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AGAR DERIVATIVES FOR CHROMATOGRAPHY, ELECTROPHORESIS AND GEL-BOUND ENZYMES

II. CHARGE-FREE AGAR*

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SUMMARY

A method is described for the preparation of charge-free agar by alkaline desulphation in the presence of sodium borohydride, followed by reduction with lithium aluminium hydride in dioxane. Charge-free agar can be used successfully for the electrophoresis of very basic proteins, for which commercially available agars and agaroses are unsuitable.

INTRODUCTION

Agar, or preferably agarose, has achieved increasing importance as a supporting medium in many types of chromatography of large molecules and viruses, as well as in electrophoresis, immuno-electrophoresis and electrofocusing. QUAST[1] has described certain advantages of agar compared with polyacrylamide gels in the latter technique.

The main disadvantages of agar derive from the more or less negatively charged polysaccharide matrix. The charge causes both irreversible adsorption of basic substances and electro-osmosis.

Detailed studies of the chemistry of agar, mainly by ARAKI[2], have shown that agar is a mixture of two polysaccharides: completely neutral agarose, composed of alternating residues 1 → 3-linked β-D-galactopyranose and 1 → 4-linked 3,6-anhydro-α-L-galactopyranose, and negatively charged agaropectin containing the above sugars substituted with sulphate and carboxylic groups in varying amounts. The sulphate groups are bound to the polysaccharide matrix by ester linkages, mainly in the 6-position. Carboxylic groups are present as pyruvated galactose (4,6-O-(carboxyethylidene)-O-galactopyranose).

This knowledge led to attempts to fractionate agar to obtain the neutral agarose, which would lack the disadvantages of agar itself. Many separation methods have

* Part I in this series of papers, "Desulphated and reduced cross-linked agar and agarose in spherical bead form", by J. PORATH, J.-C. JANSON AND T. LÅÅS, was published in *J. Chromatogr.*, 60 (1971) 167–177.

been developed, such as acetylation followed by extraction with chloroform[3], precipitation of the agaropectin with cetylpyridinium chloride[4], precipitation of agarose with polyethylene glycol[5] and ammonium sulphate[6] as well as adsorption of agaropectin on chitin and chitosan[7], on Dowex-2 and hydroxylapatite[4] and on DEAE-cellulose[8]. The combined use of extraction, batch ion exchange and electrophoresis for the preparation of agarose has recently been investigated[9]. All of these methods give agarose with better gelling ability and a lower charge density than the original agar. However, there are still considerable amounts of sulphate and carboxylic groups left in the agarose. Until recently, this was thought to be due to contamination with agaropectin. Studies in which agar and fractions of agar have been subjected to anion-exchange chromatography have shown that this is an over-simplification. IZUMI[10] states that the agar from *Gelidium amansii* (probably the most important Japanese agar) consists of a family of polysaccharides that have similar macromolecular structures, with continuously variable portions of acidic substitutents such as sulphate, pyruvic acid and uronic acid residues. He also suggests that the designation agaropectin should be discontinued since it can no longer have its original meaning. DUCKWORTH AND YAPHE[11] point out three extremes of structure: (a) neutral agarose, which is the idealized structure proposed by ARAKI (DUCKWORTH AND YAPHE concluded that this molecule is present in very small amounts, if at all, in Difco Bacto agar); (b) pyruvated agarose with little sulphation, where the D-galactose is substituted by 4,6-O-(1-carboxyethylidene)-D-galactose until the substitution reaches approximately one in twenty; (c) sulphated galactan containing little or no 3,6-anhydro-L-galactose or 4,6-O-(1-carboxyethylidene)-D-galactose residues: this extreme is composed of molecules with a sulphate content of 5–10%.

EXPERIMENTAL

Materials

Unless otherwise stated, the agar used was a gift from Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium borohydride (97–98%) was obtained from Schuchardt. Other chemicals were from Kebo, Stockholm, Sweden. Except for dioxane and lithium aluminium hydride, only analytical grade chemicals were used. Proteins for the electrophoretic experiments were from Sigma Chemical Company. MBI Electrophoresis Power Supply Type 264 and MBI Immuno-Electrophoresis Tank from Medical and Biological Instrumentation, Ashford, Kent, Great Britain, were used in the electrophoresis experiments.

