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The Enzymic Degradation of Porphyran

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1. The algal galactan, porphyran, was incubated with enzymes from a *Cytophaga* sp. and the products were examined. 2. Only about 30% of the porphyran was recovered in the form of oligosaccharides, the remainder being of high molecular weight. 3. Among the saccharides were D-galactose, 6-*O*-methyl-D-galactose, neoagarobiose, neoagarotetraose and oligosaccharides containing 6-*O*-methyl-D-galactose, the principal of which has been tentatively identified as 6^3-*O*-methyl-neoagarotetraose. Fragments containing sulphate were also isolated but not identified. 4. Within the alternating sequence of the D- and L-forms of derivatives of galactose in porphyran, 6-*O*-methyl-D-galactose replaces D-galactose in a random manner.

In the preceding paper (Turvey & Christison, 1967) an enzyme system from a marine *Cytophaga* was described that caused depolymerization of certain algal galactans. This enzyme system was most active on agarose, a polysaccharide component of agar, but it also had considerable action on porphyran, the galactan sulphate of the red alga *Porphyra umbilicalis*. Agarose is known to have a linear structure in which 1,3-linked β-D-galactopyranosyl residues alternate with 1,4-linked 3,6-anhydro-α-L-galactopyranosyl residues (Araki, 1956). Porphyran has a similar alternating structure, but the alternation is between either D-galactose or 6-*O*-methyl-D-galactose on the one hand and either 3,6-anhydro-L-galactose or L-galactose 6-sulphate on the other (Turvey & Williams, 1964; Anderson & Rees, 1965). Thus parts of the porphyran molecule could be identical in structure with agarose, but other parts will be different from it. In an attempt to gain information on the relative distribution of the various sugar residues in porphyran, the products of action of the *Cytophaga* enzyme on porphyran have been studied.

MATERIALS AND METHODS

The enzyme system, the substrate porphyran and many of the analytical methods are described in the preceding paper (Turvey & Christison, 1967). A mixture of neoagarodextrins, prepared by agarase action on agarose, was kindly supplied by Dr W. Yaphe.

Chromatography. For paper chromatography, Whatman no. 3MM was used with the following solvents: *A*, butan-1-ol–ethanol–water (3:1:1, by vol.); *B*, butan-1-ol–pyridine–water (2:1:1, by vol.); *C*, as solvent *A* but containing in addition 3% (w/v) of cetylpyridinium chloride; *D*, butan-1-ol–ethanol–water (2:1:1 by vol.); *E*, as solvent *D* but in the proportions 1:1:1 (by vol.). Spray reagents used were $AgNO_3$–NaOH (Trevelyan, Procter & Harrison, 1950) and *p*-anisidine hydrochloride (Pridham, 1956). Thin-layer chromatography on cellulose (Whatman CC41) used the solvents named above. A modified Seliwanoff spray (Yaphe, 1957) was used for the detection of 3,6-anhydrogalactose and oligosaccharides containing it. Whatman no. 54 filter paper was used for electrophoresis at 40 v/cm. in a buffer prepared by adding pyridine to 0·1 N-acetic acid to pH 6. Electrophoretic mobilities (M_g) are compared with that of D-glucose 6-sulphate, taken as unity.

Analytical. The 3,6-anhydrogalactose content of an oligosaccharide was determined by the method of Yaphe (1960), with 3,6-anhydro-D-galactose diethyl dithioacetal as standard. The total sugar content of a fraction was determined by a modification of the colorimetric method of Dubois, Gilles, Hamilton, Rebers & Smith (1956) with the phenol–H_2SO_4 reagent. The modification was necessary because the molar extinction of 3,6-anhydrogalactose with this reagent is much greater than that of either galactose or 6-*O*-methylgalactose. A preliminary determination of the anhydrosugar content enabled an adjustment to be made to the total extinction for the contribution due to the anhydrosugar.

A modification of the method of Peat, Whelan & Roberts (1956) was used to determine the degree of polymerization of a reducing oligosaccharide. In this method the anthrone–H_2SO_4 reagent is used to determine total sugar, but 3,6-anhydrogalactose produces a more intense colour with the reagent than does galactose. The 3,6-anhydrogalactose content of the oligosaccharide was therefore determined before and after reduction in aqueous solution with $NaBH_4$ to calculate what proportion of this unit constituted terminal reducing groups. The appropriate allowances were then made to the extinctions produced with the anthrone reagent to calculate the degree of polymerization.

