0273-2289/95/5401-03—0159$06.80

Recent Progress in the Electrochromatography of Proteins†

KENNETH D. COLE* AND HERIBERTO CABEZAS, JR.

Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, MD 20899

ABSTRACT

We have constructed a column for the application of an electrical field to a flowing packed bed of chromatographic media (electrochromatography), and we have studied the effect the electrical field on the elution of bovine serum albumin, β-lactoglobulin A and B, α-lactalbumin, and myoglobin from packed beds of Sephadex beads. The elution behavior of the model proteins on Sephadex with a high degree of crosslinking (Sephadex G-25) and with a lower amount of crosslinking (Sephadex G-75) was measured. We confirmed that proteins exhibited an unexplained electrically driven retention on the column packed with G-75. The electrically driven retention effect was greatly reduced or absent in columns packed with the more highly crosslinked G-25. The potential of electrochromatography for high-resolution separations was shown by the partial separation of β-lactoglobulin A and B by using short columns with the electric field polarity such that electrophoresis opposed convective flow in the column.

Index Entries: Electrochromatography; proteins; Sephadex; electrophoresis; counteracting chromatographic chromatography; serum albumin.

INTRODUCTION

Electrophoresis of proteins in a column packed with chromatography media has a long history. A variety of packing materials have been used in electrochromatography. Haglund and Tiselius (*1*) used glass powder to

*Author to whom all correspondence and reprint requests should be addressed.

†Certain commerical equipment, instruments or materials are identified in this article to specify adequately the experimental procedure. Such identification does not imply recommendation by NIST, nor does it imply that the materials or equipment are necessarily the best available for the purpose.

stabilize electrophoresis against convection in a U-tube. Starch has been used as a stabilizing medium by Flodin and Porath (*2*) and Carlson (*3*). This work was reviewed by Bloemendal (*4*). Proteins, peptides, and amino acids have been separated on columns packed with cellulose (*5–7*). Porath et al. (*8*) designed an electrochromatography column with a second smaller column that delivered a counterflow of buffer to the bottom of the major column where components were eluting. The eluted zones were collected from the counterflowing buffer. Porath (*9*) stated that Sephadex was not as satisfactory as highly purified cellulose for most proteins. Porath treated the cellulose to remove impurities and to block charged groups that would result in adsorption. Sephadex was also treated to block the residual carboxyl group to prevent adsorption.

Nerenberg and Pogojeff (*10*) used electrochromatography to separate the serum proteins on Sephadex G-100 superfine. They performed simultaneous elution and electrophoresis, and found an increase of resolution of the serum proteins from 3 peaks in the absence of a field to 16 peaks with an applied field. Luzzio (*11*) isolated a hepatitis B antigen from serum using electrochromatography in columns packed with Sephadex G-200. Epstein (*12*) described a device for continuous separation of solutes by gel filtration in a packed bed with an electrical field at right angles to the flow.

O'Farrell (*13*) developed a type of electrochromatography termed counteracting chromatographic electrophoresis (CACE). In this technique, a restrictive size-exclusion gel medium is packed above a less restrictive size-exclusion gel medium. Convective flow down the column is opposed by electrophoresis in the opposite direction. As the solute of interest migrates from the region of the column in which it is excluded from the gel into the region where it is included, the solute velocity owing to convective flow is decreased. By careful selection of the convective flow, electric field, and exclusion limits of the gels, proteins can be concentrated and purified in such a column. Hunter (*14*) developed a mathematical model of CACE to predict concentrations and electrical fields derived from an analogy with isotachophoresis. Locke and Carbonell (*15*) have done further experimental and model development on CACE. Ivory and Gobie (*16*) developed an apparatus for continuous CACE and modeled the process using linear chromatography theory.

Rudge et al. (*17*) studied the behavior of model proteins in Sephadex G-75 under an applied field. They observed an electrically driven retention of large solutes (proteins and blue dextran) with both negative and positive fields. Rudge et al. (*17*) developed a model to predict the electrochromatography elution time of proteins in Sephadex G-75. The model is linear, and it neglects dispersion in the column. One should note that neglecting dispersion has only a small effect on solute velocity, although the chromatography peaks calculated from such a model will be rather narrow when compared to experiment. The defining differential equation for the model is:

$$\alpha\,(\partial c_s / \partial t) + v\,(\partial c_s / \partial x) + (1 - \alpha)\,(\partial c_g / \partial t) + \alpha\mu_s E\,(\partial c_s / \partial x) + (1 - \alpha)\mu_g E\,(\partial c_g / \partial x) + (1 - \alpha)\,(\partial c_a / \partial t) = 0 \quad (1)$$

where α is the void fraction of the packed bed, c_s is the solute concentration in free solution, t is time, v is the superficial velocity, x is the linear distance down the axis of the column, c_g is the concentration of solute inside the chromatographic gel beads, μ_s is the solute electrophoretic mobility in free solution, E is the electric field, μ_g is the solute electrophoretic mobility inside the gel, and c_a is the solute concentration in an adsorbed layer on the surface of the gel beads. In addition, the concentrations in free solution and inside the gel beads are related by a constant thermodynamic partition coefficient (K_{av}).

$$c_g = K_{av}\,c_s \quad (2)$$

and the concentrations in free solution and in the "adsorbed" layer are related by an adsorption expression.

$$c_a = k_a\,(c_s - c_g) = k_a\,(1 - K_{av})\,c_s \quad (3)$$

where k_a is an adsorption coefficient. There are two issues to be noted in relation to the "adsorbed" layer. First this is a hypothetical adsorption, since it is not completely clear that adsorption does occur in electrochromatography. Second, the adsorption expression in linear, since the solute concentration is low, and all adsorption isotherms reduce to a linear form under these conditions.

