Properties of Periodate-oxidised Polysaccharides. Part IV. The Products obtained on Reaction with Phenylhydrazine.*

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Periodate-oxidised polysaccharides condense with phenylhydrazine to give amorphous products. When heated with phenylhydrazine in the presence of acetic acid, the oxypolysaccharides and their phenylhydrazine derivatives yield mixtures of osazones the separation of which has been achieved by chromatography on alumina. Thus potato oxystarch gave glyoxal bisphenylhydrazone and D-erythrosazone, and oxyxylan (*Rhodymenia palmata*) gave, in addition to the glyoxal derivative, glycerosazone and D-xylosazone. This confirms the existence of 1 : 3-linkages in this xylan. Oxidised Floridean starch gave, on the other hand, only the osazones of glyoxal and D-erythrose, and no glucosazone was detected.

DEGRADATION of oxystarch with dilute mineral acid leads to extensive changes in the molecule and has not proved of value as a method of preparation of erythrose. When the degradation is carried out with phenylhydrazine in acetic acid, much information regarding the fine structure of polysaccharides has been obtained (Barry, *Nature*, 1943, **152**, 537). The method has been applied with success to snail galactogen (O'Colla, *Proc. Roy. Irish Acad.*, 1953, **55**, *B*, 165), gum arabic (Dillon, O'Ceallacháin, and O'Colla, *ibid.*, p. 331), and nigeran (Barker, Bourne, and Stacey, *J.*, 1953, 3084). At room temperatures, phenylhydrazine forms amorphous condensation products with oxypolysaccharides (Jackson and Hudson, *J. Amer. Chem. Soc.*, 1937, **59**, 2049) and Jayme and Sätre (*Ber.*, 1944, **77**, 242, 248) used the nitrogen content of the phenylhydrazine polymer obtained from periodate-oxidised wheat straw xylan to calculate the degree of oxidation of the xylan. They formulated the oxyxylan–phenylhydrazine derivative as containing two phenylhydrazine molecules condensed on each dialdehyde group. With periodate-oxidised trehalose and raffinose, condensation is complete when one phenylhydrazine molecule has condensed for each dialdehyde group (Okui and Sujuki, *J. Pharm. Soc. Japan*, 1952, **72**, 891) and this agrees with the results obtained when isoniazid or thiosemicarbazide condenses with various oxypolysaccharides (Barry, McCormick, and Mitchell, *J.*, 1954, 3692). The phenylhydrazine–oxypolysaccharide products described in this paper appear to have a somewhat less precise composition.

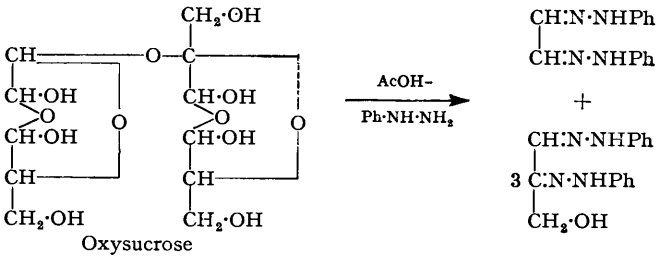
In the phenylhydrazine–acetic acid degradation experiments of oxypolysaccharides hitherto carried out, the only fragment of the molecule isolated as a crystalline derivative has been glyoxal as the bisphenylhydrazone. In general the triose and tetrose fragments seem to have been very elusive. For example, Overend, Stacey, and Wiggins (*J.*, 1949, 1358), following Jackson and Hudson (*loc. cit.*), obtained D-erythronolactone in a yield of only 7%, by hydrolysis of oxystarch with mineral acid followed by oxidation with bromine water. On the other hand, Smith and his co-workers (*J. Amer. Chem. Soc.*, 1952, **74**, 4970) have shown that reduction of oxystarch with Raney nickel or sodium borohydride followed by acid hydrolysis leads to a quantitative yield of glycollaldehyde and erythritol and suggested this proceedure as another means of investigating polysaccharide structures.

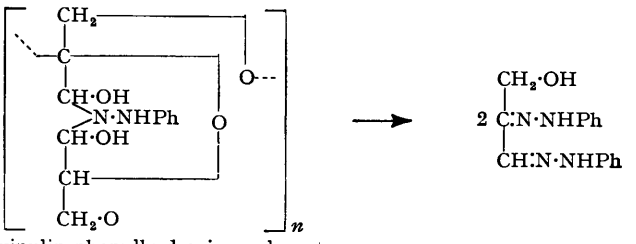
It appeared, therefore, that a successful degradation of the oxypolysaccharides would only be achieved when the aldehyde groups were protected or modified. It was of interest

* Part III, *J.*, 1954, 3692.