A porphyran fragment was completely hydrolysed by being heated in 1·5 N-H_2SO_4 at 100° for 3 hr. Since this treatment caused degradation of 3,6-anhydrogalactose to

5-hydroxymethylfuran-2-aldehyde, a milder method was used where scission of 3,6-anhydrogalactosidic linkages only was required, with minimum destruction of the anhydrosugar. This involved heating the sugar at 100° for 3hr. in 0·02N-oxalic acid, followed by cooling and neutralizing with $CaCO_3$.

Enzymic hydrolysis of porphyran. Porphyran (10g.) in 0·033M-phosphate buffer, pH 7·0 (1l.), was incubated with freeze-dried enzyme (1·05g.) at 25° under a layer of toluene. After 72hr., ethanol (2·5vol.) was added to the digest and the precipitated material was recovered on the centrifuge. This precipitate was redissolved in the buffer (500ml.) and incubated with a further portion (0·56g.) of enzyme at 25°. After 66hr., high-molecular-weight material was recovered by precipitation with ethanol (2vol.) and was washed with ethanol. The combined supernatant solutions and washings from the digests were concentrated to dryness at 40°. This product (12·9g.) was desalted and partly fractionated on a column (60cm.×5cm.) of charcoal (Ultrasorb SC, 120–240)–Celite as described by Hughes & Whelan (1958). The eluates from the column were tested for carbohydrate with the phenol–H_2SO_4 reagent (Dubois *et al.* 1956). The column was eluted with (*a*) water, until phosphate ions were no longer detected in the eluate (fraction 1), (*b*) aq. 5% (v/v) ethanol until no further carbohydrate material was eluted (fraction 2), (*c*) an ethanol gradient (Lindberg & Wickberg, 1954) produced by adding aq. 30% (v/v) ethanol (8l.) to aq. 5% (v/v) ethanol (8l.) (fractions 3–6), and (*d*) aq. 50% (v/v) ethanol (2l.) (fraction 7). Fractions eluted from the column were separately evaporated to dryness at 40° and pH 6. Separation of mixtures of components was effected by preparative paper chromatography and electrophoresis. For the separation of neutral from acidic (sulphated) saccharides in a fraction, the fraction in aqueous solution was percolated through a column of the resin De-Acidite FF (acetate form) and the neutral saccharides were eluted with water. Acidic saccharides were then desorbed by elution of the column with 1·5M-Na_2SO_4 until no further carbohydrate was detected in the eluate. The eluate was concentrated and then desalted on a column of charcoal–Celite (Hughes & Whelan, 1958) to yield the acidic saccharides.

RESULTS

The extent of hydrolysis of the porphyran was 6·8% (as galactose) after 70hr. with the first portion of enzyme, and increased to 7·2 and 7·36% after 22hr. and 66hr. respectively with the second portion of enzyme. After this time, 70% of the original polysaccharide was recovered as high-molecular-weight material by ethanol precipitation and 30% as relatively low-molecular-weight oligosaccharide fragments mixed with salts. Fractionation of these oligosaccharides by charcoal chromatography did not give pure components but effected some separation.

Examination of fractions. Fraction 1 (8g.). This consisted mainly of salts but paper chromatography (solvent *A*) indicated the presence of traces of sugars with almost zero mobility. In solvent *C*, these sugars moved perceptibly, indicating that they were sulphated, but still had a mobility only 0·01 of that of L-galactose 6-sulphate. On paper electrophoresis the sugars migrated as an elongated zone.

Fraction 2 (50mg.). This contained two major components, which were separated by preparative paper chromatography in solvent *A*. One component was identified as D-galactose by isolation in crystalline form (10mg.), m.p. 149–151°; $[\alpha]_D^{18}$ +80·03° (equilibrated in water). The derived methylphenylhydrazone had m.p. 175–176°, and the mixed m.p. with authentic D-galactose methylphenylhydrazone (m.p. 177–179°) was 177–178°.

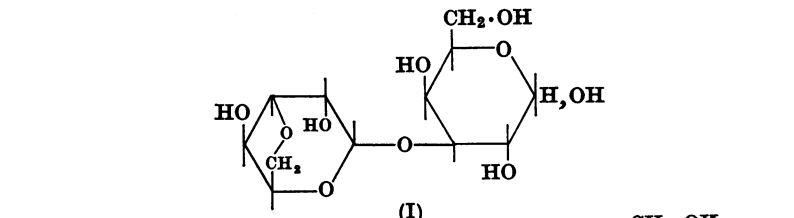
The second component of fraction 2 had R_{Gal} 0·07, but migrated on electrophoresis as a disaccharide monosulphate with M_S 0·77. On complete acid hydrolysis, it gave galactose and 6-*O*-methylgalactose (identified chromatographically).