Removal of water from agar beads

Agar beads (6%) were washed on a glass filter funnel with 6 portions of ethanol followed by 3 portions of methanol. Each portion had the same volume as the sedimented agar. The eluted solvent was collected and analysed for water by the Karl Fischer titration. The water content in the remaining product was determined by titration of the agar gel itself. The results are given in Table I and Fig. 1.

Cleaning of dioxane

To determine the best reduction conditions it was necessary to use extremely clean and water-free dioxane. Dioxane was purified by passing it through a cation

TABLE I

WATER CONTENT ACCORDING TO KARL FISCHER TITRATION IN DIFFERENTLY TREATED SOLVENTS AND IN "WATER-FREE" AGAR

Substance	*Water content (%)*
Agar after washing with 6 portions of ethanol and 3 portions of methanol (Fig. 1)	0.03
The last portion of methanol (Fig. 1)	0.03
Absolute methanol	0.02
Dioxane "purum"	0.18
Dioxane, anion-exchange treated	0.02
Dioxane, anion-exchange treated, distilled	0.02

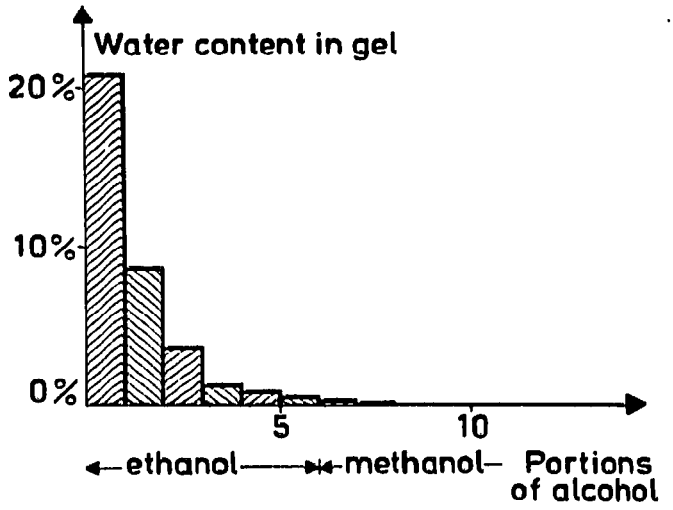

Fig. 1. Percentage of water in the suspending solvent, after washing 6% ECD-agar beads with alcohol. The volume of each portion of alcohol was equal to the volume of sedimented agar.

exchanger (Dowex 50W-X8 (H), 20–50 mesh)[12], followed by distillation in the presence of sodium borohydride. After the ion-exchange step only dried glassware was used.

Approximately 125 g of the dry exchanger were converted into the potassium form by washing twice with the calculated amount of potassium hydroxide. The ion exchanger was then washed with distilled water until neutral and dried overnight at 150°. After cooling to room temperature, the ion exchanger was put into a 250-ml cylindrical separation funnel and 350 ml of dioxane of "purum" quality* were slowly passed through the funnel. The dioxane was slightly contaminated with material from the ion exchanger (UV absorption) but the water content was greatly reduced (Table I).

In order to reduce peroxides, 0.3 g of sodium borohydride was added to the dioxane and followed by distillation. The first 10–15 ml of distillate were discarded and about 100 ml of residue were left in the distillation bottle. The distillation interval

* Minimum 98%, less than 0.5% water.

was 101 ± 0.5°. The distilled solvent was colourless and showed no UV absorption. The water content was determined (Table I).

Determination of the optimum amount of lithium aluminium hydride

Agar beads containing 6% agar were washed with 8–9 times their volume of absolute ethanol, followed by dioxane "purum" and then purified dioxane. From these beads were taken 10-ml portions which were quantitatively transferred to 50-ml round bottles. 10 ml of purified dioxane were added to each bottle, followed by various amounts of lithium aluminium hydride. Reduction was carried out for 4 h under reflux (101°). While cooling the bottle with ice water the reaction was stopped by adding ethyl acetate, water and finally 1 *M* hydrochloric acid until all the precipitated lithium aluminium hydroxide was dissolved. The gels were then immediately washed on a glass filter with ice-cold 0.1 *M* hydrochloric acid and water. The degree of reduction was checked by measuring the absorption of cytochrome C in 0.001 *M* ammonium formate buffer at pH 3.8 as earlier described in detail[13].

One 10-ml portion of agar was lyophilized and weighed. The weight was found to be 0.303 g. Using this value, the amount of added lithium aluminium hydride was calculated as % w/w of dry agar. The results are given in Fig. 2.

