Journal of Chromatography, 242 (1982) 33–40
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,765

OBSERVATIONS ON ACID–BASE TITRATIONS OF SUBSTITUTED AGAROSE GELS BY FRONTAL ANALYSIS

T. K. KORPELA
Department of Biochemistry, University of Turku, SF-20500 Turku 50 (Finland)
(First received November 20th, 1981; revised manuscript received January 25th, 1982)

SUMMARY

Breakthrough capacities of weak agarose-based ion exchangers were studied by using 10 m*M* HCl or KOH eluents to which different amounts of KCl were added. The salt significantly affected the breakthrough capacities at concentrations between 0.01 and 0.1 *M*. When gels containing carboxyl groups were titrated with HCl the calculated capacities were higher than when KOH was used as the titrant (constant KCl concentration). Conventional acid or base titration yielded capacities corresponding to the mean of those obtained with the frontal method. Similar results were obtained with gels having weakly basic groups. It was concluded that leakage of the acidic or basic species into the mobile phase was an essential factor contributing to the results observed.

INTRODUCTION

From a practical point of view, an ion exchanger can be considered as a reservoir of exchangeable counter-ions. These ions are put to use in ion-exchange operations, and hence the counter-ion content is one of the most important characteristics of an ion exchanger. The amount of fixed charge and the counter-ion content are not necessarily the same, and therefore various definitions of the ion-exchange capacity are needed for different experimental conditions[1].

Agarose-based ion exchangers have become popular in biochemical chromatography owing to their macroporous structure and excellent stability. Most affinity chromatographic purifications have been carried out with derivatized agarose. Commonly, either the affinants are ionic, or ionic functions are generated in the gel during the activation process. Recently Sluyterman *et al.*[2–5] have developed a new, very promising technique, termed chromatofocusing. It utilizes buffering properties of weak, conventional, agarose ion exchangers or those of specially modified agarose[5]. The content of ionic groups needs to be accurately known, particularly with the chromatofocusing method.

Agarose-based ion exchangers clearly differ in their properties from those having a synthetic backbone, but little is known about them. The problem is aggra-

0021-9673/82/0000–0000/$02.75

vated because the ion-exchange capacities of the gels change from manufacturer to manufacturer, and probably from batch to batch as well[5].

Acid–base titration of ion-exchange gels is the most important method of studying their properties. Such things as the salt concentration of the titrant, the swelling of the gel, the volume of the solvent in which the titration is carried out, the time used for the titration, etc. affect the shapes of the resulting titration curves.

This paper reports the titration of weak agarose ion exchangers by frontal analysis or the breakthrough of appropriate acid and base solutions.

EXPERIMENTAL

Materials

Potassium hydroxide, potassium chloride and hydrochloric acid were purchased from E. Merck, Darmstadt, G.F.R., and the reagents were of pro analysis grade. Sepharose 4B CL, DEAE- and CM-Sepharose 6B CL were from Pharmacia, Uppsala, Sweden. The manufacturer reported capacities of 13 ± 2 and 12 ± 2 mequiv./100 ml for the latter two gels, respectively. N^6-(Aminohexyl)adenosine-5′-monophosphate Sepharose was purchased from Sigma, St. Louis, MO, U.S.A. All the solutions were prepared in distilled and ion-exchanged water. Cycloserine was attached to CNBr-activated agarose as described previously[6]. CM-Sepharose was derivatized with ethanolamine by the method of Cuatrecasas[7], and a *ca.* five-fold excess of aminoethanol over the carboxymethyl functions of the gel was used.

Methods

Fig. 1 shows schematically the system used in the titrations; *ca.* 7–10 ml of gel were applied to a column of 8 mm I.D. The exact gel volume was observed after the gel had been washed for a few hours with 0.1 *M* KCl at a flow-rate of *ca.* 10 ml/h. This gel volume was considered constant in the later calculations in spite of different salt concentrations causing some changes within the gel volumes. The design of the apparatus was essentially as described previously[8], except that a Datex Model 555 pH

