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Abstract

The operating characteristics of hollow-fiber electropolarization chromatography (or electrical field-flow fractionation) of proteins are surveyed. Particular attention is given to the dependence of protein retardation and electroretention on field strength, protein loading, buffer concentration, and pH. Both mixtures of human serum albumin with Cohn fraction II (γG) and pure human serum albumin were studied. The results confirmed earlier findings that this process is highly efficient for separation of these two dissimilar proteins, but process dependence on the parameters studied is unexpectedly complex. Available models for predicting column performance are found adequate only as a rough guide, and strong interactions are shown between the effects of the above parameters. Much more work is needed to characterize this deceptively simple process, and it is particularly important to study the physics and chemistry of concentrated protein solutions.

INTRODUCTION

The purpose of this work was to survey the practical operating characteristics of hollow-fiber electropolarization chromatography* (EPC) for the processing of globular proteins. We were particularly interested

*This process is similar in concept to the electrical field-flow fractionation of Giddings (*1*).

here in studying the effects of field intensity, pH, ionic strength, and protein loading on transit time and dispersion. Effects of equipment configuration and voltage programming will be discussed in a later communication. EPC is a relatively new separation procedure of considerable complexity and will require extensive systematic study before its full potential can be realized.

As discussed at some length in the next two sections, hollow-fiber EPC is based upon a very simple concept. Solutions of solutes to be separated are introduced as a relatively compact bolus to one end of a hollow fiber, which is filled with a suitable electrolyte and subjected to a transverse electrical field. The fiber wall must be impermeable with respect to the feed solutes but freely permeable to at least some of the electrolyte species present, so that a finite field can be maintained in the fiber lumen. Under these circumstances the feed solutes will be concentrated in a region of slow-moving fluid near the fiber wall. If an electrolyte solution is now pumped axially through the fiber, the polarized solutes will move more slowly than the volume-average velocity of the solvent. Furthermore, this retardation will increase with the degree of polarization, so that species polarizing to different degrees can be separated.

The feasibility of this process has been amply demonstrated (*1–3*) and first-order quantitative descriptions of retardation (*3*) and dispersion (*4*) are available. However, it remains to determine the range of applicability of the available theory and the importance of second-order effects. Experience with this new process already shows that many of these exist and that they can be of considerable importance.

Of particular importance are quantitative departures of observed protein retardation from the predictions of the first-order theory. These departures can take an extreme form in an essentially total but reversible field dependent immobilization of significant portions of the protein feed. This phenomenon, which we refer to as *electroretention*, can be either a source of inefficiency or an effective means for enhancing separation. It was first observed by Reis and Lightfoot (*3*) but has yet to be explained in any detail. Also of interest are thermodynamic interactions of proteins with one another and with the fiber wall.

We shall see that each of these effects depends in a complex way on the chemical nature of the system and the magnitudes of such parameters as protein loading, flow rate, field strength, and system dimensions. The method of introducing the feed and changes of voltage with time can also be very important.

We are particularly concerned in this paper with the effects of pH, ionic strength, and protein loading on human serum albumin (HSA) and mixtures of HSA and immunoglobulins (γG, more specifically Cohn fraction II).

These proteins were initially chosen for convenience and because EPC appears well suited for both analytical and commercial-scale fraction of blood serum proteins. For example, if the productivity of presently functioning single-fiber units (*3*) could be maintained in a multifiber system, the albumin and immunoglobulins of 1 kg of serum proteins per day could be fractionated in a 10,000 fiber bundle 280 cm long, even though we are far from an optimized process. It could be done at room temperature with a protein holdup time of the order of 1 hr. Power costs would be very substantially smaller than those of conventional processing plants.

APPARATUS

The experimental system is shown schematically in Fig. 1. The heart of this system is the test cell containing the hollow fiber and electrodes. Two test cells were used; a linear system described previously (*3*) and a new system developed by the Amicon Corp. of Lexington, Massachusetts, that can accommodate longer fibers. In these studies a 280-cm fiber was used in the new cell. Important auxiliary equipment includes the electrical power supply, a worm-gear syringe pump, the sample injection valve, the recirculating buffer system, and the UV monitor.

