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SOME NEW METHODS FOR THE PREPARATION OF AGAROSE

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SUMMARY

The possibility of preparing agarose from agar by extraction, batchwise ion exchange, and electrophoresis has been investigated. The efficiency of each of these purification steps has been established by analyzing the product obtained for sulfur content and for adsorption of the basic substances crystal violet and cytochrome C, both of which are strongly adsorbed to agar and many other anticonvection agents. These analyses also indicate that the sulfur content is not a reliable indicator of the adsorption capacity of agarose.

These investigations showed that a combination of extraction (or, although somewhat more laborious, electrophoresis) with an ion-exchange step gives an agarose of an extremely low sulfur content (0.04–0.06 %). This agarose exhibits no adsorption of crystal violet or cytochrome C.

The author has previously reported that chromatography* of agar yields an agarose showing low electroendosmosis and adsorption, but the low capacity of the method made it unsuitable for preparative purposes. This problem has been circumvented by the introduction of the above extraction step.

INTRODUCTION

Agar has two main constituents: agarose and agaropectin. The former component is a linear polysaccharide consisting of alternating residues of D-galactose and 3,6-anhydro-L-galactose, while the latter is a poorly defined polysaccharide containing sulfuric and carboxylic acid residues[1]. Because of its neutral character, agarose is superior to agar as an anticonvection agent for electrophoresis[2–5], immunodiffusion experiments[5,6], and as bed material in chromatographic molecular sieving[7,8].

Agarose has chiefly been prepared from agar according to the three methods discussed below:

One method is based upon the different solubilities of acetylated agarose and acetylated agaropectin[2,9] in chloroform. This method has served well the purpose for which it was introduced, namely the elucidation of the structure of agar[9], but is

* *Footnote added after the paper had been accepted.* A chromatographic procedure for the preparation of agarose has also been utilized by B. A. ZABIN[17], S. J. BARTELING[18] and M. DUCKWORTH AND W. YAPHE[19]. The last authors also state that the concept that agar is made up of two polysaccharides, neutral agarose and charged agaropectin, is an oversimplification (see also the very interesting papers on the structure of agar, mentioned in refs. 20 and 21).

impractical for the routine preparation of agarose[2]. In addition, the agarose obtained is somewhat degraded.

The present author has reported a simpler method which involves the precipitation of agaropectin by quaternary ammonium compounds, such as cetyl pyridinium chloride[10,11]*.

Agaropectin can also be precipitated with polyethylene glycol, as shown by RUSSELL *et al.*[12]; but it is apparently difficult to remove completely the excess of polyethylene glycol.

The alternative methods to be described in this paper are easy to perform. Some of them give an agarose showing less adsorption and having a lower sulfur content than that obtained by any of the three methods described above; and these new procedures are therefore superior for the preparation of agarose for use as a supporting medium in electrophoresis, chromatography, and immunodiffusion.

METHODS

(*I*) *Extraction of agaropectin with phosphate buffer*

(*a*) *Extraction from a granulated gel of agar.* Difco Bacto agar (20 g) is dissolved by boiling in 1 l of 0.03 *M* sodium phosphate buffer, pH 6.8. The solution is allowed to stand at room temperature until a firm gel has formed. The gel, which has an agar concentration of 2%, is pressed through a 60-mesh net, as described in ref. 13. About 3 l of 0.03 *M* sodium phosphate buffer, pH 6.8, is added to the gel grains. After stirring at room temperature overnight the grains are allowed to sediment and the buffer is drawn off and replaced by another 3-l portion. The slurry is stirred overnight. The extraction process is repeated twice over a period of two days with a change of buffer each day. The extracted agar is washed with water on a Büchner funnel.

This simple extraction procedure reduces the sulfur content to about 0.17% (see Table I), which is still too high for many purposes (crystal violet and cytochrome C are still adsorbed). In such cases the product can be purified further as described in section IIIa.

