499. *The Constitution of Barley Starch.*

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Barley starch has been found to contain 19% of amylose, a highly purified specimen of which was obtained by repeated precipitation with butanol. Methylation studies revealed that the amylose consisted of unbranched chains containing an average of *ca.* 400 glucose units. No amylopectin fraction was obtained free from amylose. The purest (containing 2% of amylose) was obtained by precipitation with aqueous pyridine saturated with butanol. The presence in the amylopectin of one non-reducing terminal group for 26 ± 2 non-terminal glucose residues was found by the methylation technique. This result was confirmed by periodate oxidation which also indicated that over 86% of the branching linkages between the unit chains involve 1 : 6-linkages.

The structures of various starches have been the subject of recent study (Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27; Hirst, Jones, and Roudier, *J.*, 1948, 1779; Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488, 3774; Potter, Hassid, and Joslyn, *ibid.*, 1949, **71**, 4075; Meyer *et al.*, *Helv. Chim. Acta*, 1940, **23**, 845, 854, 865). In the present paper these investigations have been extended to the starch from the barley grain (" Pioneer " variety). The starch was separated by a purely mechanical process and the use of reagents likely to cause degradation was avoided.

The amylose content of the starch was 19% as determined by the iodine potentiometric method (Bates, French, and Rundle, *J. Amer. Chem. Soc.*, 1943, **65**, 142; Wilson, Schoch, and Hudson, *ibid.*, p. 1381). Barley starch is, therefore, similar to most other cereal starches. A separation of the amylose and amylopectin components of the starch was carried out by four different methods (see Tables I, I*a*) as follows, with (1) thymol (Haworth, Peat, and Sagrott, *Nature*, 1946, **157**, 19; Bourne, Donnison, Haworth, and Peat, *J.*, 1948, 1687), (2) *n*-butanol (Schoch, *J. Amer. Chem. Soc.*, 1942, **64**, 2957; Higginbotham and Morrison, *Chem. and Ind.*, 1947, **25**, 45; *Shirley Inst. Mem.*, 1948, **22**, 141), (3) pyridine (*idem*, *loc. cit.*), and (4) 20% aqueous pyridine saturated with butanol (*idem*, *loc. cit.*). The results (Table I) show that in no case was fractionation complete. In all the fractionations described (Table I) the " amylose " fraction generally contained 25—40% of amylopectin, and the " amylopectin " fraction contained 5—8% of amylose. It, therefore, appeared that no single method of fractionation was capable of yielding pure amylose and amylopectin from barley starch. Reprecipitations of the " amylose " fraction with thymol failed to effect further purification but by repeated precipitation with butanol (*idem*, *loc. cit.*), with low concentrations of amylose (0·2%), fractions were obtained which took up 20·9% of their own weight of iodine. Further reprecipitation (amylose concentration, 0·1%) failed to raise this figure. Since precipitation with pyridine (Table I) yielded the " amylose " fraction containing the greatest proportion of the total amylose a larger quantity of starch was fractionated by this method. The crude amylose was then (Table II) reprecipitated several times with butanol (amylose concentration 0·2%). The product was finally obtained in good yield (representing about 80% of the total amylose present in the starch) and took up 20·9% of its own weight of iodine (Table II). Further reprecipitation (0·1% concentration) failed to raise this value which compares favourably with those found by Higginbotham and Morrison (*loc. cit.*) for other amyloses. On examination under the microscope the amylose–butanol complexes appeared similar to those already described for such complexes from other starches, and it is concluded that the barley amylose–butanol complex is approaching its purest state when it separates in the form of short needles (cf. Kerr, " Chemistry and Industry of Starch," New York 1944, p. 147).

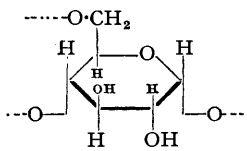
The barley amylose was methylated by repeated treatments with methyl sulphate and sodium hydroxide in nitrogen, to give a product containing OMe, 44·7% (calc. : OMe, 45·6%). Previous workers have detected small quantities of tetramethyl glucose (or tetramethyl methylglucoside) in the hydrolysates of methylated amyloses. Meyer, Wertheim, and Bernfeld (*Helv. Chim. Acta*, 1940, **23**, 865; 1941, **24**, 378) estimated that the chain lengths of maize and potato amyloses were 200—300 units. Similar results were found by Hess and Krajnc (*Ber.*, 1940, **73**, 976) and Hassid and McCready (*J. Amer. Chem. Soc.*, 1943, **65**, 1157) for potato amylose. Brown, Halsall, Hirst, and Jones (*loc. cit.*), estimated sago amylose to have a chain length of 250 ± 100 glucose units, and Bourne, Fantes, and Peat (*J.*, 1949, 1109) and Barker, Bourne,

and Wilkinson (*J.*, 1950, 3027) found chain lengths of about 200 glucose units for samples of potato amylose.

The hydrolysed methylated barley amylose was separated on a cellulose column (Hough, Jones, and Wadman, *J.*, 1949, 2511). The main product of hydrolysis was identified as 2 : 3 : 6-trimethyl D-glucose. This was accompanied by small quantities of tetramethyl glucose and a mixture of dimethyl glucoses. The amount of tetramethyl glucose was estimated to be 0·24—0·29% of the methylated glucoses produced on hydrolysis, corresponding to the presence of one non-reducing terminal group per 400 ± 40 glucose residues. Paper chromatography indicated the presence of at least two different dimethyl glucoses corresponding (1) to 2 : 3-dimethyl glucose and (2) to 2 : 6- and/or 3 : 6-dimethyl glucose (cf. Bell, *J.*, 1948, 992; Hirst, Hough, and Jones, *J.*, 1949, 928; Barker, Bourne, and Wilkinson, *loc. cit.*, for methylated starches and amylopectins). The dimethyl glucoses amounted to *ca.* 1·6% of the total hydrolysate and the proportion of 2 : 3-dimethyl glucose was estimated to be only *ca.* 0·05%. The mixture of dimethyl sugars was converted into the methylglycosides and the amount of 2 : 6-dimethyl methylglucoside was estimated to be *ca.* 1·3% by oxidation with sodium metaperiodate (Bell, *loc. cit.*). These dimethyl glucoses do not appear to have any structural significance for the reasons outlined below.

