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THE STRUCTURE OF AGAR

PART I. FRACTIONATION OF A COMPLEX MIXTURE OF POLYSACCHARIDES

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

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ABSTRACT

Fractionation of Difco Bacto agar on DEAE Sephadex (Cl^-) has shown that the concept that agar is made up of two polysaccharides, neutral agarose and charged agaropectin, is an over-simplification. The results indicate that agar is a complex mixture of polysaccharides all having the same backbone structure but substituted to a variable degree with charged groups. There are three extremes of structure in this spectrum of polysaccharides.

INTRODUCTION

Agar, the polysaccharide complex extracted from the agarophytes of the Rhodophyceae, is of great economic importance because of its ability to form a gel at low concentrations which can be used as a solid, usually inert, support in bacteriological media. Early chemical investigations[1,2] on the structure of agar were carried out by using the complete polysaccharide complex, and it was found to be a galactan made up of alternating (1→3) and (1→4) linkages. Araki[3] fractionated agar from *Gelidium amansii* by acetylation into two fractions, a virtually neutral polymer (agarose) and a charged fraction (agaropectin).

From the results of methylation[4] and enzymic hydrolysis[5,6], Araki deduced the structure of agarose to be repeating sequences of (1→4)-linked 3,6-anhydro-α-L-galactose and (1→3)-linked β-D-galactose residues. Agaropectin is thought to have the same repeating units[7], although some of the L-galactose residues can be replaced with sulphated galactose residues[8] and there is also partial replacement of the D- galactose residues with the pyruvic acid acetal 4,6-*O*-(1-carboxyethylidene)-D-galactose[9]. D-Glucuronic acid has also been reported to be present[10].

Araki's concept is therefore of two polysaccharides, neutral agarose and charged agaropectin. It has been generally accepted that the presence of the few charged groups always found in commercial agaroses must be due to inefficient fractionation procedures, but we now present evidence that this is not necessarily the case.

Initially, the separation of agarose and agaropectin was only a step in the structural determination of agar, but as agaropectin has been found to interfere in

several biological techniques (*e.g.*, antibiotic assays[11], immunoelectrophoreses[12], agar gel electrophoreses[13], and viral plaque formation[14]), the separation of agarose from agar has become commercially important. The acetylation procedure of Araki is expensive and difficult on a large scale and so simpler methods have been sought. Hjerten[15] precipitated the charged complex by using a quaternary ammonium salt, leaving the agarose in solution. Blethen[16] modified this process by adding carrageenan to agar in order to co-precipitate the agaropectin as the quaternary ammonium salt. Russell *et al.*[17] fractionated agar by adding polyethylene glycol (mol. wt 6000) to an agar solution to precipitate the agarose fraction. The use of DEAE cellulose in a batch process to selectively adsorb agaropectin has recently been patented by Zabin[18]. Barteling[19] found that an agarose of high purity could be prepared by washing Difco Bacto agar with EDTA and then adsorbing the agaropectin onto aluminium hydroxide. Egorov *et al.*[20] have prepared agarose by the fractionation of Difco Bacto agar with ammonium sulphate. This last process is difficult to evaluate since no analytical data are given for the product.

All these procedures were developed without a full understanding of the nature of agar. Before an optimal preparation scheme for agarose can be evaluated, more-detailed knowledge of the structure of agar is necessary.

RESULTS AND DISCUSSION

The predominant genera which give gelling, agar polysaccharides are *Gelidium*, *Gracilaria*, *Acanthopeltis*, *Ceramium*, and *Pterocladia*. Agar is extracted from an agarophyte by boiling the alga in water. On cooling, the filtered solution sets to a gel which can be purified by freezing and thawing. The thaw water contains salts, pigments, and polysaccharides. No information is available on the polysaccharide fraction removed by this initial, purification step, but it is likely that it is highly charged. The agar which has been used throughout this investigation is the commercial preparation Difco Bacto agar. This product is thought to be prepared from *Gelidium cartilagineum* by the above procedure.

Charge-dependent solubility of polysaccharides at various temperatures has been used to fractionate agar[21] and other polysaccharide complexes[22], and this technique was investigated first. Three fractions were collected and analysed. Fraction **1** was obtained by aqueous extraction at 20°, fraction **2** at 50°, and fraction **3** was the remaining polysaccharide; the analytical data are shown in Table I.