From the above equations, one can obtain an expression for the solute velocity (u) in terms of the other quantities. The expression is:

$$u = (dx / dt) = [v + \alpha\mu_s E + (1 - \alpha)K_{av}\,\mu_g E] / [\alpha + (1 - \alpha)\,K_{av} + (1 - \alpha)\,(1 - K_{av})k_a] \quad (4)$$

This expression indicates that a plot of u/v vs E/v should be linear if no other effects are present, i.e., if K_{av}, k_a, and perhaps α are independent of v. There is an indication that the adsorption coefficient is dependent on the superficial velocity (*17*). Our experimental results demonstrate that the aforementioned linearity does not always hold as detailed in the Results and Discussion section.

MATERIALS AND METHODS

Solutions and Buffer

The buffer used in this study was made up as a concentrated stock of 39 m*M* tris(hydroxymethyl)aminomethane (Tris) and 470 m*M* glycine (10X buffer). The diluted buffer (1X) had a pH of 8.3 and a conductivity of 102 μS/cm at 25°C. The ionic strength of this buffer was calculated to be 1.5 m*M* by Rudge et al. (*17*). Protein solutions were dissolved in 1X buffer.

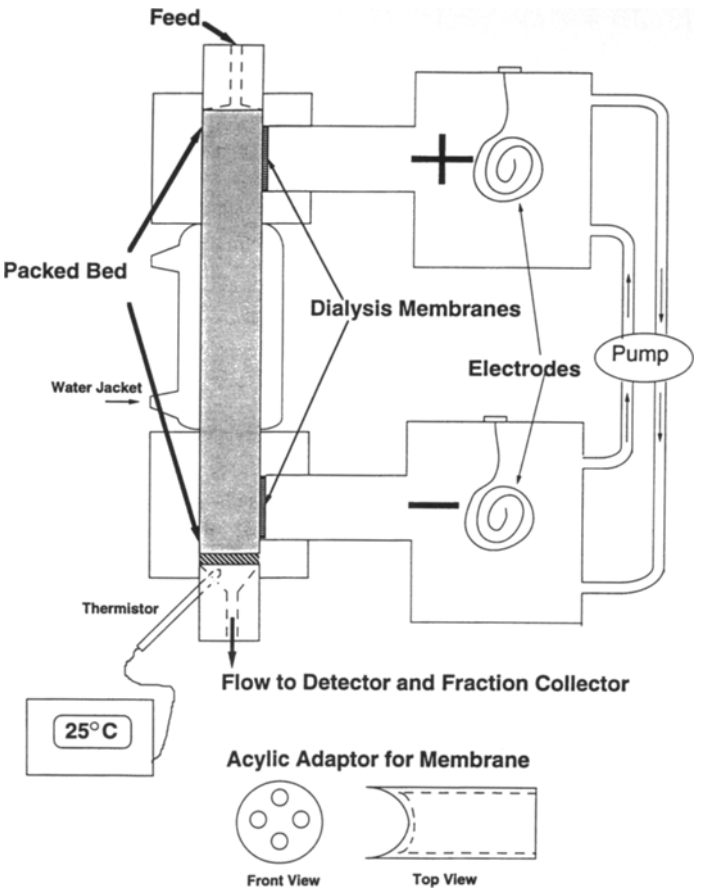

Fig. 1. Diagram of electrochromatography column and acrylic membrane adaptor used.

Electrochromatography Apparatus

The glass water-jacketed columns had inside and outside diameters of 22 and 25 mm, respectively. Dialysis membranes with a mol-wt cutoff of 6000–8000 (Spectrapor 1, Spectrum Medical Industries, Los Angeles, CA) were bonded to circular acrylic adaptors (shown in Fig. 1) using cyanoacrylate ester glue. These adaptors were connected to side-arm reservoirs (aprox 1.4 L) that contained a coil of platinum wire that was 17 cm from the axis of the column. The short column had an overall length of 25.6 cm (volume of 101 mL) and a total current path length of 55.6 cm. The very short column had an overall length of 20 cm (volume of 77 mL) and a total current path of 50 cm. An acrylic adaptor containing a porous polyethylene frit (100-μ pore size) was fitted to the outlet of the bottom block (Fig. 1). A glass-insulated thermistor (Cole-Parmer, Chicago, IL) was placed just below the frit, where it continuously monitored the temperature of the

column eluent. A dual-channel peristaltic pump circulated the buffer between the two-electrode buffer containers. A peristaltic pump (Minipuls 3, Gilson Medical Electronics, Middleton, WI) was connected to the outlet of the column and used to monitor flow. The eluent was monitored at 280 nm (Holochrome Detector equipped with 0.05-mL flow cell, Gilson Medical Electronics, Middleton, WI) and collected by a fraction collector. Fractions were weighed to obtain a flow rate profile. The top adaptor was removed, and the sample applied to a porous polyethylene frit that rested on the top of the packed bed. Samples were applied with the field on during the electrochromatography runs.