Attempts to determine the molecular size of barley amylose itself have given somewhat conflicting results. Oxidation with potassium periodate (Brown, Halsall, Hirst, and Jones, *loc. cit.*) and titration of the formic acid released, indicated the production of one mole of formic acid per 72 ± 7 units. If it is assumed that two molecules of formic acid are released from the reducing end-group, this result corresponds to the presence of one non-reducing terminal group per 216 ± 20 glucose residues. The colorimetric method of estimating the reducing power, in which 3 : 5-dinitrosalicylic acid is used in alkaline solution (Meyer, Noelting, and Bernfeld, *Helv. Chim. Acta*, 1948, **31**, 103) and the intensity of the colour is compared with that developed by maltose solutions of known concentration, indicated the presence of one aldehyde group per 135 ± 20 glucose units.

After hydrolysis of the amylose oxidised by periodate and examination by paper chromatography, no glucose was observed to be present. This indicated that there were in the amylose no glucose residues joined to each other through $C_{(2)}$ and $C_{(3)}$ (see Hirst, Jones, and Roudier, *loc. cit.*). All residues must, therefore, be joined through $C_{(1)}$, $C_{(4)}$, or $C_{(6)}$. Since the hydrolysed methylated derivative yielded only a very small proportion of 2 : 3-dimethyl glucose it is concluded that the greater part of the amylose consists of straight chains of glucose units united by 1 : 4-α-linkages. The appearance of 2 : 6- and 3 : 6-dimethyl glucoses in the hydrolysate must, therefore, be due to incomplete methylation of the polysaccharide or to the demethylation of 2 : 3 : 6-trimethyl glucose, and the presence of the small amount of 2 : 3-dimethyl glucose detected may also be attributed to these causes. From the available evidence, it is justifiable to consider that barley amylose consists for the most part of long unbranched chains of D-glucose residues united by 1 : 4-α-linkages. It is not yet possible to assign a precise length to the chain although it is certainly of the order of several hundred glucose units.



The fractionations employed (Table I) did not yield an amylopectin fraction containing less than 5% of amylose. The subjection of these fractions to further treatment with the precipitants did not yield any significant amounts of the amylose complex.

In their studies on fractionations of starches, Higginbotham and Morrison (*loc. cit.*) isolated only one sample of amylopectin which was free from amylose. This was obtained by dispersing the starch in water containing pyridine (20% by volume) followed by saturation with *n*-butanol; the yield of the non-precipitated fraction was low, however, since as much as 50% of the total amylopectin was precipitated with the amylose. Fractionation of barley starch by this method yielded an " amylopectin " fraction containing only 2% of amylose but representing only 50% of the total amylopectin present in the starch (cf. Table I). This result seemed to indicate that in barley starch, as in other starches, there exists a portion of the amylose which is not readily separated from the amylopectin.

The " amylopectin " (containing 2% of amylose) was methylated by repeated treatments with methyl sulphate and sodium hydroxide followed by methyl iodide and silver oxide. The methylated product (OMe, 45·0%) was hydrolysed and, by filter-paper chromatography, the presence of the following sugars was indicated: (*a*) 2 : 3 : 4 : 6-tetramethyl glucose (4·2%), (*b*) 2 : 3 : 6-trimethyl glucose (90·5%), (*c*) dimethyl glucose (5·3%); and (*d*) traces of monomethyl glucose and of glucose. The proportion of tetramethyl glucose corresponds to the

presence of one non-reducing terminal group per 25 glucose residues. A similar result was obtained when a quantity of the methylated amylopectin hydrolysate was separated on a cellulose column (Hough, Jones, and Wadman, *loc. cit.*) although two distinct dimethyl glucoses were produced. From the amount of tetramethyl glucopyranose isolated (*ca.* 4%) it was calculated that the amylopectin contained one non-reducing terminal group per 26 ± 2 glucose units. The proportion of glucose units per non-reducing terminal group in barley amylopectin is, therefore, similar to those found for other amylopectins (see Brown, Halsall, Hirst, and Jones, *loc. cit.*). The yields of both the end-group and 2 : 3 : 6-trimethyl glucose (*ca.* 90%) could not have been seriously affected by losses caused by demethylation during the acid hydrolysis (cf. Bell, *loc. cit.*). A similar experiment carried out on chromatographically pure 2 : 3 : 4 : 6-tetramethyl glucose and 2 : 3 : 6-trimethyl glucose showed (paper chromatography) that the former gave 1% of trimethyl glucose whilst the latter gave 0·7% of dimethyl glucoses.

