[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF MINNESOTA]

Reduction of the Products of Periodate Oxidation of Carbohydrates. III. The Constitution of Amylopectin[1]

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Amylopectin (waxy corn starch) has been subjected to prolonged periodate oxidation with sodium periodate and the polyaldehyde so formed reduced with sodium borohydride to the corresponding polyalcohol. Hydrolysis of the latter gives a small amount of glucose. Oxidations were carried out at high concentration of periodate and at different temperatures for varying lengths of time. In one instance the polyalcohol was subjected to further periodate oxidation, reduced, hydrolyzed and glucose was identified chromatographically. The β-limit dextrin also gives rise to glucose when subjected to the above procedure. The structural significance of these findings is discussed.

In previous communications[2,3] a new method has been reported for the determination of the average repeating unit of amylopectin. This general method, based upon periodate oxidation followed by reduction and hydrolysis of the waxy corn starch has been extended in order to gain further insight into the fine structure of this molecule. It is generally accepted that amylopectin is a highly ramified polysaccharide in which the anhydroglucose residues comprising the average repeating units are joined by 1,4α-glucosidic bonds. Branching of the molecule is located at other positions, and of these it is position 6 that is thought to form the main branch point,[4] and by some investigators, to form the only branch point. The isolation of 2,3- and 2,6-di-*O*-methylglucose leads to the conclusion that branches other than the 1,6-type are present,[2,5] although the possibility of demethylation of 2,3,6-tri-*O*-methyl-D-glucose to form 2,6-di-*O*-methyl-D-glucose cannot be overlooked.[6]

The prolonged periodate oxidation of waxy corn starch and its β-limit dextrin, followed by reduction and hydrolysis of the derived polyalcohols appears to offer an alternative method for the study of their fine structure. The possibility that not all the branch points in amylopectin are due to 1,6-linkages has been discussed by Hirst and co-workers[7] as a result of experiments with periodic acid. It is a well established fact that this reagent and its alkali salts specifically attack α,β-glycol groups.[8,9] Hence in the case of amylopectin, if 1,6-linkages are the only points of branching, the hydroxyl groups on C_2 and C_3 must be oxidized to the corresponding dialdehyde. If, therefore, all the linkages in amylopectin are of the 1,4- and 1,6-type, oxidation with periodate followed by reduction and hydrolysis should give no free glucose. If, however, 1,3-linkages are present or if branching of the 1,4-linked glucose chains involves C_2 or C_3, or in the event of multiple branching, no free α,β-glycol groups would be open for attack and the particular glucose units concerned would survive periodate oxidation and would appear as glucose when the periodate oxidized molecule was reduced and subsequently hydrolyzed.

It has been shown[7] that the hydrolyzates of various oxidized starches, such as potato, acorn, sago, waxy maize and the β-limit dextrin of waxy maize starch, when analyzed using paper chromatography, give rise to small amounts of glucose amounting to less than 1% of the starch used. Thus, this would indicate that more than 75% of the branches are 1,6 and that a small proportion involve the hydroxyl groups on C_2 and/or C_3. However, these workers and others do not deduce that this small amount of glucose is significant, but indicate that it may arise from incomplete oxidation of the amylopectin by periodate.

This paper is concerned with the isolation of glucose following the prolonged periodate oxidation followed by reduction and hydrolysis of waxy corn starch and its β-limit dextrin. "Over-oxidation" was considered advantageous and certain of the oxidations were carried out at relatively high temperatures and high concentrations of periodate, while others were carried out for longer periods of time (up to 5 months) at high concentration of periodate but at low temperatures. Every effort was made to eliminate the possibility of incomplete oxidation. The glucose which remains intact when the waxy corn starch is given a prolonged treatment with sodium periodate could arise from incomplete oxidation or because of fixed "*trans*" hydroxyl groups.[10] It is also possible that the ring conformation of certain of the anhydroglucose residues is such that attack by periodate is hindered or prevented. However, the fact that the glucose still survives when the derived polyalcohol itself is treated with periodate points very strongly to the presence of glucose units in waxy corn starch beside the 1,4- and the 1,4,6- linked residues. In all cases the amount of glucose obtained following the oxidation, reduction and hydrolysis was in the range of 0.2 to 0.5% based on the weight of amylopectin used. In the case of the β-limit dextrin the amount of unoxidized glucose was found to be 0.33%.

