359. *Structure of Acorn Starch.*

By E. L. Hirst, J. K. N. Jones, and A. J. Roudier.

The proportion (3%) of terminal to non-terminal glucose residues in acorn starch has been determined by four different methods which give concordant results. This starch contains 80% of amylopectin, and it follows that in the amylopectin component there are 24 glucose residues per terminal group. The special features of the acetyl and methyl derivatives of acorn starch are their high viscosities in *m*-cresol and their high molecular weights. A colorimetric method is described for the determination of methylated amylose in the presence of methylated amylopectin. The periodate oxidation procedure has been extended to provide evidence of the mode of attachment of the side chains in amylopectin, and it is shown that at least 75% of these are linked to the main chain at C_6 of one of the glucose residues in the latter.

The proportion of non-terminal to terminal glucose residues has already been determined for many starches, the values obtained both by the methylation procedure and by the periodate oxidation method (cf. Brown, Dunstan, Halsall, Hirst, and Jones, *Nature*, 1945, **156**, 785) varying for the whole starches between 20 and 30 glucose units per end group, according to the type of starch under examination. Since methods are now available for determining the proportion of amylose present in any starch, it is possible to calculate the proportion of non-terminal to terminal residues in the amylopectin component even where complete separation of the two components is difficult or impossible. For most starches this ratio is about 20 to 1, but for some amylopectins a slightly higher ratio of 24 to 1 has been observed. In the present paper these investigations have been extended to the starch from the acorn of the oak-tree (*Quercus*

robur, Linn.). The starch was separated from the macerated cotyledons by the sulphite process, and the properties of both the acetate and the fully methylated derivatives were studied. It was found that both these derivatives possess initial high specific viscosities in *m*-cresol, and although the values lie so much beyond the range of permissible extrapolation from the figures recorded for the relationship between the viscosity and molecular weight of starch derivatives (Hirst and Young, *J.*, 1939, 1471) it is clear that the amylopectin of acorn starch must have an extremely high molecular weight. In this connection it may be significant that the ordinary method for the separation of the amylose and the amylopectin components (cf. maize starch, Higginbotham and Morrison, *Chem. and Ind.*, 1947, 45) was unsatisfactory, only slight separation being effected. At present, however, there is insufficient evidence to decide whether this difficulty is due to some kind of association between the two components or to physical protection of the amylose by the highly colloidal amylopectin.

It has been found in connection with other investigations that with suitable concentration of the reagents fully methylated amyloses gave a blue colour with iodine whilst methylated amylopectins under the same conditions gave no appreciable colour. It is possible, therefore, by modification of the method of Hassid and McCready (*J. Amer. Chem. Soc.*, 1943, **65**, 1154) to determine by means of the blue value the proportions of methylated amylose and amylopectin in a methylated starch. Such determinations were used in the present work as a test of the separation of the methylated amylose and methylated amylopectin in the course of fractionating experiments. These were carried out by extracting methylated acorn starch with mixtures of ether and acetone, but, as happened also with the original starch, no appreciable separation of the constituents could be effected, the blue value being approximately the same for all fractions. A similar failure was recorded when attempts were made to fractionate methylated acorn starch by passing a solution of it in a mixture of benzene and light petroleum through a column of alumina. All the fractions recovered gave blue colours with iodine, but the blue value of the first (0·47) was slightly lower than that of the original methylated starch (0·57), indicating that some slight degree of separation had been achieved.

The proportion of terminal groups in acorn starch was estimated by the following methods:

(*a*) Determination of the tetramethyl methylglucoside content on methanolysis of the methylated derivative by partition between water and light petroleum (Brown and Jones, *J.*, 1947, 1344).

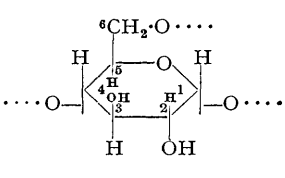
(*b*) Determination of the tetramethyl methylglucoside by chromatography on alumina (Jones, *J.*, 1944, 33; Brown and Jones, *loc. cit.*).

