826. α-1,4-*Glucosans. Part XVIII.*[1] *The Periodate Oxidation of Amylopectin.*

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Acid hydrolysates of the polyalcohols prepared from three samples of periodate-oxidised amylopectin did not contain glucose. This indicates the absence of a significant proportion of periodate-resistant structures in the polysaccharide, for example, 1,3-glucosidic linkages.

Although the majority of periodate oxidation studies of amylopectin have been concerned with measurement of the production of formic acid as a method of end-group assay, examination of the periodate-oxidised polysaccharide may also yield structural information. For example, Hirst, Jones, and Roudier [2] pointed out that the presence of 1,2- or 1,3-inter-chain linkages would prevent oxidation of branch-point residues so that on acid hydrolysis

[1] Part XVII, Kjølberg and Manners, *Comp. Biochem. Physiol.*, 1963, **8**, 353.
[2] Hirst, Jones, and Roudier, *J.*, 1948, 1779.

of periodate-oxidised amylopectin (oxoamylopectin), such residues would be released as glucose. Application of this method to acorn starch [2] gave less than 1·3% of glucose, indicating that at least 75% of the inter-chain linkages involved C-6 rather than C-2 or C-3 of the branch-point residue. With potato amylopectin, 0·1% of glucose was obtained,[3] equivalent to the presence of 97—98% of 1,6-inter-chain linkages.

It must be emphasised that in addition to 1,2- or 1,3-inter-chain linkages, 1,3-inter-residue linkages would also yield glucose by this treatment. However, hemiacetal formation between an oxidised 1,4-linked glucose residue and an unoxidised 1,4-linked residue in an adjacent chain might also produce a periodate-resistant structure.

F. Smith and his co-workers [4,5] studied the acid hydrolysis of the polyalcohol prepared by borohydride reduction of oxoamylopectin. Non-reducing end-groups gave glycerol (and its estimation provided a method of end-group assay [6]), whilst non-terminal residues produced erythritol and glycollaldehyde. In addition, small amounts of glucose (0·2—0·5%) were present, and it was suggested [4,5] that this arose from 1,3-glucosidic linkages. Whether these represented inter-chain or inter-residue linkages was not specified. In the absence of other evidence, this glucose might also have arisen from 1,2-inter-chain linkages, provided that the initial oxidation was complete.

TABLE 1.

Experimental conditions used by Hamilton and Smith for the periodate oxidation of amylopectin.[5]

Experiment	Time (days)	Temp.	Periodate concn. [M]	Reduction of periodate (mole per glucose unit)	Residual glucose (%)
I	48	2—4°	0·4	1·1	0·4
II	5·5	2—4	0·2	1·04	0·22
IV	9	26	0·4	1·51	0·52, 0·44

Smith and Hamilton [5] described three different conditions for the oxidation of waxy maize starch (see Table 1), but the yield of glucose in the polyalcohol hydrolysate was greatest in the experiment with the largest reduction of periodate. The lack of correlation between the extent of oxidation and the amount of periodate-resistant residues lowers the significance of the latter observation. We have, therefore, oxidised several amylopectin samples in an attempt to assess the structural significance of periodate-resistant glucose residues.

A 2% solution of purified potato amylopectin was oxidised with 0·4M-sodium meta-periodate at room temperature (18—20°), and samples removed at intervals (*a*) for measurement of the reduction of periodate, (*b*) for measurement of the production of formic acid, (*c*) for reduction of the oxoamylopectin to the polyalcohol, followed by acid hydrolysis and determination of the liberated glucose. The results (Table 2) show that after 8 days the extent of oxidation was similar to that found by Hamilton and Smith,[5] but that after this time oxidation continued and the amount of residual glucose decreased. The yield of about 0·1% obtained after oxidation for 24 days is similar to that reported by Gibbons and Boissonas,[3] but the structural significance of this is doubtful. After 24 days, the pH of the oxidation mixture was 2·9 and the extent of the reduction of periodate and production of formic acid was greatly in excess of that expected from a Malapradian periodate oxidation [7,8] (about 1·05 and 0·05 mole per glucose residue, respectively).