Either the new test cell or the linear cell containing a 50-cm fiber is connected to our system as illustrated in Fig. 1. Teflon and polyethylene microbore tubing are used for the lines leading to and from the ultrafiltra-

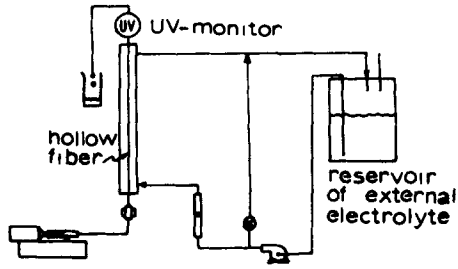

FIGURE 1

tion fiber, and a Harvard model 901 syringe pump creates the internal axial flow. The sample injection valve is installed in the line just below the fiber, and a Perkin-Elmer model LC-55 spectrophotometer, with an 8-μl flow cell, is connected to the line leading from the fiber. For the shorter, 50 cm fibers, a 10 μl injection valve is used while a 200-μl injection valve is used with the longer fibers. A Teflon-gear micropump circulates buffer from the electrodes to the reservoir through Tygon tubing in what we call the external flow.

All ultrafiltration fibers used were received from Amicon Corp. They are of the YC or YM type, with an i.d. of 0.79 mm, wall thickness of 0.076 mm, and a molecular weight cut-off below 22,000.

PROCEDURE

The essence of the procedure is to introduce a small mass of proteins into the buffer stream flowing through the hollow fiber in the presence of a transverse electric field, and to monitor its passage through the system via a UV monitor at the column outlet. However, the system is sensitive to many experimental variables, the action of which we do not fully understand. In this section we present our methods of controlling these variables and our techniques for processing the data.

The dc electric field was adjusted to the nearest volt of potential difference across the electrodes. The polarity of the electric field was reversed for each new run. All runs were performed without any temperature control, but due to the low electric currents in the test cell (measured to the nearest milliampere), little temperature rise was observed over the course of several experiments.

Ten minute average flow rates through the fiber were measured by measuring the volume output of the fiber in 10 min intervals. This internal flow rate was adjusted by changing the static pressure of the external buffer with a valve on the bypass line of the micropump. This action controlled the amount of ultrafiltration through the fiber and determined the net axial flow for a given speed of the Harvard pump. Ultrafiltration was limited to under 20% of the net axial flow in order to avoid spurious protein retardations, as reported by Reis (*4*). Internal flow rates are normally in the range of 40 to 200 μl/min. The external buffer flow rate was measured to $\pm$0.1 ml/min with a flowmeter and was set in the range of 10 to 20 ml/min.

Buffers used for these experiments were Tris, obtained as Trizma base from Sigma Chemical Co., and sodium acetate, obtained in analytical

grade from the Mallinckrodt Chemical Co. Tris buffers were made to known concentration and adjusted to pH 8.3 with either glacial acetic acid or concentrated HCl solution. Sodium acetate buffer was made from stock solutions of sodium acetate and acetic acid of the same molarity as our final solution, and mixed to give the correct pH in the range 4.6 to 5.0. Deionized water was used in all solutions. The pH and conductivity of the buffers were measured periodically and replaced when the pH changed by more than 0.1 pH unit.

HSA was purchased from Miles Laboratories under the Pentex label, and was also purchased from Nutritional Biochemicals Corp. as 4 times crystallized. Human gamma globulin was purchased from ICN Pharmaceuticals. Sample protein solutions were normally made to a 0.5% solution which was dialyzed overnight in our system buffer and centrifuged to remove undissolved solids before use. The absorbence of the solutions at 280 nm was used for calculation of yields.

Proteins were monitored at 280 nm with a Perkin-Elmer spectrophotometer as they pass through the system. The absorbence reading, which was measured to 0.001 OD, was recorded on a chart recorder for later analysis. A protein injection was not made until the absorbence was stable under a constant applied voltage and flow rate.

Data were analyzed in terms of the retardation number, r, and the area of a protein absorbence peak. The retardation number is expressed as the residence time of the protein in the fiber divided by the residence time of the protein under no applied electric field. It was calculated from

$$r = \frac{\langle t_e \rangle - \langle t_1 \rangle}{\langle t_0 \rangle - \langle t_1 \rangle}$$

where $\langle t_e \rangle$ is the time from protein injection to the center of mass of the protein absorbence peak, $\langle t_0 \rangle$ is the time from injection to the center of mass of the protein peak under no applied voltage, and $\langle t_1 \rangle$ is the residence time in the connections to the injection valve and to the flow cell on the spectrophotometer, which has been corrected to the same flow rate. The retardation number is a measure of the magnitude of electroretardation.