If the extracted agar is desired in the form of a dry powder the gel grains can be freeze-dried or, alternatively, liquefied by heating in a water bath and then precipitated by 3–4 volumes of ethanol. The efficiency of the precipitation is improved by including some sodium chloride (about 5 g)[11]. After some hours a firm precipitate is formed on the bottom of the container. The supernatant is decanted. (If necessary, the last traces of sodium chloride can be removed by washing on a Büchner funnel with 70% ethanol[11].) After washing with ether the agar(ose)** is air-dried in a hood at room temperature. The yield is about 15 g.

If water is substituted for phosphate buffer in the extraction the product

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* *Footnote added after the paper had been accepted.* J. BLETHEN[22] has modified this process by adding carrageenan to agar in order to co-precipitate the agaropectin as the quaternary ammonium salt. G. Y. AJITSKY AND G. V. KABAZEV[23] and A. M. EGOROV *et al.*[24] have also prepared agarose by a precipitation method; ammonium sulphate was used as the precipitating agent. G. G. ALLAN *et al.*[25] have described a method for the precipitation of agaropectin with chitosan and chitin.

** As the sulfur content of the extracted agar is relatively high (see Table I), it is questionable whether this product should be called agar or agarose.

TABLE I

SULFUR CONTENT AND ADSORPTION PROPERTIES OF AGAR(OSE) PREPARED BY DIFFERENT METHODS

The adsorption of crystal violet was tested by electrophoresis in agar(ose) suspensions[4] and the adsorption of cytochrome C by chromatography on a 2% gel, granulated as described in ref. 13. The sulfur determinations were performed as described by GUSTAFSSON[14,15]; the combustion of the agar(ose) samples was carried out according to the method introduced by SCHÖNIGER[16]. The headings in the table are similar to those under METHODS to simplify correlation of the table with the text.

	Sulfur content (%)	*Adsorption of crystal violet*	*Adsorption of cytochrome C*
Difco Bacto Agar (untreated)	1.03	+	+
(I) Extraction of agaropectin with phosphate buffer			
(a) Extraction from a granulated gel of agar			
(1) gel concentration: 2%	0.17	(+)	+
(2) gel concentration: 4%	0.20	(+)	+
(b) Extraction from a dry powder of agar	0.25	(+)	+
(c) A combination of method Ia and method Ib	0.16	—	(+)
(II) Electrophoretic removal of agaropectin			
(a) Electrophoresis of a granulated gel of agar			
(1) gel concentration: 2%	0.08–0.12[a]	—	(+)
(2) gel concentration: 4%	0.09–0.16[a]	—	+
(b) Electrophoresis of a homogeneous (not granulated)		—	(+)
2% gel of agar	0.14–0.20[a]	+	+
(c) Electrophoresis of a powder of agar, extracted as described in method Ib	0.14	—	(+)
(III) Adsorption of agaropectin on DEAE ion exchanger			
(a) Starting material: 2% gel grains, extracted by method Ia	0.17	(+)	+
(1) 0.5 g DEAE-Sephadex per g dry starting material	0.078	—	—
(2) 0.25 g DEAE-Sephadex per g dry starting material	0.094	—	—
(3) 0.13 g DEAE-Sephadex per g dry starting material	0.129	—	(+)
(4) 0.06 g DEAE-Sephadex per g dry starting material	0.130	—	+
(5) 0.03 g DEAE-Sephadex per g dry starting material	0.142	(+)	+
(b) Starting material: agar powder, extracted by method Ib	0.25	(+)	+
(1) 0.5 g DEAE-Sephadex per g dry starting material	0.039	—	—
(2) 0.25 g DEAE-Sephadex per g dry starting material	0.058	—	—
(3) 0.13 g DEAE-Sephadex per g dry starting material	0.087	—	(+)
(c) Starting material: 2% granulated agar gel, purified by electrophoresis according to method IIa	0.08–0.12[a]	—	(+)
(1) 0.5 g DEAE-Sephadex per g dry starting material	0.060	—	—
(2) 0.25 g DEAE-Sephadex per g dry starting material	0.069	—	—
(3) 0.13 g DEAE-Sephadex per g dry starting material	0.078	—	—

[a] The lower sulfur value refers to the top of the electrophoresis column and the higher value to the bottom of the column.

obtained has a higher sulfur content (0.21 %). An increase of the concentration of agar in the gel grains to be extracted also results in a higher sulfur content: after extraction with phosphate buffer a 2 % gel exhibits a sulfur content of 0.17 %, while the corresponding figure for a 4 % gel is 0.20 % (see Table I).

