Properties of Periodate-oxidised Polysaccharides. Part III. Estimation of α-Glycol Groupings in a Polysaccharide.*

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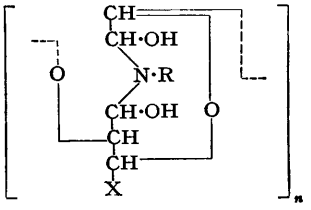
The nitrogen and sulphur contents of the polymers obtained by the condensation of periodate-oxidised polysaccharides with *iso*nicotinhydrazide and thiosemicarbazide give a measure of the proportion of sugar units vulnerable to periodate oxidation in the polysaccharides.

IN Parts I and II,* a description was given of some new derivatives of oxystarch (periodate-oxidised starch) obtained by condensation with carbonyl reagents, *e.g.*, thiosemicarbazide, hydrazine, *iso*nicotinhydrazide (isoniazid), and with various aromatic bases, *e.g.*, *p*-aminobenzoic acid. Analysis of the polymers obtained from oxystarch and thiosemicarbazide or isoniazid showed that one molecule of reagent had condensed with each potential dialdehyde group and accordingly a cyclic structure was proposed for these condensation products. Products of a similar kind have been obtained by condensation of periodate-oxidised trehalose and raffinose with *p*-nitrophenylhydrazine (Akiya, Okui, and Suzuki,



R = NH·CO⟨⟩N or NH·CS·NH_2.

X = CH_2·OH (amylose, amylopectin, waxy maize starch),

or CO_2H (alginic acid).

J. Pharm. Soc. Japan, 1952, **72**, 891; *Chem. Abs.*, 1953, **47**, 7447); these also contained only one condensed molecule of reagent for each oxidised α-glycol group. The present paper is concerned with the estimation of the proportion of α-glycol groups in various

* Parts I & II, Barry and Mitchell, *J.*, 1953, 3610, 3631.

polysaccharides from the composition of the thiosemicarbazide and isoniazid condensation products prepared from the periodate-oxidised polysaccharides, and the comparison of these values with those obtained by previously known methods.

It is now well established that amylose, amylopectin, and waxy maize starch are glucose polymers in which the hexose units are almost all joined by α-1 : 4-linkages and that the mannuronic acid units in alginic acid are β-1 : 4-linked (Hirst, Jones, and Jones, *J.*, 1939, 1880). In these polysaccharides, therefore, after oxidation with periodate, each hexose or hexuronic acid unit will provide a reactive centre for the condensation of one molecule of

TABLE 1.

	Thiosemicarbazide polymer				Isoniazid polymer	
	Reqd. (%)		Found (%)		Reqd. (%)	Found (%)
Polysaccharide	N	S	N	S	N	N
Amylose	16·7	12·7	16·1	12·1	14·1	13·9
Amylopectin	16·7	12·7	15·9	11·8	14·1	14·1
Waxy maize starch	16·7	12·7	15·2	11·8	14·1	14·4
Alginic acid	15·8	12·1	17·5	13·0	13·5	13·1

carbonyl reagent. Gravimetric analyses of the condensates will then permit the calculation of the proportion of α-glycol groups in the original polysaccharide. Table 1 shows the nitrogen and sulphur contents of the thiosemicarbazide and isoniazid polymers derived from the oxypolysaccharides, together with the values required by the theory assuming that all the linkages present in the molecules are 1 : 4. The deviations are small, particularly for the isoniazid polymers, and confirm the large preponderance in these polysaccharides of 1 : 4-linkages. It was of particular interest, therefore, to apply this method to some polysaccharides which are claimed to contain 1 : 3-linkages, *e.g.*, xylan from *Rhodymenia palmata*, nigeran from *Aspergillus niger*, Floridean starch and laminarin, and Table 2 gives the appropriate results. In the last part of the Table the α-glycol contents calculated from the analytical figures are compared with those derived from the periodate consumption.

TABLE 2.

	Thiosemicarbazide polymers				Isoniazid polymers		α-Glycol content (%)		
	Required for 100% α-glycol		Found		Required for 100% α-glycol	Found	From periodate	From thiosemicarbazide polymer [a]	From isoniazid polymer
Polysaccharide	N	S	N	S	N	N			
Xylan	19·0	14·5	14·5	11·9	15·7	13·2	69	72, 73	72
Nigeran	16·7	12·7	10·6	8·3	14·1	10·3	48 [b]	53, 55	60
Laminarin	—	—	—	—	14·1	1·5	—	—	6·1
Floridean starch	16·7	12·7	16·4	12·7	14·1	14·1	97	97, 100	100

[a] The first figure is based on the nitrogen analysis and the second on the sulphur. [b] Value obtained by Barker, Bourne, and Stacey, *J.*, 1953, 3084.