Samples of waxy maize and waxy sorghum starches, and of potato amylopectin, were

[3] Gibbons and Boissonas, *Helv. Chim. Acta*, 1950, **33**, 1477.
[4] Abdel-Akher, Hamilton, Montgomery, and Smith, *J. Amer. Chem. Soc.*, 1952, **74**, 4970.
[5] Hamilton and Smith, *J. Amer. Chem. Soc.*, 1956, **78**, 5910.
[6] Hamilton and Smith, *J. Amer. Chem. Soc.*, 1956, **78**, 5907.
[7] Jackson, *Org. Reactions*, 1944, **2**, 341.
[8] Bobbit, *Adv. Carbohydrate Chem.*, 1956, **11**, 1.

TABLE 2.

The course of oxidation of amylopectin by 0·4M-sodium metaperiodate at 18—20°.

Time (days)	4	8	13	17	20
Reduction of periodate *	0·4	1·5	1·6	2·0	2·2
Production of formic acid *	—	—	0·81	0·85	0·89
Residual glucose (%)	0·64	0·54	0·20	0·08	0·11

* Expressed as mole per glucose residue.

dispersed in water at 100°, filtered, and then oxidised with 0·4M-sodium metaperiodate at 20—26°, for 14 days. The oxoamylopectins were reduced with sodium borohydride, and portions were hydrolysed with acid. Examination of the neutralised hydrolysates by paper chromatography under various conditions (see Experimental) failed to reveal the presence of glucose. An attempt to oxidise waxy maize starch at 2° (cf. ref. 5) was unsuccessful owing to precipitation of both polysaccharide and oxidant.

The absence of glucose from the hydrolysates would indicate the absence of 1,3-linked or 1,2,4- or 1,3,4-linked glucose residues in amylopectin. However, since the oxidation conditions were relatively severe (although they were comparable with those described in Table 1), the possibility that 1,3-linked glucose residues were eliminated by " over-oxidation " was examined.

Lichenin, an essentially linear polymer containing 30% of β-1, 3- and 70% of β-1,4-glucosidic linkages [9] was oxidised with 0·4M-sodium metaperiodate. The results (see Table 3) showed that considerable over-oxidation occurred with the loss of periodate-

TABLE 3.

The course of oxidation of lichenin with 0·4M-sodium metaperiodate at 18—20°.

Time (days)	4	8	12	16	20	24
Reduction of periodate *	1·12	1·35	1·75	2·03	2·10	2·29
Production of formic acid *	0·41	0·52	0·71	0·82	1·02	1·13
Residual glucose (%)	—	28	22	—	16	14

* Expressed as mole per glucose residue.

resistant glucose residues. This process may involve either random fragmentation of the polysaccharide chain or stepwise oxidation from the reducing end-group.[10] Since amylose or oxoamylose in the presence of iodate and formic acid at pH 2·9 showed no change in viscosity, random breakdown of the lichenin molecule due to the acidity developed during over-oxidation is unlikely. The decrease in residual glucose is, therefore, due to the stepwise degradation. However, since this process cannot occur to an appreciable extent with amylopectin (this polysaccharide is virtually non-reducing, and the reaction is arrested by 1,6-glucosidic linkages), we conclude that 1,3-glucosidic linkages are not hydrolysed by the acid formed during periodate over-oxidation. (Since amylose can be degraded stepwise from the reducing group, it is not possible to study the stability of oxoamylose at pH 2·9 in the presence of periodate.)

The mechanism by which large amounts of formic acid are produced during the over-oxidation of an essentially non-reducing α-1,4-glucosan, without concomitant reduction of periodate, is not yet known. Hamilton and Smith [5] (Table 1, experiment IV) reported the production of 1 mole of formic acid per glucose residue, whereas we have found that methyl β-maltoside gave 1·6 mole after oxidation for 24 days. Perlin [11] also observed the production of large quantities of formic acid during periodate oxidation of glycogens at 17°.

