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AGAR DERIVATIVES FOR CHROMATOGRAPHY, ELECTROPHORESIS AND GEL-BOUND ENZYMES

I. DESULPHATED AND REDUCED CROSS-LINKED AGAR AND AGAROSE IN SPHERICAL BEAD FORM*

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SUMMARY

A method for producing alkali- and thermo-stable, insoluble spherical agar particles with very low adsorption capacity (tested with cytochrome C) is described. Sulphate ester groups present in the original agar are removed by alkaline hydrolysis of cross-linked agar, in bead form, at an elevated temperature. The reaction is performed in the presence of sodium borohydride to prevent simultaneous oxidation.

Further reduction of the adsorption capacity may be accomplished by treatment of the gel with lithium aluminium hydride in dioxane.

Desulphated and reduced agar and agarose gels can be packed in beds with excellent flow and molecular sieve properties.

INTRODUCTION

Hydrophilic polymers, whether cross-linked and/or substituted, constitute valuable separation media in biochemistry and related branches of science. It will suffice to mention the rôle of CM- and DEAE-celluloses for protein fractionation based on ionic adsorption; Sephadex and Bio-Gel (polyacrylamide) for molecular sieving and as matrices for gel-bound enzymes; and starch and polyacrylamide gels as anti-convection media in electrophoresis.

In spite of many desirable properties, cellulose, starch, dextran and polyacrylamide all have disadvantages, some of which derive from undesirable affinities for the substances to be fractionated when employing these polymers. The affinity may depend on sparsely distributed adsorption centres in the polymer. There is strong evidence that these centres usually consist of carboxylic groups which lead to adsorption by electrostatic attraction.

Furthermore, the matrix-fixed charged groups are partly responsible for the zone spreading in the gel filtration of proteins and particles. Loss of material resulting from irreversible adsorption is often encountered, especially at low ionic strengths

* Patent pending.

and low pH. Insoluble polymers for use as biospecific adsorbents in affinity chromatography etc., should not contain groups which would cause a decrease in specificity. When zone electrophoresis is performed with gel particles to prevent convective material transport, electroosmosis is a disturbing hindrance factor in addition to adsorption. A completely neutral matrix would also be preferable for gel-bound enzymes.

Experience at this Institute and elsewhere has shown that the ionic groups fixed to the matrix, even when present at a very low density, contribute substantially to the adsorption of high molecular weight polyelectrolytes, and indeed quite often make the polymeric gel medium useless for gel filtration or electrophoresis.

A gel polymer of wide applicability should permit formation of a bed with good flow properties. The best beds for chromatography and electrophoresis are obtained with hard, homogeneous spherical beads. Contaminating substances, *e.g.* antigens, should not leak from the gel. It should be possible to introduce desirable substituents, if necessary under drastic conditions. In conclusion the ideal polymer should fulfil the following requirements. (1) It can be wetted and permeated by water; (2) it is insoluble; (3) it is chemically resistant under the operation conditions; (4) it should not contain charged or ionogenic groups; (5) it should form mechanically stable, hard, incompressible particles; (6) it should contain functional groups (*e.g.* hydroxyl groups), and (7) its molecular permeability should be variable within wide limits.

Agar is the natural product known to approach these properties most closely. However, agar gels of matrix density lower than 2 % are quite elastic and even at fairly low applied pressures tend to form beds with a high resistance to flow.

Agar is considered to consist of the polysaccharides agarose and agaropectin. The agaropectin has a higher content of sulphur and charged groups than agarose. ARAKI[1] has made valuable contributions to our knowledge of the chemistry of agar.

Agar was first introduced by POLSON[2] as a molecular sieving gel medium. HJERTÉN[3] described the use of agarose as a more suitable molecular sieve and stabilizer for convection in electrophoresis. Agarose, like agar, has excellent gel properties but is a weak ion exchanger. By cross-linking agarose, SCHELL AND GHETIE[4] obtained a gel suitable for the synthesis of ion exchangers for protein chromatography.

Two kinds of groups causing adsorption are present in agar: (1) sulphate monoester groups[5] and (2) carboxyl groups[6,7]. The number of sulphate and carboxyl groups vary considerably in technical agar. It should presumably be possible to remove the sulphate ester groups by strong alkaline hydrolysis, for example.

