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The Action of a Bacterial Agarase on Agarose, Porphyran and Alkali-Treated Porphyran

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1. A purified extracellular agarase from a *Cytophaga* species was used to hydrolyse agarose, porphyran and alkali-treated porphyran. 2. The hydrolysate from agarose was separated by gel filtration into the series of neoagarosaccharides, the predominant member of which was the tetrasaccharide. 3. Enzyme action on alkali-treated porphyran gave neoagarosaccharides and other oligosaccharides containing 6-*O*-methyl-D-galactose units. From the composition of these oligosaccharides it is deduced that action of the enzyme on a D-galactosidic linkage is four to five times faster than on the 6-*O*-methyl-D-galactosidic linkage. 4. Enzyme action on native porphyran gives a similar series of oligosaccharides but in smaller yield, much of the polysaccharide being either not degraded or only degraded to a series of large, highly sulphated oligosaccharides. 5. For porphyran, it is concluded that 6-*O*-methyl ether groups are distributed randomly on half the D-galactose units, but that the 6-sulphate groups on L-galactose units tend to occur in blocks.

In a previous paper (Duckworth & Turvey, 1969*a*) we reported the isolation from a *Cytophaga* species of a purified extracellular enzyme, which caused depolymerization of agarose and related polysaccharides, such as porphyran (the galactan sulphate from *Porphyra umbilicalis*) and alkali-treated porphyran. To define more clearly the action of the enzyme on these polysaccharides, and to gain more information on the structure of porphyran, the products of enzyme action on these polysaccharides have now been studied. Since the nature and configuration of the glycosidic linkages in both agarose (Araki & Arai, 1957) and porphyran (Turvey & Williams, 1964; Anderson & Rees, 1965) have been clearly defined, it has been assumed that these linkages will survive in the derived oligosaccharides, and the structures deduced rely on this assumption. A preliminary account of part of this work has been given (Duckworth & Turvey, 1968).

MATERIALS AND METHODS

The enzyme from culture filtrates of a *Cytophaga* (N.C.M.B. 1327) was purified and its activity determined viscometrically as described previously (Duckworth & Turvey, 1969*a*). The sources and preparation of the polysaccharides have also been described (Turvey & Christison, 1967*a*). Complete hydrolysis of an oligosaccharide was achieved by heating in 0·75M-H_2SO_4 at 100° for 3hr., neutralizing ($BaCO_3$) and concentrating the supernatant solution at 30°. Reduction of a reducing sugar to the corresponding alcohol was achieved by treating the sugar (1mol.prop.) in water with $NaBH_4$ (2mol.prop.) for 16hr. The solution was then carefully neutralized (HCl) to pH7, concentrated and desalted as described below.

Chromatography. T.l.c. of oligosaccharides was performed by multiple development on plates of cellulose powder (Whatman CC41; 0·5mm.) with the solvent systems: *A*, butan-1-ol–pyridine–water (2:1:1, by vol.); *B*, butan-1-ol–ethanol–water (3:1:1, by vol.); *C*, butan-1-ol–ethanol–water (2:1:1, by vol.). For monosaccharides, the method of Kringstad (1964) was employed. All R_F values are expressed relative to that of galactose taken as unity (R_{Gal}). For paper electrophoresis, Whatman no. 54 paper at 40v/cm. in 0·1M-acetic acid–pyridine buffer, pH6, was employed and all electrophoretic mobilities are expressed relative to that of D-galactose 6-sulphate taken as unity (M_S). The spray reagent generally used for detecting reducing sugars, or those containing 3,6-anhydrogalactose, on plates or paper was the modified Seliwanoff spray (Yaphe, 1957). For reducing monosaccharides the *p*-anisidine hydrochloride spray (Pridham, 1956) was used, and for non-reducing sugars and glycitols the periodate–permanganate spray reagent (Lemieux & Bauer, 1954) was used.