The area measurement of the protein absorbence peak is proportional to the mass of protein in the peak, providing Beer's law is obeyed. Center of masses of absorbence peaks were found by the simple paper-cutting and weighing technique. Peak area measurements were made with a planimeter, which easily gave accuracies above 95%. Often, absorbence peaks did not return to the original base line. This ambiguous base-

line shift was treated as a vertical shift at the peak of the curve when making area measurements.

When fractions were collected from the EPC system for disk gel electrophoresis studies, the following procedure was used: 25 to 50 μl samples were applied to 5.6% gels with SDS according to Fairbanks et al. (*5*). Protein samples were first boiled for 2 min in 2% SDS reagent without reducing agent before application. These runs were made at room temperature with an electric current of 3 to 4 mA/gel.

THEORY

The primary purpose of this paper is to determine the effect of operating parameters on retardation and electroretention and to look for the possible influence of protein interactions on resolution. For these purposes the most useful point of departure is the first-order retardation prediction of Reis and Lightfoot (*3*):

$$r = \frac{\mathscr{E}}{4}\frac{I_1(\mathscr{E})}{I_2(\mathscr{E})} \tag{1}$$

where

$$\mathscr{E} \equiv mER/\mathscr{D}_m$$

m is the electrical mobility of the protein, $\mathscr{D}_m$ is its mean diffusivity, E represents the transverse electric field inside the fiber, and R is the hollow fiber inner radius. It may be noted that for high fields this result takes the simpler asymptotic form (*3*)

$$r \Rightarrow \mathscr{E}/4 \tag{2}$$

which will frequently suffice.

Here, the retardation number indirectly measures the degree of polarization of proteins, and retention corresponds to $r = \infty$.

RESULTS

Presented here are representative data taken in our laboratory over about the past year for HSA and albumin–γ-globulin mixtures. We begin by describing albumin–γ-globulin separations to demonstrate the basic utility of the process and to show the desirability of systematic studies of individual proteins. We then show the results of such studies on HSA. Several different cellulosic fibers were used to obtain these data, as de-

scribed earlier, and in all cases protein concentration was monitored via the Perkin-Elmer LC 55–505. Because the dead volume of this instrument is large relative to the effective active volume of the system, much of the observed dispersion is incidental to the monitoring operation. Attention will therefore be confined to retardation and electroretention, and it should be understood that EPC is inherently capable of much higher resolution than that shown.

Albumin–γ-Globulin Mixtures

Representative data on the resolution of albumin–γ-globulin mixtures are shown in Figs. 2 through 5. These are chosen to demonstrate the effects of mass loading and field strength and to show that effective separations are in fact being achieved. The specific conditions of each run are summarized in Table 1.

The separation of albumin–γ-globulin mixtures under conditions identical except for total mass loading is shown in Fig. 2. In each case the feed contained approximately equal amounts of the two proteins, and all runs were made in the same fiber using 1 m*M* sodium acetate buffer at pH 4.6. Total masses of protein fed were 1, 2, and 5 mg, respectively, for Curves 2a, 2b, and 2c. All experiments represented in Figs. 2 and 3 were made from the same stock solution.

For each run the first two peaks representing HSA and γG respectively were obtained under constant fields, indicated by the voltages shown across the electrodes. The third peak, obtained on eliminating the applied field, represents electroretained material, primarily γG. (It may be noted from Figs. 4 and 10 that very little HSA is electroretained under these conditions.) Our experience shows that this electroretained material would be held indefinitely (many hours) in the presence of the polarizing field and always be released immediately on its suppression.

It is readily apparent that resolution is decreased by an increase in protein loading, and it appears that γ-globulin may tend to move faster in the presence of albumin.

It may be seen from Fig. 3 that an increase in field strength at least partially offsets the effect of higher protein loading. Both curves of this figure were obtained for 5 mg of total protein under conditions generally similar to those for Fig. 2. However, for Curve 3b the field was increased relative to those of the previous runs, and apparent resolution was substantially increased as a result. The second sets of peaks represent electroretained material which are released almost immediately on eliminating