However, agar cannot be treated directly with strong alkali at the temperatures necessary for complete hydrolysis since a brown-coloured, more or less soluble, product is then formed. In this paper a method is described which enables cross-linked agar to be extensively desulphated. A gel product superior to agarose as a material for chromatography is thus obtained, circumventing the cumbersome isolation of agarose from technical agar. A prerequisite is agar with low uronic acid content.

EXPERIMENTAL

Materials

Agar was obtained from Kadoya, Japan, and Difco (Bacto-agar); Sepharose from Pharmacia Fine Chemicals; Bio-Gel A from Bio-Rad; epichlorohydrin (technical,

98 %) from Kebo, Stockholm; bisepoxides from Schuchardt; sodium borohydride (97–98 %) from Schuchardt; thyroglobulin and cytochrome C from Sigma, albumin from Kabi, Stockholm. Tobacco mosaic virus and "satellite virus" were gifts from Dr. STEFAN HÖGLUND, Dr. STURE BRISHAMMAR, and Dr. BROR STRANDBERG; poliovirus and adenovirus were gifts from Dr. Bo ÖBERG and Dr. LENNART PRAGE, respectively. Other chemicals were of analytical grade.

Analysis

Carbohydrate determinations were made according to the orcinol method[8]. Sulphur analyses were performed by Mrs. ALINE STRAUTMANIS at the Institute for Analytical Chemistry, Uppsala.

Determination of adsorption capacity

The gel was packed as a bed of 0.5 cm diameter and 2.5–3.5 cm height, and was equilibrated with 0.01 *M* ammonium acetate, pH 4.1. A 0.1 % cytochrome C solution (with the same buffer) was introduced until saturation. After washing until no more protein appeared in the eluent, the cytochrome adsorbed was displaced by 0.15 *M* ammonium acetate buffer of pH 4.1. The amount of cytochrome was determined in the eluate (absorptivity of cytochrome C is 1.54 $cm^2\ mg^{-1}$ at 280 nm).

After each determination the gel was washed with 0.5 *M* sodium hydroxide followed by distilled water until the eluate was neutral. The gel was lyophilized and weighed but no correction was made for residual water.

Production of agar particles

Spherical agar particles were produced by emulsifying a hot agar solution of the desired concentration in an organic solvent in the presence of a suitable emulsifying agent. The procedure has been described in detail by HJERTÉN[9].

Development of preparation technique

About 1 g swollen agar or agarose particles was suspended in (approximately) 5 ml 1 *M* NaOH and the suspension was heated in a boiling water bath. The sample became yellow in colour which progressively deepened. The particles dissolved and a dark brown solution was obtained.

5 g portions of swollen agar or agarose particles were transferred to 25-ml erlenmeyer flasks followed by 5 ml of 1 *M* NaOH and various amounts of epichlorohydrin. The stoppered flasks were placed on a thermostated shaking board and kept at 60°. After 2 h the temperature was increased to 80° and finally to 93° with a 2 h reaction time at each temperature. The gel in each sample was filtered on a Büchner funnel and washed thoroughly with hot water. The concentration of carbohydrate in the washings was determined. The gel was autoclaved in 10 ml 1 *M* NaOH at 120°. The solution was collected and the amount of carbohydrate extracted was determined. Usually 25–35 % of the original carbohydrate was lost in the cross-linking reaction. A higher yield (*i.e.* less extraction) can be obtained with a preincubation at a lower temperature.

The following experiments were carried out with Sepharose 2B, 6B and Bio-Gel A-1.5 m to find the dependence of the adsorption capacity on the extent of oxidation.

(1) In a series of experiments 20 g of gel, swollen in water, were treated with 18 ml of 1 *M* NaOH and 2 ml of epichlorohydrin at room temperature. The suspension was heated to 60° with stirring and the reaction stopped after 2 h. The gel was washed free of alkali with distilled water and heated in an autoclave for 1 h with 1 *M* NaOH at 120°. The gel became weakly yellow in colour.

