236. *A Modified Method for the End-group Assay of Amylose and Other Long-chain Starch Fractions.*

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The partition method of Bell (*J.*, 1944, **473**) for the end-group assay of methylated starches is not applicable to the determination of the chain-length of amylose, in which the proportion of end-group is small, for the reason that a non-crystalline impurity accompanies the crystalline end-group (tetramethyl glucose) and is not separated from it in the silica column. This syrupy impurity is probably a pentamethyl disaccharide, and is formed from trimethyl glucose but not from tetramethyl glucose under the action of aqueous acid. To minimise the proportion of impurity formed, the method of Bell has been modified as follows. The methylated amylose is submitted to methanolysis and the resulting mixture of methyl glucosides is fractionally distilled. The first fractions, which contain all the tetramethyl methylglucoside and only a small proportion of trimethyl methylglucoside, are then hydrolysed with mineral acid and the products are separated by Bell's procedure. Minimum formation of the syrupy impurity is thus assured.

Starch and its components are best methylated in suspension in liquid ammonia.

There was a dual purpose in this investigation, first to measure the average chain-lengths of the fractions derived from potato starch by selective precipitation with thymol (Haworth, Peat, and Sagrott, *Nature*, 1946, **157**, 19; Bourne, Donnison, Haworth, and Peat, *J.*, 1948, 1687), and secondly to find a reliable method for the end-group assay of 1 : 4-α-glucosidic polysaccharides which would be applicable to small quantities (2—4 g.) of both amylose and amylopectin. The latter object was to facilitate the complete examination of starch-type polysaccharides produced from glucose-1 phosphate by the agency of P- and Q-enzymes (Bourne and Peat, *J.*, 1945, **877**) by eliminating the laborious synthesis of some 30 g. of these polysaccharides.

The thymol-precipitated amylose fraction of potato starch, which was shown by Bourne, Donnison, Haworth, and Peat (*loc. cit.*) to be blue-staining and to give rise to 96—99% of maltose by β-amylolysis, is now shown to have an average chain-length of 190 ± 10 units. The red-staining amylopectin fraction, which is not precipitated by thymol and gives maltose (*ca.* 50%) and a limit dextrin under the action of β-amylase (Bourne, Donnison, Haworth, and Peat, *loc. cit.*), is shown to have an average chain-length of 18 ± 1 units. These assays, which were made by a modification of the method published by Bell (*J.*, 1944, **473**), were performed on 2·5 g. of methylated amylopectin and 2·5—4·2 g. of methylated amylose.

The polysaccharides were methylated with sodium and methyl iodide in liquid ammonia suspension at −70° (Freudenberg, Boppel, and Meyer-Delius, *Naturwiss.*, 1938, **26**, 123), a method which is less laborious and quicker than that involving the use of methyl sulphate in the presence of aqueous sodium hydroxide. Although some physical disaggregation occurs during methylation by the liquid ammonia method, as is shown by the low viscosities of the products, there is no shortening of the unit-chains (Freudenberg, Boppel, and Meyer-Delius, *loc. cit.*). Contrary to the observations of Hess and Krajnc (*Ber.*, 1940, **73**, 976), according to whom whole starch is more easily methylated by methyl sulphate–sodium hydroxide than are either amylose or amylopectin, we found amylose to be most readily methylated, amylopectin least readily, and starch at an intermediate rate. Thus after one methylation, involving six alternate additions of methyl iodide and sodium, the methoxyl contents of the products from amylose, starch, and amylopectin were, respectively, 43·5—44·8, 37·6, and 34·9%. This order of reactivity would be expected if steric hindrance effects were operative in the branched amylopectin structure. When defatted waxy-maize starch was subjected to the same treatment, either before or after "activation" by precipitation with alcohol from an aqueous paste, a sticky gel was formed, and this prevented the dispersal of the starch so effectively that no methylation occurred. When, however, the liquid ammonia was added to a suspension of waxy-maize starch in a small volume of anhydrous ether there was no swelling tendency and the methylation proceeded quite smoothly. All the polysaccharides were methylated until their methoxyl contents exceeded 43·2%. The trimethyl amylose preparations were tough filmy substances, which were resistant to grinding, whereas the starch- and amylopectin-ethers were brittle and readily powdered.

