Solute Retention in Electrochromatography by Electrically Induced Sorption

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Column chromatography and electrophoresis are combined in electrochromatography, where an electric potential is applied to a chromatography column in the axial direction. These studies utilized a dextran gel stationary phase and an eluent of low ionic strength, which were chosen to minimize electric current and therefore column heating and undesirable dispersion effects. The gel, with a small ion exchange capacity of several microequivalents per mL, turned out to be more conductive than the eluent and was able to concentrate macromolecules in the presence of combined electric and flow fields. The model presented describes solute retention due to electrically induced concentration polarization of solute on the resin surfaces, as well as electrophoresis in the mobile and stationary phases. The polarization effect explains differences between retention of high-molecular-weight solutes with exclusion coefficients of less than 1 and that of a charged low-molecular-weight solute, which is hypothesized to pass through the gel matrix in the presence of an electric field and does not experience concentration polarization. It also shows the application of this effect for protein separation in a liquid chromatography system with a superimposed electric potential.

Introduction

Separations in batch one-dimensional or continuous two-dimensional systems exploit differences in the velocity of solutes to obtain resolution of the solutes. Chromatographic systems employ a fixed column length, in which differing solute velocities result in differing retention times, while zone electrophoresis systems use a fixed time and result in differing migration distances for each component. Free-flow electrophoresis and continuously rotating annular chromatography, both continuous systems, exploit differing velocities to create different elution positions for each solute in a direction perpendicular to the flow. The retention time of solutes in chromatographic systems is critical to unit performance, since if individual retention times are not controllable or predictable, separation cannot be assured. Dispersion or band broadening becomes important only when differing solute velocities have been established.

We report retention behavior of blue dextran, proteins and bromphenol blue in a size exclusion chromatography system to which an axial electric field is applied. Invigorated by the work of O'Farrell (1985), there has been a great deal of work with packed beds to which a potential has been applied. These systems have a long history, as reviewed by Rudge and Ladisch (1988) and Ivory (1988). We have found these systems to display complex behavior, particularly for protein separations. The separation of proteins by combining electric fields with a gel permeation column was published first by Nerenberg and Pogojeff (1969), with separation of blood plasma proteins on Sephadex G-100. The technique was later extended to hepatitis antigen separation by Luzzio (1975) on Sephadex G-200 and to separations of nucleotides, and pyrimidine bases and their derivatives by Tsuda (1987) on reverse-phase (octadecylsilane) bonded resins. Electrochromatography models assume a number of processes to be important, including coion and counterion migration, Joule heating, electroosmosis, and other convection effects. Other models have addressed the complex heating and heat-transfer effects in electrophoresis and electrochromatography systems (Ivory and Gobie, 1989; Lynch and Saville, 1981).

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Anomalous retention behavior of large solutes was observed at the conditions which were selected for our experiments, which minimized the band broadening caused by heating and natural convection effects. Investigation of this phenomenon showed that the application of an electric field to a chromatography column packed with dextran gel particles may lead to concentration polarization on the surface of the gel matrix. Electrically induced polarization could have a profound effect on the manner in which mechanisms of electrochromatography are interpreted, the types of stationary phases which are selected for use in an electrochromatography column, and the manner in which operating conditions might be manipulated to achieve separations of macromolecules. The discovery of this phenomenon led to a model that relates solute retention behavior to the electric field strength and eluent flow rates.

Model

A simple equilibrium model was developed for the purpose of calculating component retention times in the presence of convection, or convection and electric forces. This development started from conventional chromatography theory, into which a term for simultaneous application of an electric field was incorporated (Ivory and Gobie, 1990).

Solute migration due to mass transport in electrochromatography

Solutes migrate in electrochromatography columns under convective forces and electric fields. In a local equilibrium analysis, the migration is described by a mass balance:

$$\alpha \frac{\partial c}{\partial t} + v \frac{\partial c}{\partial x} + (1-\alpha) \frac{\partial n}{\partial t} + \alpha \mu_1 E \frac{\partial c}{\partial x} + (1-\alpha)\mu_2 E \frac{\partial n}{\partial x} + (1-\alpha) \frac{\partial p}{\partial t} = 0 \quad (1)$$

where n is the average concentration of the solute in the gel phase, α is the extraparticle void fraction, and p represents the average concentration of solute immobilized on the surface of the resin by the electric field. Two equilibrium relations used with Eq. 1 are:

$$n = K_{av} c \quad (2)$$

and for $K_{av} < 1$

$$p = k_p (c - n) = k_p (1 - K_{av}) c \quad (3)$$

In the case where $K_{av} = 1$, complete penetration of the solute is implied, and therefore $k_p = 0$. For $K_{av} > 1$, adsorption is occurring and thus, $(V_e - V_o)/(V_t - V_o) > 1$. For ideal size exclusion chromatography, K_{av} cannot be larger than 1, hence some other effect is indicated. This condition was observed in our experiments for bromphenol blue.

Equation 3 is a key relation since it gives a simple, but effective, representation of the electrically induced concentration polarization effect. The polarization potential, k_p, defines the extent to which the electric field holds a molecule against the surface of a gel particle and out of the flow field. The term $(1 - K_{av})$ is the probability that a molecule will not enter the gel at the surface. For large molecules, such as blue dextran, the probability is 1 ($K_{av} = 0$); for small molecules, the probability is 0 ($K_{av} = 1$). The expression for k_p is a new component of this type of model and represents the surface of the resin as a leaky membrane and the electric field as analogous to a pressure driving force. A solute, which reaches the resin surface at a pore so that it can penetrate, will enter; a solute, which reaches the surface at a pore too small to penetrate, may be held there by the electric field against back-diffusion. Whether or not the solute molecule will be held depends on the strength of the electric field, the diffusivity of the molecule, and the partition coefficient, just as the concentration polarization layer in filtration depends on the pressure drop, the solute diffusivity, and the rejection coefficient. Combining Eqs. 1, 2 and 3 results in:

$$[\alpha + (1-\alpha)K_{av} + (1-\alpha)(1-K_{av})k_p] \frac{\partial c}{\partial t} + [v + \alpha\mu_1 E + (1-\alpha)K_{av}\mu_2 E] \frac{\partial c}{\partial x} = 0 \quad (4)$$

The method of characteristics, applied to Eq. 4, gives (Courant and Hilbert, 1953):

$$u = \frac{dx}{dt} = \frac{v + \alpha\mu_1 E + (1-\alpha)K_{av}\mu_2 E}{\alpha + (1-\alpha)K_{av} + (1-\alpha)(1-K_{av})k_p} \quad (5)$$

This equation reduces to a linear form of u vs. E for small solutes which have $K_{av} \geq 1$:

$$u = \frac{\alpha\mu_1 + K_{av}(1-\alpha)\mu_2}{\alpha + (1-\alpha)K_{av}} \cdot E + \frac{v}{\alpha + (1-\alpha)K_{av}} \quad (6)$$

For a large solute that is excluded from the stationary phase ($K_{av} = 0$), Eq. 6 reduces to:

$$u = \frac{\alpha\mu_1}{\alpha + (1-\alpha)k_p} \cdot E + \frac{v}{\alpha + (1-\alpha)k_p} \quad (7)$$

Equation 7 can be used to obtain values of k_p from the measured value of u for a large solute whose free solution electrophoretic mobility μ_1 is known or can be estimated.