TABLE I
Experimental Conditions for Figures

Fig. no.	Total load (mg)	Voltage (V)	Current (mA)	Buffer		pH	Flow rate (μl/min)	Fiber type	D (mm)	L (cm)
				Conc (mM)	Type					
2a	1	15	12	1	Acetate	4.6	190	YC	0.79	280
b	2	15	12	1	Acetate	4.6	190	YC	0.79	280
c	5	15	12	1	Acetate	4.6	190	YC	0.79	280
3a	5	20	18	1	Acetate	4.6	190	YC	0.79	280
b	5	15	12	1	Acetate	4.6	190	YC	0.79	280
4a	5	20	18	1	Acetate	4.6	190	YC	0.79	280
b	5	20	15	1	Acetate	4.6	200	YC	0.79	280
c	5	20	17	1	Acetate	4.6	200	YC	0.79	280
5	1	8	18	4	Acetate	4.8	80	YC	0.79	280
6	0.05	0–25	—	4	Tris acetate	8.3	40	YC	0.79	50
7	0–1	40	37–46	4	Tris acetate	8.3	200	YM	0.79	280
8	0.014–0.05	0–40	—	1–4	Tris acetate	8.3	40	YC	0.79	50
9a	0.050	10	<1	0.1	Tris chloride	8.3	40	YC	0.79	50
b	0.050	10	6	1		8.3	40	YC	0.79	50
c	0.050	10	20	4		8.3	40	YC	0.79	50
d	0.050	0	0	4		8.3	40	YC	0.79	50
10	1	0–30	—	4	Acetate	4.6–5.0	190	YC	0.79	280

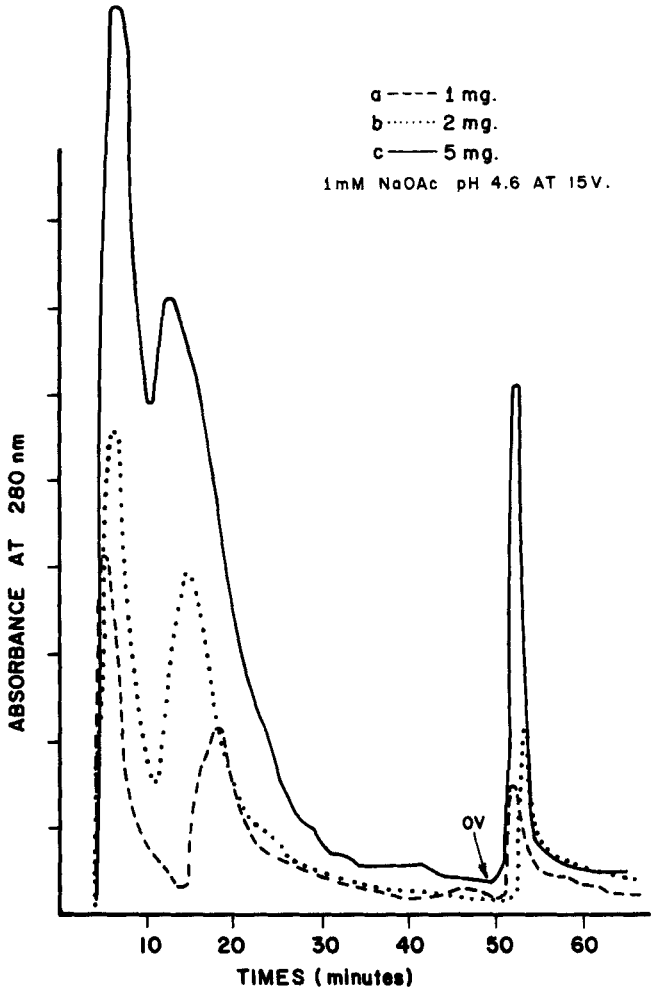

FIG. 2. Albumin–γ-globulin separations at different mass loadings.

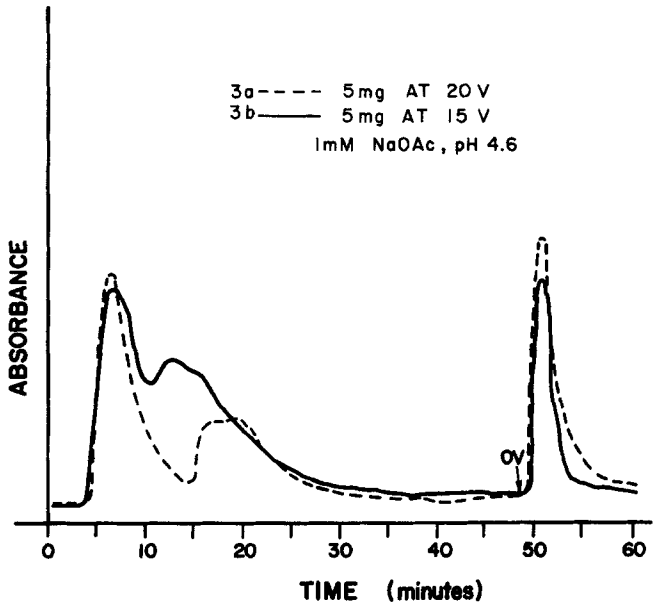

FIG. 3. Albumin–γ-globulin separation at different field strengths.

the electric field. For the conditions of this figure this occurred at about 48 min, as marked on the figure.