(2) Experiments were conducted as in (1) except that 40 mg of sodium borohydride were added to the reaction mixture. As a result white gels were obtained. The adsorption capacity for cytochrome C in 0.01 *M* ammonium acetate buffer, pH 4.1, was determined as well as the sulphur content (Table I).

TABLE I

ADSORPTION CAPACITY FOR CYTOCHROME C (CONDITIONS DESCRIBED IN THE TEXT) AND SULPHUR CONTENT OF VARIOUS AGAROSE AND AGAR GELS

Gel type	*Adsorption capacity*	% *S*
Commercial agarose A 2 %	0.124	0.179
ECD-agarose A	0.007	0.028
Commercial agarose B 6 %	0.102	0.182
ECD-agarose B	0.005	0.026
Commercial agarose C 6 %	0.080	0.118
ECD-agarose C	0.008	0.021
Commercial agar 6 % (beads)	0.240	0.371
ECD-agar 6 % (beads)	0.060	0.049
Reduced ECD-agar 6 % (beads)	0.004	0.012

(3) 10 g of wet agar gel (2 %) was placed in each of 10 flat-bottomed flasks. A series of ice-cold sodium hydroxide solutions, 2, 4, ... 20 % in 1,3-bis-(2,3-epoxypropoxy)butane, was prepared in the flasks and 0.5 % $NaBH_4$ was added to each sample.

The contents were shaken in an ice bath for 15 min and then in a water bath at 60° with shaking. After 7 h heating the gel products were washed with hot distilled water. No discoloration was observed and the beads showed no change in appearance.

The samples were autoclaved at 120° in 1 *M* NaOH and 0.5 % $NaBH_4$. The beads retained their shape and white colour perfectly.

Adopted methods of preparation

(*A*) *Epichlorohydrin cross-linked desulphated agar* (*"ECD-agar"*)

1 l of swollen agar or agarose beads was mixed at room temperature with 1 l of 1 *M* NaOH containing 20 ml epichlorohydrin and 5 g sodium borohydride. The gel had previously been thoroughly washed with distilled water. The mixture was heated to 60° for 1 h with adequate stirring.

The cross-linked gel so obtained was washed with *hot* distilled water to neutral reaction. 500 ml 2 *M* NaOH and 2.5 g $NaBH_4$ were added to 500 ml of the suspension and the mixture was heated in an autoclave at 120° for 1 h. The gel was washed with 1 *M* NaOH containing 0.5 % $NaBH_4$, in the first plate with 1.5 l of hot solution and then with 1.5 l cold solution. The gel was quickly transferred to a beaker containing finely crushed ice and acetic acid was added to pH 4.0. The gel was again transferred

to a Büchner funnel and washed with hot distilled water (to remove the remaining traces of boric acid) and finally with ice water. The gel was stored as a suspension in 0.02 % sodium azide.

(*B*) *Bisepoxide cross-linked desulphated agar* (*"BCD-agar"*)

250 g of wet gel, Sepharose 6B, were chilled in crushed ice and mixed with an ice-cold solution of 25 g 1,3-bis-(2,3-epoxypropoxy)butane in 225 ml 1 *M* NaOH containing 100 mg of $NaBH_4$. The contents were shaken, first in the cold for 15 min, then at room temperature for 30 min and finally at 60° for 2 h. The gel was autoclaved as in (A).

(*C*) *Reduced ECD-agar*

300 ml ECD-agar were lyophilized and the dry gel was treated with 600 ml of a mixture of pyridine and acetic anhydride 1:1 (v/v). The acetylation was carried out with 40 ml acetyl chloride at 60° for 75 min. Perfect spherical beads were obtained which swelled in chloroform and were light yellow in colour.

The acetylated ECD-agar was transferred to a 500-ml round-bottomed flask and 250 ml dry dioxane were added. 10 g of lithium aluminium hydride were added in portions to the reaction mixture under a stream of nitrogen, through the flask, with cautious stirring. The temperature was slowly elevated. At 45° the reaction started and tended to be vigorous. To control the reaction temporary cooling is desirable. The temperature was finally raised to 80° and kept there for 2 h.

The reaction was stopped by the addition of ethyl acetate followed by water. The reaction mixture was cooled. During cooling in an ice bath 1 *M* HCl was added until the precipitate was completely dissolved. The beads were briefly washed with chilled 0.1 *M* HCl followed by water.

