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

Reduction of the Products of Periodate Oxidation of Carbohydrates. X. Methylation Studies on Amylopectin Polyalcohol

BY I. J. GOLDSTEIN, J. K. HAMILTON AND F. SMITH[1]

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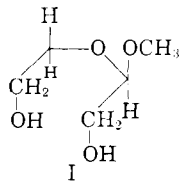
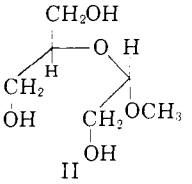
Amylopectin has been transformed into the corresponding polyalcohol IV (R = H) by periodate oxidation followed by reduction. Methylation of IV (R = H) has been effected and the product IV (R = CH_3) subjected to methanolysis; the following cleavage products have been identified: 1,4-di-*O*-methylerythritol (VI), D-(1-*O*-methylerythritol) (VII) and methoxyacetaldehyde (VIII). The molecular ratio of VI and VII has been determined and shown to provide a new approach to the average size of the repeating unit of amylopectin.

In a previous communication[2] it was shown that amylopectin could be oxidized with periodate and the resulting polyaldehyde reduced to the corresponding polyalcohol. Since model experiments have shown[3,4] that the alcohols I, II and III prepared in the same manner, respectively, from methyl β-D-xylopyranoside, methyl α-D-glucopyranoside and methyl β-cellobioside and methyl β-lactoside could be transformed into the corresponding fully methylated derivatives, it became feasible to study polysaccharide polyalcohols by the methylation technique. This paper is concerned with methylation studies on amylopectin polyalcohol (IV, R = H). The methylation



I



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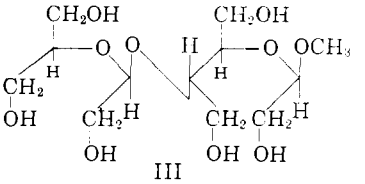

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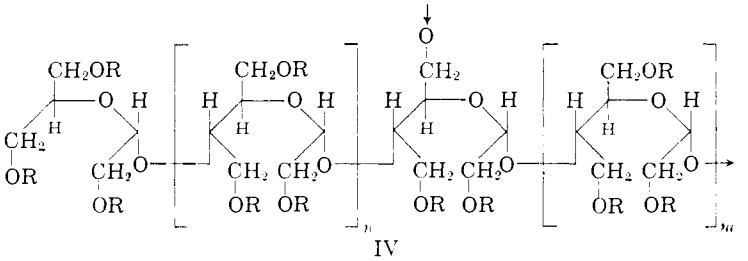
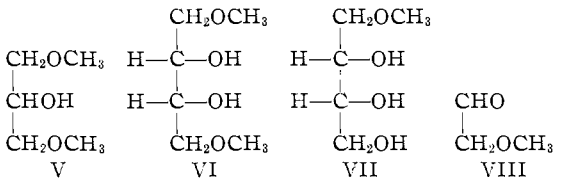


IV

studies on other polysaccharide polyalcohols will form the subject of later communications.

Amylopectin was subjected to prolonged periodate oxidation and the polyaldehyde so formed was reduced to the polyalcohol IV (R = H) with sodium borohydride.[5]

The amylopectin polyalcohol IV (R = H) was methylated with methyl sulfate and 40% potassium hydroxide in the usual way. After the first methylation the product which was readily extracted by chloroform was treated with acetic anhydride and pyridine to eliminate any borate complexes[5] and to facilitate complete methylation of the amylopectin polyalcohol. After five additional treatments with methyl sulfate and alkali the partially methylated polyalcohol was subjected to the action of sodium and methyl iodide in liquid ammonia and then two methylations with silver oxide and methyl iodide.



V VI VII VIII

Hydrolysis of the fully methylated amylopectin polyalcohol (IV, R = CH_3) with boiling methanolic hydrogen chloride afforded a mixture of 1,4-di-*O*-methylerythritol (VI), D-(1-*O*-methylerythritol) (VII) and methoxyacetaldehyde (VIII).