Convection in electrochromatography

There are two important sources of convection to consider in the analysis of electrochromatography. Axial convection is the result of a pressure drop across the column, as in liquid chromatography. Pressure-driven convection is inversely related to eluent viscosity, which itself is inversely related to eluent temperature. Therefore, increasing the temperature of the column by Joule heating results in an increased flow rate for a constant pressure drop. Convection may also result from electroosmotic flow along the column wall and resin surfaces. We have found, however, the contribution of electroosmosis to the total flow to be very small. By measuring peak velocity, rather than retention time, we were able to eliminate flow rate effects from our data. Over the range of linear velocities investigated, 0.3 to 1.1 cm/min, the flow rate had no effect on

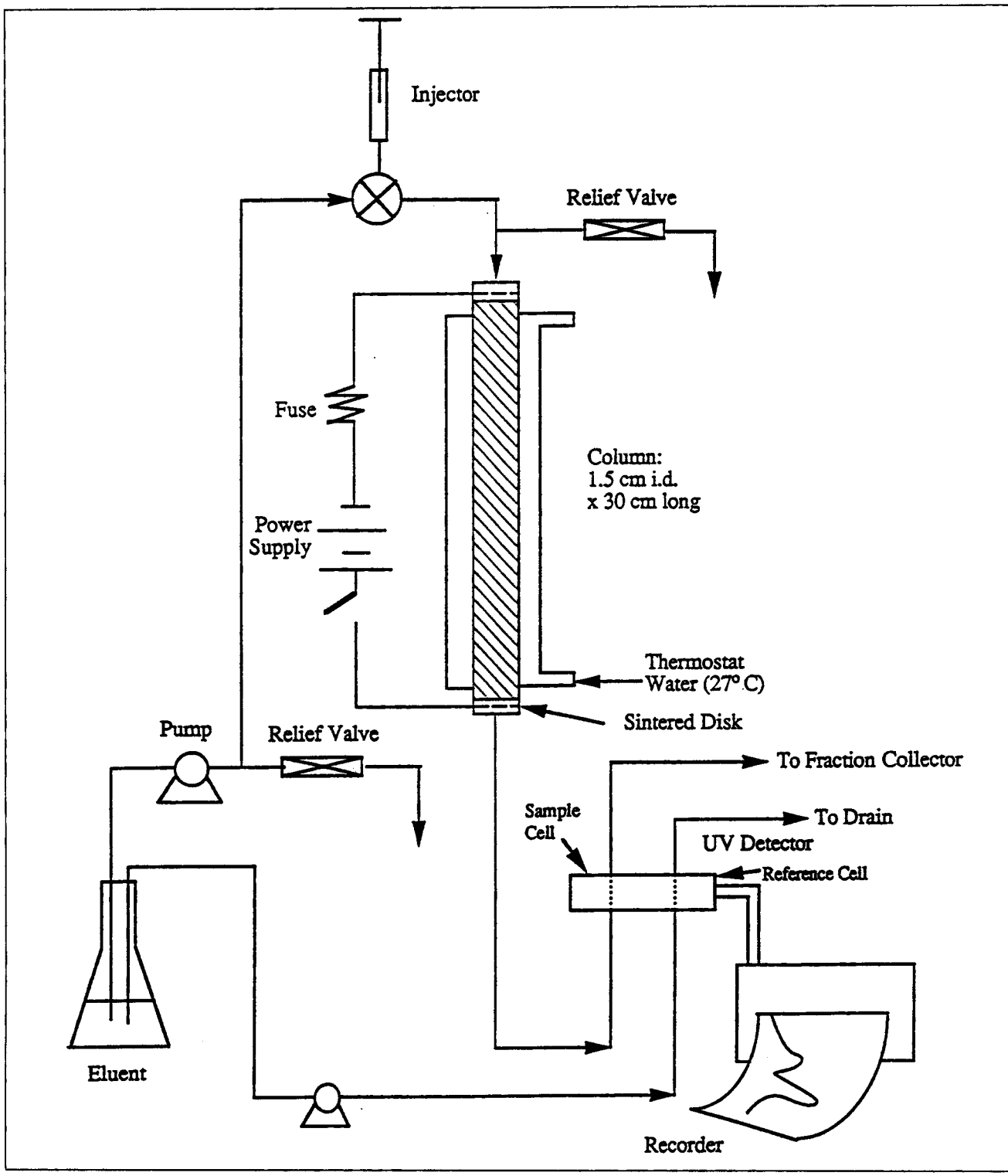

Figure 1. Apparatus: positive electric field obtained with the anode (+) at the column inlet.

solute elution volume, and the relation between solute velocity and linear velocity (u and v) was linear, as indicated by Eq. 6.

The second important type of convection is radial convection, which may be induced by Joule heating, leading to natural convection, or by electroosmosis in a closed system. Radial convection would lead to backmixing and contribute significantly to band dispersion (Allen et al., 1977). Since our peaks eluted in essentially the same shape with or without the electric field, we assumed that radial convection due to Joule heating or electroosmosis did not significantly affect axial peak velocity.

Experimental Studies

Column and electrodes

An Ace Glass jacketed column (15 × 300 mm) with Teflon end caps (Ace Glass, Inc., Vineland, NJ) was modified to accommodate platinum electrodes. The configuration for the column and detectors is shown in Figure 1. The end cap, with electrode, is shown in Figure 2. Eluent is delivered to the column head at rates of up to 2 mL/min via a peristaltic pump (Masterflex, Cole Parmer, Chicago). A two-way valve (Type 50 Teflon rotary valve, Rheodyne, Cotati, CA) served as an injector in the line 4 cm upstream from the column head. All tubing used was Tygon food grade size 14 (1.6 mm ID) (Cole Parmer) or Teflon spaghetti tubing (1.5 mm ID, 2.3 mm OD) (Ace Glass). Effluent from the column traveled to a UA-5 UV spectrophotometric absorbance fluorescence detector with a Type 6 Optical unit, (280 nm, ISCO, Lincoln, NE) with deionized, boiled and filtered water as reference, and then to a fraction collector (model 328, ISCO), if necessary. The column was an Ace Glass 15 × 300 mm jacketed chromatography column packed with Sephadex G-75 (Sigma Chemical Co., St. Louis) size exclusion gel which was previously swollen in a solution of 47-mM glycine in 3.9-mM Trizma buffer at pH 8.1. A constant temperature of 27°C was maintained in the column jacket with an MGW Lauda C20 (Germany) recirculating water bath.

The electrodes were placed in the end caps as shown in Figure 2. The electrode was a 0.38-mm-OD platinum wire, 6 cm of which was coiled behind the glass frits of the end fittings.

The Sephadex was packed so that it would fit tightly against the glass frits (2 to 5 micron cutoff) under flow conditions. The packing procedure consisted of filling the column with slurry containing between 20% to 40% (by volume) preswollen Sephadex in a buffer solution consisting of 47-mM glycine and 3.9-mM Trizma. The buffer was allowed to partially drain from the column, and more Sephadex slurry was added to bring the slurry level up to the top of the threads, as the liquid was draining. This was repeated over a period of one to two hours until the Sephadex level was flush with the inlet distributor. A well packed column gives symmetrical peaks with plate counts on the order of 900 to 1,500 per meter for blue dextran at 1 mg/mL concentration, when the column is run in the absence of an applied electric potential. The Sephadex itself has a slight charge, with a cation exchange capacity of 10 to 30 microequivalents/gram (Eaker and Porath, 1967). Ion exchange effects, however, are negligible in the presence of buffer ions.