Deacetylation was accomplished by heating the beads in 1 *M* NaOH and 0.1 % $NaBH_4$ at 80° for *ca.* 15 min.

The adsorption capacity for cytochrome C was determined in 0.001 *M* ammonium formate, pH 3.8 (see Table I).

STABILITY OF THE PARTICLE SHAPE

Particle shape is an important factor with respect to the flow properties of chromatographic columns. Spherical agar beads are easily made but it is not known to what extent the beads could resist harsh treatment without being damaged and disintegrated.

Samples were taken at various stages during the preparation of commercial, uncross-linked and cross-linked agar (or agarose) and were inspected under the microscope. It was found that the cross-linked agar (and agarose) in contradistinction to Sepharose and Bio-Gel A resisted not only strong alkali and high temperature but could also be freeze-dried and be expanded again in water to the original size while retaining their spherical shape. No damaged particles were observed in the preparations.

By washing water-swollen ECD-agar beads in the following order: propanol, dimethyl sulphoxide, dioxane and hexane, it was possible to retain the spherical shape. The volume contraction of the beads was found to be reversible.

Solvent stability

In order to study the stability of ECD-agar as compared to Sepharose gels in dimethyl sulphoxide (DMSO) and in concentrated solutions of the two chaotropic ions, iodide and thiocyanate, the following experiments were performed:

ECD-agar (2 %) and Sepharose 2B were extensively washed with distilled water on Büchner funnels. 30 g samples of well drained gel were then transported to small Büchner funnels and each type of gel was treated three times with 60 ml of the following solvents: concentrated DMSO (60 ml at 20°, 2 × 60 ml at 100°), 4 *M* KI and 3 *M* KSCN. Finally, the gels were washed with 100 ml distilled water. The wash-liquids were dialysed extensively against distilled water and the carbohydrate content was determined after lyophilization of the dialysates. The results are presented in Table II.

TABLE II

SOLVENT STABILITY OF ECD-AGAR AS COMPARED TO AGAROSE GEL

Gel	*Amount of carbohydrate dissolved under conditions given in the text (g/100 g dry substance)*		
	DMSO, 100°	*4 M I^-*	*3 M SCN^-*
ECD-Bacto agar 2 %	0.5	0.2	<0.03
Agarose gel 2 %	100	100	*ca.* 90

CHROMATOGRAPHY

*Chromatography of viruses on 2 % ECD-agar**

A chromatographic column (1.5 × 78.5 cm) was packed by allowing a suspension of 2 % ECD-agar particles in 0.05 *M* Tris–HCl, pH 7.5, with 0.02 % NaN_3 to settle. 1 ml of a mixture of tobacco mosaic virus (1 mg TMV/ml) and satellite necrosis virus (0.8 mg STNV/ml) were introduced into the column. The chromatogram was developed with the buffer at a flow rate of 7 ml per h (*ca.* 4 ml $cm^{-2}h^{-1}$). Fractions were collected and the absorption at 280 nm measured (Fig. 1). Similarly, a mixture of ^{32}P-labelled adenovirus (50000 c.p.m.) and poliovirus (200000 c.p.m.) were passed through the same column and the radioactivity of the eluate was measured (Fig. 1).

Protein fractionation on 6 % ECD-agar

A column (1.5 × 83 cm) of 6 % ECD-agar was prepared in a 0.05 *M* Tris–HCl buffer solution, pH 7.5, containing 0.02 % NaN_3. A solution containing thyroglobin, cytochrome C and phenylalanine, 10 mg of each, was introduced and chromatographed on the column at a flow rate of 10 ml per h. The absorption at 280 nm and the ninhydrin colour at 570 nm were measured in the collected fractions (2.2 ml each).

In another run a 3 ml sample containing albumin, chymotrypsin and glucose, 10 mg of each, was similarly chromatographed.

The two chromatograms are superimposed in Fig. 2a.

* Throughout this paper percentages refer to the agar content of the original uncross-linked beads.