The 2 : 3 : 4 : 6-tetramethyl, 2 : 3 : 6-trimethyl, and 2 : 3-dimethyl glucoses (*ca.* 4·5%) were obtained crystalline. The tetramethyl glucose was identified as the crystalline anilide, the trimethyl glucose by its inversion of optical rotation in cold methanolic hydrogen chloride solution (Irvine and Hirst, *J.*, 1922, **121**, 1213), and the 2 : 3-dimethyl glucose as the crystalline 2 : 3-dimethyl gluconophenylhydrazide (Evans, Levi, Hawkins, and Hibbert, *Canad. J. Res.*, 1942, **20**, *B*, 175). The second dimethyl glucose fraction (*ca.* 1%) did not crystallise but the inversion of the optical rotation in cold methanolic hydrogen chloride solution indicated that the hydroxyl groups on $C_{(4)}$ and $C_{(5)}$ were free. Treatment of the mixture of derived methyl glycosides with sodium metaperiodate by Bell's method (*loc. cit.*) indicated that this fraction contained 79% of 2 : 6-dimethyl glucose in admixture with 3 : 6-dimethyl glucose.

Oxidation of the barley amylopectin by potassium periodate indicated the presence of one non-reducing terminal group per 24 glucose residues. Similar oxidation of the whole starch indicated the presence of one non-reducing terminal group per 30 glucose residues. Since the starch contains 19% of amylose, it can be calculated from this result that the amylopectin fraction contains one non-reducing terminal group per 24 glucose residues which agrees with the result calculated from the experiment with amylopectin itself.

Estimations of the reducing power of the amylopectin with 3 : 5-dinitrosalicylic acid did not yield significant results. This, together with other evidence, indicates that the amylopectin of barley has a high molecular weight and a highly branched structure. The presence of 2 : 3-dimethyl glucose in amount only slightly greater than the yield of tetramethyl glucopyranose indicates that branching takes place through $C_{(6)}$ of one of the 1 : 4-linked glucose residues of a unit chain, and additional evidence has been found as in the experiments on acorn starch (Hirst, Jones, and Roudier, *loc. cit.*) to support this view. On hydrolysis of the barley amylopectin after periodate oxidation the free glucose released (0·59%) indicated that at least 86% of the linkages between the unit chains were 1 : 6-linkages. It was not possible to decide whether in fact all the branch linkages were of this type or whether the remainder were 1 : 3- or, less probably, 1 : 2-junctions because the residual glucose might have arisen by incomplete oxidation, and the 2 : 6- and 3 : 6-dimethyl glucoses by a combination of incomplete methylation and demethylation.

Experimental.

Preparation of Starch.—Barley (Pioneer variety; 1 kg.) was roughly ground in a "Raymond" laboratory mill and extracted with light petroleum (b. p. 40—60°) to remove fatty substances. After being dried at a low temperature, the grist was shaken with water (5 l.) for 6 hours. Most of the fibrous material was removed from the suspension by passage through 40- and 150-mesh sieves. The screened suspension was set aside overnight and a deposit of material rich in starch settled out. The supernatant liquid was siphoned off and fresh water added to *d* 1·030 (by hydrometer). After being stirred for 1 hour, the suspension was centrifuged (1500 r.p.m.). The deposit consisted of two well-defined layers—the lower of almost pure starch and the upper of fine fibrous material together with insoluble protein and some associated starch. The upper layer was removed as completely as possible by scraping with a spatula and by washing with a stream of water. Water was added to the remaining starch layer (to give *d* 1·030) and the process was repeated until all traces of impurity were removed. The starch was filtered off, dried at 40°, and ground with pestle and mortar. The fat content was further reduced by boiling the starch under reflux with methanol containing water (15% by weight). After three such treatments, the starch was filtered off, washed with water, dried at 40°, and reground.

The white powder (137 g.) was observed to be composed of granules of a moderate size (10—35 μ.) and ovoid to spheroidal. A suspension of the starch in water gave a deep blue colour with iodine. On 7 hours' heating with boiling 2% sulphuric acid (250 parts), the starch (1 part) gave 96% of the theoretical amount of glucose. No visible residue was left after hydrolysis. No other sugar could be detected on the paper chromatogram. This material had $[\alpha]_D^{18}$ +157° (*c*, 1·0 in N-sodium hydroxide), $[\alpha]_D^{18}$ +188° (*c*, 0·69 in 30% perchloric acid) (Nielsen, *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 176) [Found : sulphated ash, 0·2; N, 0·04% (Kjeldahl)]. The blue value determined by Hassid and McCready's method

(*J. Amer. Chem. Soc.*, 1943, **65**, 1154) as modified by Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924) was 0·27—0·28 (potato starch under similar conditions gave a blue value of 0·28).

Potentiometric Determination of Amylose.—The method of Bates, French, and Rundle (*J. Amer. Chem. Soc.*, 1943, **65**, 142), as modified by Wilson, Schoch, and Hudson (*ibid.*, p. 1381), was used. The amount of iodine taken up by 1 g. of starch was 0·0402 g. If 21·5% is the amount of iodine taken up by pure amylose (Higginbotham and Morrison, *loc. cit.*), the amylose content of the sample of barley starch was 18·7%.

Acetylation of Barley Starch.—Pacsu and Mullen's method (*J. Amer. Chem. Soc.*, 1941, **63**, 1487) was used. Air-dried starch (15 g.) gave a chloroform-soluble acetate (21·5 g.), η_{sp}^{20}/c 1·13 (*c*, 0·4 in *m*-cresol), $[\alpha]_D^{14}$ +170° (*c*, 0·5 in chloroform) (Found : $CH_3{\cdot}CO$, 43·3. Calc. for $C_{12}H_{16}O_8$: $CH_3{\cdot}CO$, 44·8%).

The acetyl groups were removed by the method of Zemplén and Pacsu (*Ber.*, 1929, **62**, 1613), using sodium methoxide. Acetylated starch (10 g.) was dissolved in dry chloroform (250 c.c.). Dry methanol (50 c.c.) containing dissolved sodium (50 mg.) was slowly added with shaking. The mixture was shaken for 1 day and set aside for 2 days more. The fine white precipitate was filtered on a G4 sintered-glass filter and washed with several portions of ethanol until free from sodium, and finally with ether. This material (5·5 g.) had blue value (see above) 0·283 and $[\alpha]_D^{18}$ +192° (*c*, 0·6 in 30% perchloric acid) (Found : $CH_3{\cdot}CO$, nil). The sample was later used in the fractionation experiments (see Table I below, Method I*b*).