During normal operation, the electrodes were continuously bathed in eluent consisting of 47-mM glycine in 3.9-mM Trizma buffer, which flowed in and out of the column through the end fittings. Gas generated in the column dissolved into the buffer or was flushed out of the column during runs where the current was applied for 10 minutes to 2 hours.

Electrolyte solution equilibria and pH

The buffer used for electrochromatography is critical for the success of the separation. It must be of high enough ionic strength to dissolve the solute of interest and screen ion exchange sites on the resin, but low enough to minimize the amount of current in the system. Jovin et al. (1964) used a tris-glycine buffer of pH 8.3 in their preparative gel electrophoresis apparatus to separate hemoglobin fractions. Orr et al. (1972) investigated a tris-glycine buffer for use in analytical electrophoresis and predicted good resolution could be obtained using it. They correlated the electrophoretic mobility of some proteins at their isoelectric points. These included α-amylase, aldolase, and pepsinogen. Bushey and Jorgenson (1989) reported that high concentrations of zwitterionic buffer additives improved protein separations in capillary zone electrophoresis, because they do not contribute to the conductivity of the buffer, thus making possible the use of higher voltages.

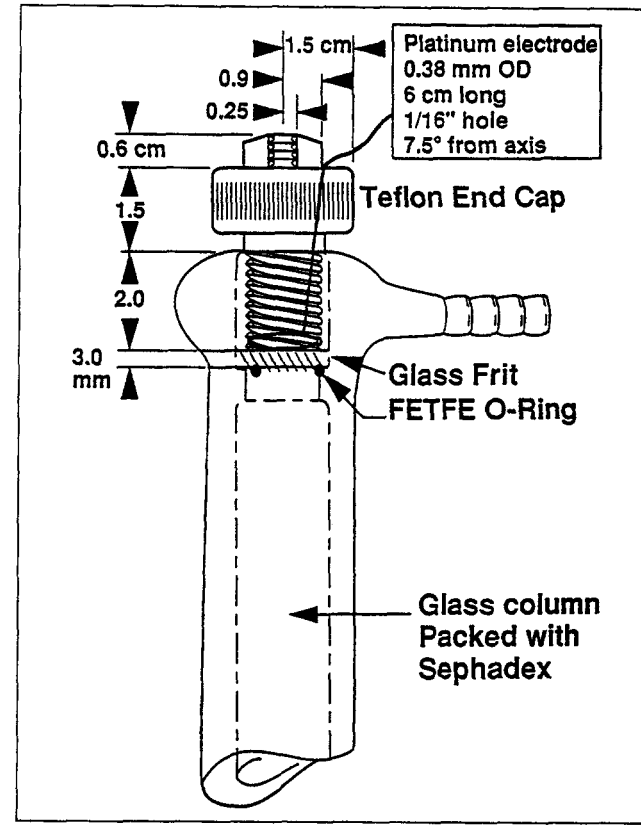

Figure 2. Design of end cap to accommodate an electrode in the flowing buffer.

The hole for the electrode was cut at an angle of approximately 14° from the axis of the piece. After the electrode was inserted, the hole was sealed with silicone rubber and capped with epoxy. The Teflon surface was treated with Chemgrip (Auburn Chemicals, Indianapolis, IN) to promote epoxy adhesion.

The 3.9-mM tris/47-mM glycine buffer used in these experiments was adopted from electrophoresis experiments with milk proteins (Rudge, 1989) and was consistent with a similar composition proposed by Jovin (1973). The desired combination of buffering capacity and low ionic strength, and therefore low conductivity and minimal heating upon application of an electric potential, was obtained with this buffer.

The pH and ionic strength of the eluent buffer can affect protein electrophoretic mobility, protein distribution in the resin, and local electric field strength. Experiments were conducted to determine the relative importance of ionic equilibrium in protein migration in electrochromatography using the tris-glycine buffer. Trizma base [$C_4H_{11}NO_3$, tris(hydroxymethyl)aminomethane] is the primary cationic

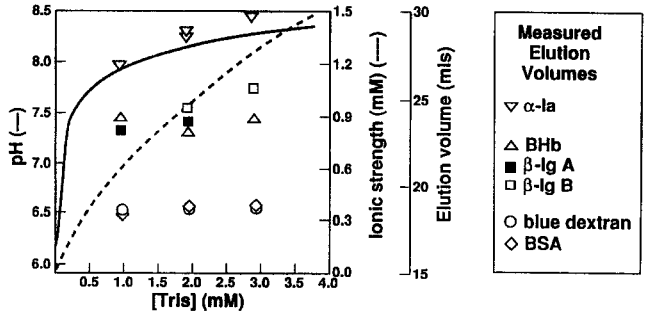

Figure 3. Effect of tris concentration on pH, ionic strength, and protein elution volume in a 47-mM glycine solution.

Column dimensions were 15 × 300 mm (53 mL) and packed with Sephadex G-75.

species in the system, with a temperature-dependent pK given by the expression $[8.08 - 0.03(T\text{-}25)]$, where T is the temperature in °C (Sigma, 1978). Glycine ($C_2H_5NO_2$), an α amino acid, is the primary anion in the system. Glycine has both a positive and negative group, the carboxyl group having a pK of 2.4, and the amino group having a pK of 9.8 at 25°C (Stryer, 1981). The hydrogen ion concentration [H] is given by the expression (Basak et al., 1992):

$$[H]^2 = \frac{\dfrac{K_{am}[gly]}{\dfrac{[H]}{K_{car}} + \dfrac{K_{am}}{[H]} + 1} + K_{oh}}{\dfrac{(tris/K_{tris})}{1 + \dfrac{[H]}{K_{tris}}} + \dfrac{1}{K_{car}} \dfrac{[gly]}{\dfrac{[H]}{K_{car}} + \dfrac{K_{am}}{[H]} + 1} + 1} \tag{8}$$

where [gly] is the glycine molarity, [tris] is the Trizma molarity, and $K_{car} = 3.98 \times 10^{-3}$ M, $K_{am} = 1.58 \times 10^{-10}$ M, $K_{tris} = 7.76 \times 10^{-9}$ M, and $K_{oh} = 1 \times 10^{-14}$ M^2 (Rudge, 1989). The effects of trisconcentration on buffer pH and ionic strength are shown in Figure 3.

The relative ionization of both the protein solutes and the stationary phase can be affected by the pH and ionic strength of the buffer. Consequently, the interactions between the resin phase and protein are also affected by the effect of the electric field on the local buffer composition. We measured the effect of the buffer composition on the distribution coefficient by measuring solute elution volume at different Trizma concentrations. The elution volumes of β-lactoglobulin (β-lg), α-lactalbumin (α-la), bovine serum albumin (BSA), bovine hemoglobin (BHb), and blue dextran increase less than 7% when Trizma concentrations increase from 1.0 to 3.0 mM (Figure 3). When the Trizma component is absent, the elution volumes of proteins eluted in 47-mM glycine alone are significantly different. However, when NaCl is added to 47-mM glycine buffer to reach an ionic strength of 1.48×10^{-3} M at pH 6.1 (equivalent to the ionic strength of 47-mM glycine, and 3.9-mM tris), all the solutes again elute in volumes similar to the 47-mM glycine, 3.9-mM tris-buffer. Consequently, an ionic strength greater than that of distilled water is important to obtaining reproducible retention volumes.

