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THE ELECTROOSMOTIC FLOW IN AGAROSE GELS AND THE VALUE OF AGAROSE AS STABILIZING AGENT IN GEL ELECTROFOCUSING

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SUMMARY

All twelve commercial preparations of agarose tested for electroosmosis showed electroosmotic flow. Considerable differences were found between the gels. Preliminary experiments suggest that it is possible to reduce electroosmosis of a certain batch by treating it with anion-exchange resin. Measurement of the conductivity of an agarose solution or gel is of no help when searching for a product with low electroosmosis. Recommendations for the application of agarose and polyacrylamide in gel electrofocusing are based on the theory of electroosmosis, on the properties of the gels, and on the practical aspects of isoelectric focusing.

INTRODUCTION

Isoelectric focusing in gels (gel electrofocusing) increasingly attracts the attention of experimental scientists working in the field of molecular biochemistry. Several investigators who used agarose as an anticonvective agent[1-3] described electroosmotic effects which differed, depending on the use of Ampholine* for pH gradient 3–10 or Ampholine for a gradient having a narrower range. In order to assess the usefulness of agarose as a supporting substance in gel electrofocusing experiments, the electroosmotic behavior of different preparations of agarose was determined in a series of electrophoresis experiments in which dextran with three different molecular weights served as marker substances[4,5].

EXPERIMENTAL

Various brands of agarose were employed, as shown in Table I. With the exception of Sepharose 6B, all samples were used without treatment prior to dissolving for the preparation of gel slabs. Sepharose 6B was dialyzed against distilled water for more than 24 h in the cold to remove the preservative; it was then partially dehydrated with absolute ethanol and finally dried at 50°. All agaroses were used as 1% (w/v) gels.

* Trade mark of LKB-Producter, Bromma, Sweden.

TABLE I

AGAROSE PREPARATIONS USED FOR THE DETERMINATION OF THE ELECTROOSMOTIC VELOCITY

Manufacturer	*Product*	*Lot No.*
BioRad Laboratories (Richmond, Calif.)	Agarose Powder	Contr. No. 5102
Calbiochem	Agarose B Grade	801450
EGA-Chemie KG. Steinheim		[a]
Koch-Light Lab. Ltd.	Agarose pure	42652
L'Industrie Biologique Française	Agarose F.F.	3018
Mann Research Lab. (N.Y.)	Agarose special grade	U 2184
Pharmacia (Uppsala)	Sepharose 6B	7639
Seakem (Marine Colloid Inc.)		752107
Serva (Heidelberg)	Agarose, reinst	11397
British Drug Houses Ltd.	Agarose for electrophoresis	Prod. No. 33006
Paines and Byrne Ltd.	"Meath"-Agarose	538-431
Behringwerke (Marburg)	Agarose Oreo	F 2217

[a] Ordered as product of Aldrich Chemical Co., Inc.

Dextran (Serva, Heidelberg) having three different molecular weight ranges was used: Dextran 15–20, mol. wt. 15000–20000; Dextran 40, mol. wt. 40000; and Dextran 500, mol. wt. 500000. Each dextran sample was dissolved as a 62.5 mg/ml solution in the buffer used for electrophoresis (0.005 *M* Sörensen phosphate, pH 7.0).

Conductivity measurements in agarose solutions and gels were carried out with a Philips conductivity meter, Type PW 9501, and a conductivity cell, PW 9512/00, having a cell constant of 1.27 cm^{-1}. The conductivity cell was kept at constant temperature with a Lauda thermostat, type K2D. A precision pH meter (Knick, Berlin, Type 350) and a combined glass electrode (Schott & Gen.) were used for pH determinations.