The 1,4-di-*O*-methylerythritol (VI) which arises from the 1,4-linked-D-glucose residues was identified as the characteristic 2,3-di-*p*-toluenesulfonate.[4] The D-(1-*O*-methylerythritol) (VII), derived from those glucose residues at which branching occurs, was identified readily as the crystalline 2,3,4-tri-*p*-nitrobenzoate. The methoxyacetaldehyde VIII which arises from C_1 and C_2 of all the building units in IV, R = CH_3, readily formed the characteristic crystalline *p*-nitrophenylhydrazone.[3]

Since the 1,4-di-*O*-methylerythritol (VI) arises from the non-terminal glucose units and the D-(1-*O*-methylerythritol) (VII) from the glucose residues at which branching occurs, then the molecular ratio of VI to VII should correspond to the average value of the repeating unit (the ratio of non-terminal to terminal non-reducing residues)

(1) Paper No. 4095, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, Minn. Presented at the 131st A.C.S. Meeting, Miami, Fla., April, 1957.

(2) J. K. Hamilton and F. Smith, THIS JOURNAL, **78**, 5907, 5910 (1956).

(3) J. K. Hamilton, G. W. Huffman and F. Smith, *ibid.*, **81**, 2173 (1959).

(4) J. K. Hamilton, G. W. Huffman and F. Smith, *ibid.*, **81**, 2176 (1959).

(5) M. Abdel-Akher, J. K. Hamilton and F. Smith, *ibid.*, **73**, 4691 (1951).

in amylopectin. The molar ratio of VI to VII was calculated from the periodate consumed by VI and VII in the hydrolyzate of IV (R = CH_3) and the formaldehyde which is formed from VII at the same time. Since the molar proportion of formaldehyde produced from VII is equal to half the molar periodate consumed by VII, the difference between the total molar periodate consumed by VI and VII, and the periodate consumed by VII corresponds to the number of moles of VI present in the mixture. The average of four determinations of the ratio of VI to VII was about 17, a result in good agreement with those obtained by direct methylation studies[6] and by periodate oxidation and determination of the formic acid produced.[2,7]

The amount of 1,3-di-*O*-methylglyceritol (V) formed from IV (R = CH_3) would also provide a value for the average repeating unit in amylopectin. This cleavage fragment (V) is, however, relatively volatile,[3] it is not readily detected on paper chromatograms and its determination therefore is much more difficult to accomplish than that of either VI or VII.

These findings therefore demonstrate that methylation studies on polyalcohols can be used to throw light on the structure of polysaccharides. A search is now being made to ascertain whether those glucose units of amylopectin that are stable to periodate can be detected as a minor component in the hydrolyzate of the methylated amylopectin polyalcohol.

Experimental

Oxidation of Amylopectin with Sodium Periodate.—Waxy corn starch amylopectin (40.0 g.) was defatted by two three-hour extractions with boiling methanol (200 ml.). The amylopectin was filtered and dried first in air and then *in vacuo* (moisture content 7.6%).

The amylopectin was suspended in water (450 ml.) and heated with stirring to 75° to effect gelatinization. After cooling to room temperature the solution was treated with a cooled (5°) aqueous solution (500 ml.) containing sodium periodate (85.6 g.) and the volume quickly adjusted to 2 liters with cold (5°) water. The reaction mixture was kept in the dark at 5°. After 10 weeks the periodate consumption had reached 1.08 moles per glucose residue; the formic acid production,[2,7] 0.052 mole per glucose residue, corresponded to an average repeating unit of 19.

The amylopectin polyaldehyde which had separated from solution was recovered (centrifuge) and washed with ice-cold water until the washings no longer gave a positive test for iodate (tested with acidified potassium iodide). The product was washed (centrifuge) successively with ethanol, diethyl ether, petroleum ether and dried in air (yield 40.0 g.). The polyaldehyde was a fine, white powder which closely resembled the original amylopectin.

Reduction of Amylopectin Polyaldehyde with Sodium Borohydride.[5]—To a suspension of the polyalcohol (20.0 g.) in water (200 ml.) was added rapidly with stirring a solution of sodium borohydride (10 g.) in water (100 ml.). The reaction mixture foamed considerably and a gel-like semi-solid formed momentarily. After standing 20 min. the solution was opalescent and after standing overnight a clear pale yellow solution resulted which did not reduce Fehling solution.