Methylation studies previously conducted with the xylan have indicated the presence of 1 : 4- and 1 : 3-linkages in this polysaccharide, and periodate consumption indicated that about 80% of the linkages are 1 : 4 [(*a*) Percival and Chanda, (*b*) Barry, Dillon, Hawkins, and O'Colla, *Nature*, 1950, **166**, 787]. The figures for this xylan (Table 2) lend support to the findings of these authors. Also, an important conclusion may be arrived at as to the homogeneity of the xylan. If it were a mixture of two polysaccharides built from 1 : 3- and 1 : 4-linked xylose anhydride residues respectively, then the polymer separating after condensation of the oxidised material with isoniazid or with thiosemicarbazide would be derived solely from the 1 : 4-linked polyoses and would thus contain a higher nitrogen content.

The polyglucose, nigeran, has been shown in methylation studies by Barker, Bourne, and Stacey (*loc. cit.*) to contain approximately equal proportions of 1 : 3- and 1 : 4-linked units. As seen in Table 2, the analyses of the derivatives prepared confirm these findings and also the conclusion of these authors that the two types of linkage are present in the one polysaccharide molecule.

The result obtained with laminarin is interesting. If one assumes that condensation of the carbonyl reagent takes place only at the non-reducing end of the periodate-oxidised polysaccharide, then the chain-length calculated from the nitrogen content works out at approximately 16 glucose units This is the degree of polymerisation found in this polysaccharide by Barry's method (*J.*, 1942, 578) and is of the same order as that found by Percival and his co-workers from methylation studies (Connell, Hirst, and Percival, *J.*, 1950, 3494 ; Percival and Ross, *J.*, 1951, 720).

Examined in this way Floridean starch has given results which do not agree in some respects with previous reports. Barry, Halsall, Hirst, and Jones (*J.*, 1949, 1468) suggested from the periodate consumption and from the fact that glucose could be identified, by paper chromatography, among the hydrolysis products of the oxidised starch, that their polysaccharide contained a proportion of 1 : 3-linked glucose residues. More recently, O'Colla (*Proc. Roy. Irish Acad.*, 1953, **55**, *B*, 321) reported failure to find conclusive evidence of the presence of 1 : 3-linkages; however, as his material contained about 20% of a galactan which he was unable to remove, it must be admitted that there remained a considerable uncertainty as to the broad structural characteristics of this polysaccharide. The sample of Floridean starch described in the present report was isolated as previously described (Barry *et al.*, 1949, *loc. cit.*) from *Dilsea edulis* collected on the Kerry coast in August 1953. It was purified by repeated precipitation with alcohol followed by dialysis. On hydrolysis this sample yielded only glucose. As Table 2 shows, there is no evidence that this polysaccharide contains 1 : 3-linkages any more than does amylose. Further, when the periodate-oxidised polysaccharide was hydrolysed by acid, paper chromatography of the hydrolysis products failed to show the presence of glucose. The oxidised xylan and nigeran on the other hand, when similarly treated, were shown to contain xylose and glucose respectively.

It might be argued that the failure to detect 1 : 3-linkages in Floridean starch was due to over-oxidation. The composition of the thiosemicarbazide and isoniazid derivatives however, shows that this was unlikely since it gives a measure of the α-glycol content of the portion of the polysaccharide molecule remaining after oxidation. The α-glycol content deduced from analysis of these derivatives could only be too high if the units removed by over-oxidation were 1 : 3-linked. Hexose units removed in this way are generally believed to come from the reducing end of the molecule (Halsall, Hirst, and Jones, *J.*, 1947, 1429; Neumuller and Vasseur, *Arkiv Kemi, Min., Geol.*, 1953, **5**, 235; Head, *J. Text. Inst.*, 1953, **44**, T209), and a chain of 1 : 3-linked units at such a position in a polysaccharide seems improbable. In any event removal of each hexose molecule, whether 1 : 3- or 1 : 4-linked, consumes according to Neumuller and Vasseur six mols. of periodate as compared with the consumption of one mol. in normal oxidation, and, if over-oxidation of the Floridean starch had taken place to any considerable degree, the overall consumption of periodate would have been greater than the 1 mol. per glucose mol. actually obtained for our Floridean starch. It seems justifiable, therefore, to conclude that this material contained no significant proportion of 1 : 3-linked glucose units.

Experimental

All polysaccharide materials were dried at 100°/10 mm.