Fraction 3. This consisted of one main component, which was separated from traces of others by paper chromatography (solvent *A*) to yield a syrup (12mg.). Paper and thin-layer chromatography in solvents *A*–*E* indicated that this was 6-*O*-methylgalactose.

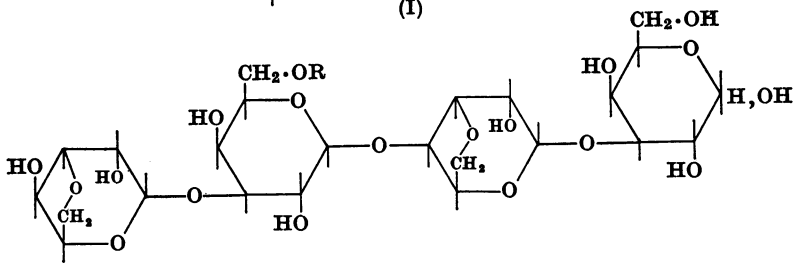
Fraction 4. The main constituent of this fraction was separated from impurities by preparative paper chromatography in solvent *B* (yield, 19mg.). In this solvent it had an R_{Gal} value (1·31) identical with that of neoagarobiose [3,6-anhydro-*O*-α-L-galactopyranosyl-(1→3)-D-galactose (I)]. Mild hydrolysis with oxalic acid gave D-galactose, 3,6-anhydro-L-galactose and 5-hydroxymethylfuran-2-aldehyde. The 3,6-anhydrogalactose content was unchanged after reduction with sodium borohydride and the molar ratio of galactose to the anhydrosugar in the original sugar was 1·19:1. The degree of polymerization was calculated to be 2·4; authentic neoagarobiose gave a value of 2·3 under the same conditions.

Fraction 5 (51mg.). This was a mixture of several components, of which one was identical with the main component of fraction 4 and others were present in fraction 6.

Fraction 6 (0·70g.). On electrophoresis in neutral buffer, this fraction was resolved into a group of neutral sugars and another group of sulphated sugars. Separation of these two groups was achieved on an ion-exchange resin. The neutral sugars consisted of seven components, of which only three (components 6*a*, 6*b*, 6*c*) were isolated by preparative paper chromatography in sufficient purity for further study. Component 6*a* (2mg.) migrated as a compact zone on paper chromatography with R_{Gal} 2·01 (solvent *A*) and 1·71 (solvent *B*), values that are greater than those for 6-*O*-methylgalactose and akin to those of 3,6-anhydrogalactose, which, however, migrates as a diffuse zone). Component 6*a* contained 6-*O*-methylgalactose residues and gave a positive test for



(I)



(II) R=H
(III) R=CH_3

3,6-anhydrogalactose with the spray reagent of Yaphe (1957). Component 6*b* had the same R_F value as neoagarobiose in three solvent systems and was not examined further. Component 6*c* (65mg.) was the major neutral constituent of fraction 6. It migrated on thin-layer chromatograms in solvent *B* at the same rate as the tetrasaccharide neoagarotetraose [4^2-β-neoagarobiosyl-neoagarobiose (II)]. When completely hydrolysed, only galactose and 5-hydroxymethylfuran-2-aldehyde were detected in the hydrolysate. The molar ratio of galactose to 3,6-anhydrogalactose was 1·0:1 and the 3,6-anhydrogalactose content was not changed by reduction with sodium borohydride. Allowing for this fact, the degree of polymerization was calculated to be 4·1.

Fraction 7. This fraction (1·66g.) was again complex, with both sulphated and neutral sugars present. Separation into two groups by ion-exchange chromatography was followed by preparative paper chromatography (solvent *A*) of the neutral sugars to give pure components 7*a*, 7*b* and 7*c*, together with a mixture of higher oligosaccharides 7*d*. Component 7*a* (about 1mg.) had an R_F value on thin-layer chromatograms identical with that of component 6*a* and was not examined further. Component 7*b* was identical with component 6*b* and was indistinguishable from neoagarobiose by chromatography. The major component, 7*c* (40mg.), had R_{Gal} 1·05 on thin-layer chromatography in solvent *B*. Complete hydrolysis gave galactose and 6-*O*-methylgalactose in comparable amounts (estimated visually on chromatograms), together with 5-hydroxymethylfuran-2-aldehyde. The 3,6-anhydrogalactose content was unchanged by reduction with sodium borohydride. Thin-layer chromatography of a hydrolysate of the reduced fraction revealed only 6-*O*-methyl-D-galactose and the furan compound (when sprayed with *p*-anisidine reagent), suggesting that D-galactose was the reducing end unit of this saccharide. Mild hydrolysis of the original component 7*c* with oxalic acid liberated D-galactose, and faster-migrating components that streaked on chromatograms but all of which reacted rapidly with the modified Seliwanoff reagent. No free 6-*O*-methyl-D-galactose was detected. The ratio of galactose plus 6-*O*-methyl-galactose to 3,6-anhydrogalactose in the original sugar was 1·15:1, and the calculated degree of polymerization was 4·2, consistent with this sugar being a tetrasaccharide.