(*c*) Fractional distillation of the tetramethyl methylglucoside (Hirst and Young, *J.*, 1938, 1247).

(*d*) The periodate oxidation method on the unmethylated starch (Brown, Dunstan, Halsall, Hirst, and Jones, *loc. cit.*).

By these methods it was found that the proportion of non-terminal to terminal glucose residues was approximately 30 to 1 in the whole starch, and since the amylose content was 20%, as determined by the iodine potentiometric method, it follows that the proportion of non-terminal to terminal sugar residues in the amylopectin component of acorn starch was approximately 24 to 1. Acorn starch is, therefore, essentially similar in this respect to the many other starches for which figures have been published.

Notwithstanding its high molecular weight, the amylopectin of acorn starch contains one terminal glucose residue for every 24 non-terminal glucose units, and it is clear, therefore, that the molecule of the amylopectin must have a branched-chain structure. There is some evidence that in other starches the branching occurs through C_6 of one of the 1 : 4 α-linked glucose residues in the main chain (Freudenberg and Boppel, *Ber.*, 1940, **73**, 609; Barker, Hirst, and Young, *Nature*, 1940, **147**, 296). The evidence from which these conclusions are drawn is based on the identification of 2 : 3-dimethyl glucose amongst the hydrolysis products of fully methylated starch. In the course of the present investigation another method of approach to this problem was attempted by means of an extension of the periodate oxidation method. If branching occurs through C_6 (inset formula) of any particular glucose residue, the juxtaposed hydroxyl groups on C_2 and C_3 of this residue remain unsubstituted. Attack with periodate would therefore disrupt the glucose residue, and, if all the branchings occur through C_6, oxidation of the amylopectin by periodate would yield a product which on subsequent hydrolysis would give no free glucose. On the other hand, if branching occurs through C_2 or C_3 the glucose residue in question contains no α-glycol grouping and would not be disrupted by periodate. The final hydrolysis product obtained from



the oxidised starch would in this case contain free glucose. In carrying out this method of attack experimental difficulties were encountered in the identification and determination of small quantities of glucose admixed with a large excess of oxidation products. It was found, however, that the chromatographic technique of Partridge (*Nature*, 1946, **158**, 270) could be used effectively to solve the problem, and for quantitative work the chromatographic procedure developed by Flood, Hirst, and Jones (*Nature*, 1947, **160**, 86) was found to be effective. The results showed that, with acorn starch as also with potato starch, a very small proportion of glucose (less than 1·3% of the weight of starch) was found after oxidation by periodate and subsequent hydrolysis. The significance of this small yield of glucose is difficult to assess at the moment, and we defer for later consideration the question whether this is of structural importance or is due to incomplete attack by the periodate. The proportion of glucose residues remaining unoxidised is very similar to the proportion of glucose residues involved in the disaggregation process when methylated amylopectin is subjected to the mild hydrolytic action of methyl alcoholic oxalic acid (Hirst and Young, *J.*, 1939, 1471) : what is abundantly clear, however, is that by far the greater proportion of the side chains, at least 75%, must be attached to the main chains through the 1 : 6 linkages.

Experimental.

Preparation of Starch.—The outer husks were removed from freshly collected acorns (1 kg.), and the cotyledons soaked for 7 days in water containing sulphur dioxide. The skins were then removed and the cotyledons passed through a mincing machine. The resultant slurry was rubbed through linen, and the starch allowed to separate out. The crude material was washed by decantation and freed from fibrous matter by allowing it to settle. The first material to separate was mainly fibre, and this was followed by a deposit of practically pure starch.