In Fig. 4 a comparison is made between an albumin–γ-globulin mixture and the behavior of the individual proteins separately. Curve 4a is a replot of 3a, while 4b and 4c are for HSA and γG, respectively, individually at the same loading of 5 mg total protein.

It may be seen that the albumin, which is essentially unretarded at this near-isoelectric pH, has the same position in both situations. However, the γ-globulin peak occurs significantly later for the mixture, and a larger proportion is electroretained. The decreased retardation of pure γG may result simply from the increased loading with regard to this protein. The effect of loading is discussed later with respect to HSA in Fig. 7.

Our primary evidence for albumin–γ-globulin separation is the comparison of effluent curves for mixtures with those for individual components as in Fig. 4. In addition to this, the actual protein composition of each peak for an EPC separation of HSA and γG was examined by SDS disk gel electrophoresis (GEP). The complete conditions of the run

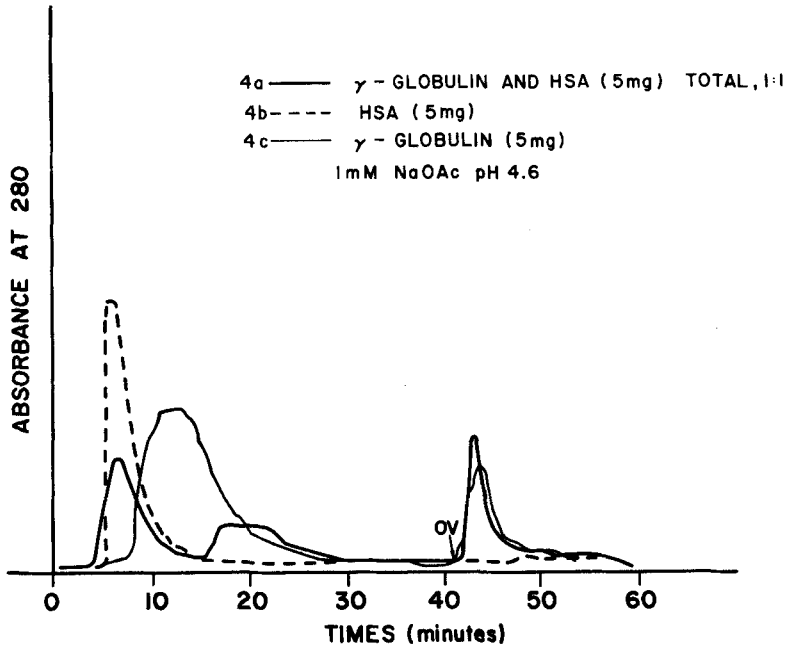

FIG. 4. Evidence for protein–protein interaction.

are included in Table 1, and the conditions for GEP are detailed in the Procedure section.

Photographs of the GEP results for an EPC run of a 50/50 mixture of HSA and γG at pH 4.8 are shown in Fig. 5. This EPC run was made at 8 V in 4 m*M* acetate buffer, as described in Table 1. Gel *a*, in Fig. 5, shows the protein banding patterns for pure HSA applied directly to the gel. The band with its front at 3.7 cm corresponds to the 68,460 molecular weight chain of HSA. The other bands observed on this gel are due to the presence of HSA oligomers. The bands on Gels *a* and *b* are broad and smeared due to overloading of the gels, which was done to make visible these oligomers. The feed mixture for the EPC run was directly applied to Gel *b* to show the protein banding pattern for the 50/50 mixture of HSA and γG. Note that γG appears as a major band at about 1.25 cm and a minor band at 0.3 cm. Gels *c* and *d* are the first and second peaks respectively off the EPC column. Gel *c* has a major band in the 68,460 molecular weight range which corresponds to HSA. The oligomers of