Reverse-Phase High-Performance Liquid Chromatography

Protein samples were analyzed by reverse-phase high-performance liquid chromatography (HPLC) using a C18 protein and peptide column (4.6 × 25 cm, #218TP1104, Vydac, Hesperia, CA) using a mobile phase composed of a gradient of 0.1% trifluoroacetic acid (pump A) and acetonitrile (pump B). Flow rate was 1.0 mL/min at ambient temperature and initial conditions of 20% B. A linear gradient was run to 40% B in 2 min after injection. A second gradient was immediately run to 50% B in 25 min. Detection was at 280 nm.

Materials

Myoglobin (horse heart), bovine serum albumin (BSA) (fraction V), α-lactalbumin (bovine milk), β-lactoglobulin (bovine milk), glycine, Sephadex G-75 regular (40–120 μ dry bead diameter), Sephadex G-75 superfine (10–40 μm bead size), Sephadex-25 coarse (100–300 μm bead size), and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Blue Dextran 2000 was obtained from Pharmacia (Uppsala, Sweden). BioGel P-4 (200–400 mesh size) was obtained from Bio-Rad (Richmond, CA).

RESULTS AND DISCUSSION

Electrochromatography

The configuration of the columns is shown in Fig. 1. The column is modular, and components plug into the plastic blocks containing captured O-rings. This column is a modified version of the Boltz-Todd column (*18,19*) used for density gradient electrophoresis of cells. The properties of the model proteins used in this study are shown in Table 1. The mobilities of these proteins shown in Table 1 were measured using density gradient electrophoresis (*19*). These mobilities were measured at 25°C in 1X buffer, so they should be directly related to μ_s, in the free solution phase of the column.

Table 1
Properties of the Model Proteins Used

Protein	Isoelectric point, ref.	Molecular mass, ref.	Mobility, $cm^2/V/s$, $\times 10^{-5} \pm$ SD, data from ref. *19*
BSA	4.9, 4.7 (*20*)[a]	68,000 (*21*)	28.9 ± 1.3 (N = 5)
β-Lactoglobulin A	5.2 (*22*)[b]	17,500 (*22*)[c]	27.2 ± 1.3 (N =9)
β-Lactoglobulin B	5.3 (*22*)[b]	17,500 (*22*)[c]	25.3 ± 1.3 (N =9)
α-Lactalbumin	4.8 (*22*)	14,400 (*21*)	19.4 ± 2.6 (N =8)
Myoglobin	7.4 (*22*)[b]	17,800 (*21*)	5.8 ± 0.7 (N =5)

SD is the standard deviation of the mean of the number of measurements (N).
[a]Measured at 25°C.
[b]Measured at 15°C.
[c]Molecular mass of monomer.

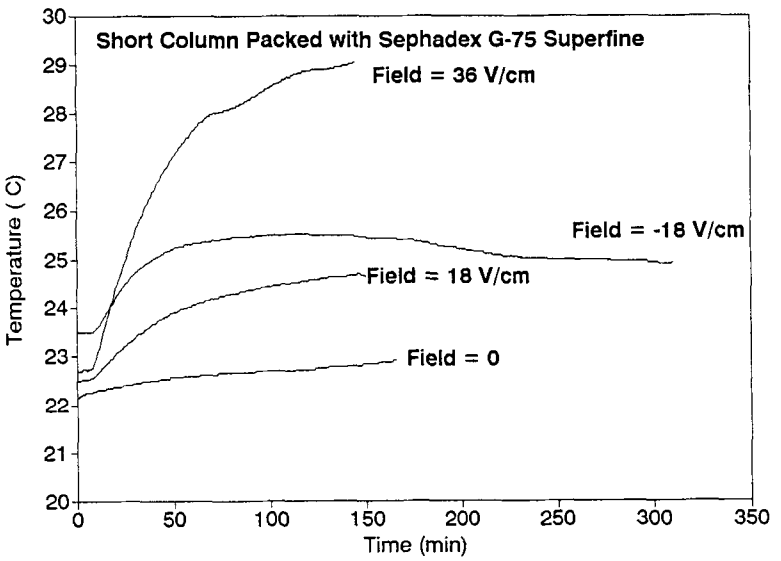

Fig. 2. Column eluent temperature profile. The short column was packed with Sephadex G-75 superfine with a flow rate of 0.38 mL/min (linear velocity of 6.0 cm/h).

The temperature increase of the column eluent is shown in Fig. 2. As can be seen from the data, the increase is only a few degrees with the electrical fields used (18 V/cm). However, the temperature increase can become significant when the field is increased to about 36 V/cm. Rudge et al. (*17*) observed similar temperature increases with their experimental apparatus. The buffer used in these studies results in relatively low currents owing to its low ionic strength and conductivity, and choice of buffer is particularly critical to the success of the separation process. Efficient heat removal from the glass walls of the column and the low currents helped to prevent excessive heating in this column.