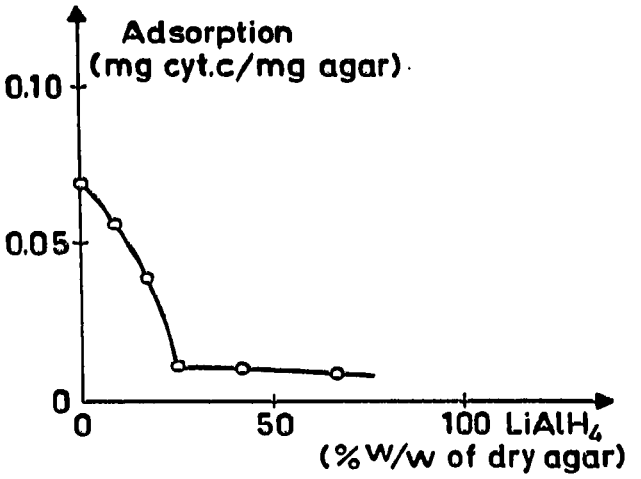

Fig. 2. Reduction of 6% ECD-agar beads with $LiAlH_4$ in dioxane. The degree of reduction was estimated by measuring the capacity of the gel to bind the positively charged protein, cytochrome C.

Alkali treatment of agar

The procedure was a modification of REES' method[14,15]. A 2-l round bottle equipped with a reflux condenser and magnetic stirrer was placed in an oil bath on a magnetic stirrer with heater. Sodium hydroxide (100 g) and sodium borohydride (1.2 g) were dissolved in 700 ml of water and poured into the bottle. While continuously stirring, 20 g of agar were added. The oil bath was brought to 80°, and this temperature maintained for 2 h.

Isopropanol (2 l) in a 3-l round bottle equipped with an anchor stirrer was chilled in an ice-bath to a temperature below 10°.

When the agar was completely dissolved and two hours had elapsed the temper-

ature was adjusted to about 50°. The agar solution was then neutralized with about 150 ml of concentrated acetic acid.

Immediately before neutralization the agar solution became more viscous and when the point of neutrality was passed the viscosity decreased. This may be used as an indicator of pH in this particular case.

Under vigorous stirring (at least 300 r.p.m.) the agar solution was added to the chilled isopropanol in small portions. The temperature must not exceed 20°. The agar precipitates as a granular–fibrous mass that can easily be washed on a glass filter or Büchner funnel.

The agar was carefully washed with water and lyophilized. The normal yield by this treatment was 85–90% of the original amount of agar. After the desulphation the agar should be white.

Batch reduction of agar

20 g of dry agar (previously alkali treated) were allowed to swell in water. The agar should preferably be in bead form or granulated for easy filtration. The water was removed by washing with approximately 2 l of ethanol on a glass filter funnel. The ethanol was replaced with dioxane of "purum" quality. About 1 l is necessary. That the replacement of solvent was complete could easily be checked with a small amount of lithium aluminium hydride. No reaction should occur, since lithium aluminium hydride does not react with agar in dioxane at room temperature. The agar was transferred to a 2-l round bottle and about 100 ml of dioxane were added to allow suspension of the agar. 10 g of lithium aluminium hydride were suspended in 50–100 ml of dioxane and poured into a dropping funnel. The bottle, equipped with dropping funnel, thermometer, reflux condenser and magnetic stirrer, was placed in an oil-bath. The temperature was raised and at approximately 75° the lithium aluminium hydride was added in small portions. The temperature rose during the reaction. When all the reagent had been added the temperature was held at 90° for 1 h. The reaction was stopped by cooling the reaction mixture to 10° in an ice-bath. Excess lithium aluminium hydride was reacted by adding 50 ml of ethyl acetate. The reaction with ethyl acetate was strongly exothermic and must be performed very slowly, as degradation of the agar may otherwise occur. The reaction products were hydrolyzed by careful addition of ice-cold water until no further reaction occurred. The precipitate of lithium aluminium hydroxide was dissolved by adding ice-cold 1 *M* hydrochloric acid until acidic pH. About 1 l is necessary. The agar was then immediately filtered and washed with cold 0.1 *M* hydrochloric acid followed by copious quantities of water.