By changing the conditions of the washing procedure, products having different values for their 3,6-anhydro-L-galactose, sulphate, and pyruvate contents were obtained. Hence, it became evident that these fractions were not different polysaccharides having homogeneous structures but just fractions of a whole polysaccharide complex. The high value for 3,6-anhydro-L-galactose in Fraction **2** was found not to be due to contamination with agarose (which presumably makes up part of Fraction **3**), since when Fraction **2** was added to a column of DEAE Sephadex A-50 (Cl^-), no polysaccharide could be eluted from this anion exchanger with distilled water.

TABLE I

FRACTIONATION OF DIFCO AGAR BY WASHING AT VARIOUS TEMPERATURES[a]

Polysaccharide component	*Yield* (%)	*3,6-Anhydro-L-galactose*[23] (%)	*Sulphate* (SO_4^{2-})[24] (%)	*Pyruvic acid*[25] (%)
Difco Bacto agar	100	30.0	2.96	0.91
Fraction 1, material soluble at 20°	15	5.3	7.00	0 55
Fraction 2, material soluble at 50°	25	19.4	4.10	1.30
Fraction 3, the remaining polysaccharide	50	45 0	0 95	0 21

[a]The methods of analysis are given in the appropriate references.

This suggested the second method for fractionation of agar by column chromatography using DEAE Sephadex A-50 in the chloride form. Sephadex was chosen in preference to cellulose because of its more-open structure. Difco Bacto agar was added to the column and the gel eluted with distilled water until the eluant became polysaccharide free. A gradient concentration of sodium chloride solution was then used to elute the charged polysacharides. The elution curve is shown in Fig. 1. From the analyses of selected fractions, it was shown that, with increasing ionic strength of the eluant, the sulphate content of the eluted polysaccharide increased. In a larger scale experiment, a step-wise increase in the ionic strength of the eluant

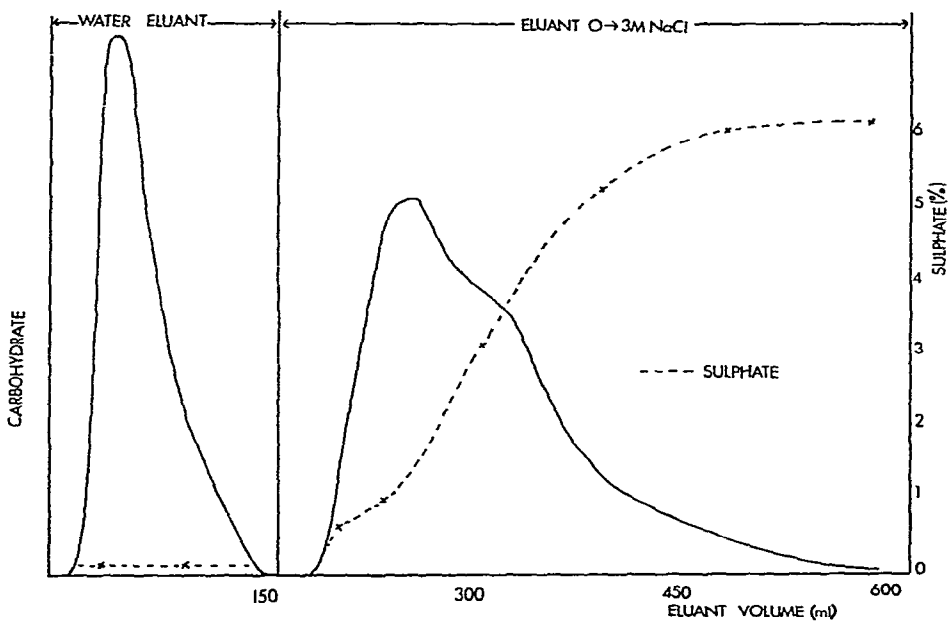

Fig. 1. Elution of Difco Bacto agar from DEAE Sephadex A-50 with a gradient concentration of sodium chloride. (———) Fractions assayed for carbohydrate with phenol–sulphuric acid, (– – – –) fraction assayed for sulphate.

was used, and the analytical data and weights of eight fractions thus obtained are expressed in Table II.