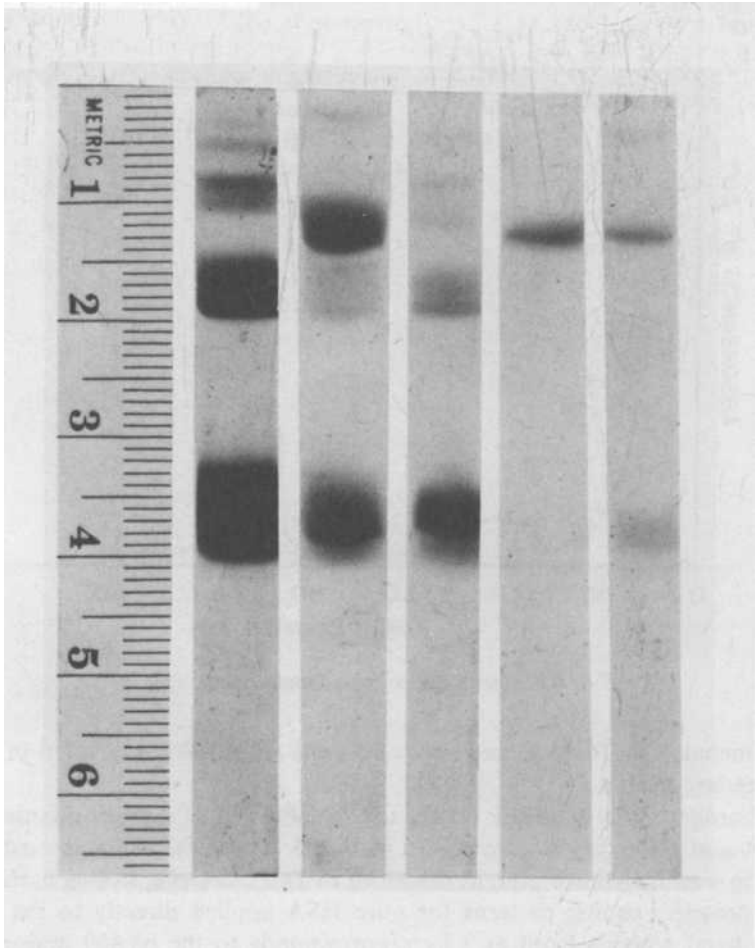

FIG. 5. Disk gel electrophoresis patterns: (*a*) concentrated HSA, (*b*) feed mixture of HSA and γG, (*c*) sample from region around first peak, (*d*) sample from region around second peak, and (*e*) electroretained material.

HSA observed on Gels *a* and *b* are also observed on Gel *c*. It is possible that a trace amount of γG may be visible at about 1.2 cm. The only band observable on Gel *d* comes at the same position as γG in the feed mixture (Gel *b*). The electroretained fraction was run on Gel *e*. Both γG and HSA are visible on this gel.

These gel studies, when looked at in conjunction with the retardation studies on individual components, clearly show that HSA and γG are well resolved by EPC. However, there is a significant portion of both HSA and γG being electroretained.

Behavior of Single Proteins

It may be seen from the above discussion that EPC is sensitive to process conditions and also that mass loading effects, though they may be appreciable, do not dominate system behavior. We shall now see that the effects of process conditions are both pronounced and complex, and that studies of single proteins are much needed. In this paper we shall confine ourselves to reviewing the response of HSA to field intensity, protein loading, buffer concentration, and pH.

Field Intensity

This is the dominant system parameter, and it is in fact the only one explicitly considered in the first-order analysis of Reis and Lightfoot (*3*). It may further be seen from the characteristic results of Fig. 6 that system response is qualitatively as predicted: retardation does increase with field intensity, here represented by voltage across the electrodes of the apparatus. However, the retardations are smaller than those predicted by Eq. (2), the asymptotic approximation, which is also shown in Fig. 6 for reference. In addition, the incremental response appears to fall off with increased voltage, and the observed retardation at 15 V is anomalously low.

The predicted curve is drawn for a fiber of negligible wall resistance, and this may explain much of the discrepancy between observation and prediction. We know that the electrical resistance of the fiber wall is appreciable, but we have not yet developed a satisfactory means for characterizing it.