There are two possible explanations for the difference between the present results and those of Hamilton and Smith.[5] First, their sample of amylopectin was atypical in that the β-limit dextrin had a chain length of only 6 glucose residues.[5] This indicates an unusually

[9] Chanda, Hirst, and Manners, *J.*, 1957, 1951.
[10] Hough and Perry, *Chem. and Ind.*, 1956, 768.
[11] Perlin, *J. Amer. Chem. Soc.*, 1954, **76**, 4101.

short interior chain length of only 3 glucose residues; this value is similar to that of many glycogens, whereas most samples of amylopectin, including ours, have interior chain lengths of 6—9 glucose residues and the rate of penetration of periodate into the interior of their sample would be reduced. In this respect, it is significant that the β-limit dextrin examined by Hamilton and Smith, which amounted to only 30% of the original polysaccharide, contained 0·33% of periodate-resistant residues.[5] If periodate-resistant glucose residues were a natural feature of the molecule, a random distribution would be expected. Secondly, we noted that dispersion of our amylopectin samples at 68—75°, as described by these authors, was not complete, and that a higher temperature followed by filtration was required to give a homogeneous solution of polysaccharide and eliminate any insoluble and potentially periodate-resistant material.

It is significant that the claim [4] that glycogen contained a small proportion of 1,3-glucosidic linkages, based on the presence of 1% of glucose in hydrolysates of glycogen polyalcohol, has not been confirmed in later experiments.[12] This result is now in agreement with our own observations.[13] In general, we consider that the utmost caution should be used in assessing the structural significance of small quantities of products in hydrolysates of periodate-oxidised polysaccharides.

Experimental

Methods and Materials.—The general methods were as described in previous papers of this series, or as reported by F. Smith and his co-workers.[5,6] The following solvents were used for paper chromatography: (A) ethyl acetate–pyridine–water (10 : 4 : 3 v/v), (B) butan-1-ol–pyridine–water (6 : 4 : 3 v/v), (C) ethyl methyl ketone–acetic acid–water (9 : 1 : 1 v/v, saturated with boric acid). Alkaline silver nitrate and aniline oxalate spray reagents were used.

The glucose content of polyalcohol hydrolysates was determined by applying portions (equivalent to about 20 mg. of polysaccharide) to the centre part of a sheet of Whatman No. 1 paper, together with a glucose reference at the sides of the sheet. After 18 hours' development in solvent A, the probable glucose-containing strips were cut out as 3″ wide bands and eluted with distilled water, and the glucose contents of aliquot portions were estimated with the phenol–sulphuric acid reagent.[14] The lower limit of this reagent is about 10 μg. of glucose; the presence of 0·1% of periodate-resistant glucose residues would yield about 20 μg. of glucose. Reagent blanks were prepared by elution of a similar area of Whatman No. 1 paper with water.

Potato starch (var. Great Scot) was fractionated with thymol and butanol, to yield an amylopectin with an average chain length of 24 glucose residues (by potassium periodate oxidation) and β-amylolysis limit of 54%; the interior chain length was therefore 7—8 glucose residues. Waxy maize and waxy sorghum starches had the following properties: average chain lengths 24 and 23, respectively; β-amylolysis limits 57 and 58%; interior chain lengths 7 and 6.

Attempts to assay [15] the amylopectins by 25 hours' oxidation with sodium metaperiodate at 2° were unsatisfactory since the production of formic acid was not complete at this time and limited overoxidation then occurred. For example, after 60 hr., the production of formic acid was equivalent to average chain lengths of 20, 22, and 20 for the potato amylopectin, waxy maize starch, and waxy sorghum starch, respectively. This result is to be expected since the concentration of polysaccharide (2 mg./ml.) and periodate (about 0·1M) are similar to those used, at 16—17°, by Perlin [11] and found to cause extensive overoxidation.[16] A lowering of the temperature to 2° will reduce the rate of, but not completely eliminate, overoxidation.