TABLE II

FRACTIONATION OF DIFCO BACTO AGAR BY COLUMN CHROMATOGRAPHY ON DEAE SEPHADEX A-50

Polysaccharide fraction	*Eluant*	*Yield*[a] (%)	*3,6-Anhydro-L-galactose* (%)	*Sulphate* (%)	*Pyruvic acid* (%)	*Gel formed*[b]
a	distilled water	25.5	47.8	0.08	0.02	++++
b	0.2M NaCl	1.9	44.1	0.94	0.06	+++
c	0.5M NaCl	25.5	44.0	1.07	0.09	++
d	0.8M NaCl	3.8	19.3	3.40	0.97	−
e	1.2M NaCl	1.9	12.0	5.80	0.64	−
f	1.8M NaCl	0.3	9.9	6.00	0.70	−
g	2.4M NaCl	0.3	9.0	6.00	0.50	−
h	3.0M NaCl	0.3	5.0	7.00	0.50	−

[a]Total yield, 59.5% of the polysaccharide added to the column; [b]+ is indicative of gel strength of a 1% solution, − no gel at a concentration of 1%.

As can be seen from the yields in Table II, a considerable proportion of material was irreversibly bound to the column. This phenomenon has been observed for other charged polysaccharide complexes[26]. The polysaccharide bound to the column possibly comprised highly charged galactans not removed in the freezing and thawing process. When this experiment was repeated with agar which had been washed at room temperature (Fractions 2 plus 3, Table I), the elution curve was the same as in Fig. 1 but the total yield from the column was 90% instead of 59.5%. When washed agar (Fraction 3) was fractionated on DEAE Sephadex (Cl^-), there was again almost total recovery with elution of components corresponding to Fractions *a*, *b*, and *c* in Table II. The total charge content of the polysaccharides eluted in Fraction *a* is least when a purified agar of low charged content is added to the Sephadex, but there is a

TABLE III

THE YIELD OF POLYSACCHARIDE VERSUS CHARGE CONTENT

Polysaccharide fraction	*Yield*[a] (%)	*Sulphate* (%)	*Pyruvic acid* (%)
Difco Bacto agar	100	2.96	0.96
Remaining polysaccharide after washing at 20° and 50° (Fraction 3)	50	0 95	0 21
Fraction 3, precipitate with polyethylene glycol (Russell *et al.*[17])	31	0.65	0.05
Polysaccharide in water eluate when Difco Bacto agar is added to DEAE Sephadex	25	0.09	0.01
Polysaccharide in water eluate when Fraction 3 is added to DEAE Sephadex	10	0.05	[b]

[a]Yield expressed as % of total agar. [b]Not detectable.

corresponding decrease in yield when expressed as a percentage of the total agar (Table III).

It was also observed that there is decreasing gel strength with increasing sulphation and decreasing content of 3,6-anhydro-L-galactose, but some of the highly charged fractions are still capable of forming a firm gel. Both these observations are in agreement with Rees' concept[27] that the gel-forming capability is due to the three equatorial hydrogen atoms on the 3,6-anhydro-L-galactose residues which constrain the polysaccharide into a helix. Interactions of helices cause gel formation. Any replacement of a sulphated galactose for a 3,6-anhydro-L-galactose residue changes the conformation of the L residue, hence causing a kink in the helix, and gelling ability is therefore impaired. From Table II, it can be seen that both the percentage of pyruvic acid and sulphate do not increase with the ionic strength of the eluant, the masking of the basic unit with 4,6-*O*-(1-carboxyethylidene)-D-galactose reaching a peak when the ionic strength of the eluant is 0.8M sodium chloride. Similarly, Fraction 2, eluted with dilute saline at 50°, has a higher pyruvic acid content than Fraction 1 which was eluted at 20° (Table I). This indicates that the masking of the basic chain with 4,6-*O*-(1-carboxyethylidene)-D-galactose groups occurs away from the sulphated galactose residues. Whether the masking occurs as 4,6-*O*-(1-carboxyethylidene)-D-galactose on some polysaccharide molecules and sulphated galactose residues on others or whether the 4,6-*O*-(1-carboxyethylidene)-D-galactose residues are laid down away from the sulphated galactose residue on one particular chain is difficult to decide.