"*Amylose*" *fraction.*

Separation of Amylose.—Method I. By thymol. (*a*) The standard procedure of Bourne, Donnison, Haworth, and Peat (*loc. cit.*) for potato starch was applied though on a smaller scale (5—10 g.). Results (Table I) seemed to indicate that dispersion of the starch was incomplete. Various modifications, (*b*), (*c*), and (*d*) (Table I), were then applied to effect better dispersion.

Method II. By butanol (Schoch, *loc. cit.*; Higginbotham and Morrison, *loc. cit.*). Dry starch (5 g.), suspended in cold water (20 c.c.), was slowly added at 90° with continuous stirring to water (200 c.c.), saturated with *n*-butanol (40 c.c.). The stirring was continued at this temperature for 2½ hours. The paste was cooled to 70° and centrifuged (10,000 r.p.m.) for 5 minutes. The temperature of the paste was readjusted to 90°. The solution was transferred to a Dewar flask and allowed to cool very slowly. After 60 hours, the amylose–butanol complex was removed in a centrifuge (5000 r.p.m.) and washed with several portions of water saturated with butanol. The precipitate was dehydrated and freed from

TABLE I.

Conditions of dispersion.							Amylose fraction.		Approx. total amylose in precipitated fraction, %	Amylopectin fraction.		Approx. total amylopectin in non-precipitated fraction, %
Conc. of starch.	Temp.	Time, hrs.	Solvent.	Starch dissolved, %.	Method.	Precipitant.	B.V.	Amylose, %.		B.V.	Amylose, %.	
3	100°	⅓	Water (cont. NaCl)	58	I*a*	Thymol	0·80	60 †	32	0·150	8·0 †	75
3	100	⅓	,,	98	I*b*	,,	0·91	66	63	0·141	7·4	89
3	100	⅓ *	,,	96	I*c*	,,	1·03	74	56	0·141	7·4	94
3	100	3	,,	96	I*d*	,,	1·03	74	72	0·128	6·6	92
2	90	2½	Water satd. with butanol	96	II	Butanol	0·90	65	68	0·137	7·1	87
2	90	2½	Water containing pyridine (15%)	96	III	Pyridine	0·99	71 †	76	0·111	5·3	89

TABLE I*a*.

2	90	½ *	Water cont. pyridine (20%) satd. with butanol	94	IV*a*	Pyridine–butanol	0·41	29	85	0·087	2·8 †	52
2	90	1 *	,,	96	IV*b*	,,	0·42	30	89	0·067	2·0 †	51
2	90	3 *	,,	98	IV*c*	,,	0·43	31	91	0·067	2·0	50

* 5 Minutes' high-speed stirring in an "Ato-Mix" disperser was given in addition to the time stated.

† The amylose content was determined by potentiometric titration. All other amylose contents were calculated from the blue values.

butanol by being repeatedly triturated with alcohol and then ether. The amylopectin fraction was precipitated with alcohol in the usual way.

Method III. By pyridine. The procedure of method II was followed, but pyridine (15% by wt.) was used. The complex was washed with water saturated with butanol as above.

Results are recorded in Table I.

Reprecipitation of the " amylose " fraction from methods I(*c*) and I(*d*) with thymol failed to increase the purity. Reprecipitation of the " amylose " fraction (B.V., 0·90), prepared by method II, with butanol gave a product (B.V., 1·06) richer in amylose.

Purification of the Amylose.—As indicated in Table I separation by method III using pyridine produced the " amylose " fraction containing the greatest proportion of the amylose present in the starch (76%). A larger quantity (40 g.) was, therefore, fractionated by this method. A small quantity of the amylose–pyridine complex was broken down by trituration with alcohol for analysis, from which it was calculated that the complex contained 9·0—9·5 g. of crude amylose (B.V., 0·99). The remainder was washed with water saturated with butanol and subjected to reprecipitation with butanol from 0·2% solution. Several reprecipitations were carried out (Table).

No. of reprecipitation	0	1	2	3	4	5	6	7 *
Uptake of iodine detd. by potentiometric titration, %	15·3	17·1	18·0	19·2	20·1	20·8	20·9	20·9
Blue value	0·99	1·09	1·15	1·20	1·25	1·29	1·30	1·30

* This reprecipitation was carried out at an amylose concentration of 0·1%. After this reprecipitation the complex was stored in water saturated with butanol. It was calculated that the complex contained 6 g. of amylose (representing approx. 80% of the total amylose present in the starch).

Examination of the Amylose–butanol Complex.—After the second reprecipitation (Table II) the complex was examined under the microscope and appeared similar to the maize amylose–butanol complex (Schoch, *loc. cit.*), taking the form of six-segmented particles (approx. diameter 5—15 μ.). After the third reprecipitation, the complex appeared in the form of rectangular platelets and showed similarity to the maize amylose–butanol compound found by Kerr and Severson (*J. Amer. Chem. Soc.*, 1943, **65**, 193). After the fourth reprecipitation the complexes appeared in the form of short thin needles similar to the potato and tapioca amylose–butanol complexes described by Kerr (*J. Amer. Chem. Soc.*, 1945, **67**, 2268; " The Chemistry and Industry of Starch, New York 1944, p. 147). The complex retained this structure after 4 more reprecipitations.

