscosity measurements given in this paper are recorded only for comparative purposes, since it cannot be assumed at this stage that the viscosities of these substances are utilisable directly for the calculation of molecular weights.)

(b) *With pyridine as catalyst.* Prepared starch (3 g.) was shaken for 2 hours with pyridine (30 c.c.). Acetic anhydride (30 c.c.) and pyridine (10 c.c.) were then added and the mixture was shaken for 3 hours. The clear solution was poured into a large excess of water and the starch triacetate was isolated in the usual way (yield, quantitative). The acetylated starch was identical in properties with that prepared by method (a). When heated, it began to sinter at about 180° and to decompose at about 210°. $[\alpha]_{5780}^{18°}$ + 166° in chloroform (*c*, 1·0). The phosphorus content of the original starch was retained during the acetylation (Found : C, 49·7; H, 5·8; CH_3·CO, 46·0; P, 0·0013%).

Fractional precipitation of the acetate from acetone solution by regulated addition of light petroleum disclosed the essential homogeneity of the material. Three fractions were obtained, all of which had the properties given above and were indistinguishable from one another as regards analytical figures. The specific viscosities of the fractions in solution in *m*-cresol were almost identical. $\eta_{sp.}$ 0·48 (*c*, 0·4%) in *m*-cresol at 20°, corresponding to an " apparent " molecular weight (Staudinger's formula) of 35,000.

Acetylation of the original starch either by Barnett's method or by the pyridine method proceeded less readily and was incomplete and the product was only partly soluble in acetone.

Regeneration of Waxy Maize Starch from the Acetate.—The finely powdered acetate (4 g.) (prepared by the pyridine method) was shaken for 30 minutes with 100 c.c. of 0·5*N*-alcoholic sodium hydroxide. The alkali was then neutralised with *N*-acetic acid and the solid was separated by filtration and ground under alcohol containing a little acetic acid. This treatment was repeated, the starch was then dissolved in warm water (40°), and the solution neutralised with acetic acid, filtered, and poured into alcohol. The precipitated starch was washed with alcohol and ether and dried in a vacuum (yield, 70%). Its properties were identical with those of the original starch in its " prepared " condition, *i.e.*, after precipitation from aqueous solution by alcohol. $[\alpha]_{5780}^{20°}$ + 152° in 4% aqueous sodium hydroxide (*c*, 0·5); + 180° in 0·4% aqueous sodium hydroxide; + 214° in water (*c*, 0·1). It was soluble in cold water, it had no action on boiling Fehling's solution, and its iodine number was negligible (< 2). Like the original starch, it gave with iodine a reddish-purple colour, which disappeared on heating. On subsequent cooling, a green-blue colour, changing to a permanent blue, was observed. The viscosities of aqueous solutions of the regenerated and the original material were identical. The phosphorus content was also similar to that of the original starch (0·003%). No appreciable difference was found between the original and the regenerated waxy maize starch.

Simultaneous Deacetylation and Methylation of Waxy Maize Starch Acetate.—The acetate (in lots of 15 g.), dissolved in acetone (200 c.c.), was methylated at 55° by methyl sulphate

(180 c.c.) and 30% aqueous sodium hydroxide (600 c.c.), the reagents being added slowly with vigorous stirring. The methylation was facilitated by addition of acetone (200 c.c. in all) to replace losses by evaporation. At the end of the reaction hot water was added (400 c.c.) and the temperature was raised to 100°. After 30 minutes the methylated product was separated, washed with boiling water, dissolved in acetone, and remethylated. The yield was then 90% of the theoretical, and the methoxyl content 39%. $[\alpha]_D$ + 195° in chloroform. After six further methylations the methoxyl content had risen to 43%. The methylated starch was then dissolved in chloroform to remove insoluble inorganic impurities and was obtained as a brittle sponge-like mass on evaporation of the chloroform. Acetone condensation products were removed by extraction with boiling ether, leaving the methylated starch as a pale yellow, granular solid, which was soluble in chloroform and acetone, only very slightly soluble in ether, and insoluble in alcohol and in hot water. In cold water swelling took place and the substance became glutinous. It was non-reducing and gave no colour with iodine; m. p. 146°; $[\alpha]_D^{20°}$ + 206° in chloroform (*c*, 0·6) (Found : OMe, 43·4%).