Protein separation on reduced 6% ECD-agar

The experiment described above was repeated, the only difference being that the column material consisted of $LiAlH_4$-reduced ECD-agar. The results are shown in Fig. 2b.

DISCUSSION

Agar is degraded in strongly alkaline and acidic solutions. In this respect it is inferior to many other polysaccharides such as cellulose and dextran. The high concentration of ionogenic groups is another disadvantage. However, agar shows much promise as a matrix for molecular sieves and adsorbents.

To convert agar into gels suitable for molecular sieving or as a starting material

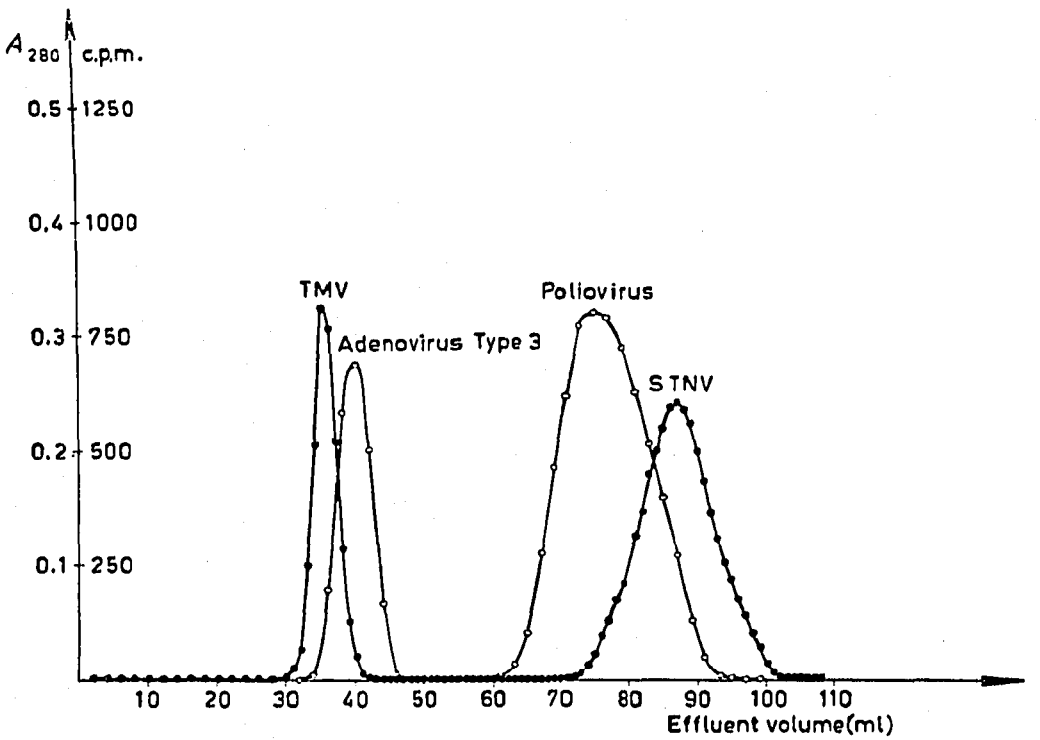


Fig. 1. Chromatogram obtained with an artificial mixture of viruses after passage through a 2% ECD-agar column. Particle weights: TMV, $39 \cdot 10^6$ (daltons); adenovirus type 3, $180 \cdot 10^6$; poliovirus, $6.8 \cdot 10^6$; STNV, $2 \cdot 10^6$. The experimental conditions are given in the text. (●—●) optical density (1 cm cuvette) at 280 nm; (○—○) counts per minute.

for the production of adsorbents and supports for zone electrophoresis and unsolubilized enzymes, it is necessary to improve substantially the physical and chemical properties of native agar.

SCHELL AND GHETIE[4,10] have converted agarose to a cross-linked product which could be used for chromatography and electrophoresis. Cross-linked agarose represents an important improvement over the materials used so far. Unfortunately, however, their product is far from ideal, the main reason being the fact that the cross-linking reaction is carried out in a large bulk phase. The procedures described here

also yield cross-linked agar products but with distinctly different properties. The procedures are simpler and yet give better defined products.