Electrophoresis experiments were done at constant voltage and nearly constant temperature—variation less than 2° during a run—employing the electrophoresis chamber of Vitatron Instruments described by WIEME[4]. The blocks on which the agarose gel slabs rested consisted of 7.5% (w/v) photopolymerized polyacrylamide gel. Octane from petroleum, b.p. 120–130°, which surrounded the gels during the run served as the cooling liquid. It was used instead of pentane because of its lower vapor pressure. The octane in turn was cooled by an ice trough on top and by a metal coil through which cold water (4°) was circulated. The whole electrophoresis chamber rested on a magnetic stirring table which effected rapid mixing of the octane. The gels were prepared according to WIEME (ref. 4, p. 69) on microscope slides. The thickness of the gel layer was 0.8 ± 0.1 mm. After ageing the gel slides for at least 24 h in the refrigerator in order to reduce possible variations of electroosmotic properties (ref. 4, p. 69) they were cut out of the layer in the petri dish. Three sample slits were punched 27.5 ± 1 mm from the anodal end of the slide and at exactly right angles to the direction of the field. This was done with an LKB punch equipped with three 6-mm-long razor blades, 2 mm apart from each other. The slits were filled with sample solution by using capillary pipettes with finely drawn tips, each slide receiving all three types of dextran. After electrophoresis for a defined time interval and with a

potential gradient of 15.4 V/cm over the length of the gel, the slides were removed from the chamber and immersed in a dextran-precipitating solution of acetic acid, ethanol, and water (5:70:25, v/v) (ref. 4, pp. 76 and 99). They were then scanned by means of a Vitatron UFD photometer and densitometer. Two 0.2-mm slit apertures in front of the photomultiplier tube, type RCA IP 22, provided for high linear resolution. A wavelength of 401 nm was selected by an interference filter. The ratio of densitometer belt movement (1.72 cm/min) to paper speed of the Vitatron UR 100 recorder (15 cm/min) enlarged the distance by a factor of 8.72. This facilitated measurement of the distance travelled by dextran during the run. From these measurements as well as from the time of the run and from the voltage drop over the length of the gel, the electroosmotic velocity in the agarose gel at unit field strength was calculated.

RESULTS

The conductivity of the agarose solutions in water decreased linearly with temperature with no deviation from linearity in the temperature range where gel formation occurred. Conductivity at 20° and electroosmotic velocity of the same agarose preparation did not correlate, which indicates that solutions of commercial agaroses in distilled water contain measurable quantities of low-molecular-weight ions.

The variation coefficient of repeated measurements of the electroosmotic flow from the same slide, *i.e.* positioning the slide on the densitometer belt, recording the absorbance *vs.* the length of the gel, measuring the distance between the start peak and the peak of absorbance of the dextran precipitate, and calculating the electroosmotic velocity of the agarose from these data, was less than 10%. Using Student's t-test, no difference in electroosmotic flow in a preparation caused by the position of the gel slide in the electrophoresis chamber during the run was indicated which, however, might be due to the small size of the sample ($n = 4$). Also, by use of the same test a difference between the mobility of Dextran 15–20 and Dextran 40 could not be found. Therefore, all calculations of electroosmotic velocity in the agarose gels were based on the distances covered by both Dextran 15–20 and Dextran 40. The values for Dextran 500 had to be omitted because they were consistently lower. The reason for this difference in mobility may be due to the markedly higher viscosity of the Dextran 500 solution resulting in reduced electroosmotic velocity and to the restriction of free diffusion of the Dextran 500 molecules caused by steric hindrance in the gel.

Considerable variations in electroosmotic velocity were found between different agarose preparations. Table II shows mean value and standard deviation of the electroosmotic velocity of each of the 12 agarose samples. They may be grouped into those with electroosmotic velocities between 1.5 and 2.0, those with velocities between 2.5 and 3.0, and those with values higher than 4×10^{-5} $cm^2 \cdot V^{-1} \cdot sec^{-1}$. Whether these differences were caused by different preparation procedures is not known. From the nature of the polysaccharide mixture of agar as described by ARAKI[6], the ionic groups remaining in the agarose gel matrix are probably carboxylic or sulfonic acid residues. In order to reduce the electroosmotic velocity, agarose in solution was treated with anion-exchange resin at a temperature above its gelling point. In a typical experiment of this kind, 1 g of agarose was dissolved in 100 ml of distilled

TABLE II

MEAN VALUES AND STANDARD DEVIATIONS OF THE ELECTROOSMOTIC VELOCITY IN 1% (w/v) AGAROSE GELS

Phosphate buffer, 0.005 moles/l, pH 7.0; 15.4 V/cm, 2.5–4°.