Fractionation of Methylated Waxy Maize Starch.—The methylated starch (16 g.), dissolved in chloroform (30 c.c.), was fractionally precipitated by addition of light petroleum (b. p. 40—60°). After systematic treatment involving re-fractionation from chloroform–ether, three principal fractions were obtained : Fraction (a), 0·6 g., m. p. 126—140°, was slightly discoloured and contained a small amount of acetone condensation products which had escaped removal by the previous ether extraction of the methylated starch. Fraction (b), 10·2 g., m. p. 156° with previous softening, $[\alpha]_D^{20°}$ + 206° in chloroform (*c*, 0·8), was further fractionated, giving three portions which had exactly similar properties, including specific viscosity in *m*-cresol solution; m. p. 153—156° with previous softening; $[\alpha]_D^{20°}$ + 206° in chloroform; $\eta_{sp.}$ 0·40 (*c*, 0·30%) in *m*-cresol at 20°, corresponding to an apparent molecular weight (Staudinger's formula) of 27,000 : the method of purification and the fractionation ensured that this material was free from ash and from traces of decomposition products [Found : C, 52·4; H, 8·1; OMe, 43·4. $C_9H_{16}O_5$ requires C, 52·9; H, 7·8; OMe, 45·6%. Methylated starch (OMe, 43·4%) requires C, 52·5; H, 7·7%]. Fraction (c), 3·8 g., m. p. 160° after previous softening, $[\alpha]_D$ + 194° in chloroform (*c*, 0·62) (Found : OMe, 39%), contained some ash (5%) and consisted mainly of incompletely methylated material.

Hydrolysis of Methylated Waxy Maize Starch.—Freshly powdered methylated starch (fraction b, 9·5 g.) was dissolved by stirring in ice-cold fuming hydrochloric acid (53 c.c.). The solution was saturated with hydrogen chloride at − 15° and left for 24 hours, first in a freezing mixture (ice–salt) and then at 0°. The excess of hydrogen chloride was removed by aëration at 15°, the solution diluted with water (60 c.c.), and the acid neutralised with barium carbonate at 15°. After filtration the solution was evaporated to dryness at 40°/12 mm. The solid residue was extracted with boiling chloroform. The material (9·0 g.) extracted by the chloroform was boiled for 7 hours with 3% methyl-alcoholic hydrogen chloride. The product (8·7 g.), which was isolated in the usual way, had $n_D^{18°}$ 1·4602. On fractional distillation a portion (A) (4·0 g.) was obtained, b. p. 136—145°/0·027 mm. (bath temp.). The first drop of distillate had $n_D^{18°}$ 1·4451, and the last drop $n_D^{18°}$ 1·4580. No tetramethyl methylglucoside remained in the still residue. Fractionation of (A) from a Widmer flask gave (B) (0·47 g.), b. p. 144°/0·03 mm. (bath temp.), $n_D^{18°}$ 1·4472 (first drop, $n_D^{18°}$ 1·4455; last drop, $n_D^{18°}$ 1·4530) (Found : OMe, 58·0%). On the basis of refractive index and methoxyl content, (B) contained 70% by weight of tetramethyl methylglucoside. This was confirmed by hydrolysis of the distillate with boiling 5% hydrochloric acid, which gave crystalline tetramethyl glucose in good yield. After (B) had been collected, another fraction (C) (0·91 g.) was obtained, b. p. 150—158°/0·03 mm., $n_D^{18°}$ 1·4552 (first drop, $n_D^{18°}$ 1·4537; last drop, 1·4557) (Found : OMe, 52·9%). This fraction contained at most 0·1 g. of tetramethyl methylglucoside. Further distillation of (A) gave pure trimethyl methylglucoside, $n_D^{18°}$ 1·4570. The total yield of tetramethyl methylglucoside was therefore 0·43 g. This requires a correction of 10% to allow for losses during manipulation (see Haworth and Machemer, J., 1932, 2270). The final yield was therefore 0·47 g. This corresponds to a yield of 4·7% of tetramethyl glucose from methylated waxy maize starch. The identity of the trimethyl methylglucoside was confirmed by its hydrolysis to crystalline 2 : 3 : 6-trimethyl glucose.