Although agar is a complex mixture of polysaccharides[1,11,12], cross-linking can be done without prior fractionation. Preferably, the agar, in the form of dispersed spherical beads, is treated with the epoxides. The conditions for cross-linking are mild enough to retain the shape of the particles. It is very important that the cross-linking is carried out on swollen gel beads. We have found that the concentration of cross-linking agent can be varied within very wide limits without altering the permeation much. It therefore appears that in the expanded matrix only some of the hydroxylic groups are available for cross-linking. In other words the matrix density of the original uncross-linked gel, rather than the conditions for the cross-linking reaction, determines the permeability properties of the final product.

Oxidation can be avoided during the reaction. The ionogenic groups can be

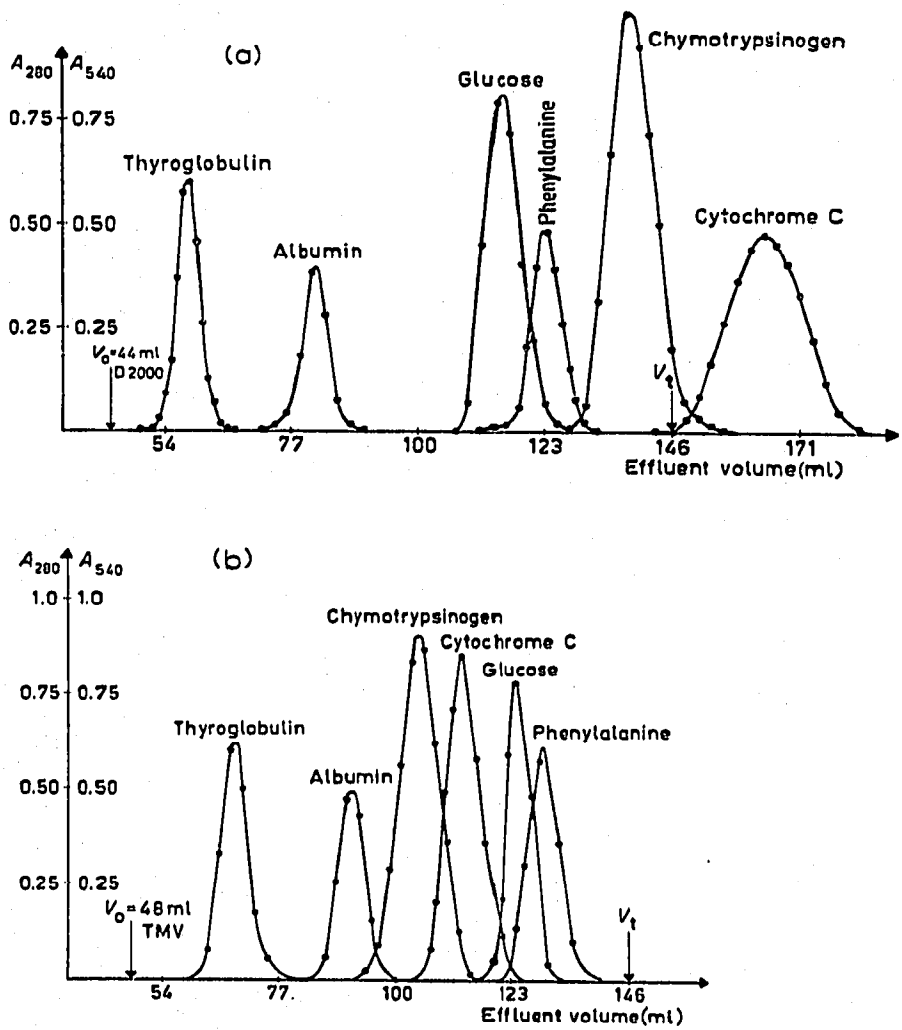
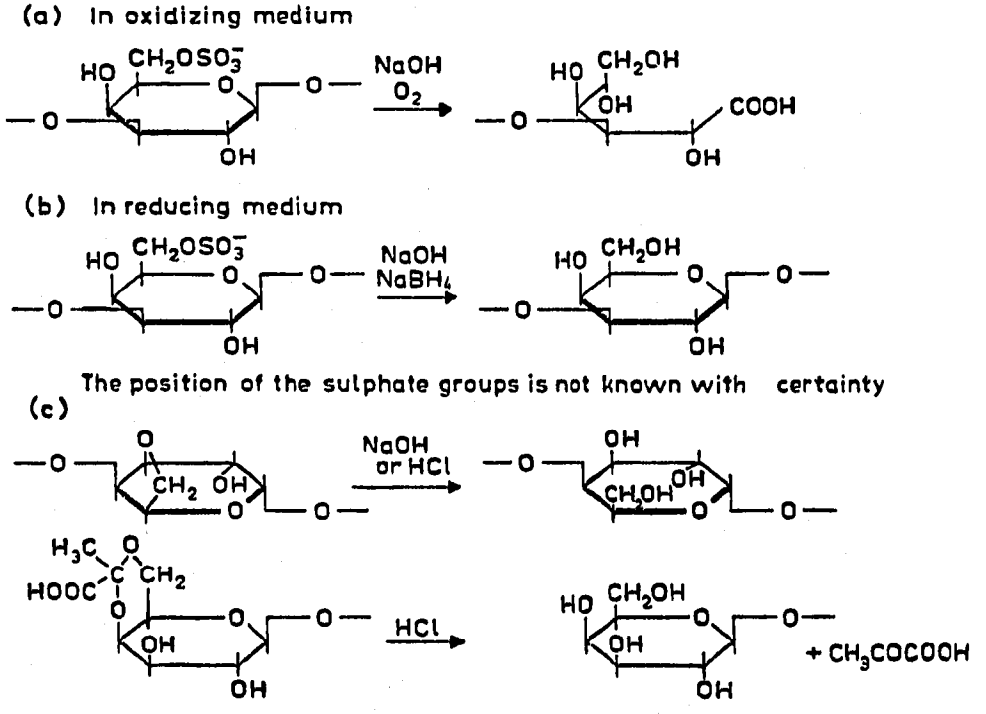