The tris-glycine buffer is continuously passed through the column in constant voltage electrochromatography experiments. During an experiment, the current varied from 2% to 10%. The tris-component of the buffer tended to migrate to the column inlet in positive fields and gave a slight decrease in the current at constant voltage. The opposite occurred in negative fields, where the tris-component migrated toward the column outlet, where it again accumulated slightly. This increased buffer conductivity and current at constant voltage. In both cases, the effects were moderated by the continuous flow of buffer and were slight, as shown by the small changes in the observed current and pH.

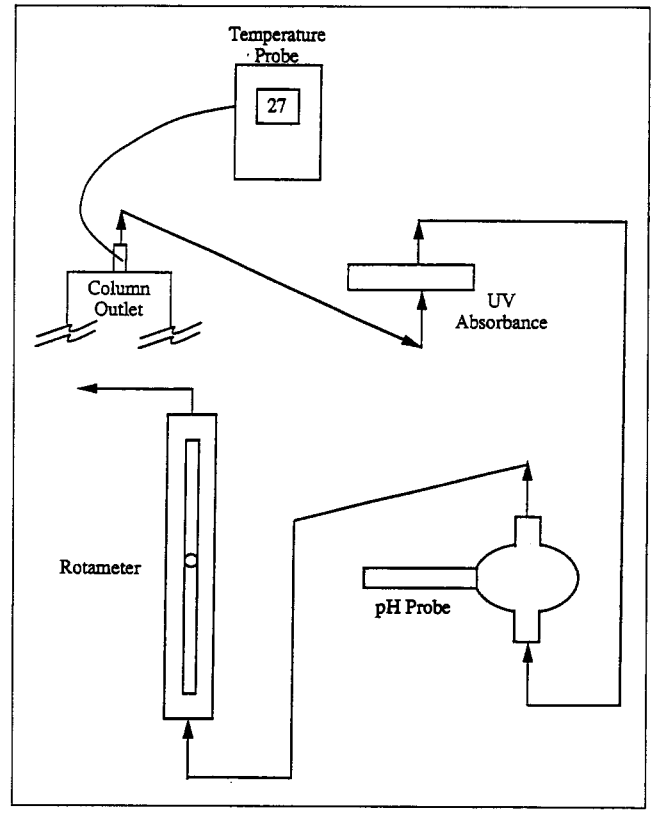

Figure 4. Column outlet detection system (temperature, absorbance, pH, and flow rate).

Experimental measurements and operating conditions

A pH probe and a floating ball rotameter were installed downstream from the optical unit for UV absorbance (Figure 4). Eluent flowed up through the pH probe cell (1.5 × 3.0 mm, Radiometer, Westlake, OH) consisting of a Radiometer A8-GK743950DR calomel electrode, connected to a Chemtrix type 50 pIon meter (Chemtrix, Inc., Hillsboro, OR). When the cathode was at the column outlet, pH could be measured continuously. However, the electric field interfered with the pH measurement when the anode was at the column outlet. In this case, pH measurements could be made only when the current to the column was disconnected. The rotameter was a TF 150-mm Teflon rotameter (Cole Parmer) and was calibrated by collecting and weighing eluent which flowed through in a measured time. Eluent flow rates were measured for each run.

Outlet temperature was monitored by inserting a thermocouple in the connection between the outlet end cap and the outlet tubing (Figure 4). The thermocouple was positioned so

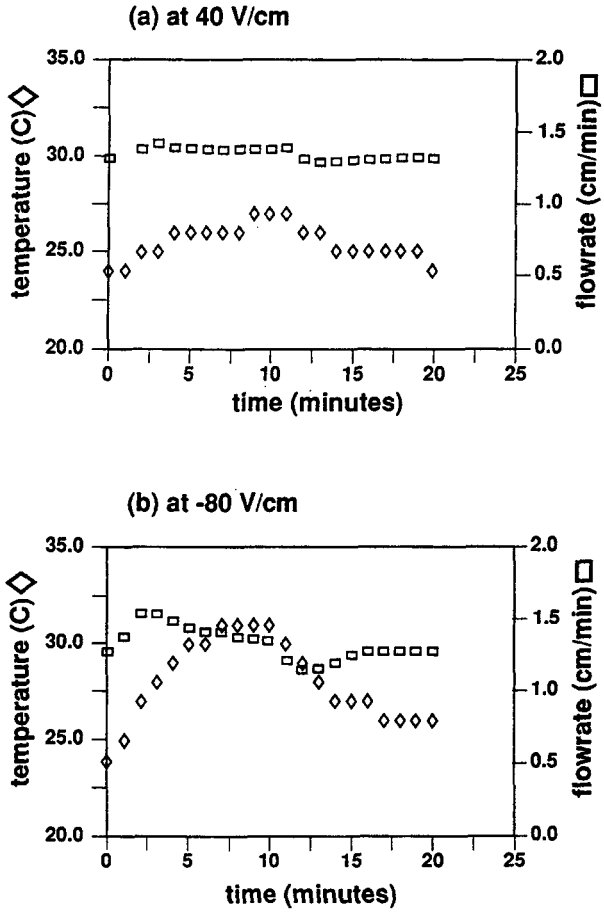

Figure 5. (a) Outlet temperature (◇) and flow rate (□) histories for a run in which a +40-V/cm field was applied to the 30-cm column for 10 min and then turned off.

The current remained constant at 3.2 mA/cm^2 for an applied potential of 40 V/cm. The applied pressure was 8,540 Pa. The column jacket temperature was 27°C.

(b) Outlet temperature (◇) and flow rate (□) histories for a run in which a −80 V/cm field was applied to the 30-cm column for 10 min and then turned off.

The current was 5.6 mA/cm^2 at an applied potential of 80 V/cm. The applied pressure was 8,380 Pa. Both runs were carried for a column packed with Sephadex G-75.

that eluent flowed directly over it. The outlet temperature was found to increase by 3°C and then level out after the electric field was applied (Figure 5a). An increased flow rate was also observed when an electric potential was applied (Figure 5b). A larger temperature rise of 7°C was obtained at the larger potential of −80 V/cm. The temperature in the interior of the column may have been somewhat higher than the outlet temperature. If so, this potential represents a practical upper limit since a higher temperature could result in protein denaturation due to heat inactivation.

Protein elution volumes in the absence of an electric field were determined by injecting 100 μL of sample containing the selected protein at a concentration in the range of 1 mg/mL and measuring the volume of eluent required for the peak to emerge from the column. The extraparticle void fraction was calculated from the elution volume of blue dextran in the absence of an applied electric potential. The value of K_{av} was then calculated using the definition $(V_e - V_o)/(V_t - V_o)$, where V_t represents the total column void volume, V_o the extraparticle void volume, and V_e the elution volume.

Measurements of protein retention were made by injecting 100 μL of protein into the column head and washing the protein band past the inlet frit into the packed bed with 400 μL of eluent. This required 1 to 4 min depending on the flow rate. A DC electric field was then applied to the column by connecting an EC 650 power supply (EC Apparatus Corp., St. Petersburg, FL) to the platinum electrodes in the end caps (see Figure 2). An electric field of 0 to 100 V/cm was applied to the column for times between 5 to 60 min as measured with a stop watch. The field was disconnected before the protein reached the outlet frit.

Experiments were performed with the eluent flowing both upward and downward. Most runs were carried out with eluent flowing downward. Comparison of results from the upward to the downward flowing configuration showed buoyancy to have no measurable effect on the retention time and dispersion of the eluting protein bands.