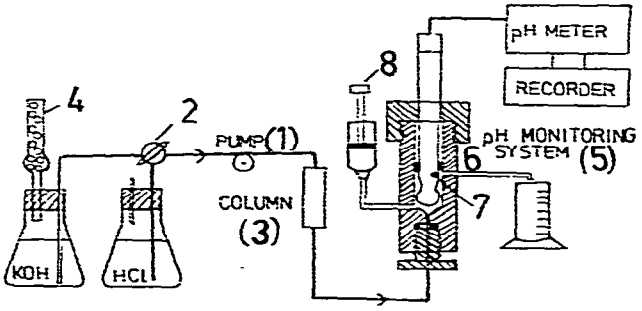

Fig. 1. Apparatus used with the acid–base titrations of the agarose gels. The acid or base solutions were pumped with an Ismatec mp-ge peristaltic pump (1) through a three-way valve (2) to the column (3) containing the gel. Potassium hydroxide solution was shielded from atmospheric CO_2 by a tube containing KOH pellets (4). The flow cell (5) was made in such a way that its internal volume was moderately small. The outlet of the cell (6) was near the hole of the reference electrode (7). The cell also contained a connection to a syringe (8) for rapid calibration and washing of the cell.

meter supplied with a recorder was used to monitor the eluent. The pH meter was calibrated at pH 7.00. The extremes of the pH values were thus not necessarily perfectly reliable. The eluent flow-rate was eventually checked with a graduated cylinder. The flow cell was made from perspex (commercial versions also available). The design of the flow cell was not critical when relatively large gel volumes (*ca.* 10 ml) were titrated.

Titrations of CM- and DEAE-Sepharoses were also carried out with normal batchwise methods. The gel titrated in column was quantitatively rinsed with 0.5 *M* KCl into a beaker. It was then titrated with 0.1 *M* HCl or KOH (both in 0.5 *M* KCl). Appropriate volumes of 0.5 *M* KCl were also titrated.

RESULTS AND DISCUSSION

Repeatability

When the same gel was successively titrated the calculated breakthrough capacities were usually very similar (range below 2%). The composition of the titrant, the chemical structure of the ion exchanger and other operational conditions affect the breakthroughs. The errors originate mostly from the measurements of the gel volume, the elution volume and the titrant concentration. Ligands did not essentially leak out from CM- or DEAE-Sepharose, even though rather drastic elution conditions (25 m*M* acid or base in 1 *M* KCl) were used in some successive titrations. Usually 10 ± 2 m*M* titrants were used in the present study. It was checked with CM- and DEAE-Sepharoses (0.1 *M* KCl) that the small variations in the titrant concentration (*ca.* 2 m*M*) did not noticeably affect the calculated capacities.

Breakthrough curves with CM-Sepharose

Fig. 2 shows an example of the breakthrough curves obtained in the titration of CM-Sepharose. At the beginning of the elution the constant pH plateau attained in the previous titration was maintained (*e.g.* region A–B in Fig. 2). This reflects the column volume and the rate of elution. Thereafter the pH smoothly changes until the breakthrough with apparent retention volume A–C occurs. The breakthrough capacities were calculated from the length of B–C, taking in account to the elution rate and the acid or base concentration.

Because the concentrations of both HCl and KOH were 10 m*M* in the titration of Fig. 2, it is clear that the breakthrough was essentially dependent on whether acid or base was used.

Usually small perturbations (see D in Fig. 2) occurred in the breakthrough curves. There was evidence that the perturbations became smaller as the salt concentration in the titration solution increased. Variation of the flow-rate between 10 and 25 ml/h (0.1 *M* KCl, 10 m*M* acid or base) did not affect the perturbation, the calculated breakthrough capacity or the pH levels of the curves. These observations indicate that an operational equilibrium existed between the ion exchanger and the mobile phase. In the context of chromatofocusing, related perturbations have been observed, and they were attributed to a "sluggishness" of the ion exchanger[4]. It is apparent that the slow reactions are connected with conformational movements of the gel which thereafter reflect the ionic properties of the gel[5]. It is known that some ion-exchange materials require very long equilibration times in their pH titrations[1]. A

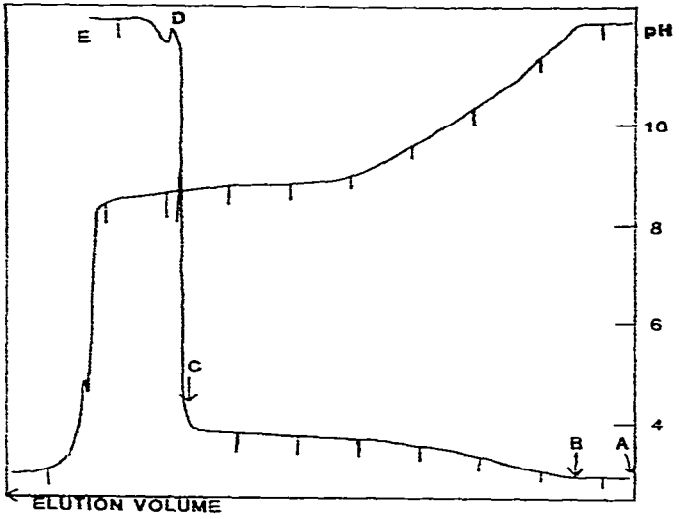

Fig. 2. Examples of the breakthrough curves obtained when *ca.* 7 ml of CM-Sepharose 6B CL were titrated with 10 m*M* HCl or KOH in 0.1 *M* KCl with the method of frontal analysis. The repeating vertical pieces of line are due to the marking points from a siphon-operated flow meter[8]. The distance between lines corresponds here to 10 ml of the eluents.

