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THE STRUCTURE OF AGAR

PART II*. THE USE OF A BACTERIAL AGARASE TO ELUCIDATE STRUCTURAL FEATURES OF THE CHARGED POLYSACCHARIDES IN AGAR

M. DUCKWORTH AND W. YAPHE
Department of Microbiology and Immunology, McGill University, Montreal (Canada)

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ABSTRACT

Agar consists of a spectrum of polysaccharides with three extremes in structure, namely, neutral agarose, pyruvated agarose having little sulphation, and a sulphated galactan. Components close to these three extremes of structure were degraded with the purified, extracellular agarase from *Pseudomonas atlantica*. Characterisation of the products of hydrolysis indicates that the masking of the basic repeating unit with 4,6-*O*-(1-carboxyethylidene)-D-galactose in place of D-galactose occurs in regions of the molecule low in sulphate content. Two new oligosaccharides containing 4,6-*O*-(1-carboxyethylidene)-D-galactose but no sulphate are described, namely, 4^3,6^3-*O*-(1-carboxyethylidene)neoagarotetraose and 4^5,6^5-*O*-(1-carboxyethylidene)neoagarohexaose.

INTRODUCTION

Agar from *Gelidium amansii* has been fractionated by acetylation into two fractions, an essentially neutral polymer (agarose) and a charged fraction (agaropectin)[1]. From the results of methylation[2] and enzymic hydrolysis[3,4], Araki deduced the structure of agarose to be (1→4)-linked 3,6-anhydro-α-L-galactose alternating with (1→3)-linked β-D-galactose. Agaropectin is thought to have the same repeating units[5], although some of the 3,6-anhydro-L-galactose residues can be replaced with L-galactose sulphate[6], and there can be partial replacement of the D-galactose residues with the pyruvic acid acetal 4,6-*O*-(1-carboxyethylidene)-D-galactose[7]. D-Glucuronic acid has also been reported to be present[8]. Recent fractionation studies[9] have indicated that agar does not consist of two major components but is composed of a complex spectrum of polysaccharides which range from a virtually neutral molecule to a highly charged galactan.

The enzymic activity of agar-digesting micro-organisms was first studied in detail by Araki and Arai[10]. An extracellular enzyme preparation from *Pseudomonas kyotoensis* was found to degrade agar into two main oligosaccharides, neoagarobiose and neoagarotetraose[3,4], which were the two smallest members of an homologous series of oligosaccharides. An extracellular agarase from a *Cytophaga species* has been

*Part I: see Ref. 9.

used more recently to obtain structural information on the related polysaccharides agarose, porphyran, and the galactan sulphate from *Laurencia pinnatifida*[11,12]. In this study, the agarase did not degrade regions of the molecule having a high sulphate content[13], and hence only portions of the molecule similar in structure to agarose were studied in detail.

We now report on the products formed when the purified extracellular agarase from *Pseudomonas atlantica*[14] is incubated with various polysaccharide fractions obtained by the fractionation of a commercial agar (Difco BACTO agar). This agarase has previously been shown to degrade agars from *Gracilaria species* to a mixture of neutral and charged oligosaccharides[15].

RESULTS AND DISCUSSION

The fractionation of agar on DEAE Sephadex A-50(Cl^-) has indicated that there are three *extremes* of structure in the spectrum of polysaccharides which compose agar: (1) neutral agarose having an idealised structure involving alternating (1→4)-linked 3,6-anhydro-α-L-galactose and (1→3)-linked β-D-galactose residues; (2) pyruvated agarose having little sulphation, for which the maximal substitution of 4,6-*O*-(1-carboxyethylidene)-D-galactose for D-galactose reaches 5%, and the degree of sulphation is 2%; (3) sulphated galactan containing little or no 3,6-anhydro-L-galactose or 4,6-*O*-(1-carboxyethylidene)-D-galactose.