Electrochromatographic velocity was calculated by measuring the total residence time of the blue dextran or protein in the column. This time was divided into two periods: the time during which an electric field was applied which was measured and the time the protein migrated in the column without the electric field, which was found by difference. The distance in the column migrated in the absence of an electric field was then calculated from:

$$d = \frac{tv}{\alpha + (1-\alpha)K_{av}} \tag{9}$$

where K_{av} is a protein distribution coefficient measured with no electric field using the method described above (Andrews, 1964). The distance migrated during the absence of an electric field was subtracted from the length of the column (30 cm). The difference was taken to be the electrochromatographic migration distance. This distance was divided by the time, during which the electric field was applied, to give solute velocity, u.

The electrophoretic mobilities of the proteins selected for this study (Table 1) are sufficiently different that they may be easily separated in a nondenaturing polyacrylamide gel (Darling and Butcher, 1975). However, different retention volumes due to differences in molecular weight would not be sufficient to obtain clear separation by size exclusion chromatography over Sephadex G-75 in the 15×300 mm column used in this work, even for proteins varying in molecular weight from 14 to 65 kdal. Consequently, these proteins provided a good test of the usefulness of the system and of our mathematical description.

Dextran is a bacterially derived polysaccharide storage product, made of α-D 1-6 polyanhydroglucose units (Merck, 1989, #2925). The blue dextran used to measure void volumes in chromatography columns has an average molecular weight of approximately 2,000 kdal and is derivatized with Reactive Blue 2 (MW 773.5), a six-ringed derivative of anthracene with three sulfate groups and seven amine groups of differing coordination (Sigma Catalogue, 1989). Since 1 mmol of Reactive

Table 1. Electrophoretic Mobilities of Proteins Used in This Study

Protein	MW kdal	Electrophoretic Mobility ($cm^2/V \cdot s$, $\times 10^5$)	pH	Ionic Strength of Protein Solution Strength ($\times 10^3$ M)	Ref.
β-lactoglobulin A (β-lgA)	18	18.6	8.6	0.02	Cannan et al. (1942)
β-lactoglobulin B (β-lgB)	18				
bovine serum albumin (BSA)	65	6.64	8.56	0.10	Longsworth and Jacobsen (1949)
α-lactalbumin (α-la)	14	4.2	8.5	0.10	Gordon and Semmett (1953)
bovine hemoglobin (BHb)	64	2.3	8.0	0.10	Pauling et al. (1949)

Blue dye reacts with 1 g of dextran, the blue dextran has ionizable groups (approximately 1 Reactive Blue 2 molecule per 60 anhydroglucose units) and therefore an electrophoretic mobility. The conductivity increment measured for blue dextran above the eluent buffer is 2.62×10^{-4} ohm^{-1} cm^{-1}/mg/mL and corresponds to an ionic mobility of 15×10^{-5} $cm^2/V \cdot s$.

Thermal effects

Electrically induced temperature rise and heating are an important consideration in any electrophoresis system. Figures 5a–5b give two typical flow rate and outlet temperature histories for our electrochromatography work. The maximum increase in flow rate after application of the electric field is matched in each case by the decrease in viscosity due to heating. According to Darcy's Law (Bird et al., 1960):

$$v = (\nabla P - \rho_g) \frac{k_r}{\eta} \tag{10}$$

The flow rate should increase as the reciprocal of eluent viscosity. In the 40 V/cm case shown in Figure 5a, the flow rate increased by 7.5%, while the reciprocal viscosity increases by 7%. Similarly, in the −80 V/cm case (Figure 5b), flow rate increases by 20% while the reciprocal viscosity increases by 17%. Thus, we feel that the major flow effect in these columns is the effect of Joule heating on eluent viscosity and that the contribution of electroosmotic flow to the overall axial convection in the column is secondary.

Perhaps more striking is the heat generated and dissipated in these columns. The experiments shown in Figure 5 were run under conditions in which 0.12 and 0.45 W/cm^3 were being generated, respectively. Significant heat removal through walls and the effluent balances this heat generation, although a measurable temperature rise is still observed.

Results

Retention

The retention of the divalent anion bromphenol blue (MW = 670 dal, Merck, 1989, #1434) on Sephadex G-75 varies linearly with the strength of the electric field, as shown in Figure 6 which plots reduced velocity u/v as a function of E/v and is given by Eq. 11:

$$u/v = \frac{\alpha\mu_1 + K_{av}(1-\alpha)\mu_2}{\alpha + (1-\alpha)K_{av}} \cdot \frac{E}{v} + \frac{1}{\alpha + (1-\alpha)K_{av}} \tag{11}$$

which is a linearized form of Eq. 6 for $K_{av} \geq 1$. The straight line for bromphenol blue in Figure 6 indicates that the chromatographic properties of the column (void fraction and distribution coefficient) remain constant in an applied electric field. As a first approximation, the electrophoretic velocity of bromphenol blue is assumed to be unaffected by the gel, that is, the bromphenol blue passes unhindered through the gel. On this basis, Eq. 11 gave an electrophoretic mobility of $\mu_1 = \mu_2 = 10.5 \times 10^{-5}$ $cm^2/V \cdot s$. This value is similar to the mobilities of other divalent anions (Jovin, 1973). Since the distribution coefficient for bromphenol blue is 2.0, the polarization term in the denominator of Eq. 5 is 0, and the observed linear behavior in Figure 6 is expected from Eq. 11. (Note that K_{av} can be greater than 1 since V_o denotes the extraparticle void volume.)

The solute velocity of a large molecule, blue dextran (MW = 2,000 kdal), did not respond linearly to the application of an electric field and decreased with either polarity of electric field. Figure 7 shows reduced velocity as a function of E/v for blue dextran for both upflowing and downflowing eluent.

Blue dextran is excluded from the gel phase ($K_{av} = 0$); consequently, the equation for velocity in the column is Eq. 12:

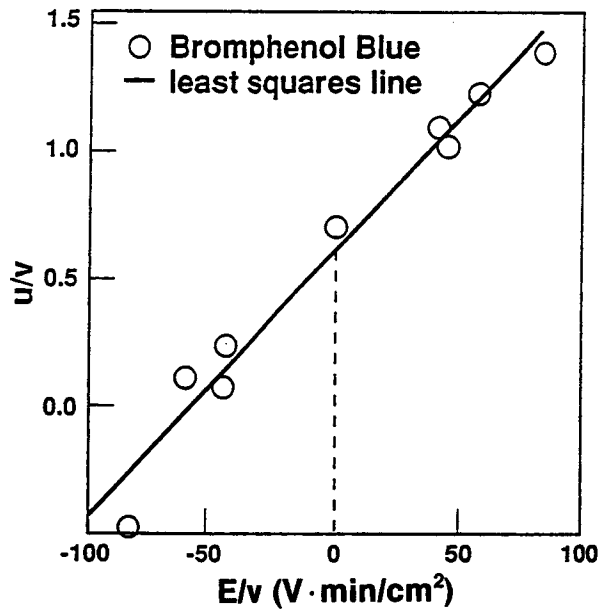

Figure 6. Reduced electrochromatographic mobility of bromphenol blue as a function of *E/v*.

All experiments were run in 47-mM glycine, 3.9-mM tris buffer, pH 8.1. Column temperature was held at 27°C, and sample size was 0.25 mL (1.4% of the column void volume). Column superficial velocities never exceeded 0.014 cm/s, and were typically $\sim 8 \times 10^{-3}$ cm/s. Column stationary phase was Sephadex G-75.