G.l.c. of sugar and glycitol acetates was performed on a Pye 104 dual-column gas chromatograph with flame ionization detectors. The carrier gas was N_2 at a flow rate of 50ml./min. Columns (5ft. × $\frac{1}{8}$in.) contained *X* [20% Apiezon M grease on silver-coated acid-washed silylated Chromosorb W (100–120 mesh), (Gunner, Jones & Perry, 1961)] or *Y* (5% Apiezon M plus 1% polyethylene glycol adipate on the same solid phase). Operating temperatures were 220° for column *X* and 200° for column *Y*. Column *X* separated glycitol acetates from one another but 6-*O*-methylgalactitol acetate was not separated from β-galactose penta-acetate; column *Y* separated these two acetates.

Analyses. The determinations of reducing power, of the 3,6-anhydrogalactose content of an oligosaccharide (before and after reduction with $NaBH_4$) and of total sugars (phenol–H_2SO_4 method) have been described (Turvey & Christison, 1967*b*). Sulphate in an oligosaccharide was determined by a flame-photometric method (Lloyd, Evans & Fielder, 1969). The ratio of D-galactose to 6-*O*-methyl-D-galactose in a neutral oligosaccharide was determined by quantitative g.l.c., on column *X*, of the derived glycitol acetates (Bowker & Turvey, 1968). To identify the sugar unit present at the reducing end of an oligosaccharide, the oligosaccharide was reduced to the alcohol with $NaBH_4$ and then hydrolysed. The derived mixture of sugars and alcohols was acetylated and examined by g.l.c. on columns *X* and *Y*. The proportion of each glycitol could be obtained quantitatively, where more than one sugar occurred at the reducing end. The D.P.* of a reducing oligosaccharide in the D.P. range 2–6 was determined by the anthrone method as described previously (Turvey & Christison, 1967*b*).

Desalting a sugar solution. Sephadex G-10 was swollen in water and packed in columns of length/diameter ratio at least 40:1. The sugar solution was concentrated to a small volume and was then added to the top of a column with a bed volume at least ten times that of the solution. The column was eluted with water and fractions (2ml. each) were tested for sugar (phenol–H_2SO_4) and for appropriate inorganic ions. If only PO_4^{3-} were present, it was more convenient to add NaCl (20mg.) to the sugar solution added to the column, the eluate then being tested for Cl^-, which were shown to be eluted with PO_4^{3-}. A complete separation of sugars from salts was usually obtained.

Enzymolysis of agarose. Agarose (7·0g.) was dissolved at 100° in 33mM-potassium phosphate buffer, pH7·0 (3l.), and the solution was cooled to 45°. Enzyme (75units) was added and the digest maintained at 45° for 30min. before being cooled to 37°. Enzyme (400units) was then added and the digest was covered with toluene and incubated at 37°, the production of reducing power being determined at intervals. After 100hr., the digest was added to ethanol (2·5vol.), the small amount of precipitate was removed on the centrifuge and the supernatant solution was evaporated to 25ml. This solution was then desalted on a column of Sephadex G-10 and the salt-free eluate was freeze-dried. The product (100mg.) in M-NaCl was eluted through a column (60cm.×2·5cm.) of Sephadex G-25 (fine grade), previously swollen in M-NaCl, the column being eluted with the same solvent. The course of elution was followed by testing fractions with the phenol–H_2SO_4 reagent (Fig. 1). Suitable fractions were combined, concentrated, desalted and freeze-dried. Where necessary, combined fractions were further resolved by preparative t.l.c. The remainder of the agarose hydrolysate was then separated into its components by a large-scale version of this procedure.

Enzymolysis of alkali-treated porphyran. The substrate (5·0g.) was dissolved in phosphate buffer (2·2 l.) and subjected to enzymolysis exactly as described for agarose. After incubation for 100hr., the digest was added to ethanol (2·5vol.) and the precipitate recovered on the centrifuge, washed with ethanol and dried (yield 45%). The supernatant solution was concentrated, desalted and then partly resolved into its component sugars by gel filtration through Sephadex G-25 (fine grade) as described above. Further resolution by preparative t.l.c. was necessary to give pure components.

* Abbreviation: D.P., degree of polymerization.