Thiosemicarbazide and Isoniazid Derivatives of Oxidised Amylose, Amylopectin, and Waxy Maize Starch.—The polysaccharide (2 g.) was kept for 48 hr. with sodium metaperiodate solution (144 c.c.; 12·547 g. per l.). The oxidised material was filtered, washed free from inorganic material, and dissolved in water (100 c.c.) on a boiling-water bath during 1 hr. The concentration of the solution in each case was determined by evaporation to dryness of an aliquot. The solutions had $[\alpha]_D^{24}$ (in H_2O) as follows: oxyamylose, +24·3° (*c* 1·088); oxyamylopectin, +20·5° (*c* 1·666); waxy maize oxystarch, +20·5° (*c* 1·999). Two portions (25 c.c. each) of the oxystarch solution were mixed with isoniazid (0·85 g.) in water (50 c.c.) and thiosemicarbazide (0·56 g.) in water (50 c.c.) respectively. An immediate precipitate was formed in each case. These were separated in the centrifuge and washed with water, alcohol, and finally ether. The yields for the isoniazid and thiosemicarbazide products were, respectively: from oxyamylose, 99, 88; oxyamylopectin, 99, 90; waxy maize starch, 100, 91%.

Thiosemicarbazide and Isoniazid Derivatives of Oxidised Alginic Acid.—Sodium alginate (Manucol) (2 g.) was treated with sodium metaperiodate solution (214 c.c.; 12·547 g. per l.). The alginate dissolved slowly and after 48 hr. ethylene glycol (5 c.c.) was added and the solution dialysed against running water for 11 days. After filtering through Celite, the filtrate had $[\alpha]_D^{18}$ +135° (*c* 0·516 in H_3O). Two 50-c.c. portions were used as described earlier for the preparation of the thiosemicarbazide and isoniazid polymers. In this case the condensation products remained in solution and were precipitated when the solutions were made slightly acid. The products were isolated and dried as above The yield of isoniazid derivative was 76% and of the thiosemicarbazide derivative 44%.

Xylan Derivatives.—The xylan was isolated from the red alga, *Rhodymenia palmata*, by Barry and Dillon's method (*Nature*, 1940, **146**, 620) and had $[\alpha]_D^{23}$ −97·1° (*c* 0·3912 in H_2O). The pentosan (2 g.) was treated in water (50 c.c.) with sodium metaperiodate (3·57 g.; 10% excess) in water (100 c.c.). Ethylene glycol (2 c.c.) was added after 72 hr. The solution, dialysed against running water for 11 days, had $[\alpha]_D^{23}$ +85° (*c* 0·88 in H_2O). The isoniazid and thiosemicarbazide condensation products were prepared in good yield as above. It was necessary to add a little sodium chloride to effect separation.

The periodate consumption and yield of formic acid were measured in a separate experiment by the method of Halsall, Hirst, and Jones (*J.*, 1947, 1430). Xylan (0·7 g.), in water, was added to sodium metaperiodate (20 c.c.; 0·2525M) and the volume brought to 200 c.c. The course of the oxidation is tabulated.

Time (hr.)	3	6	23	27	47	52	71	119
Consumption of periodate (mol. per xylose residue)	0·304	0·427	0·592	0·595	0·630	0·640	0·671	0·686
Average chain-length, calc. from formic acid produced	18·2	18·2	15·7	15·8	15·8	15·7	15·7	—

The figures in the last row represent the number of xylose units for each non-reducing end-group. Complete reliance cannot be placed on this method of end-group assay because of the difficulty of determining the end-point in the formic acid titration. (For a critical evaluation of the method, see Morrison, Knyper, and Orton, *J. Amer. Chem. Soc.*, 1953, **75**, 1502.)

Hydrolysis of Oxyxylan.—(a) *Identification of xylose.* The above oxyxylan solution (100 c.c.) was dialysed against running water for 24 hr. and then heated on the water-bath (6 hr.) with an equal volume of 6N-sulphuric acid. It was neutralised ($BaCO_3$), filtered, and concentrated under reduced pressure to a small volume. A paper chromatogram run in *n*-butanol–pyridine–water–benzene (5 : 3 : 3 : 1) gave one spot, R_g (g = glucose) 1·42. Similar treatment of the unoxidised xylan gave a spot having R_g 1·44.

(b) *Isolation of xylosazone.* Xylan (2 g.) in water (25 c.c.) was kept with sodium metaperiodate (60 c.c.; 0·25M) for 90 hr., then treated with a few c.c. of 10% aqueous ammonia (cf. Barry, Dillon, Hawkins, and O'Colla, *loc. cit.*) and neutralised with 10% acetic acid, giving a white gelatinous precipitate. This was separated, washed, and dried, as usual, to a white powder (0·5 g.) which was hydrolysed on the water-bath (1·6 hr.) with 0·5N-sulphuric acid (20 c.c.). The solution was then neutralised ($BaCO_3$), filtered, and concentrated under reduced pressure. Treatment of this at room temperature with phenylhydrazine (1 c.c.) in 33% acetic acid (3 c.c.) gave a gummy precipitate which on recrystallisation from benzene yielded glyoxalosazone, m. p. 166—167°. The filtrate after removal of the gum was warmed on the water-bath for 15 min., then kept for 1½ hr. Characteristic D-xylosazone (44 mg.) separated. This had *M* (determined spectrophotometrically) 329·4 (Required, 328) (this method will be described in a subsequent paper).