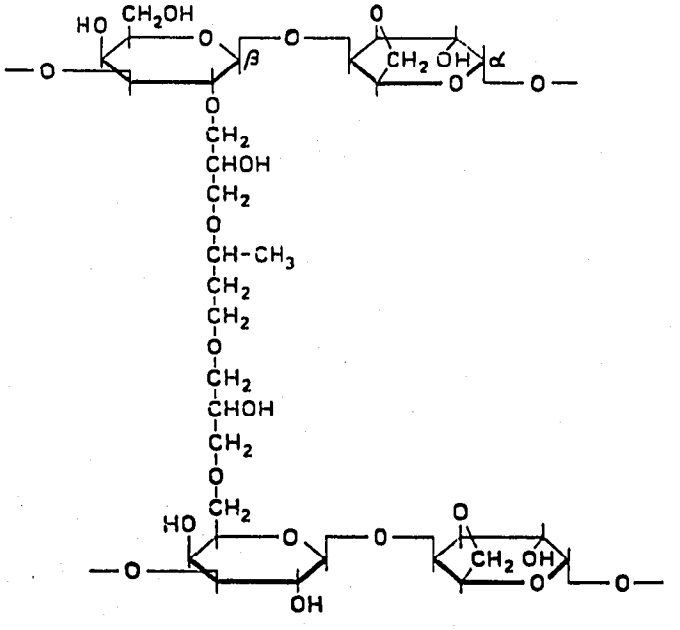
Fig. 2. (a) Chromatogram obtained with artificial mixtures of proteins and two low molecular weight substances after passage through a 6% ECD-agar column. The experimental conditions are given in the text. The void volume (V_0) was determined with a high molecular weight dextran (mol. wt. $2 \cdot 10^6$), D 2000 of Pharmacia Fine Chemicals AB, Uppsala, Sweden. (●—●) optical density (1 cm cuvette) at 280 nm for proteins and at 540 nm for glucose (orcinol reagent). V_t = total bed volume. (b) As in (a) with the following exceptions: the 6% ECD-agar gel was previously reduced with $LiAlH_4$ (see text). The void volume (V_0) was determined with a TMV suspension.

removed by the following steps. Sulphate groups are effectively removed by alkaline hydrolysis above 100° in the presence of the sodium borohydride. The remaining soluble agar is effectively removed during this treatment and in the washings following.