The observation that the structural peculiarity responsible for the immunity of certain glucose

(1) This paper, No. 3506 Scientific Journal Series, Minnesota Agricultural Experiment Station, forms part of a thesis submitted by J. K. Hamilton to the Graduate School of the University of Minnesota in partial fulfillment of the requirements of the degree of Ph.D., 1952.

(2) M. Abdel-Akher, J. K. Hamilton, R. Montgomery and F. Smith, THIS JOURNAL, **74**, 4970 (1952).

(3) J. K. Hamilton and F. Smith, *ibid.*, **78**, 5907 (1956).

(4) L. W. Georges, I. L. Miller and M. L. Wolfrom, *ibid.*, **69**, 473 (1947).

(5) L. Boggs, L. S. Cuendet, I. Ehrenthal, R. Koch and F. Smith, *Nature*, **166**, 520 (1950).

(6) *Cf.* D. J. Bell, *J. Chem. Soc.*, 992 (1948).

(7) T. G. Halsall, E. L. Hirst, J. K. N. Jones and A. J. Roudier, *Nature*, **160**, 899 (1947); E. L. Hirst, J. K. N. Jones and A. J. Roudier, *J. Chem. Soc.*, 1779 (1948).

(8) L. Malaprade, *Bull. soc. chim.*, [4] **43**, 683 (1928).

(9) P. Fleury and J. Lange, *J. pharm. chim.*, (8) **17**, 107 (1933).

(10) B. H. Alexander, R. J. Dimler and C. L. Mehltretter, THIS JOURNAL, **73**, 4658 (1951).

residues to periodate oxidation is not concentrated by β-amylolysis, prompts the suggestion that all the glucose residues stable to periodate oxidation are not located within that part of the amylopectin molecule which gives rise to the β-limit dextrin when treated with β-amylase from soybean. It also follows that the β-amylase from soybean flour is either capable of by-passing the periodate stable glucose residues in the outer chains or that the β-amylase is contaminated with an enzyme (? 1,3-glucosidase) that is specific for cleaving the linkages of these glucose residues.

It seems possible, therefore, that certain structural features of the amylopectin molecule still remain to be elucidated and that the theory of the enzymatic synthesis and breakdown of starch may require further elaboration. Similar observations are probably in order with respect to the structure of glycogen.[2]

Since this work was completed and the suggestion made[2] that 1,3-linkages might be present in amylopectin, it has been shown[11] that such linkages do indeed exist in amylopectin since graded hydrolysis has afforded D-glucopyranosyl-1→3-D-glucose (nigerose). It still remains to be determined whether, in the amylopectin molecule, the 1,3-linked glucose units are also joined through other positions.

The way in which the periodate immune glucose residues of amylopectin are joined to other units is now being investigated by methylation studies of the polyalcohol obtained from amylopectin by periodate oxidation followed by reduction.

Experimental

Periodate Oxidation of Waxy Corn Starch.—Waxy corn starch, previously defatted[12] and dried *in vacuo* to constant weight, was gelatinized in aqueous solution at 68–75°. The solution was allowed to cool and then treated with a solution of sodium periodate in water so that the concentration was 0.2 or 0.4 *M* with respect to the periodate and 2% with respect to the starch (see Table I). The reaction mixture was shaken and quickly cooled to 2–4° and kept at this temperature in the dark.

The periodate consumption was determined in the usual way[8] on an aliquot (5 ml.), removed from the reaction mixture and diluted to 25 ml.; 10 ml. of this 25 ml. was treated with an excess of 0.2 *N* sodium arsenite (10 ml.), sodium bicarbonate (1.0 g.), and water (25 ml.) in the presence of a few crystals of potassium iodide. The mixture was shaken and allowed to stand for 15 minutes and the excess arsenite back titrated with 0.01 *N* iodine using amylose as the indicator. A blank determination was carried out simultaneously. Previous experiments in this Laboratory have shown that waxy corn starch has an average chain length of 19–20 anhydroglucose units based on the formic acid production.[3]

The results of four periodate oxidations are presented in Table I.