Two methods, based on partition chromatography, were available for end-group assays of small quantities of methylated starches, involving, in the first case, separation of 2 : 3 : 6-trimethyl and 2 : 3 : 4 : 6-tetramethyl glucose on a silica column (Bell, *loc. cit.*) and, in the second case, separation of the glucosides on a column of activated alumina (Jones, *J.*, 1944, 333). Bell's method was chosen because 2 : 3 : 4 : 6-tetramethyl glucose, unlike mixtures of its α-

and β-methylglucosides, is crystalline and therefore more easily recognised in its pure form. Bell's assay is a four-stage process: Stage I, hydrolysis of the methylated polysaccharide at 100° with a mixture of glacial acetic acid (5 parts) and 5% aqueous hydrochloric acid (10 parts); Stage II, removal of the acids; Stage III, extraction of an aqueous solution of the mixed sugars with nine equal volumes of chloroform and evaporation of the chloroform extracts to a syrup which contains all the tetramethyl glucose ("tetra") and only 10% of the trimethyl glucose ("tri"); Stage IV, chromatography of a chloroform solution of the syrup on a silica–water column, under conditions such that the residue of "tri" is retained by the column, while the "tetra" passes through.

The efficiency of the partition (Stages III and IV) was ascertained by analysing artificial mixtures of tri- and tetra-methyl glucose, the molecular proportions of which ranged from 18 to 131 molecules of "tri" per molecule of "tetra." The recovered "tetra" fractions were all crystalline and had an average methoxyl content of 52·5%, which agreed well with the theoretical value, 52·6% (Table I). With one exception, the recovery of "tetra" was 88·2—95·4% and averaged 90·3%, compared with 93% quoted by Bell (*loc. cit.*) for Stage IV alone. An allowance for this consistently low recovery of tetramethyl glucose was made in all end-group assays, the factor employed being 90 ± 5%.

TABLE I.

Partition of Artificial Mixtures of "Tri" and "Tetra" by Bell's Method (Stages III and IV).

Wt. of mixed sugars (g.).	Mol. propn., "tri" : "tetra."	Recovery of "tetra" (%).	OMe of "tetra" (theory, 52·6) (%).
0·708	18	79·6	52·5
1·543	32	94·4	52·3
1·072	58	93·2	52·5
2·100	69	89·7	—
2·065	111	95·4	52·6
4·027	120	88·2	52·4
2·032	131	91·5	—
	Mean values:	90·3	52·5

TABLE II.

End-group Assays by Bell's Method (Stages I—IV).

Polysaccharide.	B.V.	Methyl ether. Wt. (g.).	Methyl ether. OMe (%).	"Tetra" fraction, OMe (%).	Chain-length.
Potato thymol-amylopectin	0·211	2·501	43·6	51·4	18 ± 1
Potato thymol-amylose	1·070	6·240	43·5	40·1	—
Potato thymol-amylose	1·160	10·076	44·3	39·0	—

Bell's end-group assay, applied, without modification, to methylated potato thymol-amylopectin (B.V., 0·211; Table II), gave an average chain-length of 18 ± 1.

Attempts to assay two samples of trimethyl amylose (Table II) by the same method yielded syrups, having OMe, 40·1 and 39·0%, instead of the crystalline samples of tetramethyl glucose which had resulted from the partition of artificial mixtures. In the amylopectin assay, previously mentioned, the "tetra" fraction had a slightly low methoxyl content (51·4%), but the error was within the accuracy limits of the assay. It is to be noted that Bell (*loc. cit.*) did not assay an amylose sample, but his "tetra" fractions derived from methylated rice starch possessed a slightly low methoxyl content, the error being insufficient seriously to affect the accuracy of his calculated chain-lengths. Thus it became clear that although the partition (Stages III and IV) was capable of yielding pure tetramethyl glucose from artificial mixtures of tri- and tetra-methyl glucose, in proportions which would be expected to result from assays on amylopectin, starch, and amylose, the whole assay (Stages I—IV) gave reasonably pure tetramethyl glucose from starch and amylopectin and very impure "tetra" from amylose. It seemed probable that Stages I and II (*i.e.*, the hydrolysis of the methylated polysaccharides and the removal of the acids) were introducing a chloroform-soluble impurity, the presence of which was more in evidence when the chain-length was high, as in an amylose.