The product (*ca.* 70 g.) was purified by extraction with aqueous dioxan to remove fats. The light grey powder was examined under the microscope, and the granules were seen to be of a very different shape and dimension from those of potato starch, being much smaller and the hilum not being eccentric as it is in potato starch. Only a very small amount of broken-down thin cell-wall membrane could be seen admixed with the starch. The starch gave with boiling water a grey paste which was much more viscous than that given by potato starch under the same conditions. It was completely soluble in N-sodium hydroxide. On addition of the sodium hydroxide solution the starch turned deep brown, and by gentle warming it gave a yellow solution. A dispersion of the starch in water gave a deep blue coloration with iodine. The blue value at 20°, determined by the method of Hassid and McCready, was 0·42—0·44 (potato starch under similar conditions gave a blue value of 0·47). On hydrolysis for 7 hours with boiling 2% sulphuric acid (250 parts), the starch (1 part) gave 95% of the theoretical amount of glucose. (Two similar determinations with pure potato starch gave 98·7% of the theoretical amount.) No visible residue was left after hydrolysis. Fehling's test, negative; $[\alpha]_D^{16°}$ + 168° (*c*, 0·33 in N-sodium hydroxide) [Found, on a dried sample : sulphated ash, 0·5; furfuraldehyde (on boiling with 12% hydrochloric acid), 0·8%; glucose under these conditions yields 0·6% of furfuraldehyde].

Separation of Amylose.—Schoch's butanol method (*loc. cit.*) gave only a very incomplete separation of amylose on the material defatted by extraction with a mixture of dioxan and water. The amylose fraction had a blue value of 0·61 only, indicating, however, that some concentration of amylose had occurred; this value was higher than that of the initial starch (0·43) but considerably lower than that of pure amylose (about 1·40). Repetition of the process resulted in little further separation of the components.

Potentiometric Determination of Amylose.—The method used was the modification elaborated by Wilson, Schoch, and Hudson (*J. Amer. Chem. Soc.*, 1943, **65**, 1381) of the method of Bates, Rundle, and French (*ibid.*, p. 142). The amount of iodine taken up by 1 g. of starch was 0·0425 g. If 21·5% is the amount of iodine taken up by pure amylose (Higginbotham and Morrison, *Chem. and Ind.*, 1947, 45), the amylose content of the sample of acorn starch was 19·8%.

Acetylation of Acorn Starch.—The starch was acetylated in a similar manner to that described by Hirst and Young (*J.*, 1939, 953) for wheat starch. The air-dried starch (12 g.) was made into a paste (3%) with hot water (400 c.c.). The paste was grey and very viscous. It was precipitated by pouring it into methylated spirit (2000 c.c.). After filtration, the product was washed thrice with methylated spirit and dried under reduced pressure over calcium chloride, and a snow-white fluffy material (11 g.) thus obtained. This was mixed with pyridine (110 c.c.) and the mixture stirred for 7 hours and kept overnight (16 hours) at room temperature (10°). It gave a transparent homogeneous jelly. Acetic anhydride (110 c.c.) was then added, and the mixture stirred for a further 45 hours at 60° and 154 hours at 10°. The viscous, light brown, homogeneous solution was diluted with glacial acetic acid (350 c.c.) and poured into ice-cold water. The acetate was washed with water until free from acid, then with alcohol and ether, and dried in a vacuum over calcium chloride. Yield, 17·5 g. (90% of the theoretical); $\eta_{sp.}^{20°}/c$ 2·57 (*c*, 0·54 in *m*-cresol) (Found: $CH_3{\cdot}CO$, 45·3%). This acetate was almost entirely soluble in acetone, and the solution gave on evaporation a milky and fragile film.

Acorn starch was much more difficult to acetylate than potato starch. A comparative experiment with the latter gave a homogeneous and much more fluid solution after only 2 hours' stirring at 25°.