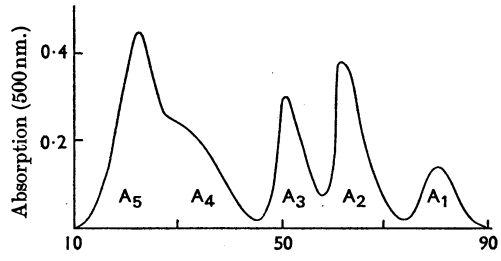
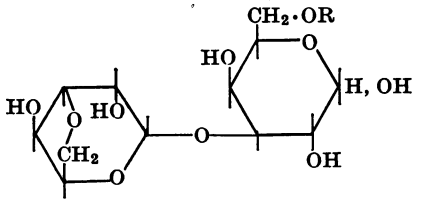


Fig. 1. Gel filtration of oligosaccharides (100mg.) from the enzymolysis of agarose on Sephadex G-25 (fine grade), eluted with M-NaCl. Fractions (5ml. each) were collected and tested for carbohydrate by the colorimetric phenol–H_2SO_4 method. The results are shown as the absorption indicated by the colorimeter.

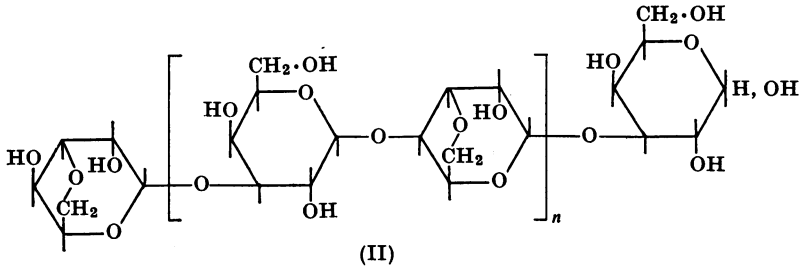
Enzymolysis of porphyran. Porphyran (15·0g.) in 33mM-potassium phosphate buffer, pH7·0 (1·5l.), was incubated with enzyme (1020units) at 37° under toluene. After 100hr., addition of ethanol (2·5vol.) gave a precipitate, which was recovered as above (yield 70%). The supernatant solution was concentrated, desalted and freeze-dried (7·1g.). The product (4·0g.) was separated on a column (70cm.×3·5cm.) of DEAE-Sephadex A-25 (Cl^- form), neutral oligosaccharides being eluted with water (500ml.). Elution with a gradient concentration of 0–2M-NaCl over 1l. then gave a series of eight bands of sulphated oligosaccharides (S_1–S_8). The combined neutral oligosaccharides were then fractionated on a column (80cm.×4cm.) of Sephadex G-25 as described above for agarose. Further resolution by preparative t.l.c. was used to obtain some of the components in a pure form.

RESULTS

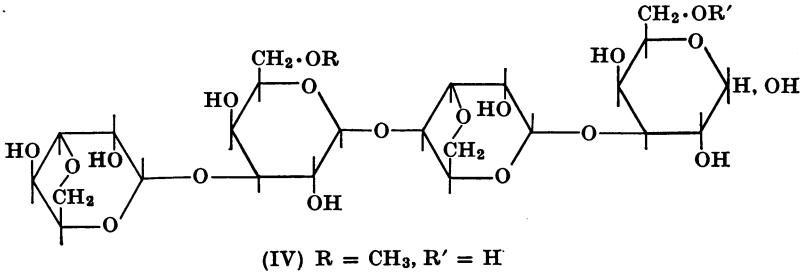
Action of the enzyme on agarose produced rapid depolymerization until the reducing power indicated an apparent conversion (as galactose) of 5% after 24hr., rising to 10% at 100hr. At this stage the reaction was so slow that the products were isolated and investigated. Addition of ethanol gave little precipitated polysaccharide and the remaining solution was examined by t.l.c. (solvent *A*, double development). Four distinct oligosaccharides separated from a slower-migrating band of oligosaccharides. Three of the oligosaccharides had R_F values identical with those of neoagarobiose [3-*O*-(3,6-anhydro-α-L-galactopyranosyl)-D-galactose, I], neoagarotetraose (II, $n=1$) and neoagarohexaose (II, $n=2$) respectively. The fourth oligosaccharide had a mobility that, when plotted according to the relationship given by Bate-Smith & Westall (1950), lay on the straight line extended from the neoagarodextrins, if it was assumed to be an octasaccharide. On a small scale, the mixture was fractionated by gel filtration on Sephadex G-25 (Fig. 1) to