Electro-osmosis measurements

Agar gels (3 ml of 1.5%) were allowed to set on microscope slides, 2.5 × 7.5 cm. The gels were stored in a moist chamber at room temperature for 20 h before use. The runs were made in 0.05 *M* barbituric buffer at pH 8.2. In all cases the sample was 3 μl of 1% bovine albumin and 1% dextran 70 (mean molecular weight = 70 000).

During electrophoresis the plates were continuously chilled from below with cold water. Experiments were run for 2 h at approximately 4 mA and 20–30 V per plate. Both the voltage and the current varied somewhat between different plates, but according to WIEME[16], and our own experience, this has little influence on the M_R values.

On completion, dextran and albumin were precipitated in a solution of ethanol–acetic acid–water, 70:7:23. In the gels with the lowest electro-osmotic flow, the albumin did not appear as a visible spot by this treatment, but had to be dyed with amido-black. Migration distances were measured with a ruler, which can be done with an accuracy of about ±0.5 mm. In this case this gives an error in the M_R value of less than 10%, except when the electro-osmosis is very low, in which case the relative error will be greater. However, it was considered satisfactory in this case. Results are given in Table II.

TABLE II

ELECTRO-OSMOSIS AND SULPHUR CONTENT OF SOME AGARS AND AGAROSES

Agar(ose)	$-M_R$	*S content (%)*[a]
Difco Bacto-Agar	0.82	0.97
Difco Special Agar-Nobel	0.65	0.436
Agar	0.43	0.330
Agarose (Miles-Seravac)	0.22	0.199
Agarose (Sepharose from Pharmacia)	0.21	0.179
Agar, alkali-treated	0.17	0.123
Agarose, Miles-Seravac, reduced	0.10	0.131
Agarose, Miles-Seravac, alkali-treated	0.06	0.014
Agar, alkali-treated, reduced	0.01–0.02	0.004 ?[b]

[a] Sulphur analyses were performed by Mrs. ALINE STRAUTMANIS at the Institute for Analytical Chemistry, Uppsala.
[b] The Analysis Laboratory's question mark.

Other electrophoresis experiments

The gel plates were made as above. About 10 µl of 0.25% protein solution were placed in a 5–6 mm long narrow slit in the gel. All the electrophoreses were carried out simultaneously for 1.5 h at a field strength of about 4 V cm^{-1} (Fig. 3). After electrophoresis the proteins were precipitated as above. The gel plates were then dried at 50° and stained with amido-black in 7% acetic acid.

RESULTS AND DISCUSSION

Referring to the introduction concerning the structure of agar, it is obvious that it is impossible to obtain a completely neutral agarose by merely fractionating agar and, if it were possible, the yield of agarose would be very small. Another way to separate a neutral agar(ose) is to change the undesirable properties by chemical means. We have described earlier the preparation and some properties of almost completely neutral 6% epichlorohydrin cross-linked desulphated agar (ECD-agar)[13]. The preparation involves desulphation by alkaline hydrolysis and reduction of the carboxylic groups with lithium aluminium hydride in dioxane. Prior to reduction, the agar beads were acetylated to make them hydrophobic and permeable to dioxane. However, it was possible to replace the water in the swollen agar gel with dioxane without shrinkage of the gel by a simple washing procedure. In this way the gel structure remains