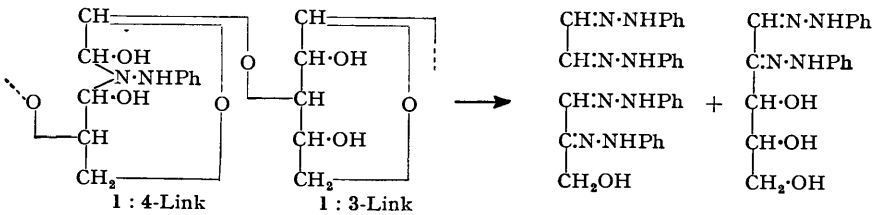
for this reason to examine the Barry degradation in more detail and to determine whether the triose and the tetrose osazones could be isolated. For this purpose it was necessary to devise a method of separating osazones of low molecular weight. This was readily achieved by chromatography on alumina using benzene and benzene–ether or ether–alcohol mixtures [chromatographic separation of osazones has also been achieved by Jorgensen (*Dansk Tidsskr. Farm.*, 1950, **24**, 1), using calcium carbonate]. The products obtained by degradation of oxystarch with phenylhydrazine in acetic acid thus yielded, first, glyoxal bisphenylhydrazone and in considerable quantity; then followed D-erythrosazone which was obtained analytically pure after one recrystallisation; and then *N*-acetylphenylhydrazine, which is readily formed when phenylhydrazine is warmed with dilute acetic acid (see, *e.g.*, Andeslini, *Ber.*, 1891, **24**, 1925).

The method was next applied to the phenylhydrazine derivatives prepared from oxysucrose, oxyinulin, and oxyxylan (for formulations see *J.*, 1953, 3631) (the xylan being obtained from *Rhodymenia palmata*; Barry, McCormick, and Mitchell, *loc. cit.*). From oxysucrose were obtained glyoxal bisphenylhydrazone and glycerosazone, while the inulin gave mainly glycerosazone together with a very small quantity of glyoxal bisphenylhydrazone. The latter could have arisen from a glucose residue at the non-reducing end of the chain.





The xylan, on the other hand, gave glyoxal bisphenylhydrazone, glycerosazone, and some xylosazone. The last-named osazone arises from 1 : 3-linked xylose units and suggests that some at least of the 1 : 3-linkages alternate with 1 : 4-linkages (cf. Part III, *loc. cit.*).



It was also found possible to separate the degradation products, rapidly and sharply, by using Rutter's circular paper chromatogram (*Nature*, 1948, **161**, 435; see also Giri, *ibid.*, 1954, **173**, 1194) and benzene(or toluene)–ethanol (9 : 1). This provides a ready method of qualitative analysis for osazone mixtures which when applied to the degradation products of the Floridean oxystarch–phenylhydrazine polymer indicated only the presence of glyoxal

bisphenylhydrazone and D-erythrosazone. No glucosazone could be detected and the conclusions reported in Part III (*loc. cit.*) regarding the structure of this starch are thus lent further support. Complete separation of the osazones could not be achieved by the usual paper chromatographic techniques, as with a variety of solvents " streaking " was always obtained.

Experimental

Rotational measurements were made in 2-dm. tubes; Brockmann standardised Merck alumina was used throughout for chromatography of the osazones.

Phenylhydrazine Derivative of Oxysucrose.—Sucrose (10·26 g.) was oxidised with sodium metaperiodate (19·5 g.) in water (300 c.c.). After being kept in the dark for 26 hr. the solution (α_D + 1·30°) was treated with lead acetate to remove iodate and periodate, and then with dilute sulphuric acid to precipitate excess of lead. The solution of oxysucrose obtained (465 c.c.) had α_D + 0·784°. 250 C.c. were treated at room temperature with phenylhydrazine (15 c.c.) in 10% acetic acid (35 c.c.). The sticky yellow solid obtained was washed well with water and dried to a yellow powder (8·52 g.), soluble in ether and alcohol, sparingly soluble in benzene, and insoluble in light petroleum. It decomposed on storage for a few days. A solution of 0·1 g. in ethanol (10 c.c.) had α_D + 0·494° ⟶ + 0·307° (5½ hr.) ⟶ + 0·29° (120 hr.).