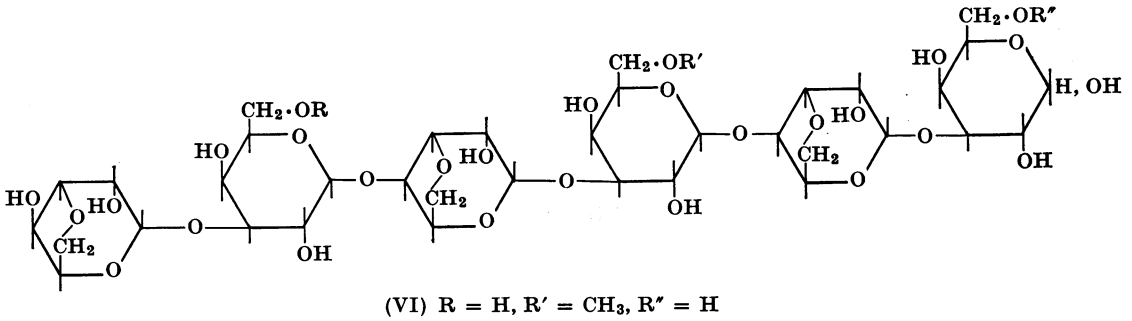
(I) R = H
(III) R = CH_3



(II)



(IV) R = CH_3, R′ = H
(V) R = H, R′ = CH_3



(VI) R = H, R′ = CH_3, R″ = H
(VII) R = CH_3, R′ = R″ = H
(VIII) R = R′ = H, R″ = CH_3

give pure samples of oligosaccharides A_1, A_2 and A_3. The fourth oligosaccharide was contaminated with material from peak A_5, from which it was separated by preparative t.l.c. (solvent *A*, double development). The yields are given in Table 1. On a larger scale, the separation on the gel was not as good as on the small scale, but pure specimens of oligosaccharides A_1–A_4 were obtained by preparative t.l.c.

For alkali-treated porphyran, enzyme action had almost ceased after 100hr., with an apparent conversion (as galactose) of 4·8%. At this stage,

Table 1. *Oligosaccharides produced by enzymolysis of agarose*

R_{Gal} values were obtained after double development in butan-1-ol–pyridine–water (2:1:1, by vol.). The weights were obtained from 100mg. of hydrolysate.

Oligosaccharide	Probable identity	R_{Gal}	Weight (mg.)
A_1	Neoagarobiose	1·30	4
A_2	Neoagarotetraose	0·80	13
A_3	Neoagarohexaose	0·46	6
A_4	Neoagaro-octaose	0·25	5

Table 2. *Oligosaccharides produced by enzymolysis of alkali-treated porphyran*

R_{Gal} values were obtained after double development in butan-1-ol–pyridine–water (2:1:1, by vol.). The weights were obtained from 5·0g. of polysaccharide.

Oligosaccharide	R_{Gal}	Weight (mg.)
D_1	1·65	~1
D_2	1·30	20
D_3	1·05	47
D_4	0·80	44
D_5	0·70	21
D_6	0·50	40

ethanol precipitation gave 45% recovery of polysaccharide material. The supernatant solution contained a complex series of oligosaccharides, including a small proportion that contained sulphate ester groups. Partial resolution was again achieved on a column of Sephadex G-25, but final purification of the smaller oligosaccharides required preparative t.l.c. The yields of oligosaccharides (D_1–D_6) are given in Table 2. A seventh band of oligosaccharides was also obtained, but this consisted of a mixture of components with R_{Gal} values ranging from 0 to 0·4 (solvent *A*, double development). Component D_6 appeared to be chromatographically homogeneous in all the solvents listed, but later evidence suggested that it was a mixture (see below).