Accordingly, prepared mixtures of tri- and tetra-methyl glucose were subjected to a complete "assay" (Stages I—IV). One mixture, corresponding to that of a trimethyl amylopectin, gave a crystalline tetramethyl glucose fraction in 87% yield, with OMe, 50·3%, while another, which corresponded to a trimethyl amylose, gave a syrupy "tetra" fraction in 232% yield,

with OMe, 40·3% (Table III). Pure samples of tri- and tetra-methyl glucose were separately submitted to a similar assay (Stages I—IV), and the results clearly revealed that the chloroform-soluble syrupy impurity arose from the action of acid on the trimethyl glucose and not on the tetramethyl glucose (Table IV). The syrup reduced Fehling's solution, was hydrolysed by aqueous acid, and had OMe, 36·2%. It was probably a pentamethyl disaccharide (OMe, 37·6%) arising from demethylation and "reversion" of trimethyl glucose (see Freudenberg and Boppel, *Ber.*, 1940, **73**, 609; Frahm, *Annalen*, 1944, **555**, 187).

TABLE III.

Partition of Artificial Mixtures of "Tri" and "Tetra" after Acid Treatment (Stages I—IV).

Wt. of mixed sugars (g.).	Mol. propn., "tri" : "tetra."	Recovery of "tetra" (%).	OMe of "tetra" (%).
1·191	19	87·0	50·3
4·011	119	232·0	40·3

TABLE IV.

Partition of Methyl Glucoses after Acid Treatment (Stages I—IV).

Sugar used.	Wt. of sugar (mg.).	Apparent "tetra" recovered. Wt. (mg.).	Nature.	OMe of apparent "tetra" (%).
Trimethyl glucose	6245	36·1	Syrup	36·2
Tetramethyl glucose	61·4	52·6	Crystalline	52·3

TABLE V.

End-group Assays by the Modified Method.

Polysaccharide.	B.V.	Methyl ether. Wt. (g.).	OMe (%).	"Tetra" fraction, OMe (%).	Chain-length.
Potato starch	0·400	2·207	45·2	51·9	26 ± 1
Potato thymol-amylose	0·915	2·596	44·8	51·8	101 ± 5
Potato thymol-amylose	1·210	4·215	43·9	—	191 ± 10
Potato thymol-amylose *	1·210	3·550	43·9	52·0	187 ± 10
Waxy-maize starch	0·104	2·500	43·6	52·1	26 ± 1

* Hydrolysed with 2N-toluene-*p*-sulphonic acid instead of hydrochloric acid.

The effect of the syrup on the assay was more marked in the case of amylose because of the much higher proportion of "tri" to "tetra," and it was therefore essential that, in such cases, the proportion of "tri" should be diminished before the hydrolysis stage was reached. To this end a modification of the method of Bell was devised, which entailed methanolysis of the trimethyl polyglucose and fractional distillation of the glucoside mixture, two small distillate fractions being collected. The first fraction, consisting of all the tetramethyl methylglucoside and a small proportion of the trimethyl methylglucoside, was hydrolysed with aqueous hydrochloric acid and the tetramethyl glucose determined by the partition method. As will be seen from Table V, this "tetra" fraction, which was crystalline in each of the examples cited, had OMe, 51·9—52·1%. It is obvious that this "tetra" specimen still contained a small amount of the syrupy impurity, produced by the action of acid on the residual trimethyl methylglucoside, but it was possible to measure the amount of this impurity by submitting a similar weight of the second distillate fraction (which consisted only of trimethyl methylglucoside) to the same acid and partition treatments, and thus to calculate, by difference, the weight of pure tetramethyl glucose in the first fraction.

The modified method of assay was first tested on methylated potato starch, and the average chain-length (26 ± 1) thus calculated was in good agreement with the accepted value. A thymol-precipitated "amylose" fraction of potato starch (B.V., 0·915), which had previously been shown by a classical end-group assay (requiring, incidentally, 28 g. of the methylated polysaccharide) to have an average chain-length of 96 ± 5, was found by the modified assay, for which only 2·6 g. of the trimethyl derivative were required, to have average chains of 101 ± 5 units. Another thymol-amylose fraction (B.V., 1·210) containing less amylopectin impurity (as shown by its higher blue value) had chains 190 ± 10 units long. There is reason to believe that even this amylose contained a little branched-chain impurity, because fractions

with B.V. as high as 1·40 can be isolated from potato starch by the aluminium hydroxide method (Bourne, Donnison, Peat, and Whelan, *J.*, 1949, 1).