Degradation of Oxysucrose–Phenylhydrazine Derivative.—The yellow powder (3 g.) was refluxed for 1½ hr. with phenylhydrazine (5 c.c.), 10% acetic acid (20 c.c.), and ethanol (50 c.c.). Removal of ethanol on the pump and addition of water precipitated an orange gum which was dissolved in ether and washed successively with dilute acetic acid, water, aqueous sodium hydrogen carbonate, and water. From the ether layer, a red oil (2·87 g.) was obtained which was dissolved in benzene and adsorbed on a column of alumina (50 g.). Fractions 1—3 (0·674 g.), eluted by benzene (170 c.c.), consisted of glyoxal bisphenylhydrazone which crystallised from benzene–light petroleum in pale yellow blades. Fractions 5—8 [0·223 g., eluted by benzene (310 c.c.); 0·288 g., eluted by 1 : 1 benzene–ether] were glycerosazone, which crystallised from benzene in yellow spears, m. p. 130—131° (Found: C, 67·4; H, 6·1; N, 20·7. Calc. for $C_{15}H_{14}ON_4$: C, 67·2; H, 6·0; N, 20·9%).

Phenylhydrazine Derivative of Oxystarch.—A solution (2%) of periodate-oxidised potato starch in water was treated at room temperature with an excess of phenylhydrazine in dilute acetic acid. The yellow precipitate which separated immediately was washed well with dilute acetic acid and water (Found: N, 12·55. Calc. for 1 phenylhydrazine per oxyhexose unit: N, 10·4. Calc. for 2 phenylhydrazines per oxyhexose unit: N, 16·4%).

Degradation of Oxystarch.—(a) *With phenylhydrazine only.* Oxystarch (2 g.) in water (40 c.c.) was refluxed with ethanol (130 c.c.) and phenylhydrazine (5 c.c.). The milky solution became clear and after 1½ hr. ethanol was removed at the pump. Addition of water gave a gum which together with the solution was extracted with ether. The ether extract yielded an oil (0·33 g.) which after adsorption on alumina and elution with benzene gave glyoxal bisphenylhydrazone (0·16 g.).

The solid (2·48 g.) remaining in the aqueous layer after extraction with ether was insoluble in benzene but soluble in ethanol, from which it was precipitated as an amorphous powder on addition of water. This was evidently an oxystarch–phenylhydrazine polymer as prepared above.

(b) *With phenylhydrazine and acetic acid.* Oxystarch (2·3 g.) in water (100 c.c.) was refluxed with ethanol (200 c.c.), phenylhydrazine (25 c.c.), and glacial acetic acid (30 c.c.) for 2½ hr. Removal of ethanol and addition of water gave a yellow solid (3·95 g.). Of this, 2 g. were dissolved in benzene (130 c.c.) and adsorbed on alumina (100 g.). Fractions 3—5 (1·21 g.), eluted with benzene (300 c.c.) and crystallised from benzene light petroleum, were glyoxal bisphenylhydrazone. Fractions 11—14 (0·062 g.), eluted with 1 : 1 benzene–ether (235 c.c.), were *N*-acetylphenylhydrazine, forming colourless plates from the above solvents. Fractions 18—24 (0·422 g.), eluted with ether (560 c.c.), gave feathery needles (from benzene), m. p. 175—177°, of D-erythrosazone (Found: C, 64·3; H, 6·5; N, 18·5. Calc. for $C_{16}H_{18}O_2N_4$: C, 64·7; H, 6·1; N, 18·85%).

Phenylhydrazine Derivative of Oxyinulin.—Inulin (10 g.) was oxidised for 48 hr. with sodium metaperiodate (16 g.) in water (400 c.c.). The solution which contained some undissolved material was filtered through Celite. The filtrate (420 c.c.; α_D − 3·0°) was then treated with lead acetate and dilute sulphuric acid as before and gave an iodate- and periodate-free solution (570 c.c.; α_D −1·95°) which was treated at room temperature with phenylhydrazine (15 c.c.)

in 10% acetic acid (35 c.c.). The pale yellow solid obtained weighed 10 g. (Found: N, 9·9. Calc. for oxyinulin with one phenylhydrazine molecule condensed per sugar unit: N, 10·4%). The filtrate had α_D − 0·04° and evidently the reaction was practically quantitative.