Native porphyran was degraded by the enzyme to an even smaller extent, reaction almost ceasing after 90hr., when the conversion (as galactose) was 2·1%. Ethanol precipitation gave a recovery of 70% of polysaccharide material. The alcohol-soluble products consisted of a complex mixture of sulphated and neutral oligosaccharides. This mixture was not resolved by gel filtration through Sephadex G-25, but a column of DEAE-Sephadex A-25 separated the neutral (29%) from the sulphated oligosaccharides (71%), and the latter were resolved into eight bands (S_1–S_8) by gradient elution from the column with increasing concentration of Cl^- (Table 4). The neutral oligosaccharides were then partly resolved by gel filtration on Sephadex G-25 and this, combined with preparative t.l.c., gave pure samples of oligosaccharides P_2–P_5 (Table 3). Oligosaccharide P_1 was obtained in only a small quantity and was contaminated with traces of P_2.

Examination of neutral oligosaccharides. Oligosaccharides A_1, D_2 and P_2 had the same R_F value as neoagarobiose in solvents *A* and *B*. They each behaved similarly in other tests. The D.P. was 2·1, and on acid hydrolysis the only products were galactose and 5-hydroxymethylfuran-2-aldehyde (a degradation product of 3,6-anhydrogalactose). The content of 3,6-anhydrogalactose was 44%, and this was unchanged after reduction with sodium borohydride. This evidence is consistent with all

Table 3. *Neutral oligosaccharides produced by enzymolysis of porphyran*

R_{Gal} values were obtained after double development in butan-1-ol–pyridine–water (2:1:1, by vol.). The weights were obtained from approx. 8·5g. of porphyran.

Oligosaccharide	R_{Gal}	Weight (mg.)	D.P.
P_1	1·70	3·5	—
P_2	1·30	31	2·1
P_3	1·05	47	3·8
P_4	0·88	44	3·7
P_5	0·64	10	5·6

of these fractions being neoagarobiose. Similarly, oligosaccharides A_2, D_4 and P_4 were all identical chromatographically with neoagarotetraose; each gave galactose and the furan derivative on acid hydrolysis, and they had D.P. 3·7–3·8; the content of 3,6-anhydrogalactose, 43–45%, was unchanged on treatment with sodium borohydride. These fractions were therefore assumed to be neoagarotetraose.

Oligosaccharides A_3 and A_5 both gave galactose and the furan derivative on acid hydrolysis, and contained 42 and 43% respectively of 3,6-anhydrogalactose (before and after reduction with sodium borohydride). The R_F value of oligosaccharide A_3 was identical with that of neoagarohexaose (solvents *A* and *B*) and oligosaccharide A_4 was assumed to be the homologous octasaccharide (II, $n=3$) from the plot of R_M value [$\log(1/R_F - 1)$] against D.P. (Bate-Smith & Westall, 1950), since its value lay on the straight line with those of the other neoagarosaccharides. Oligosaccharide D_6 had an R_F value indicating that it might be neoagarohexaose, but on complete hydrolysis it gave 6-*O*-methylgalactose as well as galactose and the furan derivative. The D.P., determined by the anthrone method, was 5·6, but the chromatographic mobility indicated that it was probably a mixture of neoagarohexaose and higher oligosaccharides containing two or more 6-*O*-methyl-D-galactose units.

Oligosaccharides D_1 and P_1 were chromatographically identical but oligosaccharide P_1 was contaminated with traces of oligosaccharide P_2 (neoagarobiose). Acid hydrolysis of oligosaccharide P_1 gave mainly 6-*O*-methyl-D-galactose together with smaller amounts of D-galactose and the furan derivative. It contained 40% of 3,6-anhydrogalactose and, after borohydride reduction, gave on acid hydrolysis 6-*O*-methyl-D-galactitol and galactitol (identified by g.l.c. of the derived acetates) but no free sugars. This evidence, together with the R_F value, indicated that the oligosaccharide P_1 was possibly 6^1-*O*-methylneoagarobiose [3-*O*-(3,6-anhydro-α-L-galactopyranosyl)-6-*O*-methyl-D-galactose, III] admixed with traces of neoagarobiose. Not enough material was available for further tests to be carried out.