Agarose	*Electroosmotic velocity (10^{-5} $cm^2 \cdot V^{-1} \cdot sec^{-1}$)*
BDH	1.56 ± 0.12
EGA (Aldrich)	1.76 ± 0.25
Koch-Light	1.78 ± 0.14
Mann	1.89 ± 0.09
Pharmacia	2.48 ± 0.21
BioRad Lab.	2.59 ± 0.12
Seakem	2.70 ± 0.16
Behringwerke	2.85 ± 0.25
L'Industrie Biologique	2.94 ± 0.19
Paines and Byrne	3.27 ± 0.25
Serva	4.19 ± 0.26
Calbiochem	6.12 ± 0.19

water by boiling. 1 g of BioRad AG 1-X1 anion-exchange resin (50–100 mesh, chloride form) was added, and the mixture was kept at 50° overnight with repeated shaking. After sedimenting of the resin particles, the supernatant was decanted and cooled to gel formation. The gel was cut, frozen, and crushed in a mortar. Part of the water was removed by the addition of absolute ethanol, and the gel particles were dried at 50°. The electroosmotic velocity of such a preparation was markedly reduced, as compared with a gel prepared from the original material (see Table III).

DISCUSSION AND CONCLUSIONS

According to ARAKI[6] agarose is a polymer of D-galactose and 3,6-anhydro-L-galactose. In aqueous solutions and at pH values usually encountered in electrophoresis or isoelectric focusing experiments, the presence of charged groups may not be expected in such polymer molecules, and, hence, it must be assumed that commercially available agarose preparations are contaminated to varying degrees by ionizable macromolecules, *e.g.* agaropectin, which are the cause of electroosmotic flow in the gel.

The influence of the electroosmotic flow on the resolution obtainable in gel

TABLE III

REDUCTION OF ELECTROOSMOTIC VELOCITY IN 1% (w/v) AGAROSE GEL BY TREATMENT WITH ANION-EXCHANGE RESIN

Experimental conditions same as in Table II.

Agarose	*Electroosmotic velocity (10^{-5} $cm^2 \cdot V^{-1} \cdot sec^{-1}$)*
BioRad Lab.	2.59 ± 0.12
BioRad exchanged	1.50 ± 0.14

electrofocusing experiments may be characterized as follows: the faster the electroosmotic flow within the gel, the greater the difference between the actual position of a zone of macromolecules and its isoelectric point on the pH gradient. This is due to the fact that the ampholytes (mol. wt. about 500) which form the pH gradient return to their respective isoelectric positions much faster after having been carried away by the electroosmotic flow than the macromolecules which are perhaps retarded by the gel if they have a molecular weight of more than 10^5 to 10^6. The latter will always lag behind their isoelectric position with a distance depending on their diffusion coefficients and the slope of their titration curves in the vicinity of their isoelectric points. Thus, the requirements for the condensation of the macromolecules in zones of Gaussian concentration distribution, namely constant conductivity and constant pH within a single zone[7] are not met, and, hence, a blurring of the bands occurs which has a diminishing effect on the resolution of neighbouring bands[8].

In order to facilitate the discussion on the magnitude of the electroosmotic flow in gels and the factors influencing it, some physico-chemical facts will be recalled. For simplification, gels are treated as capillary systems. The electroosmotic flow in capillaries of a radius much greater than $1/\kappa$, which denotes the radius of the ion cloud over the electrical charges which are fixed to the wall of the capillary, may be described by eqn. 1 which is derived from the Helmholtz–Smoluchowski equation (ref. 9, p. 406):

$$\frac{\mathrm{d}V}{\mathrm{d}t} \equiv D = \frac{I\varepsilon\zeta}{4\pi\eta_0\kappa'} \tag{1}$$

where I stands for the electrical current, ε the field strength, ζ the electrokinetic potential φ at the surface of shear[10], η_0 the viscosity of the liquid, and κ' its conductivity. In small-pore capillary systems with radii below 50 nm, deviations from the Helmholtz–Smoluchowski equation were observed[9]. In such systems, the term ζ of eqn. 1 becomes meaningless because the radius of the capillary is of the order of the radius of the ion cloud, $r \leqq 1/\kappa$, which implies that the definition of the surface of shear does not hold. The electroosmotic flow in such capillaries is described by eqn. 2 (ref. 9):

$$\frac{\mathrm{d}V}{\mathrm{d}t} \equiv D = \frac{IFc_R'}{8\eta_0\kappa''}\cdot r^2 \tag{2}$$

where F is the product of Avogadro's number and elementary charge, c_R' the concentration of counterions, and κ'' the conductivity of the fluid within the capillaries.