The presence of 4,6-*O*-(1-carboxyethylidene)-D-galactose residues would not be expected to affect the gel strength as much as the sulphated units for they do not interfere with the equatorial hydrogen atoms on the 3,6-anhydro-L-galactose residues; nor do they cause kinking in a molecule as the conformation of the molecule is not altered.

The net charge on a solely "pyruvated" chain would be considerably reduced at low pH, whereas the "sulphated" molecules would still be fully ionised. An attempt was therefore made to fractionate "pyruvated" and "sulphated" molecules. The fraction eluted at 50° from agar (2, Table I) was refractionated on a column of DEAE Sephadex A-50 at pH 3.4. Because of the need to run the column at 50°, in order to prevent gelling of the polysaccharide, control of pH was necessary to avoid excessive hydrolysis of the polysaccharide. The polysaccharides were eluted with an increasing molarity of sodium chloride as shown in Fig. 2. The analytical data for the three fractions are expressed in Table IV.

A complete separation of "pyruvated molecules" was not achieved but pyruvate-enriched and sulphate-enriched fractions were obtained. Agar cannot therefore be regarded as containing two components, neutral agarose and charged agaropectin, but is a complex range of polysaccharides varying in total charge content. Agar may be regarded in terms of extremes of structure, as has recently become apparent for the carrageenan mixture of polysaccharides[28,29].

Complete agar is thought to consist of chains having alternating α-(1→3) and

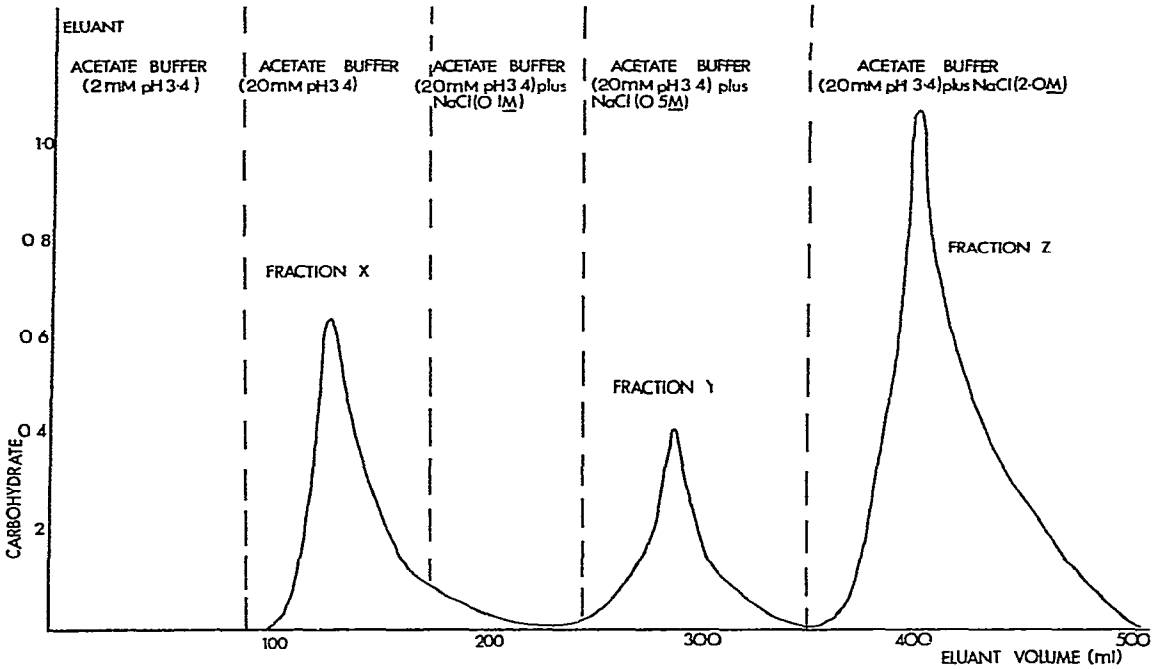

Fig. 2. Elution of Fraction 2 from DEAE Sephadex A-50 with a gradient concentration of sodium chloride in acetate buffer (pH 3.4). Assay by phenol–sulphuric acid (———).