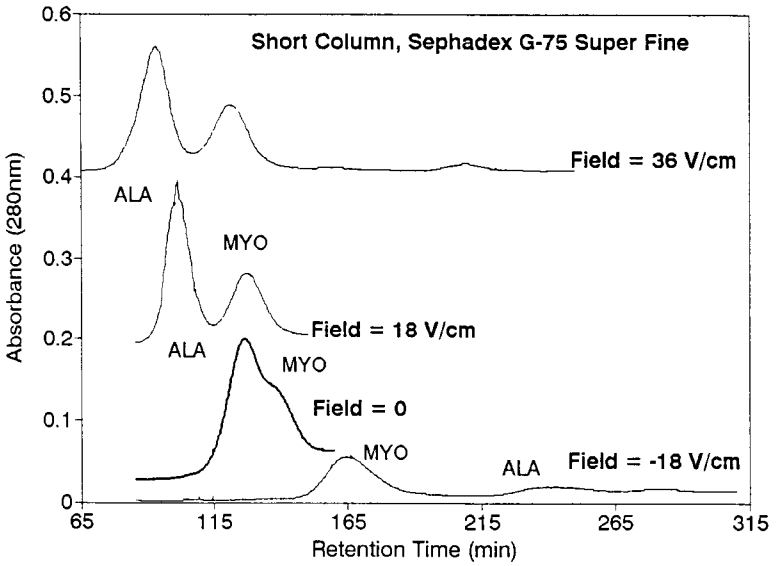

Fig. 3. Separation of α-lactoalbumin and myoglobin on the short column packed with Sephadex G-75 superfine column with and without an applied electrical field. The sample was 0.75 mL of myoglobin (1 mg/mL) and α-lactoglobulin (1.4 mg/mL) and a flow rate of 0.38 mL/min (linear velocity of 6.0 cm/h).

An example of the type of separations that can be achieved with the application of an electrical field is shown in Fig. 3. Myoglobin and α-lactoglobulin do not separate on a Sephadex G-75 superfine column of this size, but application of a positive field separates the two proteins (Fig. 3). In our work, a positive field is defined as one in which electrophoresis is in the same direction as convective flow; in this case, the proteins are negatively charged and the positive electrode is at the outlet. Doubling the positive field resulted in faster elution, but greater dispersion in the peaks possibly because of excessive heating in the column (Fig. 2). Application of a negative field results in the increased retention of both proteins (Fig. 3). As expected, the protein with the greater electrophoretic mobility (α-lactoglobulin) was retained to a much greater extent.

We used a mixture of two model proteins to study the effect of an applied electrical field on their elution from Sephadex columns. The two model proteins were bovine serum albumin and myoglobin; their properties are shown in Table 1. BSA has an electrophoretic mobility approximately fivefold greater than myoglobin. We examined their behavior on two different columns each packed with a Sephadex having different degrees of crosslinking. Both proteins are excluded from the more highly crosslinked Sephadex G-25. Myoglobin was partially included into G-75, and BSA was almost entirely excluded. In these experiments, the field was kept constant, and the superficial velocity (i.e., the free solution flow rate) of the buffer was changed. Figure 4 shows the effect of an applied field on the apparent K_{av} of myoglobin and BSA in Sephadex G-25 course.

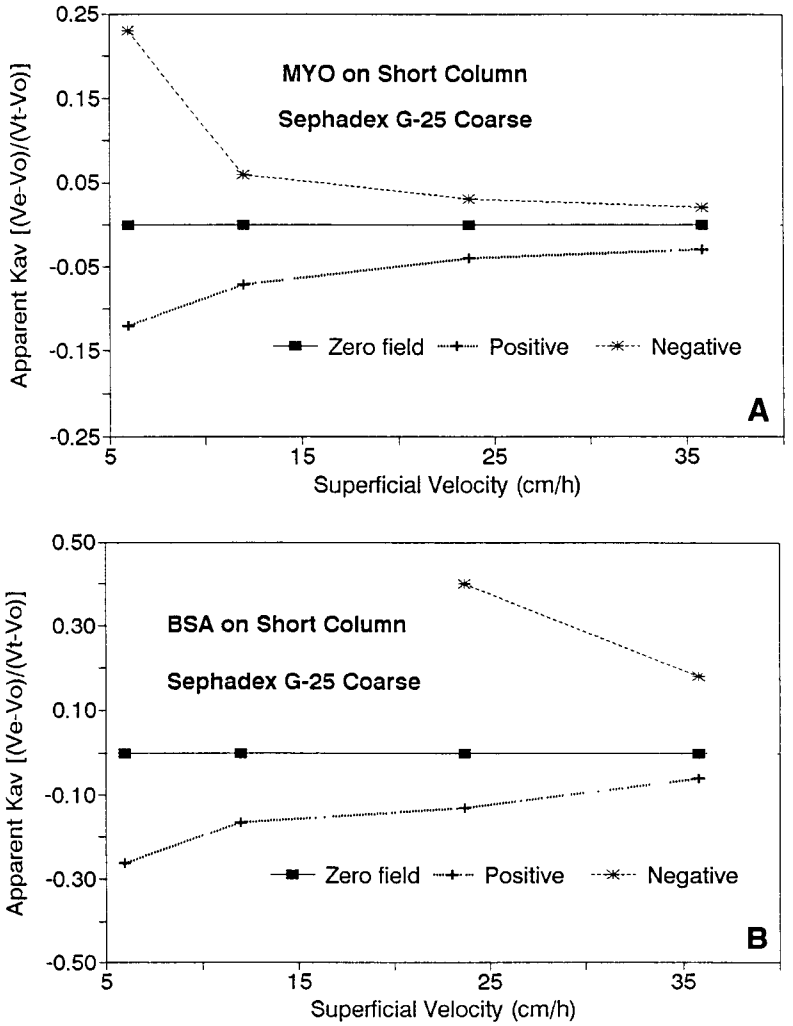

Fig. 4. The effect of applied electrical field on apparent K_{av} of myoglobin (**A**) and BSA (**B**). A 0.75-mL sample containing BSA and myoglobin (both at 1 mg/mL) was applied to the short column packed with Sephadex G-25 Coarse, with either no field, −18 V/cm, or 18 V/cm. The K_{av} is calculated by the formula: $(V_e - V_o)/(V_t - V_o)$, where V_e = elution volume of solute, V_o = void volume determined by elution of blue dextran, and V_t = total volume of column. Each point represents the average of two or three independent determinations of K_{av} at the indicated superficial velocity and electrical field.