The chain-length of waxy-maize starch, determined by means of the modified technique, was found to be 26 ± 1 units, a value which was intermediate between previously reported values: 20 units (periodate method) and 18 units (classical assay) by Brown, Halsall, Hirst, and Jones (*J.*, 1948, 27), and 26—30 units (classical assay) by Haworth, Hirst, and Woolgar (*J.*, 1935, 177). Thus it would appear that waxy-maize starch, in spite of its lower B.V., has a longer average chain-length than potato thymol-amylopectin. Perhaps this is related to the fact that waxy-maize starch is precipitated from solution by iodine under conditions which will not cause the precipitation of potato thymol-amylopectin.

EXPERIMENTAL.

Precautionary Measures.—The essential precautions with regard to the absence of grease, purity of solvents, etc., which were taken by Bell (*loc. cit.*), were observed throughout this work.

Fractionation of Potato Starch.—The amylose and amylopectin samples were obtained from potato starch by selective precipitation with thymol, according to the "Standard Procedure" described by Bourne, Donnison, Haworth, and Peat (*loc. cit.*).

Determination of the Blue Value (B.V.) of a Polysaccharide.—The blue value of a polysaccharide is a measure of the blue component of the colour produced when the polysaccharide is stained with iodine under standard conditions (Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924).

Preparation of the Silica.—The silica used in the columns was prepared by the method of Gordon, Martin, and Synge (*Biochem. J.*, 1943, **37**, 79). Each batch was tested, both qualitatively by the staining method of Bell (*loc. cit.*) and quantitatively on mixtures of 2 : 3 : 6-trimethyl and 2 : 3 : 4 : 6-tetramethyl glucose, before being employed for end-group assays.

End-group Assay of Methylated Polyglucoses by Bell's Method.—A number of methylated polysaccharides of the starch type was assayed by Bell's method (*loc. cit.*), which involved hydrolysis for 5—7 hours on a boiling water-bath with a mixture of glacial acetic acid (5 parts) and 5% (w/v) hydrochloric acid (10 parts), removal of the acids, and separation of the tetramethyl glucose from the trimethyl glucose by partition between chloroform and water, first by simple extraction and then by means of a silica column. Each calculation is based on the weight of the mixture of methyl glucoses isolated from the hydrolysate and not on the weight of the polysaccharide ether.

(a) *Potato thymol-amylopectin* (*B.V.*, 0·211). From 2·501 g. of methylated amylopectin (OMe, 43·6%), 2·401 g. (approx. recovery, 88%) of mixed sugars were obtained. The crystalline tetramethyl glucose (OMe, 51·4%) isolated by partition weighed 126·5 mg., which, after allowance for a 90 ± 5% recovery from the column, corresponded to an average chain-length of 18 ± 1 units.

(b) *Potato thymol-amylose* (*B.V.*, 1·070). 6·240 G. of trimethyl amylose (OMe, 43·5%) gave, in turn, 6·252 g. (approx. recovery, 92%) of mixed sugars and 83·9 mg. of a syrupy "tetra" fraction (OMe, 40·1%).

(c) *Potato thymol-amylose* (*B.V.*, 1·160). Partition of the mixed sugars (10·393 g.; approx. recovery, 95%) derived from 10·076 g. of trimethyl amylose (OMe, 44·3%) gave 211·5 mg. of a syrupy "tetra" fraction (OMe, 39·0%).

Partition of Artificial Mixtures of Tri- and Tetra-methyl Glucose after Acid Treatment.—Each mixture was treated in exactly the same way, including "hydrolysis" with mixed acids for 5 hours, as the above polysaccharide ethers.

(a) *Molecular ratio,* 19 "*tri*" : 1 "*tetra.*" 1·191 G. of this mixture yielded 54·9 mg. (recovery, 87·0%) of crystalline tetramethyl glucose (OMe, 50·3%).

(b) *Molecular ratio,* 119 "*tri*" : 1 "*tetra.*" 4·011 G. of this mixture gave a syrupy "tetra" fraction (82·1 mg.; OMe, 40·3%), with an apparent recovery of 232%.

Acid Treatment of 2 : 3 : 6-Trimethyl Glucose.—Trimethyl glucose (6·245 g.) was heated on a boiling water-bath for 6½ hours with 5% (w/v) hydrochloric acid. The solution was neutralised with silver carbonate and filtered. Colloidal silver was removed from the filtrate with hydrogen sulphide, and the silver sulphide separated by filtration through a charcoal pad, which was then washed with a little water. The combined filtrate and washings were analysed by the partition method of Bell (*loc. cit.*). The "tetra" fraction was a reducing syrup (36·1 mg.) (Found: OMe, 36·2. Calc. for a pentamethyl disaccharide: OMe, 37·6%).