The question now arises as to the conclusions that may be drawn from this knowledge on electroosmotic flow and to the difference which exists between both types of capillary systems with respect to the application of gels as stabilizing agents in isoelectric focusing experiments. The dependence of the electroosmotic flow, D, on the square of the radius of the capillary, r^2, in the small-pore capillary system (eqn. 2) is obvious. This makes it necessary to calculate the dimensions of the radius of the capillaries and of the ion clouds, $1/\kappa$, in the different types of gels, in order to know which model applies to the gel system under study.

The average pore radius for a 1% agar gel (Ionagar No. 2) has been determined as 90–120 nm (ref. 4, p. 117). Similar values may be calculated from data for the

selectivity of agarose gel beads for gel filtration provided by the manufacturers. The radius of the ion cloud, $1/\kappa$, is given by KORTÜM (ref. 9, p. 171) as 3.93 nm and 1.24 nm for an aqueous solution of a 1,3-salt at 25°, and for concentrations of 10^{-3} moles/l and 10^{-2} moles/l, respectively. From these data, it appears probable that eqn. 1 applies to the conditions of the electrophoretic experiments reported here. In electrofocusing experiments, the conductance of the column decreases towards the end of the run. The only free ions present in the solution of the pH gradient during the final stages of an experiment are the zwitterionic ampholytes and the focused macromolecules. Both types of molecules are multivalent ions in aqueous solutions. The average concentration of ampholytes is usually of the order of 10^{-1} to 10^{-2} moles/l, while the macromolecules are much less concentrated. With increasing valence of the ionic species and increasing concentration of the ions, the radius of the ion cloud, $1/\kappa$, decreases. It seems, therefore, reasonable to assume that eqn. 1 holds for electrofocusing experiments in large-pore gels, *e.g.* gels with an agarose concentration up to 1% (w/v). However, since nothing is known about the exact pore size distribution within the gels, deviations of the electroosmotic flow from the one expected from eqn. 1 cannot be excluded because capillaries with much smaller pore sizes than the average may be present.

DAVIES[11] has demonstrated considerable differences of conductivity along density-stabilized pH gradients which were formed by electrofocusing Ampholines of various pH ranges. In experiments with agarose as stabilizing agent, this phenomenon leads to differences of electroosmotic flow (*cf.* eqn. 1) in a gel, and hence also of electroosmotic pressure, as soon as the pH gradient is established. Under certain experimental conditions this may cause the gel to rupture. Another effect which has been observed by CATSIMPOOLAS[2] may be explained in this way. The anionic groups fixed in the gel matrix have protolytic dissociation constants which most probably lie in the range of 10^{-2} to 10^{-4}. Therefore, the acidic side of the pH gradient, pH 3–4, may exert a great influence on the degree of dissociation of such groups.

Decreasing the number of fixed charges by lowering the degree of dissociation leads to a drop in electrokinetic potential at the surface of shear, ζ, which in turn results in a reduction of electroosmotic flow in the anodic part of the gel. Since the direction of the electroosmotic flow is towards the cathode, this, in turn, leads to dehydration and to shrinkage of the anodic part of the gel. Data supporting this interpretation were presented by WALDMAN-MEYER[5] for paper, and by RAACKE[12] for starch as supporting media in electrophoresis experiments.

Electroosmosis may also occur in polyacrylamide gels[13]. Although I have not observed electroosmotic flow in control experiments with photopolymerized polyacrylamide gel in the system described above, it has been encountered during gel electrofocusing experiments with polyacrylamide by VESTERBERG (*cf.* ref. 14, p. 4) and POGACAR[15]. The average pore size in polyacrylamide gel of 7.5% (w/v) concentration is estimated as being 50 nm[16,17]. Thus it seems probable that a considerable number of pores are of the size for which eqn. 2 applies, *i.e.* $r \leqq 1/\kappa$. Since the electroosmotic flow is proportional to the square of the radius of the capillaries (see eqn. 2), it is mandatory to prepare homogeneous gels for gel electrofocusing in polyacrylamide.