A positive field decreases the elution time of the proteins, and a negative field increases the elution time of the proteins. At low superficial velocities, a negative field results in BSA being held on the column (Fig. 4B). The results for Sephadex G-75 are somewhat different. In the case of both proteins, a negative field resulted in increased retention on the column as expected (Fig. 5A and B). Application of a positive field resulted in faster

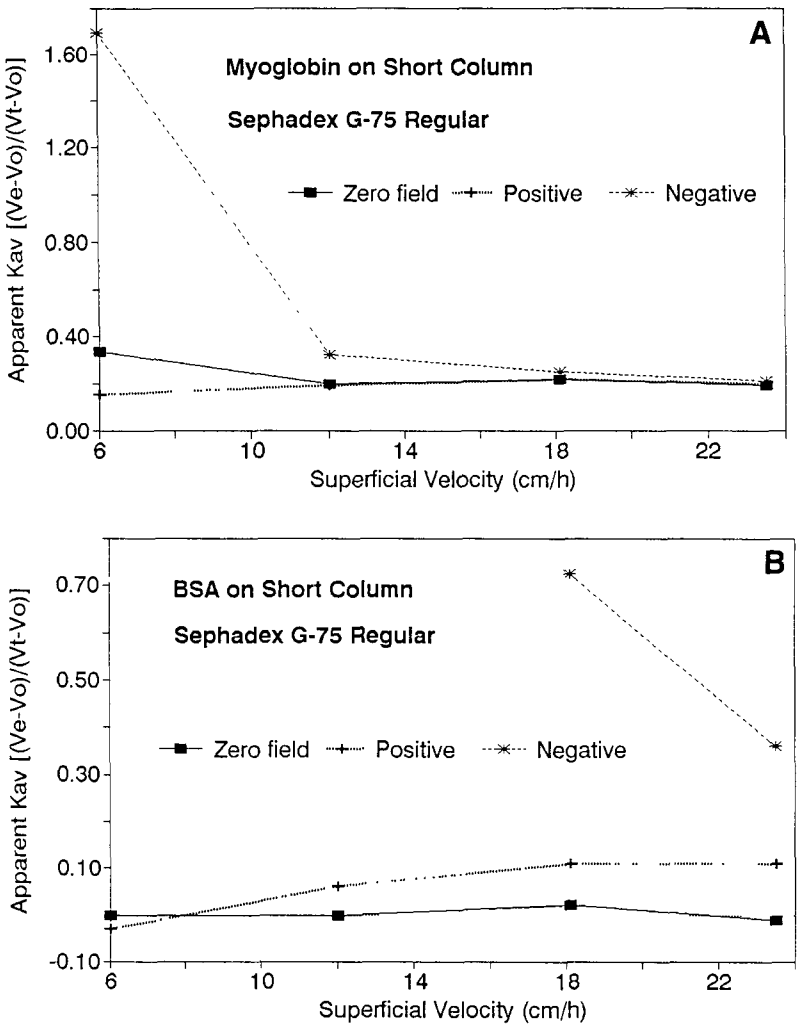

Fig. 5. The effect of applied electrical field on apparent K_{av} of myoglobin (**A**) and BSA (**B**) in the short column packed with Sephadex G-75 regular. A 0.75-mL sample containing BSA and myoglobin (both at 1 mg/mL) was applied to the column with either no field, −18 V/cm, or 18 V/cm.

elution of myoglobin at lower superficial velocities (Fig. 5A), but the application of a positive field to BSA in Sephadex G-75 resulted in increased retention at linear velocities, except the lowest value tested. This behavior was also observed by Rudge et al. (*17*).

We have plotted these data in the form suggested by Rudge et al. (*17*) using Eq. (4). Plotting u/v vs E/v should result in a straight line if only electrophoresis and convective flow are occurring. Rudge et al. (*17*) added a term for adsorption to explain the anomalous behavior of proteins and blue dextran on G-75. This transformation results in linear plots for myoglobin on Sephadex G-75 (r^2 = 0.94; Fig. 6A) and for BSA on Sephadex