Methylation of Amylopectin Polyalcohol.—The solution obtained in the previous experiment was treated with methyl sulfate (300 ml.) and 40% aqueous potassium hydroxide (900 ml.), the reagents being added during 2 hr. in 12 equal portions with vigorous stirring, half of the reagents being added at room temperature and the rest at 55°; acetone was added when necessary to keep the partially methylated polyalcohol in solution. The product was extracted from the cooled reaction mixture with chloroform. The combined chloroform extracts were washed with water to remove salts and evaporated to dryness giving a sirupy product.

Acetylation of the Partially Methylated Amylopectin Polyalcohol.—In order to cleave any borate complexes[5] and to facilitate completion of the methylation, the sirupy product obtained in the previous experiment was dissolved in pyridine (50 ml.) and treated with acetic anhydride (35 ml.). After heating to 40° the reaction mixture was kept overnight and poured with stirring into ice-water (300 ml.). The stiff sirupy product which settled immediately was separated by decantation from the upper, aqueous layer and repeatedly washed by trituration with water. The aqueous layer was extracted five times with chloroform (100 ml.) and the combined chloroform extracts were washed with sodium bicarbonate solution and with water. The chloroform extract was combined with the sirupy product and the whole evaporated *in vacuo* to give a sirup.

Complete Methylation of Amylopectin Polyalcohol.—The sirupy product obtained from the previous acetylation experiment was methylated in acetone solution with methyl sulfate (200 ml.) and 45% aqueous potassium hydroxide (750 ml.) as already described. Five methylations were applied in this manner, the product being isolated after each methylation by extraction with chloroform. The product after the fifth methylation was dissolved in chloroform and the solution washed with water and evaporated to give the methylated amylopectin polyalcohol as a yellow sirup which was dried by heating *in vacuo* at 60° for 3 hr.

The dried residue was dissolved in liquid ammonia (750 ml.) and treated with sodium (3.3 g.) and methyl iodide (9 ml.) as described previously.[3,4] After evaporation of the ammonia in a stream of dry air the methylated amylopectin polyalcohol was dissolved in chloroform and the solution was washed with water until the washings were neutral. The chloroform solution was dried ($MgSO_4$), evaporated to dryness *in vacuo* and the product retreated with sodium and methyl iodide in liquid ammonia as before.

The methylated amylopectin polyalcohol was subjected to four successive treatments with Purdie reagents, methyl iodide (100 ml.) and silver oxide (10 g.), the latter being added in five portions in the usual way. The methyl iodide was distilled and the product isolated by extraction with diethyl ether (150 ml.). Dry petroleum ether (b.p. 30–60°) was added until the solution became turbid after which centrifugation removed all inorganic impurities and a clear yellow solution resulted. Evaporation of solvent and heating at 60° at 1 mm. gave the methylated amylopectin polyalcohol IV (R = CH_3) as a viscous yellow sirup (yield 20.0 g.). A solution of the product in methanol (*c* 4) was optically inactive.

Anal. Calcd. for $C_9H_{15}O_5$: OCH_3, 45.1. Found: OCH_3, 44.1, 44.2.

Methanolysis of the Methylated Amylopectin Polyalcohol.—A solution of the methylated polyalcohol IV (R = CH_3, 1.016 g.) in 3% methanolic hydrogen chloride (40 ml.) was boiled for 18 hr. The reaction mixture was neutralized (Ag_2CO_3), filtered, and concentrated (atm. press.) to give a residue R (2 ml.) and a distillate D.

Treatment of this distillate, D, as before[3,4] with an acidified solution of *p*-nitrophenylhydrazine readily gave methoxyacetaldehyde *p*-nitrophenylhydrazone, m.p. and mixed m.p. 116°.

When the distillate D was treated with 2,4-dinitrophenylhydrazine a crystalline product was formed which was separated mechanically into two crystalline forms, orange needles, m.p. 116°, and yellow plates, m.p. 124°.