Periodate Oxidation of Potato Amylopectin.—A filtered solution of amylopectin (10 g.) in water was oxidised with sodium metaperiodate (42·8 g.) in a final volume of 500 ml., at 20° and in the dark. Portions (50 ml.) were withdrawn at intervals; periodate reduction was measured on a 1-ml. sample, after suitable dilution, by the spectrophotometric method of Aspinall and

[12] F. Smith, unpublished work cited by Barker, *Proc. Chem. Soc.*, 1962, 373.
[13] Bell and Manners, *J.*, 1954, 1891.
[14] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350
[15] Greenwood and Thomson, *J.*, 1962, 222.
[16] Manners and Wright, *J.*, 1961, 2681.

Ferrier,[17] and a 4-ml. sample was used for the estimation of formic acid. To the remaining solution (45 ml.), ethylene glycol (4 ml.) was added (to neutralise the excess of periodate), and iodate was removed by precipitation with barium acetate. Potassium borohydride (2 g.) in water (25 ml.) was added to the oxoamylopectin solution and after 24 hr. the excess of borohydride was decomposed by the dropwise addition of 6N-sulphuric acid (to pH 7·0). The solution was evaporated to dryness under reduced pressure at 30°, and the resultant material dissolved in 2N-sulphuric acid and heated at 100° for 3 hr. The neutralised (barium carbonate) hydrolysate was evaporated to a syrup and dissolved in warm 80% ethanol (100 ml.), and inorganic material was removed by filtration. This was extracted with hot 80% ethanol (30 ml.), and the combined filtrates were evaporated to a syrup and the residue was dissolved in water. Portions (equivalent to 18 mg. of polysaccharide) were applied to a sheet of Whatman No. 1 paper and developed, and aliquot portions of the eluates were treated with the phenol–sulphuric acid reagent.[14] The results, corrected for filter paper " blanks," are shown in Table 2. After 25 days, the pH of the reaction mixture was 2·9.

Periodate Oxidation of Waxy Maize Starch.—Starch (10 g.), previously defatted and dried, was dissolved in about 400 ml. of water with heating to 98° and stirring for 1 hr. The cooled solution was filtered (G3 sintered glass) to remove a small insoluble residue, and then oxidised with 0·4M-sodium metaperiodate in a final volume of 500 ml. at 26° for 14 days. The oxoamylopectin was freed from periodate and iodate and reduced with sodium borohydride (2 g.) in water (20 ml.) for 18 hr. at 20°. A second addition of borohydride (0·5 g.) was made. After 5 hr., the solution was neutralised with sulphuric acid and deionised by electrodialysis. Polyalcohol concentrate (about 0·5 g.) was hydrolysed with 3N-sulphuric acid at 100° for 3 hr., neutralised, and concentrated. Varying amounts of the hydrolysate were examined by paper chromatography in solvents B and C. Glycollaldehyde, erythritol, and a component of low R_G value were present; glucose could not be detected. The remainder of the polyalcohol was hydrolysed and examined on paper chromatograms as described for potato amylopectin. The phenol–sulphuric acid reagent did not detect glucose when portions equivalent to about 18 mg. of polysaccharide were examined.

Periodate Oxidation of Waxy Sorghum Starch and Potato Amylopectin.—Amylopectins (10 g each) were oxidised under the conditions described for waxy maize starch except that the temperature was 18°. After 14 days, the reduction of periodate was 1·4 and 1·5 mole per glucose residue, respectively. The oxoamylopectin solutions (reduced in volume to 100 ml.) were treated with sodium borohydride (4·0 g. in 20 ml. of water) for 18 hr., followed by the addition of a further 0·5 g. of borohydride. After 5 hr., the polyalcohol solution was neutralised and then adjusted to pH 1·0 with sulphuric acid. After 20 hr. at 18° the solution was neutralised with barium carbonate. Paper-chromatographic analysis showed the presence of glycollaldehyde, glycerol, and erythritol, but glucosylerythritol was absent (β-glucosylerythritol prepared by the partial acid hydrolysis of lichenin polyalcohol was used as a reference compound).