If the uronic acid content of the agar is low the ECD-agar is already a better material than any agar or agarose at present commercially available. If an extremely low adsorption capacity is desired the carboxylic group content may also be reduced. The various reactions likely to occur in the production of cross-linked desulphated agar are the following:



The cross-linking reaction with the bisepoxide gives the following structure:



It is possible that other hydroxyl groups than those shown here are involved in the cross-linking.

Cross-linked agar gels are characterized by a solute permeability which does not seem to be inferior to that of uncross-linked agarose with a corresponding matrix density. Gels of high macroporosity may be produced. Thus as a demonstration Fig. 1 shows that viruses of high particle weight are retarded in a 2 % ECD-agar bed. It should be noted that the rod-shaped TMV moves ahead of the spherical adenovirus in spite of the fact that the latter is of higher molecular weight. The shape of the distribution curves must be considered as very satisfactory in view of the relatively high flow-rate (4 ml cm^{-2} h^{-1}).

The proteins in the chromatogram on ECD-agar of higher matrix content appear in the order expected for molecular sieving as shown in Fig. 2a. However, since cytochrome C and chymotrypsin are retarded more than glucose and phenylalanine, molecular sieving alone cannot explain the chromatographic behaviour. The corresponding experiment performed in a bed of *reduced* ECD-agar is illuminating. In this case both cytochrome C and chymotrypsin move faster than the low molecular weight substances (Fig. 2b). Thus from the two chromatograms it may be concluded that ionic adsorption is indeed an important factor in the case of unreduced agar.

In practice, the limited adsorption of certain solutes on ECD-agar may be tolerated and may indeed be desirable in special cases. For example, it is evident that the chromatogram, Fig. 2b, obtained with reduced agar is more compressed than that on the ECD-agar. The separation of the proteins in the latter case is therefore substantially better. Obviously the remaining ionogenic groups in the ECD-agar improve the separation power of the gel by moving chymotrypsinogen and cytochrome C backwards. Native agar, however, is an unsuitable adsorbent for protein chromatography because it contains ionogenic groups of two different kinds with different dissociation constants and this fact makes desorption in a narrow range of pH or ionic strength impossible. It is our intention to utilize these findings and, starting from reduced ECD-agar, to introduce in a controlled manner, various kinds of ionogenic groups in order to study the requirements and limits for linear adsorption chromatography of biopolymers on agar gels.

The improvement in the chromatographic properties of desulphated cross-linked agar as compared with agar and agarose is attributed to a very much lower solubility and a lower content of ionogenic groups.

The rapid penetration of bisepoxides or epihalohydrins ensures uniform cross-linking of the agar gel in the expanded form. It was noticed that the concentration of the cross-linking agent had little effect on the volume of the gel.

The solubility is worth commenting on. As a consequence of the cross-linking, the stability towards alkali is much improved. In fact little material can be extracted from ECD-agar in boiling 5 *M* NaOH. Cross-linked agar is therefore a very suitable starting material for making ion exchangers for protein chromatography. The stability in acid solutions is, however, affected a little. After prior extraction with dimethyl sulphoxide, no leakage of carbohydrate could be detected even after extensive washing, either in the cold or at elevated temperatures, with ordinary buffer systems. Even in buffers containing high concentrations of chaotropic ions such as iodide and thiocyanate leakage is negligible (Table II). The insolubility is a necessary prerequisite in order that agar gels can be used in the purification of substances intended

for intravenous injections. Furthermore it is obvious that cross-linked, desulphated agar and agarose, reduced or unreduced, should be very superior to uncross-linked agarose for the production of adsorbents for both affinity chromatography as well as for unsolubilized enzymes.

In this context it is appropriate to point out that ECD-agar can be sterilized, *e.g.* by autoclaving, an obvious advantage over agar and agarose in the many possible applications such as the production of chemicals, vaccines etc. which are to be used clinically.

Finally another important property of agar should be noted. Since agar is a polysaccharide mixture of marine origin and with a structure very different from common carbohydrates, it is very unlikely that agar, or cross-linked agar in particular, would be attacked by agarase-producing microorganisms in the laboratory or factory.

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