Hydrolysis of the Oxyxylan–Isoniazid Polymer.—The polymer (0·6 g.) was warmed on the water-bath (1½ hr.) with 0·5N-sulphuric acid (24 c.c.). After neutralisation and the usual treatment, D-xylosazone was obtained, identity being confirmed by paper chromatography (Rutter, *Nature*, 1948, **161**, 435; *Analyst*, 1950, **75**, 37) with benzene–alcohol (9 : 1).

Derivatives of Nigeran.—Nigeran (0·178 g.) was kept with a solution of sodium metaperiodate (0·282 g. in 35 c.c.) for 72 hr. Lead acetate (in slight excess) was added and the precipitate removed by filtration. Rotational measurements before and after addition of the lead acetate showed that more than half of the material had been lost. The precipitate was washed with water, and the combined washings and filtrate were treated with sufficient sulphuric acid (10%) to remove excess of lead. The filtrate, in two parts, was treated as above for the preparation of the isoniazid and thiosemicarbazide derivatives. The precipitates were formed slowly and after 24 hr. were separated on the centrifuge, washed, and dried (about 15 mg. in each case).

Derivatives of Floridean Starch.—Floridean starch (2 g.) was treated with sodium metaperiodate solution (144 c.c.; 0·0585M). After 48 hr., ethylene glycol (5 c.c.) was added and the

solution dialysed for 12 days against running water. Flocculent material was dissolved by warming and the solution filtered through Celite. Two 50-c.c. portions of the solution ($[\alpha]_D^{20}$ +15·7°; *c* 0·838 in H_2O) were treated with isoniazid (0·828 g. in water, 50 c.c.) and thiosemicarbazide (0·56 g. in water, 50 c.c.), severally. Precipitates were formed slowly and after 24 hr. were separated on the centrifuge, washed, and dried as usual. The yield of isoniazid product was 0·716 g. (100%) and of the thiosemicarbazide product 0·571 g. (97%).

Periodate Consumption of Floridean Starch.—(a) *With sodium metaperiodate.* The starch (0·693 g.) dissolved in water was mixed with the periodate solution (20 c.c.; 0·2386M) and diluted to 150 c.c. The periodate consumption (mol. per anhydroglucose unit) was: 0·71 (2·5 hr.), 0·90 (22·0 hr.), 0·92 (26·5 hr.), 0·93 (29·5 hr.), 0·97 (50·5 hr.), 0·97 (69·7 hr.), 0·97 (143·0 hr.).

(b) *With potassium metaperiodate.* The starch (0·7 g.) was dissolved in water containing potassium chloride (7·0 g.), sodium metaperiodate (20 c.c.; 0·244M) was added, and the solution was shaken for 8 days. Estimation of the periodate present showed that 1 mol. of anhydroglucose had consumed 0·96 mol. of periodate.

Hydrolysis of Floridean Oxystarch.—The solution (108 c.c.) resulting from the previous estimation was filtered to remove potassium periodate. It was then treated with lead acetate solution and, after removal of the precipitate, excess of lead was precipitated with sulphuric acid (10%). The filtrate was concentrated to 7 c.c. under reduced pressure, mixed with an equal volume of N-sulphuric acid and warmed on the water-bath for 8 hr. After neutralisation with barium carbonate, the filtrate was concentrated under reduced pressure. A drop of this concentrated solution was run on a paper chromatogram with *n*-butanol–pyridine–benzene–water (5 : 3 : 3 : 1), and the paper developed with aniline phthalate. There was no evidence of the presence of glucose.

The remaining portion of the hydrolysate on treatment at room temperature as described above with phenylhydrazine and acetic acid gave a crystalline precipitate which proved to be mainly glyoxalosazone. After removal of the latter, the filtrate was heated on the water-bath (1 hr.). No glucosazone separated nor was any evidence of it obtained on a paper chromatogram (Rutter, *loc. cit.*).

Laminarin–Isoniazid Derivative.—An aqueous solution of laminarin was treated with excess of sodium metaperiodate solution and kept for 72 hr. The polysaccharide which had separated from solution was removed by filtration, washed with water, and dried. It was then redissolved in hot water, and an excess of an aqueous solution of isoniazid added. After a week, the off-white granular deposit was separated, washed with water, ethanol, and ether, and dried.

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