Isolation of the Waxy Corn Starch Polyaldehyde.—The reaction mixture (450 ml.) from the 150-day oxidation (experiment III) was centrifuged to remove the insoluble oxidized waxy corn starch from the periodate solution. The polyaldehyde was thoroughly washed with water, and dried by solvent exchange using absolute ethanol–petroleum ether (30–60°), the residual solvent being removed *in vacuo* over calcium chloride. The waxy corn starch polyaldehyde was a white amorphous powder (yield 6.0 g. or 60%). No attempt was made to isolate the polyaldehyde in a dry state in each experiment; rather the reduction to the polyalcohol was carried out as soon as possible.

In experiment IV, where the oxidation had proceeded to a value of 1.51 moles consumed per anhydroglucose unit, the oxidized product was soluble in the reaction mixture. The excess periodate was converted to iodate by adding ethylene glycol and the iodate was precipitated by the addition of barium acetate and removed by filtration. The filtrate was concentrated under diminished pressure to a small volume and reduced directly with an excess of sodium borohydride as described below.

TABLE I

OXIDATION OF WAXY CORN STARCH WITH SODIUM PERIODATE

Expt.	Time, days	Temp., °C.	Starch, g.	Vol. reacn. mixture, ml.	Periodate concn., *M*	Consumption periodate per anhydroglucose unit	Glucose after oxidn., %
I	48	2–4	20	1000	0.4	1.1	0.4
II	5.5	2–4	5	250	.2	1.04	0.22
III	150	2–4	10	500	.4	1.29	..
IV[a,b]	9	26	20	1000	.4	1.51	0.52, 0.44

[a] The starch was gelatinized and dispersed in water (250 ml.), treated with sodium periodate (85.6 g.) in water (650 ml.) at 40°, and the volume adjusted to 1000 ml. with water at 26°. [b] The acid produced corresponded to 1 mole of formic acid per anhydroglucose unit.

Reduction of the Waxy Corn Starch Polyaldehyde with Sodium Borohydride.—The polyaldehyde (5 g.) was suspended in water (300 ml.) and sodium borohydride (1 g. in 25 ml. of water) was added in portions with stirring. The alkaline reaction solution was left overnight when an aliquot, after acidification with dilute acetic acid, was non-reducing to Fehling solution. An additional 0.5 g. of sodium borohydride was added to the solution and the reaction mixture allowed to stand 5 hours in order to make sure the material was completely reduced. The alkaline solution was neutralized with glacial acetic acid and the solution concentrated under diminished pressure to a small volume. Hydrochloric acid was added to the concentrate from the previous experiment until the solution had a *p*H of 1.

Hydrolysis of the Waxy Corn Starch Polyalcohol.—The acidified solution was refluxed gently for 10 hours. The cooled hydrolysate was neutralized and deionized by passage first through a cation exchange column (Amberlite IR-120[13]) and then through an anion exchange column (Duolite A4[14]) respectively. The resulting neutral solution was concentrated under diminished pressure to a sirup which was dissolved in a small volume of ethanol.

Qualitative Chromatographic Analysis.—Paper partition chromatographic analysis of the hydrolysate of the above polyalcohol on Whatman No. 1 filter paper, using phenol saturated with water at room temperature as the irrigating solvent, showed the presence of glucose (R_f, 0.40), erythritol (R_f 0.63), glycerol (R_f 0.69) and small amounts of other unidentified spots.

Quantitative Determination of Glucose.—The amount of glucose present in the hydrolysates of the polyalcohols from the various experiments was determined spectrophotometrically at 490 mμ by the phenol–sulfuric acid method of Smith, *et al.*,[15] after separation by paper chromatography using 1-butanol:ethanol:water (4:1:5).