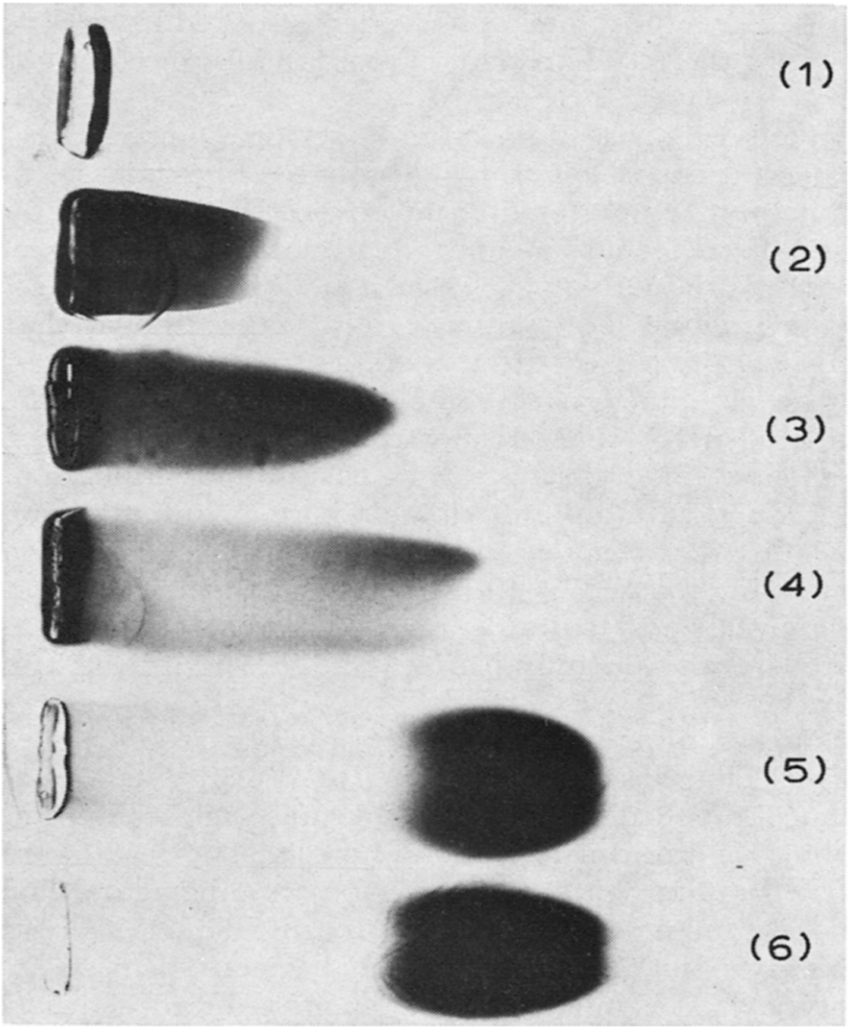

Fig. 3. Electrophoresis pattern of calf thymus histone in 0.05 *M* sodium acetate buffer at pH 4.8 in different agars and agaroses. 1 = Difco Special Agar-Noble; 2 = Agar, gift from Pharmacia Fine Chemicals; 3 = Agarose from Miles-Seravac; 4 = Alkali-treated agar (from Pharmacia); 5 = Alkali-treated agarose (Miles-Seravac); 6 = Alkali-treated and reduced agar (from Pharmacia).

expanded allowing reaction between carboxylic groups and lithium aluminium hydride. The fact that the solvent in a swollen gel can be replaced by *any* other solvent without shrinkage or other alteration of the gel structure has long been recognized[17], but nevertheless does not seem to be well known. In Fig. 1 it is seen that changing the solvent from water to alcohol in 6% agar goes rapidly and quantitatively, indicating that the hydrogen bonds are not strong enough to fix the water molecules to the gel matrix. This is of fundamental importance to the described method for reduction since the remaining water or alcohol will consume lithium aluminium hydride before the reduction of carboxylic groups to the corresponding alcohols can take place.

The next step was to determine the optimum amount of lithium aluminium hydride. In these experiments dioxane was used, which was free from water and peroxides. The water content of differently treated dioxanes is shown in Table I.

It was necessary to use 25–30% lithium aluminium hydride based on the dry weight of agar to obtain maximum reduction. This corresponds to approximately

1 mole of lithium aluminium hydride per mole of hexose. The degree of reduction was tested by measuring the adsorption of the basic protein, cytochrome C, on the agar beads (Fig. 2).

The experiments described have been based on cross-linked ECD-agar. However, it was interesting to determine if it is possible to make a charge-free, non-cross-linked agar with the original gelling ability unaltered or improved. Desulphation by alkali treatment of porphyran and carrageenan without degradation of the polysaccharides has been reported by REES[14,15]. GUISELEY[18] used a modification of REES' method for the treatment of agarose, and TSUCHIYA AND HONG[19] have shown that the gel strength of many agaroses was increased after alkaline treatment. The alkali treatment probably exerts the combined effect of alkaline hydrolysis of the sulphate ester bonds and extraction of highly charged shorter polysaccharide chains. According to PERCIVAL's rules[20], the alkaline hydrolysis of 6- or 3-bound sulphate of the L-galactose will result in the production of 3,6-anhydrogalactose by anhydro-ring formation. Sulphate groups are also to a varying extent bound to other positions on the polysaccharide. These will not be hydrolyzed by the alkaline treatment. This fact must be considered when choosing the most suitable agar for further treatment. However, it seems that sulphate groups remaining after alkaline hydrolysis are removed by the reduction (Table II).