(*b*) *Extraction from a dry powder of agar.* Of a 0.03 *M* sodium phosphate buffer, pH 6.8, 3 l are added to 20 g of agar (Difco Bacto agar). The extraction is performed by repeated washings with stirring during 4 days in a manner similar to that described in section Ia. The dry weight of the extracted agar is about 15 g, and its sulfur

content is about 0.25 % (Table I). Without dehydration, it can be purified further by the method described in section IIIb.

(*c*) *A combination of methods Ia and Ib.* If agar is first extracted as a dry powder and then in the form of a 2 % granulated gel, the sulfur content will be reduced from 0.25 % to 0.16 % (Table I). This product exhibits no adsorption of crystal violet and only slight adsorption of cytochrome C.

(*II*) *Electrophoretic removal of agaropectin*

This purification requires only very simple electrophoresis equipment. With the apparatus illustrated in Fig. 1 the procedure is as follows:

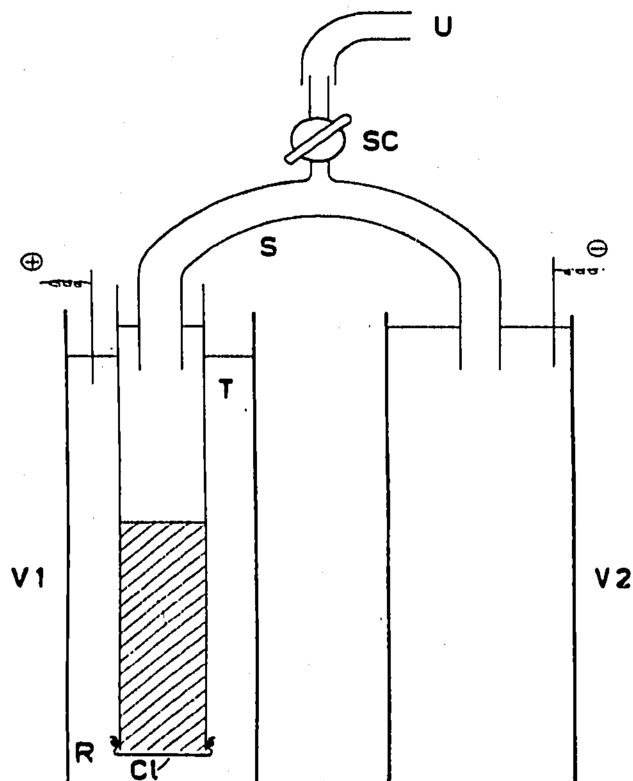

Fig. 1. T = electrophoresis tube (of glass or Perspex); S = siphon; SC = stop-cock; U = tubing; V1, V2 = electrode vessels; Cl = cellulose sponge cloth (Wettex); R = rubber bands (or O-ring).

(*a*) *Electrophoresis of a granulated gel of agar.* Difco Bacto agar (20 g) is dissolved by boiling in 1 l of 0.03 *M* sodium phosphate buffer, pH 6.8. After gelation the gel is forced through a 60 mesh net[13]. The gel grains obtained are suspended in 0.03 *M* sodium phosphate buffer, pH 6.8, and poured into the electrophoresis tube T. A piece of cellulose sponge cloth (Wettex)* is fixed to the tube by tightly stretched rubber bands (R) or an O-ring as a support for the gel bed. When the gel grains have settled the electrode vessels V1 and V2 are filled with buffer (0.03 *M* sodium phosphate, pH 6.8). Buffer is sucked up into the siphon S by coupling a water pump to tubing U (to prevent the gel bed from being disturbed its upper surface should be covered by a filter paper). If the electrophoresis tube has a diameter of 7 cm (as in our experiment), the current should be adjusted to about 70 mA. Should the buffer change its pH

* A dialysis membrane should not be employed as its pores seem to be too small to permit passage of agaropectin.

during the electrophoresis by more than one unit, it must be replaced by fresh buffer. After electrophoresis for 125 h the glass tube T is emptied onto a Büchner funnel and the interstitial water is thoroughly sucked off. The gel grains are liquefied and precipitated with ethanol as described in section Ia. The yield is about 13 g. The sulfur content of the agarose at the top of the gel column is 0.08 % and at the bottom 0.12 %.