The apparent negative second derivative of the retardation curve and the anomaly at 15 V cannot be shown to be significant from the data reported in Fig. 6 alone. However, similar behavior has been observed consistently in data to be submitted for publication shortly, and we

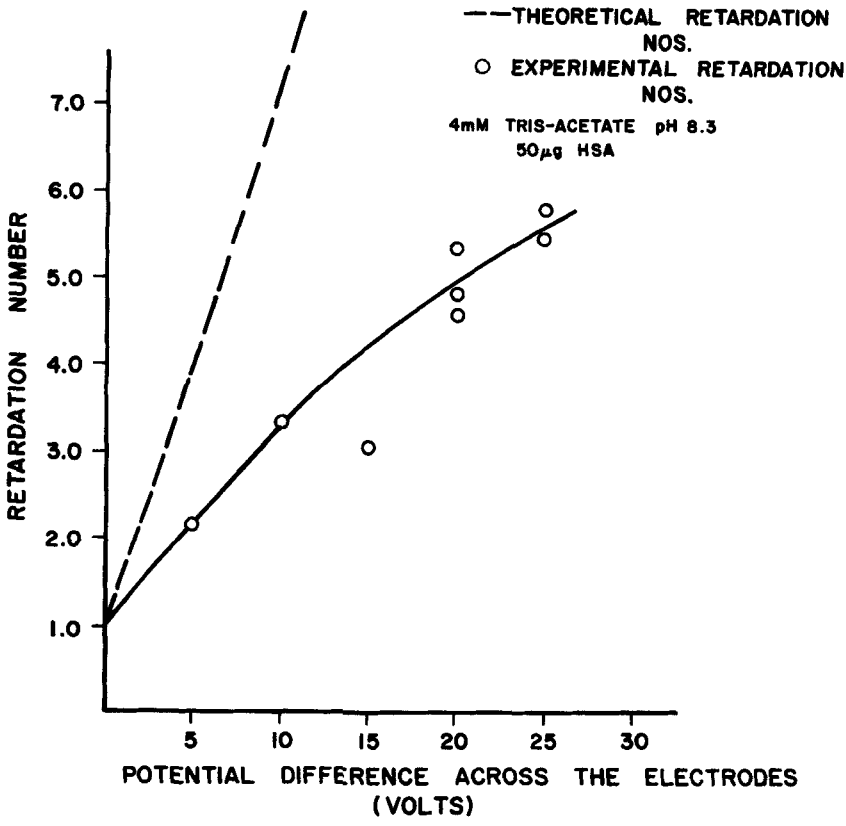

FIG. 6. Retardation number as a function of potential difference across the electrodes.

believe both results to be real in the sense of being reproducible. We have as yet no explanation for either, however, and it is entirely possible that they are artifacts specific to the apparatus used. We are currently examining field effects in some detail.

Protein Loading

It may be seen from Figs. 7 and 8 that both retardation and the fraction of the feed electroretained are sensitive to protein loading, and in fact that it may be quite important from a practical standpoint to strike a careful balance between these effects.

Figure 7 shows retardation as a function of protein loading for otherwise identical conditions, and it is clear from these results that the first-order theory is of only qualitative utility. Retardation decreases in an important way with an increase in protein loading, except perhaps in the limit of small feed mass. This effect is of primary importance for preparative separations where high loading is desirable.

The effect of loading is also important for analytical-scale separations since, as shown in Fig. 8, the fraction of feed electroretained also increases as loading is reduced. Therefore the potentially higher resolution observed for low loading carries the price of increased electroretention.

Considerable insight may be gained by calculating the total amount of electroretained protein rather than the fractional retention shown in Fig. 8. A simple linear regression of the form

$$m(ER) = bV$$

fits the Tris acetate data for both 14 and 50 μg column loads quite well, as is indicated by the summary in Table 2. Here $m(ER)$ is the total mass of (recoverable) electroretained protein and V is the voltage across the cell electrodes. The data for the two loadings give remarkably similar expressions and are clearly not significantly different. These data thus back up the earlier suggestion by Reis (*8*) that the amount of protein electroretained may be independent of column loading. Further investigation appears desirable.

For HSA and the conditions of these figures it may be seen that quite large retardation and small electro-retention can be obtained with loadings of 200–500 μg. It is clearly important to learn more about the behavior shown in these two figures.