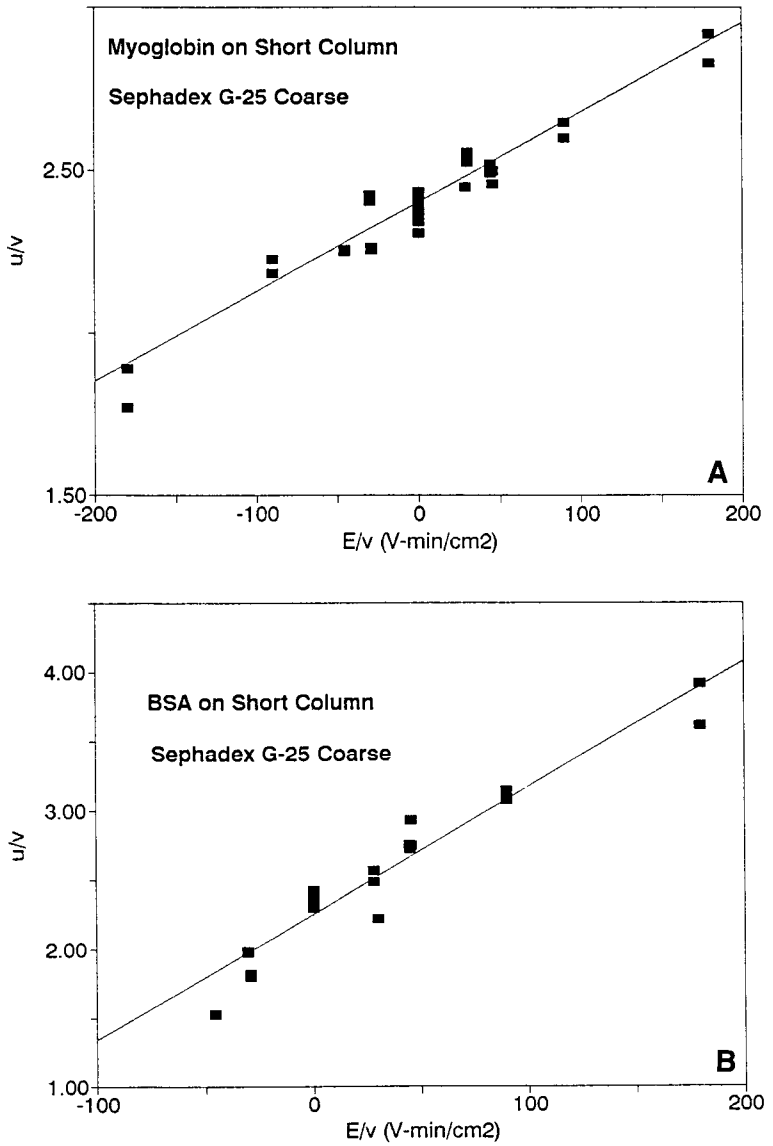

Fig. 6. Plots of reduced electrochromatographic mobility as a function of field divided by superficial velocity in Sephadex G-25 Coarse. Data are from experiments described in Fig. 4.

G-25 (r^2 = 0.90; Fig. 6B). The plots for Sephadex G-75 are quite different. Both proteins have a pronounced break in the curve in going from a negative to a positive field (Fig. 7A and B). On Sephadex G-25, myoglobin and BSA will both have a K_{av} of near 0. Then Eq. (4) will reduce to:

$$u / v = [\alpha\mu_s (E / v)] / [\alpha + (1 - \alpha)k_a] + 1 / [\alpha + (1 - \alpha)k_a] \quad (5)$$

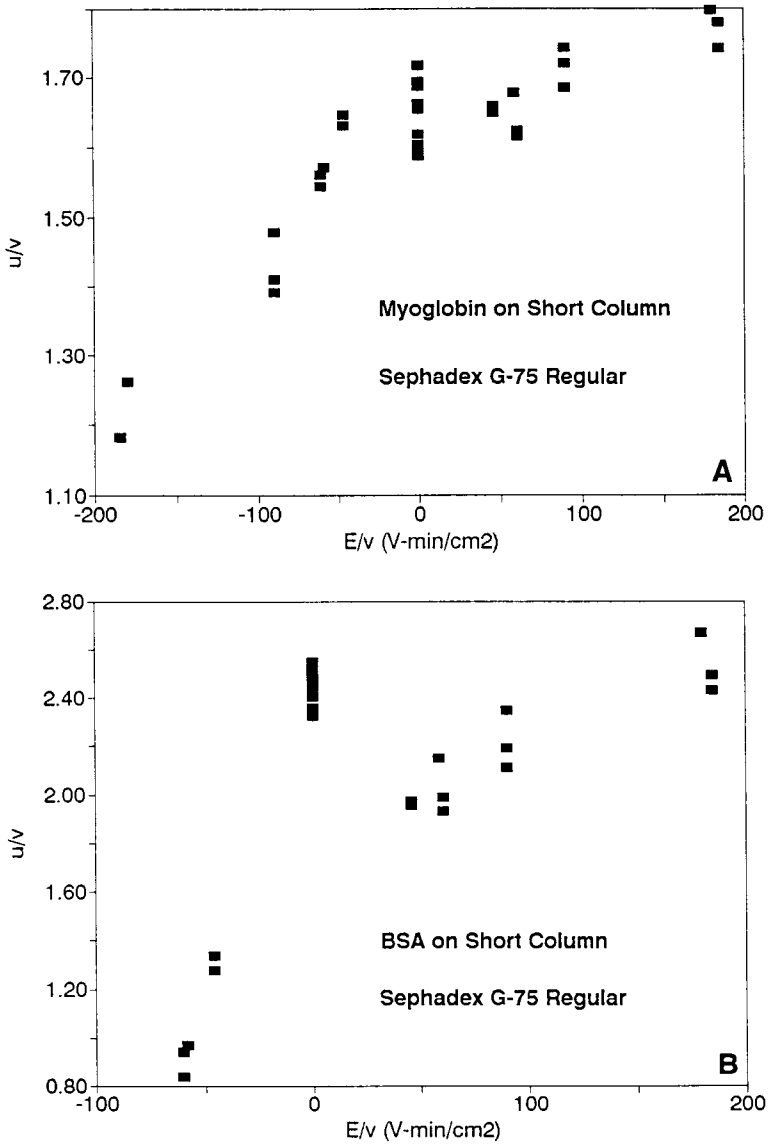

Fig. 7. Plots of reduced electrochromatographic mobility as a function of field divided by superficial velocity in Sephadex G-75 Regular. Data are from experiments described in Fig. 5.