Anal. Calcd. for $C_9H_{10}O_5N_4$: C, 42.5; H, 3.94; N, 22.03; OCH_3, 12.2. Found (for the orange needles): C, 42.99; H, 4.06; N, 21.6; OCH_3, 11.8.

The residue R (2 ml.) was extracted with acetone, filtered and evaporated (bath temp. 30–35°) at the water-pump. Paper chromatographic analysis, using butanone–water azeotrope as the irrigating solvent and Tollens spray reagent, revealed the presence of 1,4-di-*O*-methylerythritol (R_f 0.61) and D-(1-*O*-methylerythritol) (R_f 0.29).

Separation and Identification of 1,4-Di-*O*-methylerythritol and D-(1-*O*-Methylerythritol).—The sirupy product from

(6) E. L. Hirst, M. M. T. Plant and M. D. Wilkinson, *J. Chem. Soc.*, 2375 (1932); W. Z. Hassid and R. M. McCready, THIS JOURNAL, **65**, 1157 (1943).

(7) F. Brown, S. Dunstan, T. G. Halsall, E. L. Hirst and J. K. N. Jones, *Nature*, **156**, 785 (1945); A. L. Potter and W. Z. Hassid, THIS JOURNAL, **70**, 3488 (1948).

the previous experiment was dissolved in the minimum amount of butanone–water azeotrope and transferred to a cellulose–hydrocellulose column.[8] Separation of the components was effected with butanone–water azeotrope using an automatic fraction collector, fractions of 10 ml. being collected every 30 min. Examination of the contents of the tubes in the usual way using the Tollens reagent spray showed that the 1,4-di-*O*-methylerythritol was in tubes 18–27 and the D-(1-*O*-methylerythritol) in tubes 58–70.

Identification of 1,4-Di-*O*-methylerythritol.—Evaporation (*in vacuo* at 35–40°) of the eluate in tubes 18–27 gave a colorless sirup (450 mg.). Some of the product distilled with the solvent and it was detected in the distillate by paper chromatography. Treatment of the sirupy product with *p*-toluenesulfonyl chloride in pyridine in the usual manner[4] afforded crystalline 1,4-di-*O*-methyl-2,3-di-*O*-tosylerythritol, m.p. and mixed m.p. 139° (after recrystallization from ethanol).

Anal. Calcd. for $C_{20}H_{26}O_8S_2$: C, 52.4; H, 5.72. Found: C, 52.5; H, 5.72.

Identification of D-(1-*O*-Methylerythritol).—Concentration of the contents of tubes 58–70 gave D-(1-*O*-methylerythritol) as a colorless sirup (15 mg.) which showed $[\alpha]^{23}D$ −5° in ethanol (*c* 0.8). Treatment of the D-(1-*O*-methylerythritol) with *p*-nitrobenzoyl chloride in dry pyridine in the usual manner gave the 2,3,4-tri-*p*-nitrobenzoate as fine, white needles, m.p. and mixed m.p. 162.5–164°, $[\alpha]^{22}D$ + 80° in chloroform (*c* 1) after recrystallization from acetone–ethanol. An authentic specimen of D-(1-*O*-methylerythritol) tri-*p*-nitrobenzoate[9] showed m.p. 162.5–164°, $[\alpha]^{23}D$ + 85° in chloroform (*c* 1).

Determination of the Ratio of D-(1-*O*-Methylerythritol) to 1,4-Di-*O*-methylerythritol in the Hydrolyzate of Methylated Amylopectin Polyalcohol.—The D-(1-*O*-methylerythritol) was determined by periodate oxidation and determination of the formaldehyde produced from C_4. Since the molar proportion of periodate consumed in this reaction is twice the molar proportion of formaldehyde generated, then subtraction of this calculated molar amount of periodate consumed by the D-(1-*O*-methylerythritol) from the total molar amount of periodate consumed by the mixture of D-(1-*O*-methylerythritol) and 1,4-di-*O*-methylerythritol gives the molar amount of periodate taken up by the 1,4-di-*O*-methylerythritol. It was thus possible to arrive at an approximate value for the molar ratio of 1,4-di-*O*-methylerythritol to D-(1-*O*-methylerythritol) which corresponds to the average value of the repeating unit in the parent amylopectin.