Polysaccharide fractions as close as possible to these idealised extremes were chosen as substrates for the agarase, so that representative oligosaccharides would be obtained. The polysaccharide fractions were Fraction *a*, the polysaccharide in the water eluant when complete agar is fractionated on DEAE Sephadex A-50(Cl^-); Fraction 2, the polysaccharide in the eluant when granulated agar is washed with dilute saline at 50°, after first removing the 20° soluble material; and Fraction 1, the polysaccharide in the eluant when granulated agar is washed with dilute saline at 20°. For reasons of clarity throughout the text, Fractions *a*, 2, and 1, will be referred to as agarose, pyruvated agarose, and sulphated galactan, respectively. The analytical data for these three fractions are shown in Table I.

TABLE I

ANALYTICAL DATA FOR THE POLYSACCHARIDE FRACTIONS USED IN THIS INVESTIGATION

Fraction	*Sulphate* (%)	*Pyruvic acid* (%)	*3,6-Anhydro-L-galactose* (%)	*Polysaccharide remaining after enzyme action* (%)
a. Agarose	0.05	not detectable	47	0
2. Pyruvated agarose	4.10	0.99	20	24
1. Sulphated galactan	7.10	0.55	5	55

Analysis of enzymic hydrolysates. — Purified extracellular agarase was incubated with polysaccharide fractions *a*, 2, and 1. The percentage of undegraded polysacchar-

ide was correlated with the sulphate content of the initial polysaccharide (Table I) and indicates that the enzyme is not able to degrade regions of the molecules rich in sulphate. The oligosaccharides in the enzymic hydrolysates were studied by high-voltage electrophoresis and cellulose thin-layer chromatography. In the agarose hydrolysate, oligosaccharides having R_{Gal} values of 1.10, 0.80, and 0.51 (solvent *A*) were detected and, in the hydrolysates of pyruvated agarose and the sulphated galactan, oligosaccharides having R_{Gal} values of 1.10, 0.91, 0.80, 0.64, and 0.51. The M_s values at pH 6.0 of the charged oligosaccharides in the hydrolysates of pyruvated agarose and the galactan sulphate were 0.76, 0.59, 0.49, 0.31, and 0.20. The difficulties involved in the cellulose t.l.c. analysis of the mixture of neutral and charged oligosaccharides formed by the enzymic hydrolysis of agar have recently been reviewed[20].

The enzymic hydrolysis of agarose yielded mainly neutral oligosaccharides (Table II) which were separated on Sephadex G-25 (Fig. 1) into neoagarohexaose (41.2%), neoagarotetraose (49.7%), and neoagarobiose (7.1%). The structure of these oligosaccharides was confirmed by cellulose t.l.c. with different solvent systems, the absence of charged sugars on electrophoresis, and by chemical methods which have been described previously[12]. The ratio (6:7:1) of hexa-, tetra-, and di-saccharides is considerably different from that (6:13:4) reported[12] for the agarase from a *Cytophaga species*.

This is indicative of different mechanisms of enzyme action. The absolute specificity of the *Pseudomonas atlantica* agarase will be discussed elsewhere.

TABLE II

YIELDS OF NEUTRAL AND CHARGED OLIGOSACCHARIDES OBTAINED BY ENZYMIC HYDROLYSIS OF THE POLYSACCHARIDE FRACTIONS

Polysaccharide fraction	*Neutral oligosaccharides* (%)	*Charged oligosaccharides* (%)
Agarose	95	5
Pyruvated agarose	28	72
Sulphated galactan	18	82

Pyruvated agarose and the sulphated galactan formed a complicated mixture of neutral and charged oligosaccharides on enzymic hydrolysis which could not be resolved until the neutral and charged oligosaccharides had been separated from each other on DEAE Sephadex A-25(Cl^-). The yields of neutral and charged oligosaccharides obtained from each fraction are shown in Table II. With increasing charge of the parent polysaccharide, the percentage of neutral oligosaccharides formed decreases. The yield of charged oligosaccharides obtained by the enzymic hydrolysis of a commercial agarose is therefore indicative of the purity of the polysaccharide preparation with respect to charged groups.