Reoxidation of the Waxy Corn Starch Polyalcohol.—A sample of the waxy corn starch polyaldehyde (500 mg.) obtained from experiment III was reduced with sodium borohydride in the normal manner described above. The alkaline solution (25 ml.) was neutralized with a drop of sulfuric acid (1 *N*) and the neutral solution was added to an aqueous sodium periodate solution and the volume adjusted to 50 ml. giving a concentration of 0.4 *M* with respect to the sodium periodate. The solution was maintained at 2 to 4° in the dark for 15 days. At the end of this period the sample showed a formic acid production of 0.9 mole per anhydroglucose unit and the characteristic odor of formaldehyde could be detected.

(11) M. L. Wolfrom and A. Thompson, THIS JOURNAL, **77**, 6403 (1955).

(12) T. J. Schoch, *Advances in Carbohydrate Chem.*, **1**, 247 (1945).

(13) A product of the Rohm and Haas Co., Philadelphia, Pa.

(14) A product of the Chemical Process Co., Redwood City, Calif.

(15) (a) M. Dubois, K. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Nature*, **168**, 167 (1951); (b) R. B. Koch, W. F. Geddes and F. Smith, *Cereal Chem.*, **28**, 424 (1951); (c) M. Dubois, K. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956)

To the periodate oxidation solution of the polyalcohol, a solution of barium acetate (2.55 g.) was added, and the precipitate of barium iodate and barium periodate removed by centrifugation. The supernatant and washings were neutralized with 0.105 *N* sodium hydroxide and to the neutral solution sodium borohydride (200 mg.) was added in two separate additions of 100 mg. over a period of 4 hours. The solution was allowed to stand for 24 hours and neutralized with glacial acetic acid. The acidity of the solution was increased to approximately 1 *N* by the addition of concentrated hydrochloric acid. The solution was boiled under reflux for 12 hours, cooled and neutralized by successive passage through a cation exchange resin (Amberlite IR-120) and an anion exchange resin (Duolite A4), respectively. The neutral solution was concentrated under diminished pressure to a sirup. The glucose, separated from the other hydrolytic products by paper partition chromatography using 1-butanol:ethanol:water (4:1:5) as the solvent corresponded in R_f to a glucose standard.

Isolation of D-Glucose and Preparation of the *p*-Nitroanilide.—The glucose present in the hydrolysate of the waxy corn starch polyalcohol from experiment II was separated using the sheet paper chromatography technique described in a previous paper.[3] The product obtained in this way was purified by chromatographing on a single sheet of Whatman No. 3 filter paper using the same irrigating solvent. The sirupy product thus obtained was found by polarimetric observation to contain D-glucose (11.2 mg.). Following the procedure of Weygand, *et al.*,[16] there was obtained D-glucose *p*-nitroanilide, m.p. and mixed m.p. 180–182°, $[\alpha]^{23}D$ −190° in pyridine (*c* 1) (after recrystallization from methanol).

Periodate Oxidation of the β-Limit Dextrin.—The β-limit dextrin (5.0 g.) prepared as described previously,[3] was dissolved in hot water (50 ml.) and allowed to cool to room temperature, after which a solution of sodium periodate (21.4 g.) in water (150 ml.) was added. The volume was quickly adjusted to 250 ml. with water giving a final periodate concentration of 0.4 *M*. The reaction mixture was kept at 2–4° in the dark for 6 days. The formic acid production and periodate consumption were followed in the manner already described.[3] The average chain length as determined by formic acid production was 6 (constant after 24 hours) and the periodate consumed per anhydroglucose unit after 144 hours was 1.09 moles.

Sodium Borohydride Reduction and Hydrolysis of the Polyalcohol.—The polyaldehyde was reduced, hydrolyzed, deionized and concentrated under reduced pressure to a sirup. Qualitative paper chromatographic analysis was carried out for 48 hours using an irrigating solvent of 1-butanol:ethanol:water (4:1:5). The results of spraying with Tollens reagent showed the presence of a small amount of glucose and larger amounts of glycerol and erythritol.