A correction was applied for a small amount of demethylation of 1,4-di-*O*-methylerythritol that occurred during the methanolysis of the methylated amylopectin polyalcohol. By means of the chromotropic acid method[11] it was shown that 100 mg. of 1,4-di-*O*-methylerythritol gave rise to 1.3 mg. of mono-*O*-methylerythritol. That this reaction took place was confirmed in a separate experiment by paper chromatography.

The experimental procedure adopted after several trial experiments is presented: a solution of the methylated amylopectin polyalcohol (1.062 g.) in methanol (30 ml.) containing hydrogen chloride (0.6 g.) was refluxed for 3 hr. The solution was cooled and the volume adjusted to 50 ml. with water. This solution containing the mixture of 1,4-di-*O*-methylerythritol and D-(1-*O*-methylerythritol) is referred to as solution A.

Total Periodate Consumption.[10]—To an aliquot (5 ml.) of solution A was added an aqueous solution (10 ml.) containing periodic acid (0.20 g.) and the volume was adjusted to 25 ml. with water. The reaction mixture together with a "blank" was kept at 20° in the dark. The periodate consumption was determined by adding a 1-ml. aliquot to a saturated aqueous solution of sodium bicarbonate (10 ml.) containing 0.1 *N* arsenite (1 ml.). After standing 15 min. a crystal of potassium iodide was added and the excess of the arsenite back-titrated with 0.01 *N* iodine. The results showed that the periodate consumed by the 1-ml. aliquot corresponded to 3.7 ml. of 0.01 *N* iodine. Hence the periodate consumed by 5 ml. of the solution A = (3.7 × 0.01)/2 × 25/1 mmoles. Had all the hydrolyzate been treated, the periodate consumption would have been = (3.7 × 0.01)/2 × 25/1 × 50/5 = 4.62 mmoles. This corresponds to the total periodate consumed by the mono- and di-*O*-methylerythritol.

Determination of the Mono-*O*-methylerythritol.—An aliquot (1 ml.) of solution A oxidized by periodate according to the procedure of Lambert and Neish[11] was found, by reference to a standard curve for 1-*O*-methylerythritol to contain 189 mg. of formaldehyde. This corresponds to a total of 42.8 mg. or 0.315 mmoles of 1-*O*-methylerythritol in solution A. In determining the standard curve relating absorbance and concentration of 1-*O*-methylerythritol by the chromotropic acid method an amount of 1,4-di-*O*-methylerythritol was added before periodate cleavage was applied to generate formaldehyde. This ensured that the same amount of methoxyacetaldehyde was present in the standard solution as in the reaction mixture from the methylated amylopectin polyalcohol being analyzed.

Calculation of the Average Repeating Unit of Amylopectin.—The total amount of 1,4-di-*O*-methylerythritol is equal to the total molar consumption of periodate (4.62 mmoles) less the molar consumption (0.63 mmole) of periodate by the 1-*O*-methylerythritol (0.315 mmole) (since 1 mole of the latter reacts with 2 moles of periodate) = 4.62 − 0.63 = 3.99 mmoles or 3.99 × 155 = 618 mg. of 1,4-di-*O*-methylerythritol (mol. wt. 155).

Since demethylation results in the formation of 1.3 mg. of mono-*O*-methylerythritol from 100 mg. of 1,4-di-*O*-methylerythritol, then 618 mg. of 1,4-di-O-methylerythritol will give rise to 618 × (1.3/100) = 8 mg. of mono-*O*-methylerythritol. Hence the D-(1-*O*-methylerythritol) derived directly from the methylated polyalcohol = 42.8 − 8 = 34.8 mg. or 0.256 mmole. Therefore the molar ratio of 1,4-di-*O*-methyl to D-(1-*O*-methylerythritol) = 3.99/0.256 = 15.6/1. In additional experiments the molar ratio was 16.3, 18.2, 17.5; average, 17.

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