Methylated Waxy Maize Starch from the Acetate prepared with Sulphur Dioxide and Chlorine as Catalyst.—The acetate (the main portion obtained during fractionation) was methylated by the method described above. After five methylations the rotation of the product was $[\alpha]_D^{20°}$ + 211° in chloroform and the methoxyl content was 42·3%. After 6 further methylations the product was purified by washing with boiling water and after being dried was extracted

five times with boiling ether to remove acetone condensation products. The last ethereal extracts contained only a trace of material, which appeared to be methylated starch having OMe, 44·2%. The main portion of the methylated waxy maize starch was a pale yellow powder, soluble in chloroform and acetone, insoluble in alcohol and in boiling water, slightly soluble in ether. In cold water it became glutinous (yield, 85%). Fractional precipitation from chloroform solution by addition of light petroleum separated the methylated product into three main fractions : (A), 5% of the total, $\eta_{sp.}$ 0·02; (B), 12% of the total, $\eta_{sp.}$ 0·06; (C), 80% of the total, $\eta_{sp.}$ 0·12 (apparent mol. wt.—see above—approx. 6000). The viscosities in each case refer to solutions in *m*-cresol at 20° containing 0·04 g. in 10 c.c. Each fraction had OMe 44—45%, and $[\alpha]_D^{20°}$ + 204° in chloroform. Attempts to separate (C) into further fractions were unsuccessful [Found for (C) : C, 52·8; H, 8·0%].

Fraction (C) (15 g.) was hydrolysed by boiling 1% methyl-alcoholic hydrogen chloride (600 c.c.). The reaction was complete in 4 hours. The acid was neutralised with silver carbonate, and the products (15·7 g.) were isolated in the usual way and submitted to fractional distillation under diminished pressure. The first portion (6·3 g., bath temp. 125—130°/0·03 mm.) was refractionated from a Widmer flask, giving (a) 0·7 g. (bath temp. 135—144°/0·03 mm.), $n_D^{18°}$ 1·4470 (Found : OMe, 59·6%). This fraction consisted, to the extent of 80%, of tetramethyl methylglucopyranoside. After hydrolysis with 3% hydrochloric acid, tetramethyl glucopyranose was obtained in good yield. (b) 0·2 G. (bath temp. 150°/0·03 mm.), $n_D^{18°}$ 1·4535. (c) Pure 2 : 3 : 6-trimethyl methylglucoside, which crystallised in the receiver : the crystalline β-form had m. p. 57°, $[\alpha]_D^{18°}$ − 32° in water (*c*, 0·3), after recrystallisation from light petroleum. The non-crystalline portion had $n_D^{20°}$ 1·4553 (Found : OMe, 52·0%), and both this and the crystalline substance gave in excellent yield on hydrolysis 2 : 3 : 6-trimethyl glucose, m. p. 117° alone or when mixed with an authentic sample. After removal of all the trimethyl methylglucoside by distillation a fraction (0·4 g.) was obtained, b. p. 225°/0·02 mm., $n_D^{21°}$ 1·4685 (Found : OMe, 46·3%). This contained some dimethyl methylglucoside. There remained an undistillable residue (2 g.) which was not further examined.

From the above figures the corrected yield of tetramethyl methylglucoside from 15 g. of methylated starch was 0·66 g., corresponding to an estimated chain length of 29 units.

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