If the buffer level in the right-hand electrode vessel V2 is higher than that in the left-hand one, there will be a downward flow of buffer through the gel column. This is recommended, because the electrophoretic purification will then be complemented by a chromatographic purification similar to the extraction procedure described in section Ia.

If the electrophoresis is performed on an agar gel of higher concentration (4 % instead of 2 %), the sulfur content of the product obtained is somewhat higher (see Table I). (A higher sulfur content is also obtained in the extraction method, described in section Ia, when the concentration of the granulated agar gel is increased from 2 % to 4 %, according to Table I.)

(*b*) *Electrophoresis of a homogeneous (not granulated) gel of agar*. This procedure gave a product with a sulfur content of 0.14–0.20 % and is thus inferior to method IIa, as indicated in Table I.

(*c*) *Electrophoresis of an agar powder, extracted as described in method Ib*. This procedure diminished the sulfur content from 0.25 % to 0.14 % (see Table I). The product obtained exhibited no adsorption of crystal violet and only slight adsorption of cytochrome C.

(*III*) *Adsorption of agaropectin to DEAE-ion exchanger*

(*a*) *Starting material: gel grains, extracted by method Ia*. The 2 % gel grains, extracted as described in section Ia, are *slowly* washed on the Büchner funnel with about 1 l of 0.01 *M* Tris–acetic acid, pH 8.0. The interstitial water is thoroughly sucked off. The drained gel grains are then liquefied by heating in a water bath (to keep the agarose concentration high, no water should be added). About 0.25 g of dry DEAE-cellulose (Type S-D 7 from Mikro-Technik, Miltenberg am Main, G.F.R.) or DEAE-Sephadex® A-50, 200–400 mesh (from Pharmacia Fine Chemicals, Sweden) is added with stirring for each gram of agar(ose)*, *i.e.* about 3.8 g of ion exchanger** are required. The stirring is continued overnight as 65° (in a water bath or oven). The DEAE-ion exchanger is filtered off on a warm Büchner funnel***. The agarose is precipitated by addition of alcohol as described in section Ia. About 12 g of agarose with a sulfur content of about 0.09 % are obtained (Table I). In cases when a lower sulfur content is required more than 0.25 g of DEAE-ion exchanger per gram of agar(ose)* must be used (see Table I).

(*b*) *Starting material: agar powder, extracted by method Ib*. The agar, extracted as described in section Ib, is transferred to a Büchner funnel and the phosphate buffer is sucked off. The agar is washed with water and then *slowly* with about 1 l of 0.01 *M* Tris–acetic acid, pH 8.0. The extracted agar (about 15 g) is dissolved by boiling in about 0.75 l of the Tris–acetic acid buffer (the agar(ose)* concentration should

* See footnote ** on p. 74.

** DEAE-cellulose from the companies Serva, Mannex and Whatman have a lower capacity for agaropectin than that from Mikro-Technik and DEAE-Sephadex from Pharmacia.

*** The DEAE-cellulose is somewhat preferable to DEAE-Sephadex as it has a lower degree of swelling and thus leaves a smaller amount of agarose on the Büchner funnel, giving a higher yield.

be about 2 %). With stirring, 3.8 g of DEAE-ion exchanger are added, *i.e.* the same amount of ion exchanger as in the above method IIIa. The stirring, the removal of the ion exchanger, the precipitation of the agarose, etc. are done as described in method IIIa. The sulfur content will be about 0.06 %, *i.e.*, lower than for the agarose obtained by method IIIa, although the sulfur content in the starting material used in that method is lower.

(*c*) *Starting material: agar gel, purified by electrophoresis according to method IIa.* Electrophoresis of a 2 % granulated agar gel yields a product with a sulfur content which is sometimes extremely low (0.08 %). Despite the low sulfur content cytochrome C is still slightly adsorbed (see Table I), but the adsorption can be completely eliminated by treatment with DEAE-ion exchanger in a manner analogous to that described in section IIIa, as indicated in Table I. The sulfur content of the agarose thus obtained is about 0.07 % (Table I).