Acid Treatment of 2 : 3 : 4 : 6-Tetramethyl Glucose.—Tetramethyl glucose (61·4 mg.) was heated on a boiling water-bath for 6 hours with 5% (w/v) hydrochloric acid (10 c.c.). The solution was neutralised with silver carbonate and filtered. Colloidal silver was removed from the filtrate with hydrogen sulphide, and the silver sulphide collected on a charcoal pad. The combined filtrate and washings, when analysed by the partition method of Bell (*loc. cit.*), yielded 52·6 mg. (recovery, 86%) of crystalline tetramethyl glucose (OMe, 52·3%).

Modified End-group Assay of Methylated Polyglucoses.—A suspension of the dry methylated polysaccharide (*A* mg.) in dry methyl alcohol containing 1·5—2% (w/v) dry hydrogen chloride was heated under reflux for 9 hours, before being neutralised with a slight excess of lead carbonate. The lead salts were removed by filtration and washed with dry methyl alcohol. The filtrate and washings were combined and evaporated under reduced pressure in the presence of a little barium carbonate to a syrup, which was dried at 100°/200 mm. for 30 minutes. A dry ethereal solution of the syrup was filtered (in small portions) through sintered glass into a tared Widmer flask containing known weights of barium carbonate and glass wool. The solution was evaporated to a syrup, which was dried at 100°/200 mm. to constant weight (*B* mg.). The glucoside mixture was distilled at 0·01 mm., two distillate fractions (400—650 mg.; weights *C* mg. and *D* mg., respectively) being collected in tared

receivers. As a check that all the tetramethyl glucose was present in the first fraction, the refractive indices of the last drops of the first and second fractions were measured and found to be identical, within experimental error, in every case. The two distillate fractions were separately hydrolysed with 50 parts (by weight) of 5% (w/v) aqueous hydrochloric acid for 7 hours at 100°. The hydrolysates were neutralised with silver carbonate and filtered. In some cases hydrogen sulphide was passed through the filtrate and washings to remove colloidal silver, but this was not essential as the colloidal silver did not pass through the silica column. The filtrates and washings were analysed for tetramethyl glucose by Bell's partition method. In every case the first distillate fraction yielded crystalline tetramethyl glucose (E mg.) contaminated with only a trace of oily material, while the second distillate fraction invariably yielded a small amount (F mg.) of syrup.

The chain-lengths were calculated in the following way :

Wt. of tetramethyl glucose, corrected for syrupy impurity and losses in the column = $\frac{100}{90}\left[E - \frac{F(C-E)}{D}\right]$ mg. = X mg.

$$\therefore \quad \text{G.-mols. of tetramethyl glucose} = \frac{X}{236}.$$

$$\text{No. of G.-mols. in glucoside mixture} = \frac{B}{236}.$$

$$\therefore \quad \text{Chain-length} = \frac{B}{X} = \frac{0{\cdot}9\,B}{E - \frac{F(C-E)}{D}}$$

This method was applied to several methylated polysaccharides with the results shown below.

Polysaccharide.	Potato starch.	Potato thymol-amylose.	Potato thymol-amylose.	Potato thymol-amylose.*	Waxy-maize starch.
Blue value	0·400	0·915	1·210	1·210	0·104
Wt. of methylated polysaccharide (A mg.)	2207	2596	4215	3550	2500
Wt. of glucoside mixture (B mg.)	2472	2776	4724	3873	2709
Wt. of first distillate fraction (C mg.)	999·1	444·7	575·0	506·8	631·4
Wt. of second distillate fraction (D mg.)	967·8	435·1	645·0	482·0	622·1
Wt. of " tetra " from first fraction (E mg.)	96·2	35·2	27·3	22·8	99·5
Wt. of " tetra " from second fraction (F mg.)	12·9	11·0	5·9	4·1	7·2
OMe of " tetra " from first fraction (%)	51·9	51·8	—	52·0	52·1
Chain-length	26 ± 1	101 ± 5	191 ± 10	187 ± 10	26 ± 1

* In this case 2N-toluene-*p*-sulphonic acid was used instead of aqueous hydrochloric acid for hydrolysis of the glucoside mixture, and the neutralisation stage was omitted.

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