Oligosaccharides D_3 and P_3 were chromatographically identical and each gave on hydrolysis galactose, 6-*O*-methyl-D-galactose and the furan derivative. For oligosaccharide P_3, the content of 3,6-anhydrogalactose was 42%, the value remaining constant after reduction with sodium borohydride. The D.P. was 3·8, indicating a tetrasaccharide. A similar tetrasaccharide has been reported from enzymic hydrolysates of porphyran (Turvey & Christison, 1967*b*) and this was then assigned the structure (IV). The oligosaccharide P_3 was reduced with sodium borohydride and then acid-hydrolysed. The major hexose detected by t.l.c. (*p*-anisidine spray) was 6-*O*-methylgalactose, but quantitative g.l.c. of the derived acetates indicated that, although the major glycitol acetate was galactitol acetate, some 6-*O*-methylgalactitol acetate was also present. The ratio of galactitol to 6-*O*-methylgalactitol was 5:1. This evidence suggests that oligosaccharide P_3 and also probably oligosaccharide D_3 are in fact mixtures containing 5 parts of oligosaccharide (IV) to 1 part of oligosaccharide (V).

Oligosaccharides D_5 and P_5 were also identical chromatographically and contained (acid hydrolysis) about twice as much galactose as 6-*O*-methylgalactose. The 3,6-anhydrogalactose content (41%; unchanged on reduction) and the D.P. (5·6) indicated that these were probably hexasaccharides formed by two D-galactose units, one 6-*O*-methyl-D-galactose unit and three 3,6-anhydro-L-galactose units, with the anhydro sugar not at the reducing end. Borohydride reduction, acid hydrolysis and g.l.c. of the derived acetates indicated that the reducing end unit was mainly D-galactose, but that a minor component contained the 6-*O*-methyl sugar at the reducing end. The major constituents could therefore be hexasaccharides (VI) and (VII), together with small amounts of hexasaccharide (VIII).

The sulphated oligosaccharides obtained from porphyran constitute a series in which increasing mobility, indicative of increasing content of ester sulphate, is paralleled by a decrease in 3,6-anhydrogalactose content (Table 4). It was not possible to determine the average D.P. of these fractions, since the method used is inaccurate above D.P. 6. From estimations of reducing power, the lowest D.P. of any of the constituents was about 20. The sulphate content of combined sulphated oligosaccharides S_1–S_8 was 15·2% (as SO_4^{2-}). The proportion of D-galactose to 6-*O*-methyl-D-galactose present as reducing end groups in the combined sulphated oligosaccharides was determined by reduction of the oligosaccharides with borohydride, followed by acid hydrolysis and g.l.c. of the derived alditol acetates. From this it appeared that for every one 6-*O*-methyl-D-galactose there were four D-galactose residues as reducing end unit.

Table 4. *Sulphated oligosaccharides produced by enzymolysis of porphyran*

The weights were obtained from approx. 8·5g. of porphyran.

Fraction	Weight (mg.)	M_S values of components	3,6-Anhydrogalactose content (%)
S_1	150	0·53, 0·67, 0·78	35
S_2	32	0·53, 0·67, 0·78	22
S_3	146	0·67, 0·78	22
S_4	182	0·67, 0·76	18
S_5	201	0·74, 0·82	14
S_6	198	0·74, 0·86	13
S_7	370	0·86	11
S_8	70	0·90	9

The proportions of 6-*O*-methyl-D-galactose units to D-galactose units in various fractions from porphyran and from alkali-treated porphyran were determined. The ratio of 6-*O*-methyl-D-galactose to galactose was not significantly different in the native porphyran (1·1:1), the total sulphated oligosaccharides from porphyran (1·3:1), the neutral oligosaccharides from porphyran (1·1:1), the high-molecular-weight neutral oligosaccharides from alkali-treated porphyran eluted first from the Sephadex G-25 column (1·3:1) and in the combined smaller oligosaccharides D_1–D_6 from alkali-treated porphyran (1·1:1).