high concentration of salt in the titrant aids the ion-exchange process itself and, in addition, it is apparent that it stabilizes the gel structure into a conformation. Sharpness of the breakthrough boundary could be used to analyse the ion-exchange rates and to study the equilibrium of the ions between the solution and gel[1]. It could be useful to measure the counter-ion concentrations, in addition to the pH, in chromatofocusing and related experiments.

The concentration of KCl in the titrant largely affected the breakthrough, especially between 0.01 and 0.1 *M* of salt (Fig. 3), maximal capacity appearing at zero concentration. When the concentration was higher the effect of the salt was reduced. The titration reaction itself naturally produced *ca.* 10 m*M* of salt into the eluent.

Fig. 3 also shows pH values at *ca.* 10 ml before the breakthrough. These values were dependent on the added salt in parallel way as the breakthrough capacities.

Successive derivatizations and titrations of CM-Sepharose 6B CL with ethanolamine by the carbodiimide method[7] indicated that only *ca.* 2/3 of the acid groups can be masked. The masking reaction went that far at the first attempt, and repetition of the reaction did not drive it toward completion. Similar observations have been made earlier[9]. It would be interesting to know whether salts in the reaction mixture increase the substitution by "opening" the gel structure and making it more accessible to the reagents.

Breakthrough curves with DEAE-Sepharose

Principally the same kind of breakthrough curves as with CM-Sepharose were found with DEAE-Sepharose. The difference between the breakthrough capacities achieved by titration with acid or base were somewhat larger with CM-Sepharose

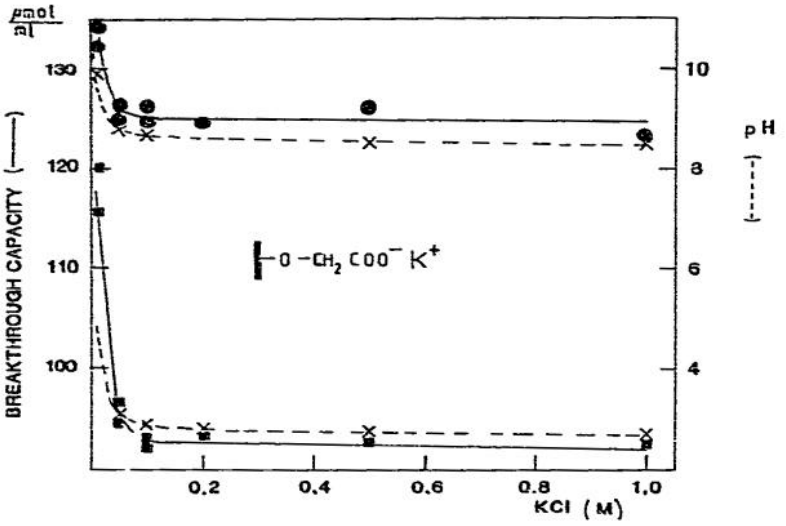

Fig. 3. The dependence of calculated breakthrough capacities (solid line) on the salt concentration in the acid or base titrant when CM-Sepharose 6B CL was titrated. The lower solid line is due to titration with KOH and the upper one with HCl. The broken lines indicate the pH values of the solutions emerging from the column as measured appropriately before the breakthrough.

than with DEAE-Sepharose (Figs. 3 and 4). As with CM-Sepharose, the titration reaction with the DEAE gel, which was based on the neutralization, produced smaller breakthrough capacities than those involving titration of charged groups. The pH values in the mobile external solutions of the anion and cation exchangers were reversed, as well.