Direct Methylation of Granular Acorn Starch.—The granular acorn starch was methylated under conditions similar to those used by Hirst and Young (*loc. cit.*) for rice starch. Starch (18·2 g.) was made into a cream with cold water (150 c.c.), and 30% sodium hydroxide (100 c.c.) was added. The gel was dispersed by addition of water (50 c.c.); 30% sodium hydroxide (400 c.c.) and methyl sulphate (200 c.c.) were then added gradually at room temperature with vigorous mechanical stirring. After 15 hours' stirring at room temperature the alkali was partly neutralised with 50% sulphuric acid (120 g.). The

solution was evaporated on the steam-bath, and the product (without separation of mineral salts) was remethylated with methyl sulphate (135 c.c.) and 30% sodium hydroxide (330 c.c.). After 15 hours' stirring at room temperature, acetone (500 c.c.) was added and the mixture was evaporated on the steam-bath. The insoluble methylated starch was removed from the surface of the mixture, dissolved in acetone (400 c.c.) and water (100 c.c.), and remethylated with methyl sulphate (140 c.c.) and 30% sodium hydroxide (350 c.c.). After this methylation procedure had been repeated a further 7 times the product was purified by dissolution in aqueous acetone followed by filtration and removal of the solvent by distillation. It was dried under reduced pressure and extracted with ether in a Soxhlet apparatus. Yield, 18·4 g. (78% of the theoretical) (Found : OMe, 42·6%).

Fractionation of the Methylated Starch.—The methylated starch (16·7 g.) was extracted successively by boiling it for 4 hours under reflux with mixtures of ether and acetone, 80 : 20, 60 : 40, 20 : 80, and 100% by volume respectively. The blue values of the fractions were determined in the following way (cf. Hassid and McCready, *loc. cit.*). The methylated starch (25 mg.) was dissolved in cold water (25 c.c.). The solution (5 c.c.) was mixed with iodine reagent (5 c.c.) (Hassid and McCready, *loc. cit.*) and diluted to 100 c.c. The intensity of the colour recorded as log I/I_0 was measured at 20° in a Spekker absorptionmeter using 1 cm. cells and a red filter. On dilution to 300 c.c. the blue colour disappeared completely and suddenly. (1) 0·37 G.; $[\alpha]_D^{16°}$ + 208° (*c*, 0·37 in acetone); $\eta_{sp.}^{20°}/c$ 0·248 (*c*, 0·46 in *m*-cresol), corresponding to an apparent *M* of 55,000 (see Hirst and Young, *J.*, 1939, 1475); OMe, 43·6%. (2) 10·7 G.; $[\alpha]_D^{15°}$ + 209° (*c*, 0·38 in chloroform); B.V. 0·57; $\eta_{sp.}^{20°}/c$ 1·97 (*c*, 0·41 in *m*-cresol), corresponding to an apparent *M* of 725,000; OMe, 43·8%. (3) 3·85 G.; $[\alpha]_D^{15°}$ + 209° (*c*, 0·68 in chloroform); B.V. 0·56; $\eta_{sp.}^{20°}/c$ 1·93 (*c*, 0·38 in *m*-cresol), corresponding to an apparent *M* of 750,000; OMe, 43·8%. (4) 1·4 G.; $[\alpha]_D^{15°}$ + 209° (*c*, 0·75 in chloroform); B.V. 0·61; $\eta_{sp.}^{20°}/c$ 2·28 (*c*, 0·45 in *m*-cresol), corresponding to an apparent *M* of 770,000; OMe, 43·1%. (5) 0·10 G.; $[\alpha]_D^{15°}$ + 209° (*c*, 0·43 in chloroform); B.V. 0·35; OMe, 38·7%. (6) A residue insoluble in acetone, 0·06 g.; this gave no blue colour with iodine. The losses during fractionation were 0·18 g. or 1·1%.

Determination of End-groups.—Three different methods were used to determine the proportion of tetramethyl methylglucoside present in the products obtained on methanolysis of the methylated starch, namely, distillation, partition between light petroleum and water, and chromatography on alumina. The methanolysis was effected by boiling the methylated starch (1 part) with 1% methyl-alcoholic hydrogen chloride (50 parts) for 7 hours. The solution was then removed by addition of an ethereal solution of diazomethane and finally concentrated to a syrup on the boiling water-bath.