Although polyacrylamide gels have been used in most experiments of gel electrofocusing, agarose may have certain advantages. For instance, dilute agarose gels may be pumped through capillary tubing, agarose gel is transparent in the UV

range[4,18] without special treatment as is necessary for polyacrylamide gel[19,20], and agarose does not seem to interact chemically with sample molecules, again in contrast to polyacrylamide which affects enzyme activity[21,22], interferon, poliovirus, and insulin[23] probably by interaction with residues of the persulfate catalyst system. Furthermore, agarose is nontoxic while the monomer solutions for polyacrylamide gels are very skin- and neuro-toxic. From these considerations, recommendations for gel electrofocusing experiments are as follows:

(1) Low concentrations of especially purified gel substances should be used, *e.g.* recrystallized acrylamide monomer or agarose treated with anion-exchange resin.

(2) The ampholyte concentration should be high in order to reduce electroosmotic flow.

(3) The anodic solution should not be a strong acid when using agarose gel (*cf.* ref. 2).

(4) Precipitates within the gel should be strictly avoided (*cf.* ref. 24).

(5) Increasing the viscosity of the liquid in the gel by adding neutral, chemically inert substances, *e.g.* sucrose, may be of help in reducing electroosmotic flow.

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REFERENCES

1 R. F. RILEY AND M. K. COLEMAN, *J. Lab. Clin. Med.*, 72 (1968) 714.
2 N. CATSIMPOOLAS, *Sci. Tools*, 16 (1969) 1.
3 N. CATSIMPOOLAS, *Clin. Chim. Acta*, 23 (1969) 237.
4 R. J. WIEME, *Agar Gel Electrophoresis*, Elsevier, Amsterdam, London, New York, 1965, p. 69, 76, 99, 117.
5 H. WALDMANN-MEYER, *Chromatog. Rev.*, 5 (1963) 1.
6 C. ARAKI, *Bull. Chem. Soc. Japan*, 29 (1956) 543.
7 H. SVENSSON, *Acta Chem. Scand.*, 15 (1961) 325.
8 H. SVENSSON, *J. Chromatog.*, 25 (1966) 266.
9 G. KORTÜM, *Lehrbuch der Elektrochemie*, Verlag Chemie, Weinheim/Bergstr., 1966, p. 171, 406, 413.
10 C. C. BRINTON, JR. AND M. A. LAUFFER, in M. BIER (Editor), *Electrophoresis, Theory, Methods and Applications*, Academic Press, New York, 1959.
11 H. DAVIES, in H. PEETERS (Editor), *Protides of Biological Fluids, Proc. 17th Coll. Bruges 1969*, Pergamon, Oxford, 1970.
12 I. D. RAACKE, *J. Am. Chem. Soc.*, 80 (1958) 3055.
13 S. RAYMOND AND M. NAKAMICHI, *Anal. Biochem.*, 3 (1962) 23.
14 O. VESTERBERG, cited in *Acta Ampholinae*, 7 (1970) 1.
15 P. POGACAR, personal communication.
16 L. ORNSTEIN, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
17 M. P. TOMBS, *Anal. Biochem.*, 13 (1965) 121.
18 R. TSANEV, *Biochim. Biophys. Acta*, 103 (1965) 374.
19 A. H. GORDON, *Electrophoresis of Proteins in Polyacrylamide and Starch Gels*, North Holland, Amsterdam, London, 1969.
20 S. RAYMOND AND L. WEINTRAUB, *Science*, 130 (1959) 711.

21 J. M. Brewer, *Science*, 156 (1967) 256.
22 B. K. Hartman and S. Udenfriend, *Anal. Biochem.*, 30 (1969) 391.
23 K. H. Fantes and I. G. S. Furminger, *Nature*, 215 (1967) 750.
24 H. Rilbe, in H. Peeters (Editor), *Protides of Biological Fluids, Proc. 17th Coll. Bruges 1969*, Pergamon Press, Oxford, 1970.