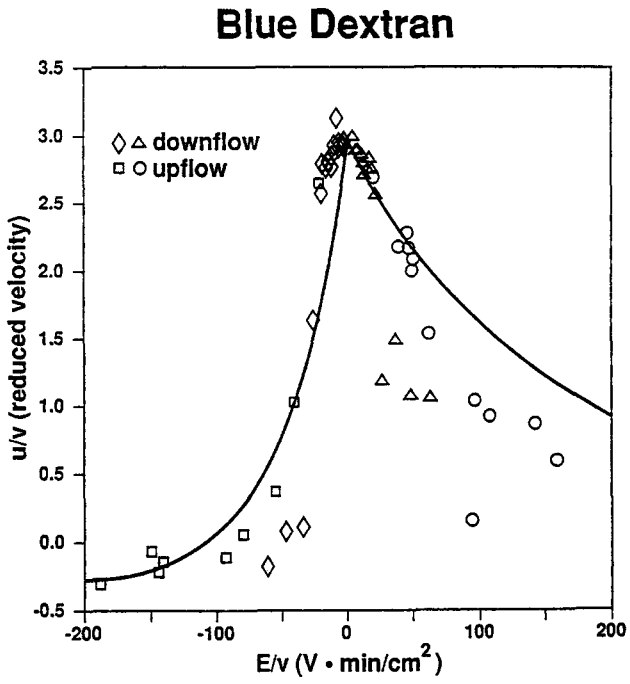

Figure 7. Reduced electrochromatographic mobility of blue dextran as a function of *E/v*.

Conditions same as given for Figure 6.

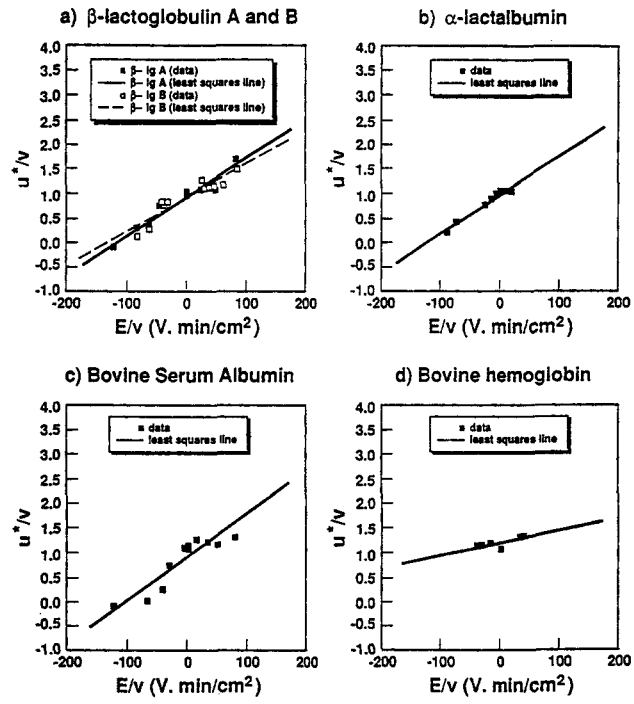

Figure 8. Reduced velocities for: (a) β-lactoglobulins *A* (■) and *B* (□); (b) α-lactalbumin; (c) BSA; and (d) bovine hemoglobin.

Conditions the same as given for Figure 6.

$$u/v=\frac{\alpha\mu_1}{\alpha+(1-\alpha)k_p}\cdot\frac{E}{v}+\frac{1}{\alpha+(1-\alpha)k_p} \tag{12}$$

which follows from Eq. 5 and includes the function k_p (Eq. 3) for electrically induced concentration polarization. The polarization adsorption, k_p, is a function of the electric field and should be zero when the electric field is zero. We chose a function,

$$k_p=\exp(a|E/v|)-1 \tag{13}$$

to describe the polarization adsorption. When combined with Eq. 12, an equation with two unknowns, μ_1 and a, results. For blue dextran, the values of μ_1 and a were found to be 30×10^{-5} and 12×10^{-5} cm²/V·s, respectively, by fitting Eq. 12 to the data with a nonlinear, two-parameter, unweighted least-squares method available in the IMSL libraries (subroutine rnlin). This routine estimates the derivative of the data at every point and, combined with the model and its derivatives, computes values for the unknown parameters. The fit of Eq. 12 to the data (solid line in Figure 7) gives a value of 30×10^{-5} cm²/V·s for the mobility of blue dextran compared to a mobility of 15×10^{-5} cm²/V·s calculated from a conductivity measurement of blue dextran in tris-glycine buffer, as described earlier.

The solute velocity of several proteins was measured and gave straight lines when plotted in Figures 8a–8d, as u^*/v vs. E/v, where

$$u^*/v=u/v[\alpha+(1-\alpha)K_{av}+(1-\alpha)(1-K_{av})k_p]$$

$$=[\alpha\mu_1+(1-\alpha)K_{av}\mu_2]\frac{E}{v}+1 \tag{14}$$

and k_p, based on blue dextran, was:

$$k_p=\exp(7.06\times10^{-3}|E/v|)-1 \tag{15}$$

where E/v has units of V·min/cm². The corresponding slopes, intercepts, and regression coefficients are given in Table 2.

The migration rates of proteins α-lactalbumin, β-lactoglobulins *A* and *B*, bovine serum albumin and bovine hemoglobin, like blue dextran, decreased with increasing electric field of both polarities as shown by the data, replotted in Figure 9. Since the column temperature, pH, and ionic strength were essentially constant at the different potentials, a fundamental difference in retention characteristics in combined convective and electric fields between macromolecules (proteins and blue

Table 2. Statistical Data from the Elution of 5 Proteins (Figures 8a–8d) from Electrochromatographs and μ_2 Calculated from Eqs. 5 and 14

	β-lg A	β-lg B	α-la	BSA	BHb
MW (kdal)	18.6	18.6	14	67	64 (dimer)
K_{av}	0.23	0.25	0.36	0.03	0.18
μ_1 (from literature) $(cm^2/V\cdot s)\times10^5$	18.6	18.6	4.2	6.64	2.3
Slope of best fit lines $(cm^2/V\cdot s\times10^3$ from Figure 8	7.8	6.8	8.0	8.6	2.4
Intercept of best fit lines from Figure 8	0.91	0.91	0.94	0.89	1.14
Correlation (r^2) for data in Figure 8	0.97	0.92	0.97	0.85	0.81
Calculated μ_2 $(cm^2/V\cdot s)\times10^5$	43	30	50	608	27

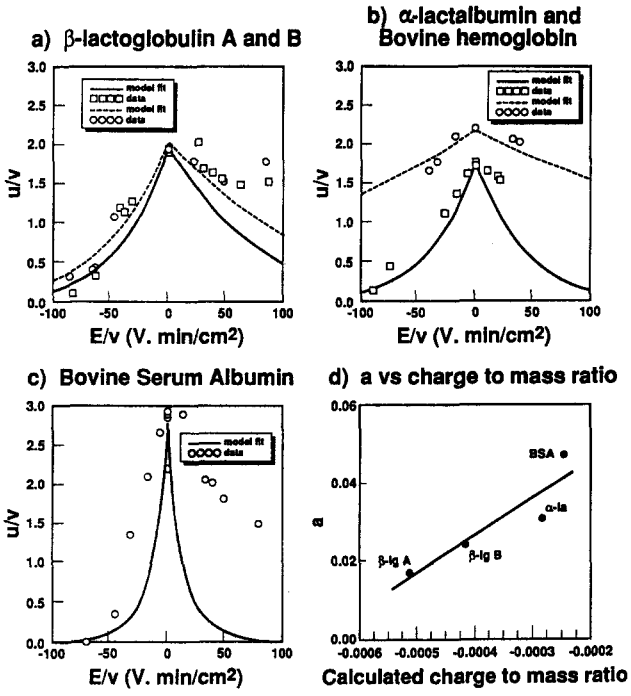

Figure 9. Velocities of: (a) β-lactoglobulin A (○) and B (□); (b) α-lactalbumin (□) and bovine hemoglobin (○); (c) bovine serum albumin fit to Eq. 15; and (d) relation between parameter *a* and the charge to mass ratio at pH 8.1 for four proteins estimated from the amino acid sequences.