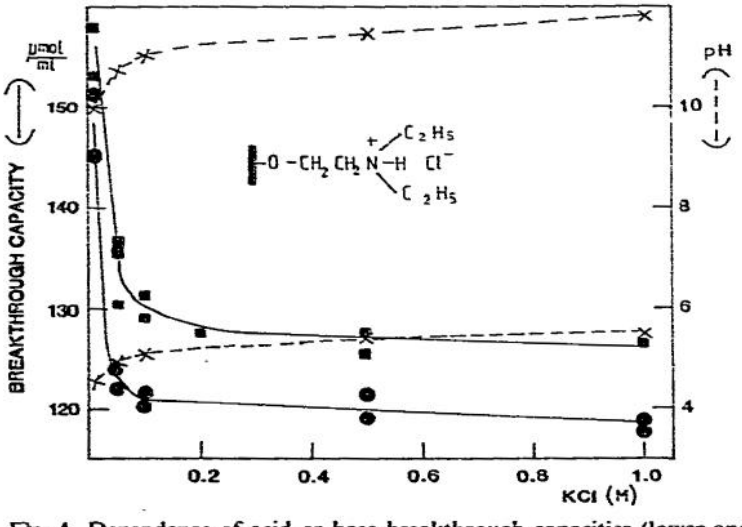

Fig. 4. Dependence of acid or base breakthrough capacities (lower and upper solid lines, respectively) when DEAE-Sepharose 6B CL was titrated by using different concentrations of KCl in the titrant. The broken lines indicate the pH values of the solutions emerging from the column measured in the same way as in Fig. 3.

Comparison of the frontal and batchwise titrations

The normal titration of CM- and DEAE-Sepharose in 0.5 *M* KCl solution resulted in approximately the same capacity when titrated either with acid or base. This capacity was (within the experimental error of *ca.* ± 5 μmol/ml) for both the gels the mean of the acid and base titrations obtained with the frontal method in 0.5 *M* KCl.

Consecutive titrations of CM- and DEAE-Sepharose

It was questioned how real were the pH values of the breakthrough curves. Fig. 5 schematically shows breakthrough curves of appropriate volumes (*ca.* 4 ml) of the ion exchangers in two successive columns when one or another was the first. Fig. 5 also shows breakthrough curves when the gels were mixed, as well as appropriate volumes (*ca.* 8 ml) of the parent ion exchangers. For the sake of clarity of the figure, the breakthrough volumes of all the gels are drawn of equal length in Fig. 5.

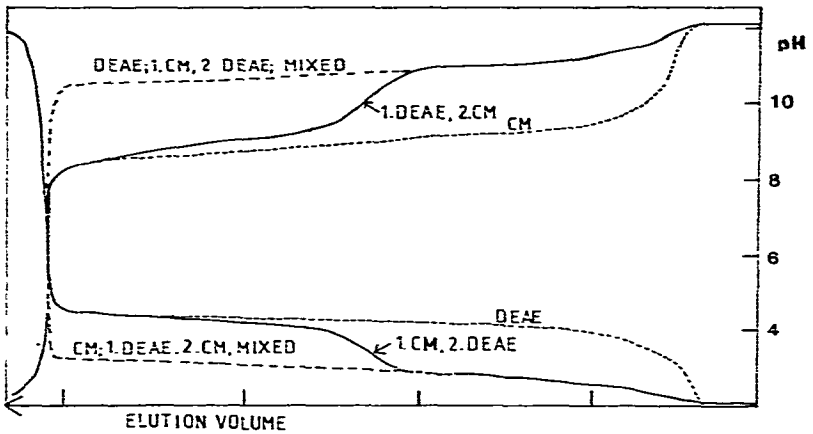

Fig. 5. Breakthrough curves of appropriate volumes of DEAE- or CM-Sepharose 6B CL when they were titrated as successive column beds, or when the beds were mixed. The numbers inside the figure indicate the order of the column beds in respect to the eluent feed.

If the gel beds were in series, the pH values of the curves were determined by the second column if it was in the uncharged form. When the first column was initially in free acid or base form, and the second in the salt form, the pH curve was determined by the first column until it was fully changed to salt form. After that the pH level of the second gel was attained (Fig. 5). Hence, for example, CM-Sepharose in acid form released protons into the external solution so that a pH of *ca.* 2–3 was maintained in the eluent. The subsequent DEAE column in salt form was not able to alter the protonic equilibrium. When a weak ion exchanger is titrated from acid toward alkali, a certain amount of protons is leaked through the gel and this amount does not consume the base.

Potassium ions in the eluent can to some degree compete with protons for the fixed functional groups and drive protons into external solution. The amount of protons leaked from the column can be integrated by collecting the solution until the breakthrough (volume B–C in Fig. 2) and titrating the solution. Another possibility is

to integrate the recorded pH. Two successively arranged ion-exchange columns could be very conveniently used so that another functions as an internal standard for the concentration of the titrant.