Investigation of the Amylose.—The butanol–amylose complex dissolved easily in warm water to give a clear solution which was coloured an intense blue on the addition of iodine solution (0·1N.). After 2—3 days, the amylose solution became cloudy and after 7 days a precipitate could be separated. Hydrolysis of the amylose with boiling sulphuric acid (2%) gave 98% of the theoretical yield of glucose. The complex had $[\alpha]_D^{18}$ +205° (*c*, 0·5 in water), +149° (*c*, 0·5 in N-sodium hydroxide), and +200° (*c*, 0·5 in 30% perchloric acid). (When preparing specimens for analysis, the usual method of precipitation from aqueous solution by alcohol was not adopted. An aliquot of the solution was evaporated to dryness and the weight of amylose determined directly.)

Methylation of Amylose.—The amylose (4·5 g.) was methylated under conditions similar to those used by Hirst, Jones, and Roudier (*J.*, 1948, 1779), 14 treatments being given. The product (3·8 g.) was isolated and purified by washing it with boiling water until free from sulphate and by precipitation from chloroform solution with light petroleum (b. p. 40—60°) (Found : OMe, 44·7%). The methylated amylose was obtained as a white solid forming rubber-like films which could not be powdered.

Fractionation of Methylated Amylose.—Fractionation from chloroform solution by the addition of light petroleum (b. p. 40—60°) gave : (*a*) 2·7 g. (Found : OMe, 44·8%), $[\alpha]_D^{20}$ +206° (*c*, 0·5 in chloroform), η_{sp}^{20}/c, 0·45 (*c*, 0·4 in *m*-cresol); (*b*) 0·8 g. (Found : OMe, 44·9%), $[\alpha]_D^{20}$ +204° (*c*, 0·5 in chloroform), η_{sp}^{20}/c, 0·335 (*c*, 0·4 in *m*-cresol); and (*c*) 0·2 g. (Found : OMe, 21·7%). Fraction (*c*) was not investigated further.

By use of Staudinger's equation $\eta_{sp} = Km.Mc$ (c = the concentration in g.-mol. of repeating units per l.) and $Km = 12 \times 10^{-4}$ (the value for the structurally similar methylated cellulose; Staudinger and Reinecke, *Annalen*, 1938, **535**, 47) the apparent values for the molecular weights of fractions (*a*) and (*b*) are found to 76,000 (D.P. 375) and 57,000 (D.P. 280) respectively, but since the true value for the constant is unknown these figures are only an indication of the order of magnitude of the molecular weights.

Hydrolysis of Methylated Amylose and Separation of Methylated Glucoses.—Fraction (*a*) (2·65 g.) was boiled under reflux in methanolic hydrogen chloride (150 c.c.; 1%) for 7 hours. After neutralisation with silver carbonate, treatment with hydrogen sulphide, etc., and concentration, a clear golden non-reducing syrup (2·93 g.) was obtained. The syrup was boiled under reflux with hydrochloric acid (100 c.c.; 2%) for 7 hours. After neutralisation with silver carbonate and concentration, a syrup (2·63 g.) was obtained which partly crystallised on storage. Examination of the syrup by paper chromatography (Hirst, Hough, and Jones, *loc. cit.*) indicated the presence of a single substance corresponding to 2 : 3 : 6-trimethyl glucose.

The hydrolysate was then separated on a column of cellulose (powdered Whatman No. 1 ashless filter tablets; 50 × 3·5 cm.) in the usual way (Hough, Jones, and Wadman, *loc. cit.*; Chanda *et al.*, *J.*, 1950, 1289). By elution with 50% light petroleum (b. p. 100—120°)–50% *n*-butanol, saturated with water, fractions (1) (22 mg.) and (2) (2·217 g.) were obtained, and by elution with water fraction (3) (39 mg.) was isolated (total recovery, 86%). [Groups of 5 tubes were combined and examined by paper chromatography before fraction (1) was detected.] The collected fractions were dissolved in water, warmed with charcoal, filtered through " Filter-Cel " and, after evaporation of the solvent, dried over phosphoric oxide at 35° in a vacuum.

Examination of the Fractions.—The R_G value of fraction (1) on a paper chromatogram was identical with that of tetramethyl glucopyranose. The fraction was dissolved in water (12·5 c.c.), and a portion

(2·5 c.c.) hydrolysed by boiling with sulphuric acid (1 ml.; 2%) for 6 hours. After neutralisation with barium carbonate and examination by paper chromatography, the presence of an additional substance was indicated, the R_G value of which corresponded to that of 2 : 3 : 6-trimethyl glucose. Quantitative determination of the mixture by the method of Hirst, Hough, and Jones (*loc. cit.*) [the more convenient phosphate buffer (Chanda *et al.*, *loc. cit.*) was used in this and other similar analyses] revealed the presence of 33·7% of tetramethyl glucose. Two portions (each 5 c.c.) of the solution were oxidised with buffered hypoiodite :

Titre of thiosulphate (c.c.) (0·0105N.)	2·062	2·159
Wt. of sugar oxidised (mg.)	2·56	2·68

From these results, the amount of tetramethyl glucose in fraction (1) was calculated to be 6·55 ± 0·15 mg., corresponding to the presence of one non-reducing terminal group per 400 ± 40 glucose residues. The solutions obtained after determination of the tetramethyl glucose were combined and neutralised with barium carbonate. After filtration and evaporation to dryness (reduced pressure) the residue was extracted several times with chloroform. The chloroform extract was evaporated to dryness and the residue hydrolysed with boiling sulphuric acid (1 c.c.; 2%) for 6 hours. After neutralisation paper chromatography showed the presence of 2 : 3 : 6-trimethyl glucose only (Found : 13 mg. by hypoiodite oxidation).