The neutral oligosaccharides in the enzymic hydrolysates of pyruvated agarose and the sulphated galactan were identical with those described above for agarose,

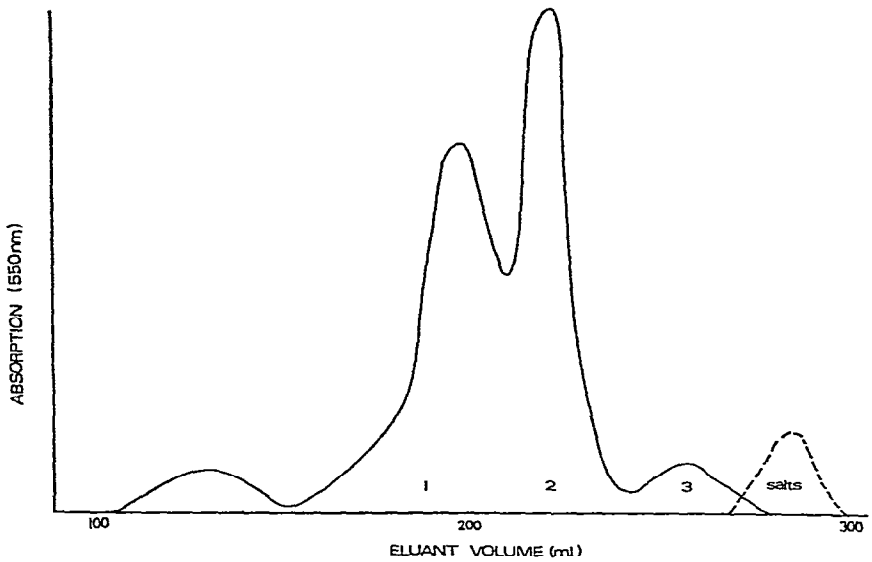

Fig. 1. The separation on Sephadex G-25 (Fine) of the neutral oligosaccharides obtained by the enzymic hydrolysis of agarose. Analysis showed that Peak 1 was neoagarohexaose, Peak 2 neoagarotetraose, and Peak 3 neoagarobiose. The eluant was distilled water.

and this may be taken as evidence that all agar components have regions which approximate to the agarose structure.

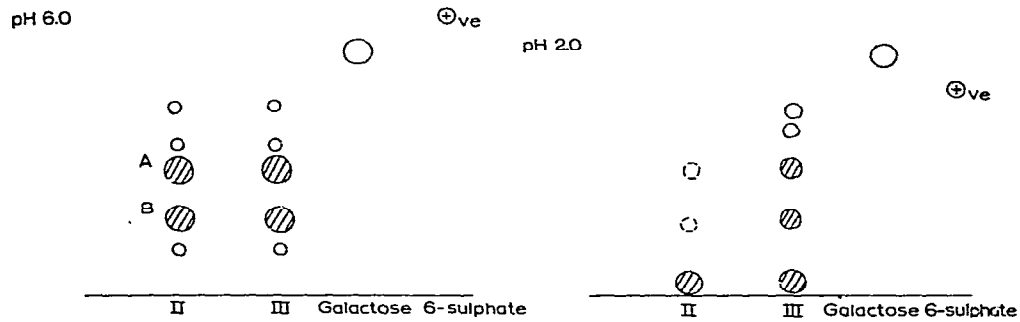

Fig. 2. Electropherograms at pH 2.0 and pH 6.0 of the charged oligosaccharides formed by the enzymic hydrolysis of pyruvated agarose (II) and the galactan sulphate (III).

Fractionation and analysis of the charged oligosaccharides obtained from pyruvated agarose. — The charged oligosaccharides derived from pyruvated agarose were studied by high-voltage electrophoresis prior to separation. Two different types of charged oligosaccharides were noted (Fig. 2), one of which migrated at both pH 2.0 and pH 6.0, and one of which had no mobility at pH 2.0. The charged oligosaccharides were then fractionated on DEAE Sephadex A-25(Cl^-) with a gradient concentration of sodium chloride (Fig. 3). Chemical analysis (Table III) showed that the oligosaccharides in peak 1 contained mainly 4,6-*O*-(1-carboxyethylidene)-D-galactose, whereas peak 2 had a preponderance of sulphate groups.