(a) *Distillation method.* A sample of methylated starch (fraction 2, 2·52 g.) was boiled with methyl-alcoholic hydrogen chloride, and the resultant methylglucosides isolated as described above. The syrup (2·7 g.) was dissolved in water (50 c.c.) and extracted with light petroleum (b. p. 40—50°, specially purified) in an all-glass continuous extractor. After extraction (30 hours) the solvent was distilled off and the residual syrup (0·952 g.) was distilled under reduced pressure (1·0 mm.). Two fractions were collected : (i) (bath temp. *ca.* 135°) 0·197 g.; $n_D^{20°}$ 1·4484; $[\alpha]_D^{18°}$ + 14·8° (*c*, 1·55 in water). (ii) (bath temp. 135—145°) 0·116 g.; $n_D^{19°}$ 1·4555; $[\alpha]_D^{18°}$ + 11·8° (*c*, 1·17 in water). Fraction (ii) was pure trimethyl methylglucoside (cf. Hirst and Young, *loc. cit.*). The proportion of tetramethyl methylglucoside in the first fraction was 46%; 10% was added for losses, giving a total amount 0f 0·10 g. of tetramethyl methylglucoside from 2·520 g. of initial methylated starch. The ratio of non-terminal to terminal groups calculated from these figures was 29 : 1.

(b) *Partition method* (Brown and Jones, *loc. cit.*). The methylated starch (fraction 2, 2·574 g.) was boiled with 1% methyl-alcoholic hydrogen chloride, and hydrogen chloride was then removed by addition of diazomethane. The syrupy glucosides (2·9 g.) isolated on concentration of the neutral solution were dissolved in water (50 c.c.), and the solution poured into the upper part of a double continuous all-glass extractor. In the lower part of the extractor water (50 c.c.) was introduced. The following fractions were obtained by successive extractions of 7 hours with light petroleum (b. p. 40—60°; free from sulphur compounds and unsaturated paraffins). Fraction 1, 0·220 g.; $n_D^{22°}$ 1·4490. Fraction 2, $n_D^{22°}$ 1·4490. Fraction 3, $n_D^{22°}$ 1·4500. Fraction 4, 0·056 g.; $n_D^{22°}$ 1·452. Fraction 5, 0·116 g.; $n_D^{20°}$ 1·455. The last fraction crystallised and was pure trimethyl methylglucoside. The first 4 fractions were combined and extracted in the same way, and the following fractions were then isolated : (*a*) 0·110 G.; $n_D^{22°}$ 1·4452. (b) 0·019 G.; $n_D^{22°}$ 1·4503; $[\alpha]_D^{21°}$ + 21° in water. (*c*) 0·024 G.; $n_D^{18°}$ 1·4553. This fraction crystallised and was pure trimethyl methylglucoside. Fraction (*a*), a pale yellow liquid, was distilled in a micro-distillation apparatus, and gave a colourless distillate (102 mg.; $n_D^{24°}$ 1·4430; $[\alpha]_D^{24°}$ + 31° in water) and a brown residue (7·4 mg.). As the distillate was not pure tetramethyl methylglucoside it was distilled in the same apparatus and gave three fractions : (*a*) 64 Mg.; $n_D^{18°}$ 1·4427; $[\alpha]_D^{19°}$ + 45° in water) (Found : OMe, 60·3%). (*β*) 30·7 Mg.; $n_D^{19°}$ 1·4480 (Found : OMe, 55·2%). (*γ*) A brown residue, 3·5 mg. (Found : OMe, 45%). Fraction (*a*) is pure tetramethyl methylglucoside. From the values of refractive indices and specific rotations it was calculated that fraction (*β*) contained 56% or 17·3 mg. of tetramethyl-glucoside and fraction (*b*) 32% or 6·0 mg. of tetramethyl methylglucoside. The total amount of tetramethyl methylglucoside was then 88 mg; 10% was added to this figure for losses during extraction and distillation. The ratio of non-terminal to terminal groups calculated from this figure was 30 : 1.