DISCUSSION

The extracellular enzyme produced by the bacterium is a typical agarase, similar to those reported from other micro-organisms (Araki & Arai, 1956; Yaphe, 1957; Swartz & Gordon, 1959), in that it is specific for the β-D-galactosidic linkage in agarose and the products of its action on agarose are the series of neoagarosaccharides. Table 1

shows that the preponderant oligosaccharide produced from agarose is neoagarotetraose, suggesting that this oligosaccharide accumulates in the digest. It is also significant that, among the small neutral oligosaccharides from porphyran and from alkali-treated porphyran, tetrasaccharides again predominate, suggesting that there is little or no enzyme action on these oligosaccharides.

Enzymic hydrolysis of alkali-treated porphyran is slower than for agarose, and almost half the molecule is not degraded to a marked extent. In an ideal case, the only structural difference between agarose and alkali-treated porphyran would be that the latter contains 6-*O*-methyl ether groups on some of the D-galactose units (Anderson & Rees, 1965), but in practice it is difficult to remove the sulphate completely from porphyran by treatment with alkali. The sample of porphyran used in these studies had 11·7% of sulphate (as SO_4^{2-}) and treatment with alkali decreased this to 1·8%. This residual sulphate, together with the methyl ether group, must present serious hindrance to enzyme action; indeed, the large effect from such a small proportion of sulphate ester groups suggests that the enzyme cannot catalyse the hydrolysis of linkages anywhere near these groups.

An estimate of the effect of 6-*O*-methyl groups in hindering enzyme action can be obtained from the distribution of these groups in the neutral oligosaccharides from porphyran. The tetrasaccharide fraction, P_3, from porphyran contained two oligosaccharides (IV and V) in the ratio 5:1. If the distribution of 6-*O*-methyl ether groups on half of the D-galactose units is random (see below) then this implies that the enzyme catalyses the hydrolysis of the 6-*O*-methyl-D-galactosidic linkage at about one-fifth of the rate for the D-galactosidic linkage. The ratio (1:4) of 6-*O*-methyl-D-galactose to D-galactose units at the reducing end of the sulphated oligosaccharides also supports this hypothesis.

That the 6-*O*-methyl ether groups are distributed randomly on the D-galactose units throughout the porphyran molecule is suggested by the fairly even ratio for the distribution of the two monosaccharide residues throughout all the groups of fractions examined. Both high-molecular-weight and low-molecular-weight neutral oligosaccharides and also the sulphated oligosaccharides have approximately the same ratio. The isolation of neutral oligosaccharides containing only D-galactose and the anhydro sugar, of others containing D-galactose and 6-*O*-methyl-D-galactose with the anhydro sugar and the suggested occurrence of others in which the 6-*O*-methyl sugar predominates (e.g. part of oligosaccharide fraction D_6) all tend to establish a more or less random distribution of the 6-*O*-methyl sugar within the repeating sequence of units in porphyran, as has been suggested previously (Turvey & Christison, 1967*b*).

The distribution of sulphate ester groups in porphyran is more difficult to define. Sulphate groups inhibit enzyme action in their vicinity, thus rendering about 70% of the porphyran molecule resistant to enzyme action. Of the 30% of porphyran degraded to oligosaccharides, about two-thirds (by weight) are present in the form of sulphated oligosaccharides. The important feature of these sulphated fragments is that they are relatively large and must contain several sulphate groups/molecule; no oligosaccharide carrying only one sulphate group/molecule was isolated. Indeed, the relatively high electrophoretic mobility of most of these fragments, coupled with the high D.P., suggests that the oligosaccharides are highly sulphated. On the other hand, about 10% of the porphyran appears as neutral oligosaccharides (fractions P_1–P_5 are examples). All these facts suggest that sulphate groups are localized in the molecule of porphyran, tending to occur in blocks rather than in a random manner.

Studies on the specificity of the enzyme are reported in the following paper (Duckworth & Turvey, 1969*b*).

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