TABLE III

ANALYSIS OF THE CHARGED OLIGOSACCHARIDES

Oligosaccharide fraction	*Relative yield* (%)	*Sulphate* (%)	*Pyruvic acid* (%)	*3,6-Anhydro-L-galactose* (%)
Peak 1	80	3.0	9.8	32
Peak 2	20	9.0	3.0	16

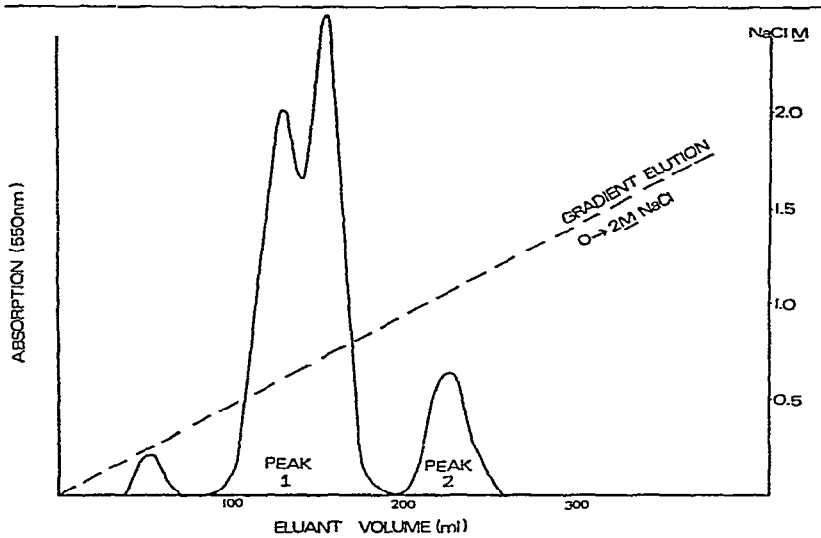

Fig. 3. Separation of the charged oligosaccharides (obtained by the enzymic hydrolysis of pyruvated agarose) on DEAE Sephadex A-25 (Cl^-). The small peak before Peak 1 was found, by analysis, to be neutral oligosaccharides which had not been completely separated from the charged oligosaccharides.

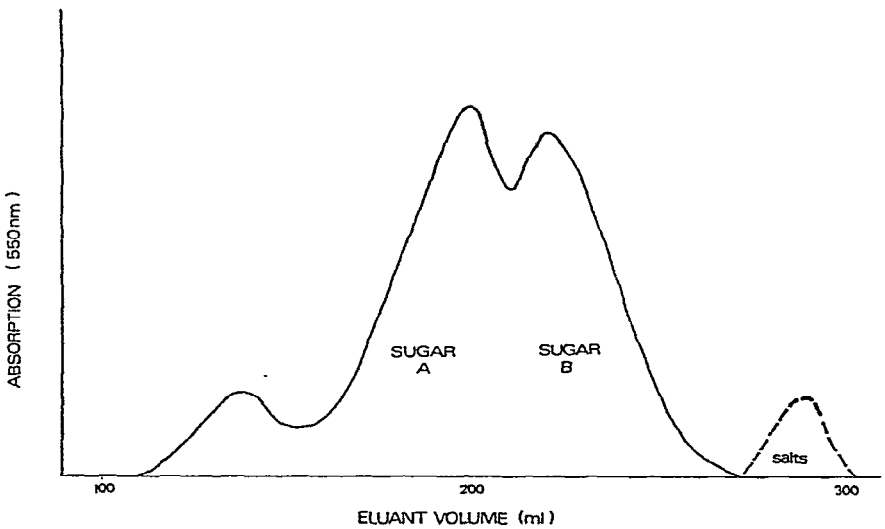

Fig. 4. Separation of the Peak 1 charged oligosaccharides [from DEAE Sephadex A-25 (Cl^-), Fig. 3] on Sephadex G-25 (Fine). The eluant was distilled water.