(c) *Alumina chromatogram method* (Brown and Jones, *loc. cit.*). The methylated starch (4·64 g., fraction 2) was boiled with 1% methyl-alcoholic hydrogen chloride, and the hydrogen chloride was removed by addition of ethereal diazomethane. The syrupy methylglycosides obtained by removal of the solvent were dissolved in water (50 c.c.), and the solution was extracted continuously with light petroleum (b. p. 40—60°, specially purified) until all the tetramethyl methylglucoside had been removed, *i.e.*, until a sample of the petroleum on evaporation left a syrup which had a refractive index corresponding to that of trimethyl methylglucoside. The mixture of methylglucosides (*ca.* 0·3 g.) was dissolved in light petroleum (50 c.c., b. p. 40°, purified) and passed through a column of activated alumina (British Aluminium Co., Burntisland) and eluted with dry alcohol-free chloroform. The following fractions were obtained. (A) 156 Mg.; $n_D^{21°}$ 1·4420; $[\alpha]_D^{19°}$ + 63·5° (*c*, 1·56 in water). (B) 78 Mg.; $n_D^{19°}$ 1·4502; $[\alpha]_D^{20°}$ + 32° (*c*, 3·41 in water). (C) 28 Mg.; $n_D^{20°}$ 1·4550; $[\alpha]_D^{19°}$ + 18·6° (*c*, 1·1 in water). Fraction (A)

is pure tetramethyl methylglucoside, (C) is pure trimethyl methylglucoside, and (B) is a mixture of the two containing 40% or 31·2 mg. of tetramethyl methylglucoside. The total yield of tetramethyl methylglucoside is 187 mg., corresponding to the occurrence of one end group in every 30 glucose residues.

Determination of End Group in Acorn Starch by the Periodate Oxidation Method.—A sample of starch (0·50 g.) was dissolved in water (80 c.c.) containing potassium chloride (5 g.) and oxidised with sodium metaperiodate (40 c.c., 0·31M). The mixture was shaken, and at intervals samples (20 c.c.) were withdrawn, ethylene glycol added to destroy the excess of periodate, and the formic acid titrated, using methyl-red as indicator (Found: G.-mol. of formic acid per 162 g. starch: 7 days, 0·035; 8 days, 0·036; 10 days, 0·036). The percentage of end group calculated from the yield of formic acid after 7 days was 29.

Determination of Glucose Residues Linked through C_1, C_4, *and* C_6.—The starch (2 g.) was mixed with 0·27M-sodium periodate (60 c.c.), potassium chloride (10 g.), and water (220 c.c.) in a 500 c.c. glass-stoppered bottle, and the mixture was shaken for 10 days at room temperature. Excess of ethylene glycol (5 c.c.) was then added, and shaking was continued for 24 hours. The oxidised starch was filtered on a G3 sintered-glass filter, washed with cold water until free from oxidant (test with potassium iodide and sulphuric acid and diphenylamine and sulphuric acid) and then with alcohol, and dried in a vacuum desiccator over calcium chloride. The dry oxidised starch was hydrolysed with N/2-sulphuric acid (200 c.c.) for 10 hours at 95°. To the hot solution aqueous barium hydroxide was added until the pH was 5. The solution was then filtered, and barium was removed from the soluble barium salts of organic acids by addition of a slight excess of N-sulphuric acid. Barium sulphate was removed on the centrifuge, and the clear solution was extracted continuously with ether in order to eliminate any glyceraldehyde and acids formed after hydrolysis of the oxidised polysaccharide. As only a small amount of organic acid was extracted, this operation was omitted in subsequent experiments. The glucose determination was carried out by the new paper chromatogram method (Partridge, *loc. cit.*; Flood, Hirst, and Jones, *loc. cit.*). The following quantities of glucose were found (calculated on 100 g. of starch): potato starch, 1·1 g.; acorn starch, 1·8 g.; sago starch, 1·2 g.

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