The dotted lines are fits for β-lg B, and α-la in Figures 9a and 9b, respectively. The solid lines are fits for β-lg A and BHb, respectively.

dextran) and a small solute (bromphenol blue) was hypothesized.

The slopes of the lines in Figure 8 were used with Eq. 14 to calculate the electrophoretic mobility, μ_2, of the protein through the Sephadex resin, for known values of the free fluid electrophoretic mobility of the protein, μ_1, given in Table 1. The calculated values of μ_2, which are hypothesized to represent electrophoretic mobilities within the gel, were 1 to 2 orders of magnitudes higher than values of the electrophoretic mobility in the eluent phase (Table 2). Such large differences between electrophoretic mobilities for the extraparticle mobile phase, compared to the more viscous environment within the gel, seemed physically unreasonable. Consequently, a second approach was taken in which μ_2 was assumed to have the same value as μ_1 and the parameter *a* in Eq. 13 was varied to fit the data to Eq. 14. Values of *a* from the nonlinear least-squares fit of the data in Figure 9 are summarized in Table 3. This approach was chosen since it is physically reasonable for the parameter *a* to reflect the overall charge to mass ratio (Figure 9d). The charge was calculated using amino acid sequences reported by Eigel et al. (1984). While different from the protein's surface charge, the overall charge still gives a measure of the protein's potential mobility in an electric field. The charge for bovine hemoglobin could not be calculated, and so is excluded from Figure 9d.

Table 3. Values of Parameter *a*

	Charge/Mass Parameter *a*	Sum of Std. Error	Deg of Freedom
β-lgA	0.017	148	12
β-lgB	0.024	19	7
α-la	0.031	29	9
BSA	0.047	6,836	9
BHb	0.004	5	5

The data for the proteins and the buffer system employed here show that an expression for electrically induced adsorption (Eq. 13) is applicable to globular proteins having lower molecular weights than blue dextran. This adsorption appears to be nonspecific and gives an explanation for the otherwise anomalous retention of large solutes ($K_{av} < 1$) in this electrochromatographic system.

Discussion of electric-field-driven concentration polarization

Electric-field-driven concentration polarization of solutes against the surface of the Sephadex gel particles may explain the nonspecific adsorption observed for the macromolecules. This effect would require the electric field lines to differ from the convection streamlines near the particle surface. The electric field is conceived as passing through the resin, while the convective field passes around the resin's surface, as shown in Figure 10. Unlike the macromolecules, small charged solutes would pass into and through the gel matrix freely under the force of the electric field. Electric-field-induced partitioning of small solutes between the resin and buffer phases is minimal, and so the thermodynamic properties of each phase would remain nearly constant when the electric field is applied.

The electric field will travel through the gel if the particle has a conductivity greater than zero. Our measurements indicate that Sephadex G-75 has a conductivity of approximately 1,300 μmho/cm when swollen in 3.9-mM Tris, 47-mM glycine buffer. The buffer itself has a conductivity of 80 μmho/cm. Therefore, the gel-phase controls the potential drop in the column and could distort the electric field to travel through the particle. We have observed indirectly that the resin particles are more conductive than the buffer used in our work in that our power supply was not able to sustain a voltage across a column filled with buffer only, while voltage drops were easily established across packed columns. The enhanced conductivity of the resin is apparently due to residual carboxyl groups, present in Sephadex gels at 10 to 30 microequivalents/g, unswollen (Eaker and Porath, 1967). Sephadex G-75 absorbs approximately 7.5 g water/g dry resin (Flodin, 1962), so that the swollen gel approximates a 1- to 4-mM solution of carboxyl groups. In comparison, a 10-mM solution of KCl has a conductivity of 1,100 μmho/cm. When the stationary phase is less conductive than the eluent, concentration polarization would not be expected since electric field lines and flow streamlines would be parallel. This is consistent with the results obtained by Tsuda (1987) on silica-based resins in an electric field: the resins used are less conductive than the buffers used, and polarization was not observed.

The solute concentration in counteracting chromatographic electrophoresis (CACE) systems may be affected by an electric

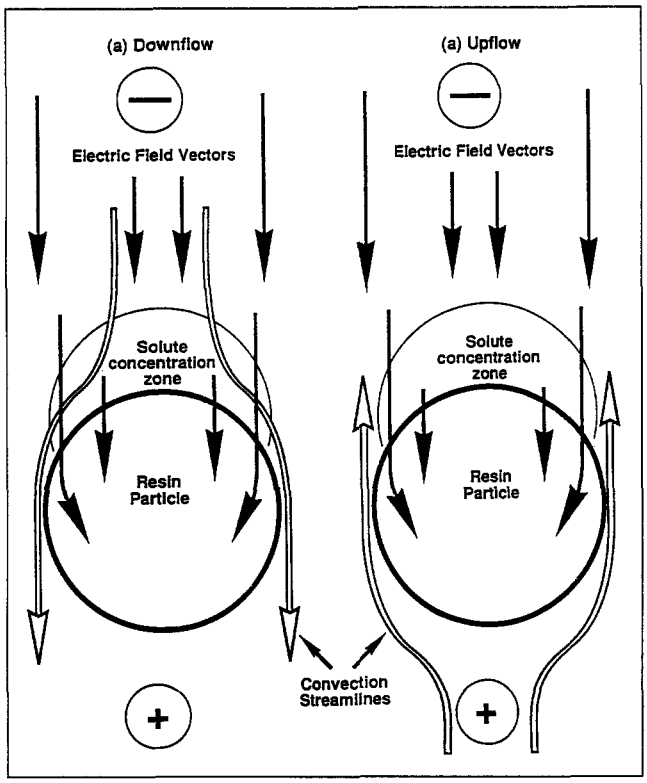

Figure 10. Concentration polarization.

The electric field gradient builds a concentrated layer of solute on the surface of the resin provided the solute does not freely penetrate the resin ($K_{av} < 1$, MW > 1,000 kdal for Sephadex G-75). Solute, which diffuses away from the resin surface may be carried downstream by convection, may be driven back to the resin surface by the electric field or may enter the resin through a pore large enough to accommodate it.