Peak 1 oligosaccharides were fractionated on Sephadex G-25 (Fig. 4) but the separation was not as complete as for the neutral series of oligosaccharides (Fig. 1). Consequently, the two major charged sugars (*A* and *B* in Fig. 2) had to be subjected to several elutions before pure oligosaccharides were obtained. These two sugars had the same elution volume on Sephadex G-25 as neutral neoagarohexaose and neoagarotetraose (Fig. 1) and had the same R_{Gal} values on t.l.c. The homogeneity of the oligosaccharides was checked by electrophoresis and by cellulose t.l.c. On high-voltage electrophoresis (pH 6.0), they had M_s values 0.31 and 0.49, respectively, but at pH 2.0 they had no mobility. On analysis, these oligosaccharides were shown to have no sulphate and a high content of pyruvic acid (Table IV). The two types of oligosaccharides (shown by high-voltage electrophoresis) arise because the carboxyethylidene group is un-ionised at pH 2.0, whereas the sulphated oligosaccharides are ionised at both pH values.

TABLE IV

ANALYTICAL DATA FOR THE MAJOR CHARGED OLIGOSACCHARIDES FORMED BY THE ENZYMIC HYDROLYSIS OF PYRUVATED AGAROSE

Charged oligosaccharides	*3,6-Anhydro-L-galactose* (%)	*Pyruvic acid* (%)[a]	*Sulphate* (%)	*D.p.*	M_s (*pH 6.0*)
A	40	9.2	0	5.8	0.31
B	41	12.3	0	3.9	0.49

[a]The theoretical percentage of pyruvic acid for *A*, a hexasaccharide with one 4,6-*O*-(1-carboxyethylidene)-D-galactose residue, would be 8.8%, and for *B*, a tetrasaccharide with one 4,6-*O*-(1-carboxyethylidene)-D-galactose residue, would 12.5%.

The data presented in Table IV indicate that these charged molecules are analogous to the hexa- and tetra-saccharide of the neutral series of oligosaccharides, but contain one 4,6-*O*-(1-carboxyethylidene)-D-galactose residue in place of a D-galactose. The carboxyethylidene group could be in one of three positions on the hexasaccharide and one of two positions in the tetrasaccharide. That 4,6-*O*-(1-carboxyethylidene)-D-galactose did not occur at the reducing end of these oligosaccharides was shown by converting each oligosaccharide into the corresponding alcohol, followed by characterisation of the sugar at the former reducing end by g.l.c. after mild hydrolysis with acid. The structure of the tetrasaccharide must, therefore, be as shown in Fig. 5. The known specificity of the enzyme was used to deduce the position of the carboxyethylidene group in the hexasaccharide. Neoagarohexaose is degraded by the purified, extracellular agarase of *Pseudomonas atlantica* by cleaving the β-(1→4) linkage nearest the reducing end[21]. Similarly, the charged hexasaccharide *A* is degraded to charged tetrasaccharide *B* plus neoagarobiose. The structures of the two charged oligosaccharides are therefore as shown in Fig. 5. The configuration of the 4,6-*O*-(1-carboxyethylidene)-D-galactose can be considered to be absolute on these oligosaccharides, because Gorin and Ishikawa[22] have shown the absolute configuration of the D-galactose monomer. These workers presented n.m.r. data for

this monomer, the *C*-methyl group giving a sharp singlet at 1.5 p.p.m. The n.m.r. spectrum (100 MHz, D_2O, external tetramethylsilane) of the tetrasaccharide containing the 4,6-*O*-(1-carboxyethylidene)-D-galactose also gave a sharp singlet at this position.