Quantitative Determination of Glucose.—The hydrolysate of the β-limit dextrin polyalcohol was concentrated under diminished pressure to a sirup of constant weight (1.464 g.) which was dissolved in ethanol and made up to 10 ml. in a volumetric flask. An aliquot of this solution (0.048 ml.) was transferred to a sheet of Whatman No. 1 filter paper (8" × 22") and separated by paper chromatography, irrigation being carried out for 46 hours with 1-butanol:ethanol:water.

The amount of glucose unattacked by periodate was found to be 0.33% based on the starting weight of β-limit dextrin. This was the average value obtained from four separate analyses carried out in duplicate which gave 0.39, 0.30, 0.33 and 0.29% glucose, respectively.

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(16) F. Weygand, W. Perkow and P. Kuhner, *Ber.*, **84**, 594 (1951).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

The Detosylation of 1,4:3,6-Dianhydrohexitol Ditosylates and Syntheses of 1,4:2,5:3,6-Trianhydro-D-Mannitol

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The two *exo*-tosylate groups in 1,4:3,6-dianhydro-L-iditol ditosylate have been removed by treatment with barium methoxide, forming the dianhydride I with retention of configuration. Reactions of 1,4:3,6-dianhydro-D-mannitol ditosylate, which has two *endo*-tosylate groups, with hydroxide, methoxide and hydride ions also resulted in retention of configuration with formation of the dianhydride II. Treatment of 1,4:3,6-dianhydro-D-glucitol ditosylate with sodium ethoxide yielded 14% of the dianhydride III and 60% of 1,4:2,5:3,6-trianhydro-D-mannitol (X), of interest as a hexitol trianhydride. Reaction of the trianhydride X with concentrated hydrochloric acid resulted in cleavage of the 1,4- and 3,6-anhydro rings, and the crystalline dichloro glycol XIV was obtained in 76% yield. The occurrence of "transtosylation," the conversion of an alcohol to the tosylate by reaction with another alkyl or aryl tosylate, has been demonstrated in this series of compounds. This reaction is analogous to the well-known transesterification reaction of carboxylic esters with alcohols.

The reaction of an "isolated" secondary sulfonate group in carbohydrates[2] with nucleophilic reagents such as hydroxide and alkoxide ions has been shown[2] to result in desulfonylation with the cleavage of the sulfur–oxygen bond, forming the alcohol with complete retention of configuration[3] as indicated

$$ArSO_2\text{—}O\text{—}R + OH^{\ominus} \longrightarrow ArSO_2OH + OR^{\ominus} \quad (S_N2S)$$

For the sake of convenience this type of displacement at a sulfur atom is designated in this paper as an SN2S reaction. The prevalence of SN2S reactions in carbohydrates is in direct contrast to the behavior of alkyl sulfonates under similar conditions,[6] except when the alkyl sulfonate group

(1) Sharp and Dohme Research Associate.

(2) A sulfonate group that has no adjacent hydroxyl or sulfonate group; S. P. Tipson, *Advances in Carbohydrate Chem.*, **8**, 207 (1953).

(3) The retention of configuration is not likely to be the result of an SN1 type solvolysis of the sulfonate group with the aid of a neighboring oxygen group participation. An "isolated" secondary sulfonate group in a carbohydrate has been shown[2] to be very unreactive under solvolytic conditions, probably due to the inductive effect of the neighboring oxygen-containing group.[4] The desulfonylation reactions are often carried out with strong nucleophilic reagents. Furthermore, desulfonylation with sodium alkoxide in dry alcohol affords the hydroxy compound, frequently in almost quantitative yield,[5] instead of the alkyl ether to be expected from solvolytic reactions.

(4) S. Winstein and co-workers, THIS JOURNAL, **70**, 816 (1948), and later papers.

(5) For example: J. W. H. Oldham and G. J. Robertson, *J. Chem. Soc.*, 685 (1935); D. J. Bell and J. Williamson, *ibid.*, 1196 (1938); W. N. Haworth, E. L. Hirst and L. Panizzon, *ibid.*, 154 (1934).

(6) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 341.