Degradation of Oxyinulin Phenylhydrazine Derivative.—The yellow powder (0·98 g.) was refluxed with ethanol (30 c.c.), phenylhydrazine (3 c.c.), glacial acetic acid (5 c.c.), and water (10 c.c.) for 4 hr. The solution, treated as previously, yielded a pale brown solid (1·91 g.) which was dissolved in benzene and adsorbed on alumina (60 g.). Fractions 1—3 (0·646 g.), eluted with benzene (240 c.c.), were a red oil, from which crystallisation from benzene–light petroleum yielded a few mg. of glyoxal osazone. Fractions 6—8 (0·339 g.), eluted by 1 : 1 benzene–ether, were glycerosazone, forming deep yellow spears, m. p. 130°, from benzene. Fractions 9—11 (0·339 g.), isolated by ether (320 c.c.), were *N*-acetylphenylhydrazine, colourless plates (from benzene–light petroleum), m. p. 127—129°.

Oxyxylan–Phenylhydrazine Derivative.—Xylan (3 g.), isolated from *Rhodymenia palmata* (Barry, McCormick, and Mitchell, *loc. cit.*), was oxidised with sodium metaperiodate (5·85 g.) in water (200 c.c.). The reaction (followed polarimetrically) was complete after 120 hr. when the solution had α_D + 2·12°. Periodate and iodate were removed as before and the oxyxylan solution obtained (360 c.c.; α_D + 1·25°) treated at room temperature with phenylhydrazine (7·5 c.c.) in 10% acetic acid (18 c.c.). The orange precipitate obtained weighed 2·53 g. (Found: N, 11·1. Calc. for each periodate-vulnerable xylose condensed with 1 phenylhydrazine molecule: N, 15·7%). The solution remaining was optically inactive.

Degradation of the Oxyxylan Derivative.—The orange powder (2·36 g.) was refluxed for 4 hr. with ethanol (60 c.c.), phenylhydrazine (7 c.c.), glacial acetic acid (9 c.c.), and water (30 c.c.). Concentration of the solution yielded crystalline glyoxal bisphenylhydrazone (1·46 g.). The filtrate on dilution with water gave a gum (2·60 g.) which was dissolved in benzene and chromatographed on alumina (80 g.), giving fractions: 1—3 (0·571 g.), eluted with benzene (130 c.c.), glyoxal bisphenylhydrazone (crystallised from benzene); 10—11, eluted with 1 : 1 benzene–ether (80 c.c.), glycerosazone (0·283 g.), forming deep yellow crystals, m. p. 130°, from benzene; 13—15 (0·462 g.), eluted by 1 : 1 benzene–ether (200 c.c.), *N*-acetylphenylhydrazine, m. p. 128° (from benzene–light petroleum); 16 (0·242 g; not crystalline), eluted by ether (150 c.c.); and 22—26 (0·346 g.), eluted by 9 : 1 ethanol–water, forming yellow needles (from methanol–water), identified as xylosazone on a circular paper chromatogram.

The solution (100 c.c.) from which the degradation products were separated had α_D − 0·416°, but no further products were isolated from it. The presence of a 1 : 3-linked oligosaccharide is therefore not ruled out.

Separation of Osazones by Circular Paper Chromatograms.—Spots of a mixture of the osazones of maltose, galactose, xylose, erythrose, glycerose, and glyoxal were made on a circle, 4 cm. in diameter, in the centre of a piece of Whatman No. 1 filter paper (30 cm. in diameter). This was clamped between two glass plates and eluted with a mixture of toluene, ethanol, and water (270 : 30 : 1) by means of a wick inserted into the centre of the paper. Separate spots of the osazones of arabinose, glucose, and rhamnose were run concurrently. After 2 hr. the osazones had separated into well-defined arcs which were easily visible. They may be darkened by spraying with ammoniacal silver nitrate. The R_F values were found to vary somewhat along different axes. The average values of a number of runs in different directions were as follows: glyoxal 0·96, glycerose 0·75, erythrose 0·55, rhamnose 0·54, arabinose 0·48, xylose 0·47, galactose 0·42, glucose 0·41, and maltose 0·046.

This method of identification was very useful as a rapid analysis of the products of the degradation of the oxypolysaccharides. It was necessary to keep the time of heating with phenylhydrazine to a minimum (15—30 min.) to avoid the formation of much *N*-acetylphenylhydrazine since this appeared as a spot at about the same point as erythrosazone and obscured this osazone especially when the paper was developed with ammoniacal silver nitrate. When this method was applied to the Floridean oxystarch–phenylhydrazine derivative prepared in the usual way, no evidence of glucosazone was obtained, whilst spots due to the osazones of D-erythrose and glyoxal were visible. Similarly the xylan derivative gave clear evidence of xylosazone, glycerosazone, and glyoxalosazone.

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