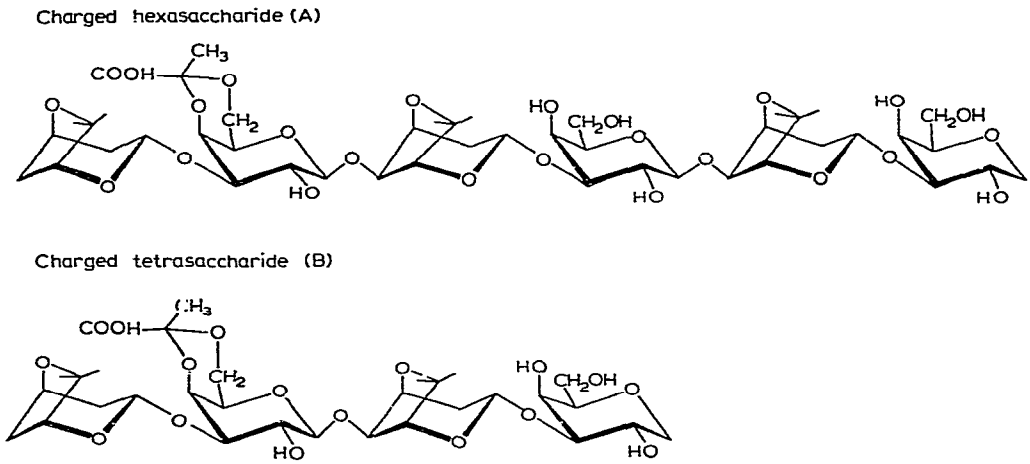

Fig. 5. (*A*) $4^5,6^5$-*O*-(1-carboxyethylidene)-neoagarohexaose. (*B*) $4^3,6^3$-*O*-(1-carboxyethylidene)-neoagarotetraose.

The degradation of the polysaccharide rich in 4,6-*O*-(1-carboxyethylidene)-D-galactose by agarase action therefore follows a pathway similar to that for agarose, *i.e.*, hydrolysis of the polysaccharide to yield a hexasaccharide and tetrasaccharide containing one 4,6-*O*-(1-carboxyethylidene)-D-galactose residue per molecule, followed by slower hydrolysis of the charged hexasaccharide to yield the tetrasaccharide containing one unit of 4,6-*O*-(1-carboxyethylidene)-D-galactose plus neoagarobiose.

The large yield of these two charged oligosaccharides containing 4,6-*O*-(1-carboxyethylidene)-D-galactose and yet free of sulphate is again indicative that replacement of the D-galactose residues with 4,6-*O*-(1-carboxyethylidene)-D-galactose residues occurs in regions of the molecule where replacement of the 3,6-anhydro-L-galactose residue by L-galactose sulphate is low. Further evidence was obtained by analysing the enzyme-resistant portion of pyruvated agarose. The residual polysaccharide has a greatly decreased content of pyruvic acid but is richer in sulphate (pyruvated agarose, 4.1% of sulphate, 0.99% of pyruvic acid; enzyme-resistant portion, 6.0% of sulphate, 0.20% of pyruvic acid).

The charged oligosaccharides of peak 2 in the fractionation of the complete charged oligosaccharides on DEAE Sephadex A-25(Cl^-) contained a much more-complex mixture of oligosaccharides than did peak 1. Cellulose t.l.c. indicated that these oligosaccharides had a higher molecular weight and chemical analysis (Table III) showed that they were extremely rich in sulphate.

Fractionation and analysis of the charged oligosaccharides derived from galactan sulphate. — The charged oligosaccharides obtained by the enzymic hydrolysis of the

galactan sulphate were first studied by high-voltage electrophoresis prior to separation. The yield of oligosaccharides mobile at both pH 6.0 and 2.0 was far greater than that of the oligosaccharides which had no mobility at pH 2.0. Attempts to separate the oligosaccharides into two fractions on DEAE Sephadex A-25(Cl^-) were unsuccessful. An attempt was therefore made to separate the charged sugars directly on Sephadex G-25 (Fig. 6). The oligosaccharides in peak 1 were shown to be sulphated oligosaccharides of high molecular weight and peaks 2 and 3 were found to contain the pyruvated hexa- and tetra-saccharide, respectively, which have been described in the enzymic hydrolysate of pyruvated agarose. The ratio of sulphated to pyruvated oligosaccharides was 79:21.