The mixture of oligosaccharides, component 7*d*, was not resolved further, but paper chromatography in solvent *A* revealed a series of three components with decreasing R_F values. A plot of R_M value (Bate-Smith & Westall, 1950) against order of decreasing R_F value was a straight line, with components 7*b* and 7*c* also on the line (Fig. 1).

The sulphated components from fractions 6 and 7 were combined and briefly examined. Paper chromatography in solvent *C* revealed no components with the mobility of monosaccharide sulphates, but components with the chromatographic mobilities of disaccharide sulphates were present in trace amounts. Paper electrophoresis confirmed that only traces of material migrated at the rate expected for disaccharide monosulphates (M_S 0·77), the bulk of the fractions being much less mobile. Preparative thin-layer chromatography, with triple development in solvent *E*, separated

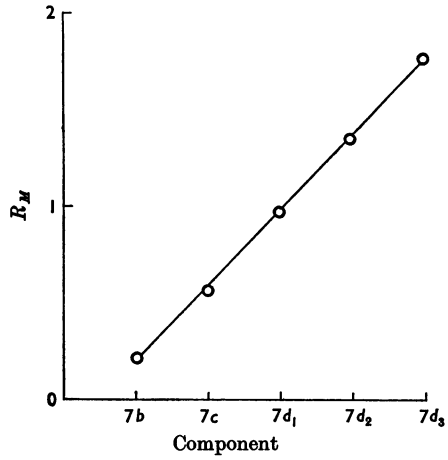


Fig. 1. Bate-Smith & Westall (1950) relationship for oligosaccharide components (fraction 7) of enzymic hydrolysate of porphyran: 7*b*, neoagarobiose; 7*c*, 6³-*O*-methylneoagarotetraose; $7d_1$–$7d_3$, higher oligosaccharides. Solvent, butan-1-ol–ethanol–water (3:1:1, by vol.).

one major component in an impure form. This had M_S 0·65 on electrophoresis and R_F 0·057 in solvent *C*, consistent with a sulphated tetra- or penta-saccharide.

DISCUSSION

That action of this enzyme system on porphyran is limited is apparent from the recovery of about 70% of the molecule as high-molecular-weight material after prolonged enzyme action. If, as has been suggested on the basis of earlier studies (Turvey & Christison, 1967), the enzyme system is specific for the agarose type of structure, i.e. for alternate 1,4-linked 3,6-anhydro-α-L-galactopyranose and 1,3-linked β-D-galactopyranose units, this limited action implies that the distribution of such an alternately linked structure in porphyran is relatively small. If regions of the molecule contained the D-galactose in its 6-*O*-methylated form or the L-galactose in its 6-sulphated form (or both), enzyme action would be inhibited at such regions.

A complete separation of all the products of the action of the *Cytophaga* enzyme has not yet been achieved. Nevertheless, certain conclusions may be drawn. The isolation of the monosaccharide D-galactose in low yield suggests that the enzyme mixture contains an oligosaccharase capable of carrying further the hydrolysis of the oligosaccharides that are the main products. 6-*O*-Methyl-D-galactose was also isolated, indicating that oligosaccharase action is not confined to those oligosaccharides containing unsubstituted D-galactose. The main oligosaccharide components of the hydrolysate were tentatively identified as neoagarodextrins. The main component of fraction 4 and a minor component of fractions 5, 6 and 7 is probably neoagarobiose, identified by its chromatographic mobility, and by other properties consistent with its structure being that of a 3,6-anhydro-L-galactopyranosyl-D-galactose. In relation to the known structural features of porphyran, namely the location and configuration of the linkages, this disaccharide is almost certainly neoagarobiose (I). Similarly, a major component of fraction 6 (component 6*c*) was provisionally identified as neoagarotetraose (II). Higher dextrins in fraction 6 were undoubtedly of this series.

Araki & Arai (1956, 1957) have isolated and characterized neoagarobiose and neoagarotetraose as products of the action of a bacterial enzyme on agar. The isolation of these oligosaccharides from enzymic hydrolysates of porphyran establishes that the molecule contains sequences of units identical with those in agarose, although these sequences must be relatively few in number.