Fraction (2) crystallised completely and after two recrystallisations from dry ether, had m. p. 115—117° (alone or admixed with an authentic sample of 2 : 3 : 6-trimethyl glucose). The R_G value on a paper chromatogram was identical with that of 2 : 3 : 6-trimethyl glucose. The substance had $[\alpha]_D^{18}$ +94° (initial), +71° (constant) (*c*, 1·0 in water), and +69° (initial), −34° (7 hours, constant) (*c*, 1·0 in cold 2% methanolic hydrogen chloride solution) (cf. Irvine and Hirst, *loc. cit.*) (Found : OMe, 41·4. Calc. for $C_9H_{16}O_6$: OMe, 41·9%).

Fraction (3) did not crystallise; it had $[\alpha]_D^{18}$ +62° (*c*, 0·5 in water), $[\alpha]_D^{18}$ +60° (initial), −10° (6 hours const.) (*c*, 0·4 in 2% methanolic hydrogen chloride) (Found : OMe, 28·9. Calc. for $C_8H_{16}O_6$: OMe, 29·8%). Examination on the paper chromatogram showed two distinct spots corresponding to (1) 2 : 3-dimethyl glucose (R_G, 0·57) and (2) another dimethyl glucose (R_G, 0·51) (cf. Bell; Hough, Hirst, and Jones; Barker, Bourne, and Wilkinson, *locc. cit.*), the latter being in greater concentration. Traces of monomethyl glucose and glucose were also observed. Quantitative determination of the mixture of dimethyl sugars by the method of Hirst, Hough, and Jones (*loc. cit.*) indicated the presence of 2·6% of 2 : 3-dimethyl glucose (1 mg.). Fraction (3) (35 mg.) was converted into the methyl glucosides and treated with sodium metaperiodate by Bell's method (*loc. cit.*). The uptake of periodate was 0·84 mole per $C_9H_{18}O_6$ unit, indicating that fraction (3) contained 84% of 2 : 6-dimethyl glucose. After the determination, the excess of periodate was destroyed by shaking it with ethylene glycol. The glucosides were extracted, hydrolysed, and examined by paper chromatography as described for fraction (1). Two spots were observed—one (R_G, 0·57) corresponded to 2 : 3-dimethyl glucose whilst the other (R_G, 0·51) corresponded to 3 : 6-dimethyl glucose.

Partial Demethylation of 2 : 3 : 4 : 6-*Tetramethyl and* 2 : 3 : 6-*Trimethyl Glucose* (cf. Bell, *loc. cit.*).—Pure 2 : 3 : 4 : 6-tetramethyl and 2 : 3 : 6-trimethyl glucose (50 mg.) were heated in sealed tubes with 1% methanolic hydrogen chloride (1 c.c.) for 7 hours at 100°. After cooling, the acid was neutralised with silver carbonate. After filtration, the solutions were evaporated to dryness, the residues hydrolysed with 1% hydrochloric acid (2·5 c.c.) by heating at 100° for 7 hours, and the mixtures of free sugars after removal of inorganic ions were isolated as syrup which were examined by paper chromatography. The tetramethyl glucose was accompanied by trimethyl hexose (R_G, 0·82) (1%). Faint traces of dimethyl and monomethyl glucoses were also observed. The trimethyl glucose was accompanied by two dimethyl hexose spots (R_G, 0·58, 0·52) (the latter in greater concentration). A trace of monomethyl glucose was also observed. Quantitative determination of the two dimethyl sugars together indicated that they were present to an extent of 0·7%.

Determination of End Group in Barley Amylose by Periodate Oxidation.—Barley amylose (0·25 g.) was treated with sodium metaperiodate and potassium chloride (Brown, Halsall, Hirst, and Jones, *loc. cit.*), and the acid titre after 150 hours' oxidation corresponded to 0·0154 g.-mol. of formic acid from 162 g. of amylose, *i.e.*, of one mole per 65 glucose units. A similar experiment yielded one mole of formic acid per 78 glucose units. These results indicate the presence of one non-reducing terminal group per 216 ± 20 glucose residues.

Determination of the Glucose Residues linked through $C_{(1)}$, $C_{(4)}$, *and* $C_{(6)}$.—After completion of the formic acid determination (see above), ethylene glycol (1·5 c.c.) was added to the remainder to destroy excess of periodate (overnight shaking). The oxidised amylose (which had been rendered insoluble) was then dialysed until free from oxidant (test with potassium iodide and sulphuric acid, and diphenylamine and sulphuric acid) and then treated with sodium metaperiodate (15 c.c.; 0·3M.) to ensure complete oxidation. After removal of the periodate as above, the solution was evaporated to dryness and the residue hydrolysed with sulphuric acid (50 c.c.; 0·5N.) at 95° for 10 hours. After neutralisation with barium carbonate, the solution was evaporated to dryness. Only a very small white residue remained. This was dissolved in water (0·1 c.c.) and examined by paper chromatography. No glucose or other sugar was found.

Estimation of Degree of Polymerisation of Amylose, from the Reducing Power.—The method of Meyer, Noelting, and Bernfeld (*loc. cit.*) was used. A standard curve was constructed for maltose (Meyer, *loc. cit.*) by treatment of known amounts of the sugar (0·1—2 mg.) in water (3 c.c.) with 3 : 5-dinitrosalicylic acid (1 c.c.; 1·5%) and sodium hydroxide (1 c.c.; 6N.) at 65° for 30 minutes, cooling, and

diluting to 25 c.c. The solution thus obtained was compared with a blank in a " Spekker " photoelectric absorptiometer, 4-cm. cells and green filters (No. 604) being used (see Table).