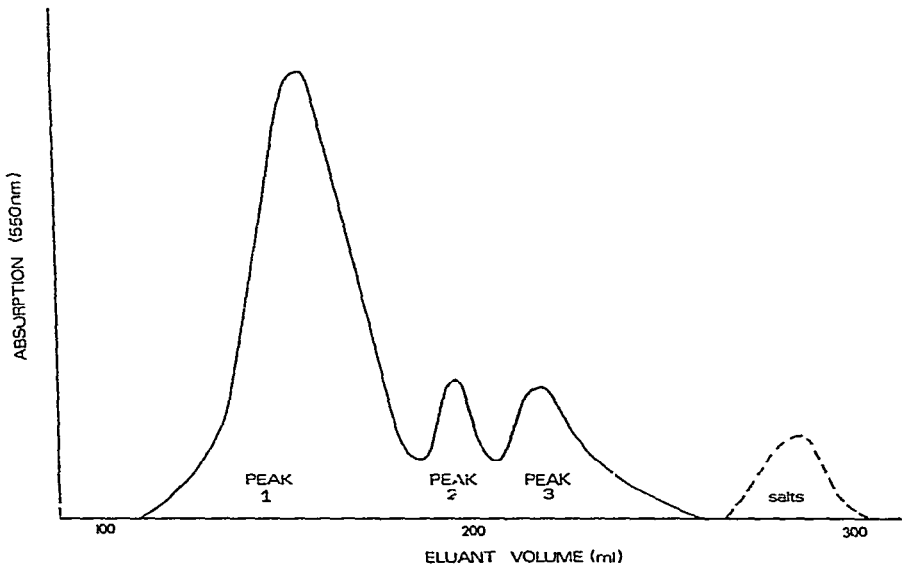

Fig. 6. Attempted separation of the charged oligosaccharides (formed by the enzymic hydrolysis of the galactan sulphate) on Sephadex G-25. The eluant was distilled water.

Only a limited amount of information can be obtained on the structure of the highly sulphated oligosaccharides produced by enzymic hydrolysis. Consequently, the information obtained on the highly sulphated polysaccharides in agar by using agarases which yield the neoagarobiose series of oligosaccharides is also limited, because the agarase will not cleave a β-D-(1→4) linkage close to a sulphated galactose. Structural analysis of the sulphated regions in agar awaits the isolation of an enzyme which will degrade a linkage close to a sulphated galactose.

The ability of the agarase to cleave a β-D-(1→4) linkage near but not next to the 4,6-*O*-(1-carboxyethylidene)-D-galactose has enabled oligosaccharides containing this residue to be formed. The yield of these oligosaccharides that are free of sulphate has provided evidence that the majority of the pyruvate groups are laid down away from

the sulphated galactose residues. The studies of the enzymic hydrolysis of agar have therefore provided further evidence for the concept that agar consists of a spectrum of polysaccharides having three extremes in structure.

EXPERIMENTAL

Chromatography. — T.l.c. of oligosaccharides was performed on cellulose powder (Camag D.S.O. 0.2 mm) with the following solvent systems. (*A*) butyl alcohol–acetic acid–water (4:1:2), (*B*) butyl alcohol–ethanol–water (3:2:2), and (*C*) butyl alcohol–pyridine–water (2:1:1) by double development. Mobilities (R_{Gal}) are expressed relative to that of galactose.

For paper electrophoresis, Schleicher and Schuell AG (No. 2043B) paper at 80 volts/cm in 0.1M acetic acid–pyridine buffer (pH 6.0) or at 45 volts/cm in formic acid–acetic acid buffer (pH 2.0) was employed. All electrophoretic mobilities (M_s) are expressed relative to that of D-galactose 6-sulphate. The spray reagent used for both cellulose t.l.c. and paper electropherograms was the modified Seliwanoff spray[23].