DISCUSSION

It is noteworthy that agarose prepared by electrophoresis of agar gel grains exhibits adsorption of cytochrome C in spite of the fact that the sulfur content can be as low as 0.08 % (Table I). Agarose prepared by precipitating agaropectin with cetyl pyridinium chloride often has a higher sulfur content (about 0.13 %), but shows no adsorption of this basic protein[10]. The sulfur content is thus not a parameter that can be used alone to decide whether an agarose product is suitable as an anticonvection agent. Other factors, including the amount of the carboxylic residues in the product obtained, also play a role. We want to stress this because it seems to be generally believed that the sulfur content of an agarose is directly related to its adsorptive properties. In order to eliminate the cytochrome C adsorption completely, both the electrophoresis method and the extraction method must be followed by a step involving the use of DEAE-ion exchanger* (Table I). After this treatment the sulfur content can be as low as 0.04 % (Table I). The sulfur content can be further reduced by increasing the amount of ion exchanger per gram of agar(ose), which, however, results in a lower yield of agarose. Owing to its simplicity, method IIIb (extraction of dry agar powder combined with ion-exchange chromatography) is the method of choice for routine preparations of high quality agarose, using 0.25 g of DEAE-ion exchanger per gram of extracted agar**. When a slight cytochrome C adsorption can be tolerated and when an extremely inexpensive purification procedure is required, method Ic is recommended (extraction of agar first in the form of a dry powder and then in the form of a granulated gel). Methods Ic and IIIb have the advantage that they can easily be scaled up.

After electrophoresis of the granular agar gel the sulfur content can decrease to 0.08 %, while the same treatment of a coherent (not granulated) agar gel lowers the sulfur content to only 0.14–0.20 %. This finding might indicate that some of the substances responsible for the sulfur content of the agar (part of the agaropectin) migrate very slowly in the coherent gel — and consequently within a gel grain — but as soon

* Commercial agar can be treated directly with DEAE-ion exchangers (*i.e.* without pretreatment by electrophoresis or extraction) to give an agarose which exhibits no adsorption of crystal violet or cytochrome C. The drawback is, however, that relatively large amounts of ion exchanger are required (often more than 1 g of ion exchanger per gram of agar).

** This simple method has been used in our Institute since 1968 and proved very efficient.

as they have left a gel grain in a granulated bed they will migrate through the interstitial spaces and not through the gel grains. This view is in accordance with our observation that electrophoresis of a mixture of proteins on a granulated polyacrylamide gel gives a separation pattern similar to that obtained in free solution, even if these proteins migrate chromatographically on the same gel bed with different R values. The gel grains thus do not seem to exhibit any molecular-sieving properties during electrophoresis. Similarly, when serum is submitted to thin-layer electrophoresis on Sephadex G-200 the separation obtained resembles that observed in paper electrophoresis. That molecular-sieving effects apparently do not occur during electrophoresis in beds of gel grains might possibly indicate that proteins do not penetrate the entire gel grains even in molecular-sieve chromatography*.

Agarose with an extremely low sulfur content (0.08 %) can be prepared from gel grains of agar by *electrophoresis* as described in this paper (Table I). This finding may be explained in different ways, for instance by assuming that a part of the agaropectin is not gel-forming (agaropectin as a gel cannot be removed by electrophoresis). In this connection it should be pointed out that agaropectin is a heterogeneous mixture of polysaccharides (all polysaccharides in agar except agarose[1]). It is, therefore, not surprising if agaropectin contains both gelling and non-gelling components.

In some experiments we have used an agar with a sulfur content lower than 1 % as starting material, instead of Difco Bacto agar. As expected the sulfur content of the agarose obtained in these cases was less than those listed in Table I.

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* Numerous illustrations in articles on this chromatographic method indicate that most researchers assume — without discussion — that the *entire* gel grain is used in the separation process. It is difficult to decide experimentally if this assumption is correct, or if the separation takes place at the *surface* of the gel grain.

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