If k_a is zero, i.e., if there is no electrically driven adsorption, then $u = \mu_s E$, and the slope of the plot of Eq. (5) gives the electrophoretic mobility of the solute in free solution. The slope of the linear regression of the data shown in Fig. 6A is 4.6×10^{-5} cm²/V/s for myoglobin. The slope of the linear regression line in Fig. 6B is 15.2×10^{-5} cm²/V/s for BSA. It is interesting to note that for myoglobin, the value of the slope is nearly within the ex-

perimental uncertainty for the independently measured electrophoretic mobility (Table 1). For BSA, however, the slope is significantly below the measured value of the electrophoretic mobility consistent with the presence of an adsorption effect, since there is a nonzero value of k_a. These observations are consistent with the preliminary results from our own mathematical modeling efforts.

We have attempted to explore the levels of resolution that can be achieved by electrochromatography in general and by our experimental setup in particular. To this purpose, we studied the model system of β-lactoglobulin A and B for which the electrophoretic mobilities differ by approx 8% (Table 1). These two nearly identical genetic variants would not be expected to be separated by size-exclusion gel chromatography. However, we were able to achieve a partial resolution of these two forms by electrochromatography on a short column of BioGel P-4 with a negative electrical field. Figure 8A shows the absorbance curve at 280 nm, indicating the separation. Figure 8B shows the distribution of β-lactoglobulin A and B in the fractions as measured by HPLC analysis of the fractions. The peaks are separated, but both forms are present in the peak tails resulting in a partial separation. Similar results were obtained with Sephadex G-25 in a short column. The best results were obtained with short columns filled with the more highly crosslinked gels, such as Sephadex G-25 or BioGel P-4.

CONCLUSIONS AND FUTURE DIRECTIONS

We have confirmed the results of Rudge et al. (*17*) on Sephadex G-75, which shows unexplained electrically driven retention with electrical fields of both polarities. We have extended this work to Sephadex G-25. The electrically driven retention appears to be greatly reduced in Sephadex G-25. The Sephadex G-75 and G-25 differ in their degree of crosslinking, but are chemically very similar. Any physical or chemical explanation of the electrically induced retention behavior must take this into account. The retention effect is the strongest with BSA, which has the highest electrophoretic mobility. We have considered the possibility that a specific adsorption is taking place on the surface of the beads. BSA is able to enter some of the pores in Sephadex G-75 (K_{av} = 0.01), but it is completely excluded from Sephadex G-25 (K_{av} = 0.00). An adsorption on the surface of Sephadex G-75 might increase the amount of BSA entering the gel phase. This would be less likely in Sephadex G-25. An increase in the amount of BSA entering the gel phase in Sephadex could explain the electrically driven retention effect.

Electrochromatography has the potential for high resolution, as shown by the partial separation of the two genetic variants of β-lactoglobulin. If the tailing of both peaks can be reduced or eliminated, the separation