The distribution of products that contain 6-*O*-methyl-D-galactose is of importance in this study. Apart from the free sugar, isolated in small amount, a component with the chromatographic mobility expected of a disaccharide constituted of 6-*O*-methyl-D-galactose and 3,6-anhydro-L-galactose was isolated in trace quantities only (components 6*a* and 7*a*). The major sugar product containing 6-*O*-methylgalactose as a constituent unit was component 7*c*. This was a tetrasaccharide constituted of D-galactose, 6-*O*-methyl-D-galactose and 3,6-anhydro-L-galactose in the proportions 1:1:2. The reducing end unit of the tetrasaccharide was galactose. A structure consistent with this evidence is 3,6-anhydro-*O*-α-L-galactopyranosyl-(1→3)-6-*O*-methyl-*O*-β-D-galactopyranosyl-(1→4)-3,6-anhydro-*O*-α-L-galactopyranosyl-(1→3)-D-galactose (6³-*O*-methylneoagarotetraose; III). Selective hydrolysis (with oxalic acid) of the 3,6-anhydrogalactosidic linkages in structure (III) should give rise to 3,6-anhydro-L-galactose, 6²-*O*-methylagarobiose and D-galactose. Galactose and components containing the 3,6-anhydrosugar were detected in such a hydrolysate of component 7*c*. Higher oligosaccharides in fraction 7 also incorporated 6-*O*-methyl-D-galactose residues, and probably constituted a polymer-homologous series, as indicated by the straight line obtained when the Bate-Smith & Westall (1950) relationship was plotted (Fig. 1).

The structure of the sulphated fragments is still in some doubt. Traces of a component with the chromatographic and electrophoretic mobilities of a disaccharide monosulphate were isolated, although the major fragments appeared to be

sulphated tetra- or higher saccharides. An important point, however, is that many of these fragments were of relatively short chain length, remained in the supernatant solution after ethanol precipitation and had very small, but finite, mobilities on a paper chromatograph. These sulphated fragments were probably flanked by regions in which the L-isomer of galactose occurred as the anhydrosugar, thus being susceptible to enzyme action.

The nature of the fragments isolated leads to the following suggestions on the structure of porphyran. Portions of the molecule contain structures identical with agarose, whereas in other portions 6-*O*-methyl-D-galactose appears as replacing some of the D-galactose. The isolation of the tetrasaccharide (III) suggests that some of the 6-*O*-methylgalactose may occur as a single unit flanked by the anhydrosugar and D-galactose. This follows from the fact that only traces of oligosaccharides, in which 6-*O*-methyl-D-galactose is probably a reducing end group, were isolated, which suggests that requirements of enzyme specificity largely preclude scission of the glycosidic linkage between 6-*O*-methyl-D-galactose and 3,6-anhydro-L-galactose. It is probable that there may be sequences in which 6-*O*-methyl-D-galactose units do occur in groups, each unit flanked by the anhydrosugar: structures of this type would not have been detected in this study, since they would be present only in higher saccharides. It is also evident that blocks of units occur in which the L-galactose is present as the sulphate, but which are flanked by sequences in which 3,6-anhydro-L-galactose units occur, thus giving rise to the sulphated oligosaccharides. That about 70% of the molecule was largely unattacked, however, suggests that the barriers to enzyme action (the 6-*O*-methyl ether or the sulphated unit) are distributed throughout the molecule.

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REFERENCES

Anderson, N. S. & Rees, D. A. (1965). *J. chem. Soc.* p. 5880.
Araki, C. (1956). *Bull. chem. Soc. Japan*, **29**, 543.
Araki, C. & Arai, K. (1956). *Bull. chem. Soc. Japan*, **29**, 339.
Araki, C. & Arai, K. (1957). *Bull. chem. Soc. Japan*, **30**, 287.
Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. biophys. Acta*, **4**, 427.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Hughes, R. C. & Whelan, W. J. (1958). *Chem. & Ind.* p. 884.
Lindberg, B. & Wickberg, B. (1954). *Acta chem. scand.* **8**, 569.
Peat, S., Whelan, W. J. & Roberts, J. G. (1956). *J. chem. Soc.* p. 2258.
Pridham, J. B. (1956). *Analyt. Chem.* **28**, 1967.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Turvey, J. R. & Christison, J. (1967). *Biochem. J.* **105**, 311.
Turvey, J. R. & Williams, T. P. (1964). *Proc. 4th int. Seaweed Symp., Biarritz*, p. 372. London: Pergamon Press Ltd.
Yaphe, W. (1957). *Canad. J. Microbiol.* **3**, 987.
Yaphe, W. (1960). *Analyt. Chem.* **32**, 1327.