TABLE IV

FRACTIONATION OF AGAR FRACTION 2 (TABLE I) ON DEAE SEPHADEX A-50 AT pH 3.4

Polysaccharide fraction	*Eluant*	*Yield*[a] (%)	*3,6-Anhydro-L-galactose* (%)	*Sulphate* (%)	*Pyruvic acid* (%)
Fraction 2 before fractionation	—	100	19.4	4.1	1.3
X	Acetate buffer (20mM, pH 3.4)	20	18.0	4.8	1.7
Y	Acetate buffer (20mM, pH 3 4) plus NaCl (0.5M)	15	33.3	2.0	2.5
Z	Acetate buffer (20mM, pH 3.4) plus NaCl (2.0M)	32	8.3	9.4	0.9

[a]Expressed as % of polysaccharide added to the column.

β-(1→4) linkages, and the extremes of structure are therefore as follows. (*a*) *Neutral agarose.* The idealised structure put forward by Araki of a molecule of alternating (1→4)-linked 3,6-anhydro-α-L-galactose and (1→3)-linked β-D-galactose residues containing no charge groups. The data presented in this paper suggest that this ideal molecule is present in very small proportion, if at all, in Difco Bacto agar. (*b*) *Pyruvated agarose with little sulphation.* From the first extreme of neutral agarose, the

masking of the basic structure with charged groups gradually increases. The D-galactose residues are substituted by 4,6-*O*-(1-carboxyethylidene)-D-galactose residues until the substitution reaches approximately one in twenty. At this point, the concentration of sulphate is 2%, indicative of substitution of the 3,6-anhydro-L-galactose residues with galactose sulphate. (*c*) *Sulphated galactan containing no or little 3,6-anhydro-L-galactose or 4,6-O-(1-carboxyethylidene-D-galactose residues.* From the second extreme, the substitution of the D-galactose residues by 4,6-*O*-(1-carboxyethylidene)-D-galactose residues gradually decreases and the concentration of galactose sulphate increases to the third extreme, a non-gelling, sulphated galactan.

The ratio of each extreme will be different for agars from different species and possibly with the time of year and stage in growth of the plant. The yield of each extreme can be expressed as in Fig. 3. This Figure explains why commercial preparations of agarose contain charged groups, and also explains why the yield becomes progressively less when preparing agarose with fewer and fewer sulphate and pyruvate groups (see also Table III).

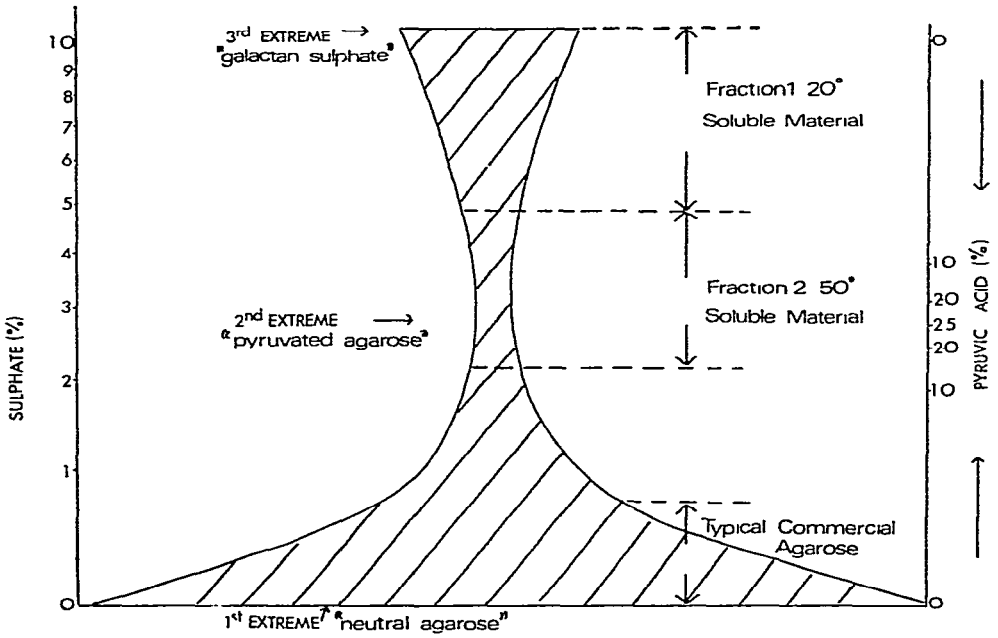

Fig. 3. The complexity of the spectrum of polysaccharides in Difco Bacto agar. The shaded area represents complete agar. The area within the curves between any lines drawn horizontally indicates the approximate amount of polysaccharide having that number of charged groups.