Titrations of some low-capacity ion exchangers

The gels designed for the affinity chromatography contain ligand concentrations of *ca.* 1–20 μmol/ml of packed gel. Because the affinants are usually ionic, acid–base titration is suitable for characterizing affinants. Generally accepted methods for the analysis of affinity gels are required because different analyses often agree poorly with each other[10].

Then *ca.* 8 ml of N^6-(6-aminohexyl)adenosine-5′-monophosphate Sepharose was titrated with 4 and 1 m*M* HCl or KOH in 0.2 *M* KCl by frontal analysis. Four successive titrations yielded an ion content of 5–6 μmol/ml of the gel. Considering that the ligands contained more than one titratable function, the result is of reasonable magnitude (the manufacturer reported 2–4 μmol/ml). It appeared that some of the ligands leaked out in alkali. Titration of Sepharose 4B CL showed 0.1–0.3 μmol/ml of the ionic functions. Titration of a cycloserine-derivatized agarose showed 4–6 μmol/ml ligands, whereas amino acid analysis yielded 10 μmol/ml of the gel[6]. The differences between the results may be due to a two-point attachment of the ligand.

When a very low concentration (0.2–0.5 m*M*) of alkali was used for the titration, very detailed "titration curves" appeared (Fig. 6) the approximate shapes of which were reproducible. They did not appear when the same concentrations of HCl were used. The ligand concentrations calculated from the titrations with low concentrations of alkali were not, however, within reasonable limits (see Fig. 6). Other affinity gels showed different shapes of the "titration curves", which could indicate that the curves were in some way related to the gel structure. Possibly a very low base concentration was capable of forming pH gradients within the gel.

Because the breakthrough titration seemed to be moderately accurate, it was applied to the measurement of immobilized enzymes. A 2-ml volume of a preparation containing 2.6 mg of immobilized aspartate aminotransferase per millilitre of gel

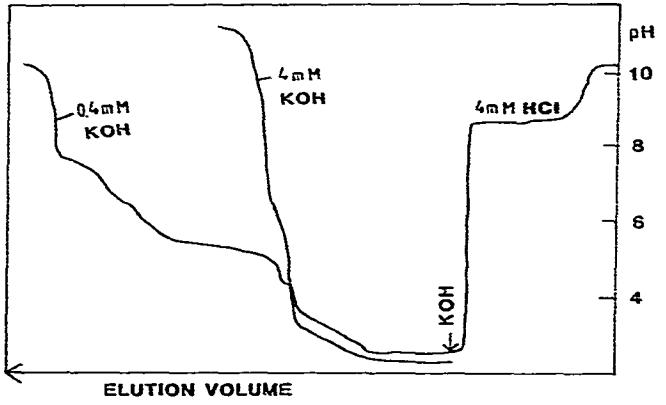

Fig. 6. The pH profiles obtained when *ca.* 8 ml of a cycloserine-derivatized agarose was titrated with acid or base solutions (indicated in the figure) containing 0.2 *M* KCl by the similar frontal method as shown in Fig. 2.

(CNBr coupling) was titrated. The protein apparently largely leaked from the column, because typically very large pH oscillations at the breakthrough boundary were observed, especially on the first few times of titration. Another possible explanation for the oscillations is slowly attained ionic equilibria. The concentration of the ionic groups within the gel was measured with 1–4 m*M* titrants in 0.1 *M* KCl to be *ca.* 4–5 μmol/ml of gel. A suitable, stably fixed protein could serve as a cheap immobilized buffer polyelectrolyte for the chromatofocusing technique.

REFERENCES

1 F. Helfferich, *Ion Exchange*, McGraw-Hill, New York, 1962, pp. 72–94, 424–427.
2 L. A. Æ. Sluyterman and O. Elgersma, *J. Chromatogr.*, 150 (1978) 17.
3 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 150 (1978) 31.
4 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 206 (1981) 429.
5 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 206 (1981) 441.
6 T. Korpela, A. Hinkkanen and R. Raunio, *J. Solid-Phase Biochem.*, 1 (1976) 215.
7 P. Cuatrecasas, *J. Biol. Chem.*, 245 (1970) 3059.
8 T. Korpela and E. Mäkinen, *J. Chromatogr.*, 166 (1978) 268.
9 J. Porath, *J. Macromol. Sci. (A)*, 10 (1976) 1.
10 J. Turková, *Affinity Chromatography*, Elsevier, Amsterdam, 1978, p. 203.