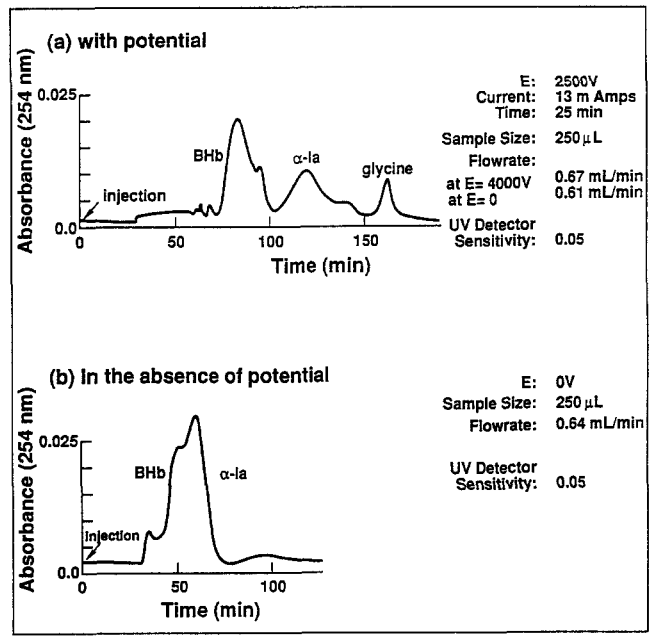

Figure 11. Separation of α-lactalbumin from bovine hemoglobin with an electric field of a) −2,500 V, 0.67 mls/min (0.38 cm/min) and b) 0 V, 0.61 mls/min (0.34 cm/min).

The field was held for 25 min. Other conditions correspond to those in Figure 6.

field polarization effect. Several recent studies of CACE (Hunter, 1988; Locke and Carbonell, 1989; Ivory and Gobie, 1990) have indicated that solute concentration occurs in the upper gel matrix, which is usually more conductive and is more likely to exclude solutes than the lower gel matrix. While it appears to be well established that the mobilities of the buffer ions in CACE systems are responsible in part for zone location, the effect of solute polarization on resin surfaces may help predict solute concentration in CACE.

Separation by electrochromatography

The fits of the lines for protein retention normalized for electrically induced concentration polarization in Figures 8a–8d and Figures 9a–9c differ for different proteins, which indicates that each protein has a distinct mobility in the electric field. To test this result, a mixture of α-lactalbumin (MW = 14 kdal) and bovine hemoglobin (MW = 64 kdal, dimer), which could not be resolved without an electric field in our column, was separated into two peaks in the presence of the electric field, as shown in Figure 11. α-Lactalbumin has a higher (negative) zeta potential at pH 8.4 than does hemoglobin and it is the smaller molecule, so a negative electric field was employed. An alternate case is presented when the larger protein is the more highly charged species. A positive electric field is then preferred. A partial resolution of β-lactoglobulin B (MW = 18 kdal) and α-lactalbumin (MW = 14 kdal) is shown in Figure 12 for which the latter condition applies. Both cases illustrate the utility of Eq. 14 in choosing appropriate separation conditions.

The proteins examined for these test cases are readily separated by ion exchange or reverse-phase chromatography. Hence, the use of electrochromatography would not be necessary, nor attractive, to achieve fractionation. These proteins were selected since their properties are relatively well known and were likely to demonstrate benefits of applying an electric potential across a size exclusion column to achieve improved resolution. Further work now seems appropriate to test this technique for more difficult separations or for solutes where other fractional methods are unsuitable. We speculate chemical mobile-phase gradients, currently used in reverse-phase, ion exchange or hydrophobic interaction chromatography may be supplanted with an electric potential. This is an attractive concept in cases where chemical gradients are used since an electrical gradient can be readily and almost instantaneously turned on or off. Further, the use of an electrical gradient would reduce the need for addition and later removal of an organic modifier in the mobile phase (often at high concentration) to obtain a separation.

Conclusions

Our work indicates that eluent flow rate and electric field strength are not linearly superimposable forces in electrochromatography. Our results suggest that solute polarization is induced on gel particles packed in a chromatography column when an electric potential is applied. The concentration polarization effect appears to be relatively nonspecific for ex-

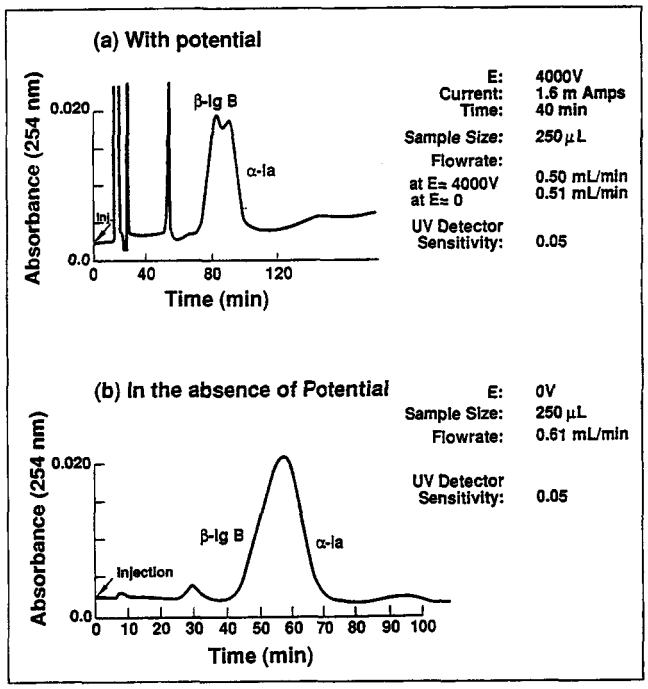

Figure 12. Partial resolution of α-lactalbumin and β-lactoglobulin with an electric field of a) +4,000 V, 0.495 mls/min (0.28 cm/min) and b) 0 V, 0.51 mls/min (0.29 cm/min).

The field was applied for 40 min. Other conditions correspond to those in Figure 6. The spikes to the left of the peak are due to gas bubbles in the detector.

cluded solutes having a size on the order of the size of pores in the Sephadex gel. Electrophoretic migration through the gel and interstitial fluid is still achieved for solutes having $K_{av} < 1$. A model has been developed to correlate measured electrochromatographic migration rates over a range of eluent linear velocities and electric field strengths. Appropriate choice of a positive or negative field, field strength, and eluent flow rate allows control of the solute velocity of a target molecule. Separation of a protein mixture was demonstrated in which the electric field was chosen to take advantage of protein electrophoretic mobilities.

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Notation

a = polarization parameter, $cm^2/V \cdot s$
c = mobile phase concentration, mol/cm^3
C_o = sample concentration, g/cm^3
d = distance migrated, cm
E = electric field gradient, V/cm
g = gravitational acceleration, cm/s^2
K_{am} = electrolyte dissociation constants, M
K_{av} = protein exclusion coefficient $= (V_e - V_o)/(V_t - V_o)$
K_{car} = electrolyte dissociation constants, M
K_{oh} = electrolyte dissociation constants, M^2
k_p = polarization adsorption function
k_r = Blake-Kozeny permeability, cm^2
K_{tris} = electrolyte dissociation constants, M
n = gel phase concentration, mol/cm^3
p = polarization concentration, $mol \cdot g/cm^3$
P = pressure, N/cm^2
t = time, s
u = solute velocity, cm/s
u^* = modified protein migration rate $= u[\alpha + (1-\alpha)K_{av} + (1-\alpha)(1-K_{av})k_p]$
v = superficial velocity, cm/s
V_c = column volume, cm^3
V_e = elution volume, cm^3
V_o = elution volume of an excluded component; equivalent to extraparticle void volume, cm^3
V_t = total column void volume, cm^3
x = axial distance, cm

Greek letters

α = void fraction
μ_1 = electrophoretic mobility of solute in eluent phase, $cm^2/V \cdot s$
μ_2 = electrophoretic mobility of solute in resin phase, $cm^2/V \cdot s$
η = viscosity, $N \cdot s/cm^2$
ρ = eluent density, g/cm^3

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