Maltose, mg./25 c.c.	Molar concn. per l. ($\times 10^4$).	" Spekker " drum readings, log I/I_0.	Maltose, mg./25 c.c.	Molar concn. per l. ($\times 10^4$).	" Spekker " drum readings, log I/I_0.
0·2	0·23	0·009	1·2	1·40	0·315
0·5	0·58	0·067	1·5	1·75	0·524
0·75	0·88	0·138	1·8	2·11	0·745
1·0	1·17	0·241	2·0	2·34	0·970

For determination of the degree of polymerisation of amylose, two equal samples (50—60 mg.) were dissolved in water (3 c.c.) containing sodium hydroxide (1 c.c.; 6N.). Water (1 c.c.) was added to one sample (A, the blank) and 3 : 5 dinitrosalicylic (1 c.c.; 1·5%) to the other (B). The samples were heated as before and cooled. 1 C.c. of the dinitrosalicylic acid solution was added to A and both were made up as before. Log I/I_0 varied between 0·112 and 0·223, corresponding to a degree of polymerisation of 135 $\pm$ 20 glucose units.

" Amylopectin " fraction.

The amylopectin fractions obtained from the various precipitation procedures (Table I) were dispersed as before and treated with the various precipitants. In no case was there any significant precipitation.

Separation of " Amylopectin " with Low Amylose Content.—The method of Higginbotham and Morrison (*loc. cit.*) was used. Dry starch (5 g.), suspended in water (20 c.c.), was added with stirring to water (180 c.c.) at 90° containing pyridine (50 c.c.). The paste was stirred for various periods of time (Table I*a*) and then subjected to 5 minutes' high-speed stirring in an " Ato-Mix " disperser. The paste was cooled to 70° and centrifuged (10,000 r.p.m.) for 5 minutes. The temperature of the paste was adjusted to 90° and sufficient butanol was added to saturate the solution. After transference to a Dewar flask, the solution was allowed to cool very slowly and the fractions separated as before (Table I*a*).

A further portion (20 g.) was fractionated by this method (1 hour's stirring), to give 11·1 g. of amylopectin (B.V. 0·067) containing 2% of amylose. The white solid dispersed readily in warm water on stirring. The solution gave a blue colour with iodine (0·1N.). The addition of further iodine yielded a reddish-purple colour. The material had $[\alpha]_D^{15}$ +168° (*c*, 0·76 in 30% perchloric acid) and +149° (*c*, 0·75 in N-sodium hydroxide).

Methylation of Amylopectin.—Amylopectin (9·1 g.) was methylated as was the amylose fraction. After 6 treatments with the reagents, the product was purified as before (Found : OMe, 44·0%) (yield, 7·3 g.).

Fractionation from chloroform solution by the addition of light petroleum (b. p. 40—60°) gave (*a*) 5·6 g., $[\alpha]_D^{17}$ +204° (*c*, 0·5 in chloroform) (Found : OMe, 44·0%), (*b*) 1·5 g., $[\alpha]_D^{17}$ +204° (*c*, 0·5 in chloroform) (Found : OMe, 43·9%), and (*c*) 0·2 g. (Found : OMe, nil).

Fraction (*a*) had η_{sp}/c 3·2 (*c*, 0·4 in *m*-cresol) corresponding to an apparent M = 485,000 (see Hirst and Young, *J.*, 1939, 1475).

Fraction (*b*) had η_{sp}/c 1·5 (*c*, 0·4 in *m*-cresol), corresponding to an apparent M = 215,000.

Fraction (*a*) (4·3 g.) was treated four times in boiling methyl iodide (100 g.) with dry silver oxide (25 g.), added in small portions during 24 hours. After filtration and extraction with hot chloroform fraction (a_1) (3·6 g.) was obtained (Found : OMe, 45·0%). The product was essentially homogeneous and had a molecular weight similar to that before treatment.

Hydrolysis of Methylated Amylopectin and Separation of Methylated Glucoses.—(a) *By paper chromatography.* Fraction (a_1) (50 mg.) was hydrolysed in a sealed tube by the method of Hough, Hirst, and Jones (*loc. cit.*). Analysis by paper chromatography revealed the following :

Sugar indicated.	R_G.	Wt., %.
2 : 3 : 4 : 6-Tetramethyl glucose	1·0	4·2
2 : 3 : 6-Trimethyl glucose	0·80	90·5
(?2 : 3-)Dimethyl glucose	0·55	5·3

Traces of monomethyl glucose and glucose were also observed. These results indicate the presence of one non-reducing terminal group per 25 glucose residues.

(b) *By a cellulose column.* Fraction (a_1) (3·22 g.) was hydrolysed as described for the methylated amylose. The mixture of methylated glucoses (3·25 g., 92%) was separated on a column of cellulose (80 × 3·3 cm.) as before. By elution with 50% light petroleum (b. p. 100—120°)–50% *n*-butanol, saturated with water, fractions (1) (152 mg.), (2) (2·679 g.), (3) (140 mg.), and (4) (32 mg.) were isolated. By elution with water, a small fraction (less than 10 mg.) was obtained; paper chromatography indicated that this fraction, which was not investigated further, contained a monomethyl glucose. The total recovery was 93%.