The practical definition of agarose will have to indicate that agarose is not a neutral polysaccharide obtained by separation from the charged polymer agaropectin. Agarose is that mixture of agar molecules having lowest charge content, and therefore the greatest gelling ability, fractionated from a whole complex of molecules, called agar, all differing in their extent of substitution with charged groups.

EXPERIMENTAL

Fractionation of agar. — (*a*) *By washing the polysaccharide at 20° and 50°.* Agar (100 g of Difco Bacto agar) was suspended in 50mM sodium chloride at room temperature in a jacketted column (50 × 8 cm). The agar was then washed with 50mM sodium chloride at 20° until the eluant became polysaccharide-free (phenol–sulphuric acid reagent[30]). The dilute solution of sodium chloride was used instead of distilled water so as to prevent excessive swelling of the polysaccharide. The polysaccharides in the eluants were precipitated with ethanol (4 volumes). Water at 50° was then circulated through the jacket, and the agar was washed with saline at 50° until the eluant was again polysaccharide-free. This fraction was also precipitated with ethanol (4 volumes). Water at 100° was then circulated through the jacket until the remaining polysaccharide was in solution. This solution was filtered to remove debris and poured into ethanol (4 volumes) to precipitate the polysaccharide. Analytical data for the fractions are given in Table I.

(*b*) *By column chromatography on DEAE Sephadex A-50.* DEAE Sephadex (Pharmacia) was prepared in the chloride form by washing with 0.5M hydrochloric acid, 0.5M sodium hydroxide, and 0.5M hydrochloric acid. The gel was then washed extensively with water to ensure that the pH of the eluant was that of distilled water. The gel was packed into a jacketted column (30 × 4 cm) maintained at 50–60°. The temperature of the eluant was also 50–60°.

A solution of Difco Bacto agar (2 g) in distilled water (90 ml) was added to the Sephadex gel. The gel was eluted with water, and fractions (10 ml) were collected until the eluant became polysaccharide-free. A gradient concentration of sodium chloride (0→3M) was then used to elute the more highly charged polysaccharide molecules. When the eluant became polysaccharide-free, the Sephadex gel was eluted with sodium chloride solution (5M) to ensure that as much polysaccharide as possible had been eluted. Appropriate fractions were combined, dialysed, precipitated in ethanol (4 volumes), and centrifuged. The polysaccharides were air-dried; the elution curve is shown in Fig. 1.

In a larger scale experiment, Difco Bacto agar (12 g) was added to a larger column of Sephadex (50 × 8 cm). The following concentrations (M) of sodium chloride were used to elute the polysaccharide fractions, 0, 0.2, 0.5, 1.8, 2.4, and 3.0 (Table II). The polysaccharides in each eluant were recovered as described above.

(*c*) *Further fractionation of Fraction 2 (Table I) on DEAE Sephadex A-50 (Cl^-) at pH 3.4.* DEAE Sephadex (Cl^- form) was suspended in acetate buffer (2mM at pH 3.4). The Sephadex gel was then stirred for 1 h before being packed into a jacketted column (30 × 4 cm) maintained at 50°. A solution of Fraction 2 (2 g) in water (50 ml) was added to the Sephadex gel. The gel was washed with acetate buffer (2mM, pH 3.4), and the polysaccharide fractions were then eluted with acetate buffer (20mM, pH 3.4) and solutions of increasing ionic strength of sodium chloride (0.1M, 0.5M, 2.0M) in acetate buffer. The fractions were dialysed overnight against warm, running water and

then precipitated with ethanol (4 volumes). The precipitates were removed by centrifugation and air-dried before analysis (Table IV).

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