To compare the charge densities in different agars and agaroses, the M_R values for electrophoresis were determined. This was done according to WIEME[16] as $M_R = -D/(A + D)$, where D is the distance travelled by a neutral molecule, such as dextran, and A is the distance travelled by albumin during electrophoresis. The results are shown in Table II together with the sulphur content of the corresponding agars. The electro-osmosis in the alkali-treated and reduced agar was hardly detectable and difficult to determine. The value given is very uncertain. The electro-osmosis in alkali-treated agarose was also very low. It is much lower than in reduced agarose, indicating that this commercial agarose has a very low proportion of carboxylic groups, but still contains a considerable amount of sulphate. This is also confirmed by the sulphur values in the same table. Alkali treatment of agarose is perhaps the easiest way of obtaining an agarose with a much lower charge density than previously described, although this treatment alone does not give a completely neutral agarose.

It is well known that basic substances are irreversibly adsorbed on agar. To demonstrate this, electrophoresis experiments were performed with cytochrome C, histone, lysozyme chloride and protamine sulphate. Runs were made in 0.05 M barbituric buffer at pH 8.2 and in sodium acetate buffer at pH 4.8. As the most illustrative example the pattern of histone is shown on some different agars and agaroses at pH 4.8 (Fig. 3). In contrast to lysozyme and protamine, cytochrome C and histone showed slight adsorption even on the most neutral gel. Whether this depends on an extreme sensitivity to the few remaining negative charges or is due to some other cause is at present unknown. On Difco Special Agar-Nobel the only substance that migrated was protamine sulphate, and on Difco Bacto-Agar no substance was observed to move, all substances remaining at the point of application.

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Finally, I wish to thank Professor JERKER PORATH for suggesting this research problem and whose advice and encouragement has been of the greatest value throughout the work.

CORRECTION

By mistake an important correction was not made in our previous paper in this series *J. Chromatogr.*, 60 (1971) 176–177. In the description of the adopted method of preparation of the ECD agar: page 170, 9th line from the bottom, "20 ml epichlorhydrin" should read "100 ml epichlorhydrin" and 7th line from the bottom "heated to 60° for 1 h" should read "heated to 60° for 2 h".

REFERENCES

1 R. QUAST, *J. Chromatogr.*, 54 (1971) 405.
2 C. ARAKI, *Proceedings Fifth International Seaweed Symposium, Halifax, Canada*, Pergamon Press, 1966, pp. 3–17.
3 C. ARAKI, *J. Chem. Soc. (Japan)*, 58 (1937) 1338.
4 S. HJERTÉN, *Biochem. Biophys. Acta*, 62 (1962) 445.
5 B. RUSSEL, T. H. MEAD AND A. POLSON, *Biochem. Biophys. Acta*, 86 (1964) 169.
6 A. M. EGOROV, A. KH. VAKHABOV AND V. YA. CHERNYAK, *J. Chromatogr.*, 46 (1970) 143.
7 G. G. ALLAN, P. G. JOHNSON, Y-Z. LAI AND K. V. SARKANEN, *Carbohyd. Res.*, 17 (1971) 234.
8 B. A. ZABIN, *U.S. Pat.* 3,423,396 (1969).
9 S. HJERTÉN, *J. Chromatogr.*, 61 (1971) 73.
10 K. IZUMI, *Carbohyd. Res.*, 17 (1971) 227.
11 M. DUCKWORTH AND W. YAPHE, *Carbohyd. Res.*, 16 (1971) 189.
12 W. RIEMAN AND H. F. WALTON, *Ion Exchange in Analytical Chemistry*, Pergamon Press, 1970, p. 33.
13 J. PORATH, J.-C. JANSON AND T. LÅAS, *J. Chromatogr.*, 60 (1971) 167.
14 D. A. REES, *J. Chem. Soc.*, (1961) 5168.
15 D. A. REES, *J. Chem. Soc.*, (1963) 1821.
16 R. J. WIEME, *Agar Gel Electrophoresis*, Elsevier Publishing Company, 1965.
17 H. R. KRUYT, *Colloid Sci.*, 2 (1949) 581.
18 K. B. GUISELEY, *Carbohyd. Res.*, 13 (1970) 247.
19 Y. TSUCHIYA AND K. C. HONG, *Proceedings Fifth International Seaweed Symposium, Halifax, Canada*, Pergamon Press, 1966, pp. 315–321.
20 E. G. V. PERCIVAL, *Quart. Rev.*, 3 (1949) 369.