The partial acid-hydrolysates were diluted to 100 ml., made 3N with respect to sulphuric acid, heated at 98° for 3 hr., cooled, neutralised, and evaporated to dryness. Paper chromatography in solvents A and C failed to reveal the presence of glucose when silver nitrate, aniline oxalate, potassium periodatocuprate,[18] lead tetra-acetate–Rosaniline,[19] or periodate–permanganate [20] were used as spray reagents. Application of a solution of the hydrolysate to a sheet of Whatman paper, development, and elution of the probable glucose strips followed by analysis with the phenol–sulphuric acid reagent indicated the absence of glucose from the waxy sorghum starch hydrolysate and the possible presence of an insignificant amount (<0·03%) of glucose in the potato amylopectin hydrolysate.

Periodate Oxidation of Lichenin.—Lichenin (1 g.; sample II prepared by Dr. F. B. Anderson, see ref. 9) was dissolved in 25 ml. of water, with heating, and sodium metaperiodate (4·28g.) was added to the cooled solution, which was then diluted to 50 ml. Samples (0·5 or 1·0 ml.) were removed for measurement of the reduction of periodate or production of formic acid. Samples (2·5 ml.) were also removed for estimation of residual glucose; these were freed from periodate and iodate (treatment with ethylene glycol and barium acetate), reduced by the addition of sodium borohydride (0·5 g.), and after 14 hr. neutralised and then hydrolysed in

[17] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.
[18] Bonner, *Chem. and Ind.*, 1960, 345.
[19] Sampson, Schild, and Wicker, *Chem. and Ind.*, 1961, 82.
[20] Lemieux and Bauer, *Analyt. Chem.*, 1954, **26**, 920.

3N-sulphuric acid. The neutralised (barium carbonate) hydrolysates were diluted with water to 100 ml., and samples (0·2 ml.) were chromatographed on sheets of Whatman No. 1 paper, the glucose regions being eluted with water and analysed by the phenol–sulphuric acid reagent. The results are shown in Table 3.

Periodate Oxidation of Methyl β-Maltoside.—Methyl β-maltoside (15·2 mg.; kindly provided by Dr. K. Hunt) was dissolved in 0·4M-sodium metaperiodate (10 ml.). The production of formic acid, measured on 1-ml. samples, was 1·03, 1·17, 1·17, 1·28, and 1·57 mol. after 4, 8, 12, 16 and 24 days at 18°.

Stability of Amylose and Oxoamylose at pH 2·9.—To a 2% solution of amylose in water (100 ml.), sodium iodate (2·5 g.) and 90% formic acid (to give pH 2·9) were added. The solution was filtered (sintered glass, G.3) into a modified Ubbelhode viscometer maintained at 25° in a constant-temperature water-bath. At 0, 10, and 20 days, the flow times were 150·26, 150·28, and 151·56 sec.

Amylose (1 g.) was dissolved in 0·4M-sodium metaperiodate (100 ml.), with shaking, and left at 18° in the dark for 50 hr. The excess of periodate was then destroyed by ethylene glycol, and the solution dialysed against running tap-water for 48 hr. The solution was concentrated, treated with sodium iodate and formic acid (to give pH 2·9), and filtered into a viscometer. The flow times after 0, 2, 4, 6, and 8 days were 697·56, 696·91, 697·51, 697·58, and 697·50 sec.

We thank Professor E. L. Hirst, C.B.E., F.R.S., for his interest in this work, and Cerebos Ltd. for the award of a research scholarship (to G. A. M.).

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