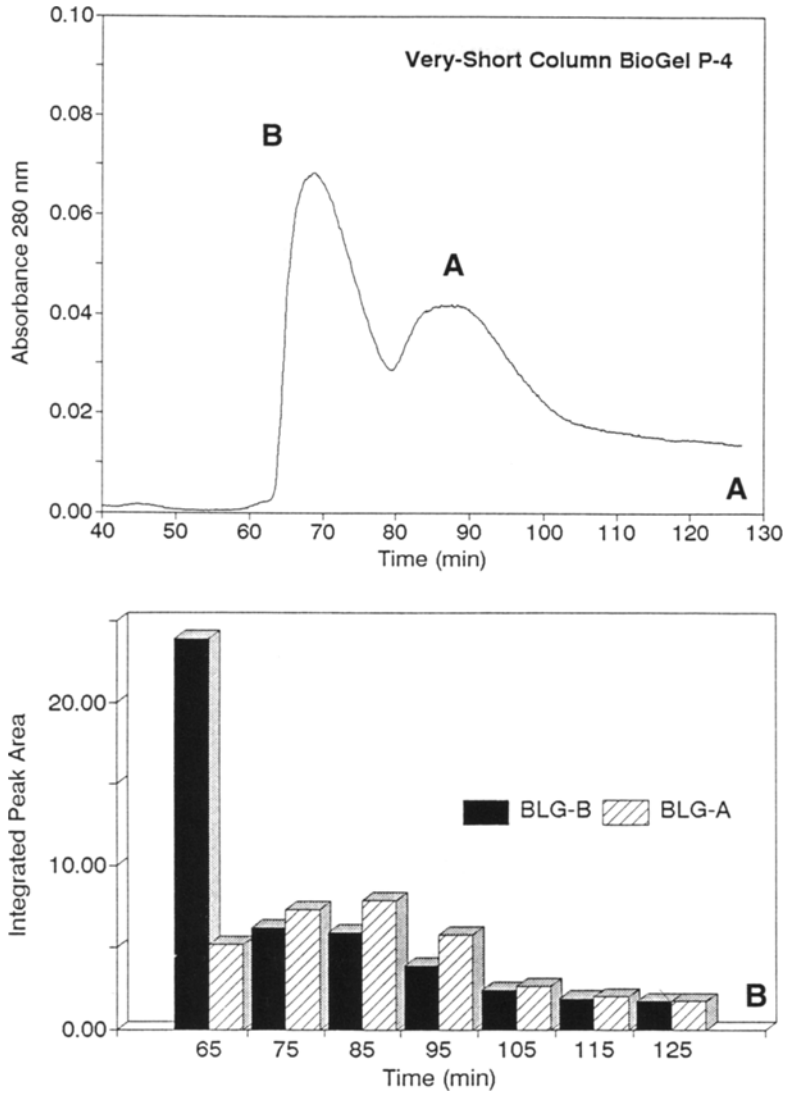

Fig. 8. (**A**) The absorbance at 280 nm of the separation of β-lactoglobulin A and B on the very short column packed with BioGel P-4 (200–400 mesh). The flow rate was 0.75 mL/min (linear velocity of 11.8 cm/h) and a field of 22 V/cm. The sample was 2.0 mL of β-lactoglobulin A and B (2 mg/mL). (**B**) The analysis of the fractions (every 10 min) of the separation of a mixture of β-lactoglobin. The fractions were analyzed by HPLC as described in the text.

would be very satisfying. The peak tailing could be the result of a nonuniform electrical field in the column, inefficient heat rejection at the ends of the column with the development of thermal gradients, or electrically driven retention of unknown mechanism. To eliminate the first two possibilities, we are designing and constructing columns that should provide

more uniform electrical fields and more efficient heat rejection. The third possibility, however, will be more difficult to address. Alternative packing materials may provide insight into this mechanism. A clue to a possible solution may be provided by the fact that Porath (*9*) obtained the best results with highly purified cellulose material. We plan to investigate the performance of such material in the future.

ACKNOWLEDGMENTS

We wish to acknowledge Dr. Scott Rudge (Synergen, Boulder, CO) for very useful advice and suggestions. We wish to thank Dr. Paul Todd (Department of Chemical Engineering, University of Colorado, Boulder, CO) and Dr. Dennis Reeder (NIST, Gaithersburg, MD) for support and encouragement.

REFERENCES

1. Haglund, H. and Tiselius, A. (1950), *Acta Chemica Scand.* **4,** 957–962.
2. Flodin, P. and Porath, J. (1954), *Biochim. et Biophys. Acta.* **13,** 175–182.
3. Carlson, L. A. (1954), *Acta Chemica Scand.* **8,** 510–520.
4. Bloemendal, H. (1960), *J. Chromatogr.* **3,** 1–10.
5. Porath, J. (1954), *Acta Chemica Scand.* **8,** 1813–1826.
6. Porath, J. (1956), *Biochim. et Biophys. Acta* **22,** 151–175.
7. Flodin, P. and Kupke, D. W. (1956), *Biochim. et Biophys. Acta.* **21,** 368–376.
8. Porath, J., Lindner, E. B., and Jerstedt, S. (1958), *Nature* **182,** 744–745.
9. Porath, J. (1964), *Sci. Tools* **11** (2), 21–27.
10. Nerenberg, S. T. and Pogojeff, B. A. (1969), *Am. J. Clin. Path.* **51,** 728–740.
11. Luzzio, A. J. (1975), *J. Infect. Dis.* **4,** 359–366.
12. Epstein, I. (1977), *Chromatographia.* **10 (2),** 65–69.
13. O'Farrell, P. H. (1984), *Science* **227,** 1586–1589.
14. Hunter, J. B. (1988), *Sep. Sci. Tech.* **23,** 913–930.
15. Locke, B. R. and Carbonell, R. G. (1989), *Sep. Pur. Methods* **18,** 65–109.
16. Ivory, C. F. and Gobie, W. A. (1990), *Biotechnol. Prog.* **6,** 21–32.
17. Rudge, S. R., Basak, S. K., and Ladisch, M. R. (1993), *AIChE J.* **39,** 797–808.
18. Boltz, R. C., Jr., Miller, T. Y., Todd, P., and Kukulinsky, N. E. (1978), in *Electrophoresis '78,* Catsimpoolas, N., ed. Elsevier North Holland, Amsterdam, pp. 345–355.
19. Cole, K. D., Todd, P., Srinivasan, K., and Dutta, B. K. (in press), *J. Chrom.*
20. Radola, B. J. (1973), *Biochim. Biophys. Acta.* **295,** 412–428.
21. Reeder, D. J. (1987), in *New Directions in Electrophoretic Methods,* Jorgenson, J. W. and Phillips, M., eds., American Chemical Society Symposium Series, vol. 335, pp. 102–116.
22. FMC Bioproducts, Rockland, ME.
23. Zittle, C. A. (1956), *Arch. Biochem. Biophys.* **64,** 144–151.