G.l.c. of aldose and alditol acetates was performed on a Hewlett–Packard 402 gas chromatograph with a flame-ionisation detector. The carrier gas was nitrogen at a pressure of 40 ml/min. Columns (6 ft × 0.125 in.) contained 3% ECNSS-M on Gas Chrom Q (Applied Science Laboratories). The operating temperatures were as follows: oven, 180°; flash heater, 230°. G.l.c. of the trimethylsilyl derivatives[24] of aldoses and alditols was performed on the same instrument. Colums (6 ft × 0.125 in.) contained 3.8% of SE-30 on Diatoport S.

Analyses. — Sulphate (SO_4^{2-}) was measured by the method of Jones and Letham[18], pyruvic acid by the definitive lactate dehydrogenase method[17], and 3,6-anhydro-L-galactose by the method of Yaphe and Arsenault[16]. The degree of polymerisation (d.p.) of a reducing oligosaccharide in the range 2–6 was determined by the anthrone method[19].

Preparation of polysaccharide fraction and purified enzyme. — All polysaccharide fractions were prepared as discussed in Part I[9], and the enzyme was prepared and purified as described by Yaphe[14].

Enzymic hydrolysis of agarose. — Fraction *a* (agarose, 1.0 g) was dissolved in boiling distilled water (50 ml), and the solution was allowed to cool to 42°. Purified agarase* was added, the mixture was cooled to 25°, and more enzyme was then added. The flask was incubated at 25° for 24 h, and then the digest was poured into ethanol (3 volumes). No precipitation took place, and the solution was evaporated to a small volume and freeze-dried. The oligosaccharides in the hydrolysate were examined by cellulose t.l.c. (solvents *A*, *B*, and *C*) and by high-voltage electrophoresis at pH 6.0. The neutral and charged oligosaccharides were separated from each other on DEAE-Sephadex A-25(Cl^-); the neutral oligosaccharides were eluted with water, and the charged with 2M sodium chloride. The neutral oligosaccharides were separated by

*The ratio of the weight of polysaccharide to the weight of enzyme was the same in each case.

elution from a column (75 × 4 cm) of Sephadex G-25 (Fine) with distilled water. The charged oligosaccharides were desalted on Sephadex G-25 and freeze-dried.

Enzymic hydrolysis of pyruvated agarose. — Fraction 2 (pyruvated agarose, 6.5 g) was dissolved in boiling distilled water (250 ml) and degraded with purified agarase, and the products were fractionated as described for agarose, except that when the digest was poured into ethanol a precipitate was formed and subsequently removed by centrifugation. The desalted charged oligosaccharides were fractionated on DEAE Sephadex A-25(Cl^-) with a gradient of sodium chloride. Each fraction was desalted on Sephadex G-25, freeze-dried, and then analysed for 3,6-anhydro-L-galactose, sulphate, and pyruvic acid. From peak 1 (Fig. 3), two chromatographically pure oligosaccharides were obtained by elution from Sephadex G-25.

Enzymic hydrolysis of the galactan sulphate. — Fraction 1 (galactan sulphate, 4.0 g) was dissolved in boiling distilled water (200 ml) and degraded with purified agarase, and the products were fractionated as for pyruvated agarose. A gradient concentration of sodium chloride failed to separate the charged oligosaccharides on DEAE Sephadex A-25(Cl^-), but they were separated into three peaks on Sephadex G-25 (Fig. 6).

Characterisation of the sugar unit at the reducing end of oligosaccharide A. — Sugar *A* (40 mg) was reduced with sodium borohydride and then hydrolysed with 0.4 M sulphuric acid at 75° for 2 h. After neutralisation with barium carbonate, the solution was evaporated to a small volume. From this solution, the 4,6-*O*-(1-carboxyethylidene) monomer was separated by high-voltage electrophoresis (neutral buffer, 3000 volts, 35 mamps for 20 min.). The monomer was then hydrolysed (0.75 M sulphuric acid, 100°, 3 h) to give pyruvic acid and the parent sugar. The hydrolysate was neutralised with barium carbonate and the product was then trimethylsilylated or acetylated and analysed by g.l.c. Only galactose could be detected.

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