Examination of the Fractions.—Fraction (1). Paper chromatography revealed the presence of a single spot (R_G, 1·0) corresponding to tetramethyl glucose. Hypoiodite oxidation revealed that the fraction was 84·9% pure. A portion (10 mg.) was hydrolysed with boiling sulphuric acid (1 c.c.; 2%) for 6 hours. Re-examination by paper chromatography showed the presence of an additional substance

(R_G, 0·81) corresponding to 2 : 3 : 6-trimethyl glucose. Fraction (1) (134 mg.) was therefore hydrolysed with hydrochloric acid (10 c.c.; 1%) for 7 hours. The hydrolysate was separated on a cellulose column (35 × 1·3 cm.) as before, to give fractions (1*a*) (106 mg.) and (1*b*) (21 mg.) (recovery, 95%). Fraction (1*a*) partly crystallised on storage. After two recrystallisations from light petroleum (b. p. 40—60°) it had m. p. 85—87° (not depressed on admixture with tetramethyl D-glucopyranose), $[\alpha]_D^{18}$ +84° (*c*, 0·45 in water) (Found : OMe, 52·1. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%). The anilide had m. p. 137—138° (alone or admixed with authentic tetramethyl D-glucopyranose anilide) (Found : N, 4·4; OMe, 39·5. Calc. for $C_{16}H_{25}O_5N$: N, 4·5; OMe, 39·9%). From the above results the amount of tetramethyl glucose was calculated to be 118 ± 12 mg., corresponding to one non-reducing terminal group per 26 ± 2 glucose residues.

Fraction (1*b*) did not crystallise. Paper chromatography indicated the presence of a single substance (R_G, 0·81) corresponding to 2 : 3 : 6-trimethyl glucose, $[\alpha]_D^{19}$ +68·4° (*c*, 0·4 in water), $[\alpha]_D^{18}$ +67·1° (initial), −36·9° (7 hours, constant) (*c*, 0·4 in cold dry 2% methanolic hydrogen chloride) (Found : OMe, 40·8. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (2) crystallised partly when kept. The material was recrystallised twice from dry ether (50% yield) and had m. p. 115—117° alone or admixed with an authentic sample of 2 : 3 : 6-trimethyl D-glucose. The R_G value on a paper chromatogram was identical with that of 2 : 3 : 6-trimethyl glucose. The crystalline material had $[\alpha]_D^{19}$ +90° (initial), 71° (constant) (*c*, 1 in water), and $[\alpha]_D^{18}$ +68·4° (initial), −35·0° (7 hours, constant) (*c*, 1·0 in cold 2% methanolic hydrogen chloride solution) (Found : OMe, 41·6. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (3) was obtained as a pale yellow syrup which partly crystallised during 2 weeks in the cold. Recrystallised once from dry ethyl acetate it had m. p. 84—86°. The R_G value (0·57) on a paper chromatogram was identical with that of 2 : 3-dimethyl glucose. The crystalline material had $[\alpha]_D^{19}$ +113° (initial), +65° (final) (*c*, 1·0 in water) (Found : OMe, 29·4. Calc. for $C_8H_{16}O_6$: OMe, 29·7%). The 2 : 3-dimethyl gluconophenylhydrazide (Evans, Levi, Hawkins, and Hibbert, *Canad. J. Res.*, 1942, **20**, *B*, 175) had m. p. 160—162° (Evans *et al.*, 166·5—167°) (Found : C, 53·7; H, 7·1; N, 9·0; OMe, 19·3. Calc. for $C_{14}H_{22}O_6N_2$: C, 53·5; H, 7·0; N, 8·9; OMe, 19·7%).

Fraction (4) was obtained as a syrup which failed to crystallise during several weeks in the cold. It had $[\alpha]_D^{18}$ +78° (initial), +62° (final) (*c*, 1·0 in water), $[\alpha]_D^{20}$ +61° (initial), −11° (final, after 8 hours) (*c*, 0·5 in cold 2% methanolic hydrogen chloride solution) (Found : OMe, 28·9. Calc. for $C_8H_{16}O_6$: OMe, 29·7%). The fraction (25 mg.) was converted into the methylglucosides and treated with sodium metaperiodate by Bell's method (*loc. cit.*). The uptake of sodium metaperiodate was 79% per $C_9H_{18}O_6$ unit, indicating that the fraction contained 79% of 2 : 6-dimethyl glucose.

Determination of End Group in Amylopectin and in Whole Starch by Periodate Oxidation.—(a) *Amylopectin.* This was carried out by the method described above for amylose. The yield of formic acid from 162 g. of amylopectin after 150 hours' oxidation was 0·0412 g.-mol., corresponding to one non-reducing terminal group per 24 glucose residues.

(b) *Barley starch.* The yield of formic acid from 162 g. of starch after 150 hours' oxidation was 0·033 g.-mol., corresponding to one non-reducing terminal group per 30 glucose residues. If the amount of formic acid yielded from the amylose fraction is neglected then the formic acid yield from the whole starch corresponds to the presence of one non-reducing terminal group per 24 glucose residues in the amylopectin fraction.

Determination of Glucose Residues in Amylopectin linked through $C_{(1)}$, $C_{(4)}$, *and* $C_{(6)}$.—This was carried out by the method described by Hirst, Jones, and Roudier (*loc. cit.*) on (*a*) the amylopectin fraction and (*b*) the whole starch. On hydrolysis and examination by paper chromatography, glucose was found in each case. Determination by the method of Flood, Hirst, and Jones (*J.*, 1948, 1679) using xylose as standard gave : for (*a*), 0·59 g. of glucose from 100 g. of amylopectin, and (*b*) 1·8 g. of glucose from 100 g. of starch. These results indicate that (i) over 86% of the linkages in the amylopectin fraction are 1 : 6-linkages, and (ii) that about 50% of the linkages in the amylopectin fraction are 1 : 6-linkages. It is thought probable that the closely packed granular structure prevented complete oxidation by the periodate in case (*b*).

Attempted Estimation of Degree of Polymerisation of Amylopectin.—The method used by Meyer *et al.* (*loc. cit.*) gave very low "Spekker" readings with separated amylopectin and with whole starch. The results indicated that the molecular weights were in excess of 240,000.

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