Buffer Concentration

Both the concentration and chemical nature of the buffer used have

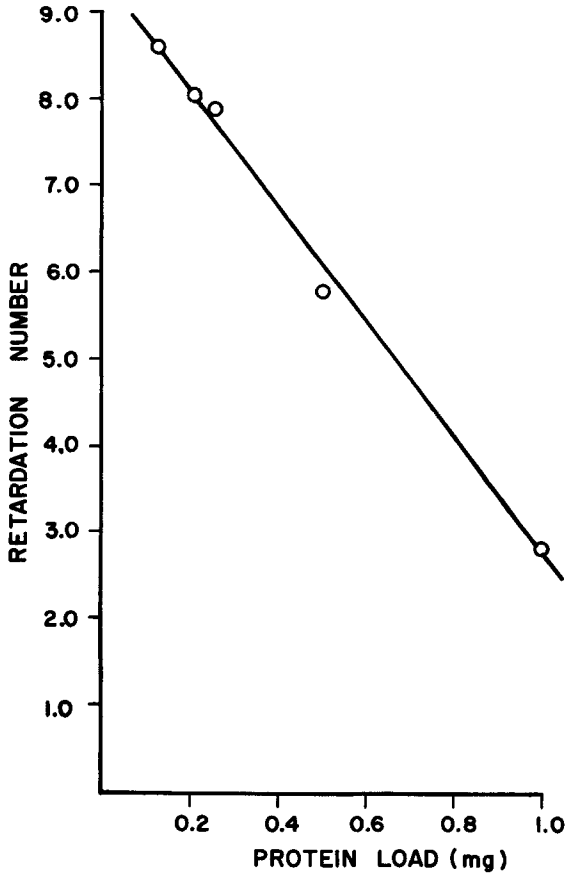

FIG. 7. Retardation as a function of protein loading. HSA in 4 m*M* Tris-acetate, pH 8.3 at 40 V.

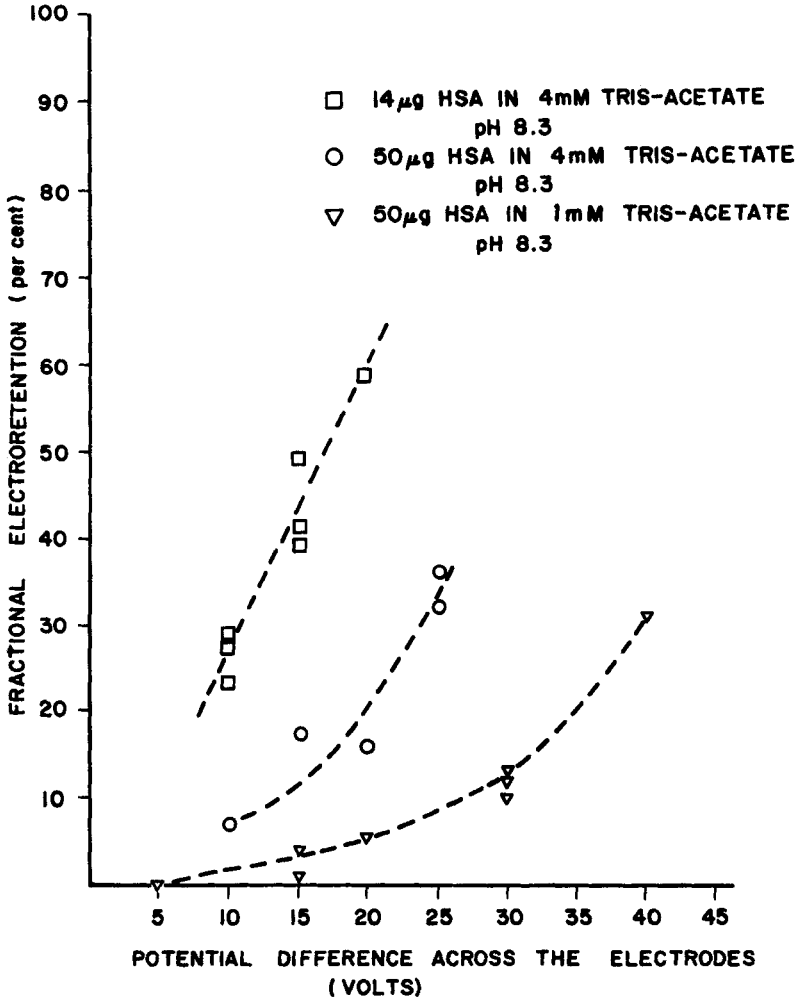

FIG. 8. Fractional electroretention as a function of the potential difference across the electrodes.

TABLE 2
Linear Approximations for Predicting Degree of Electroretention[a]

	14 μg load	50 μg load	Sum
b	2.44	2.42	2.43
r^2	0.99	0.91	0.945

[a] 4 mM Tris acetate.

$$b = \sum (x_i y_i)/\sum (x_i)^2$$
$$r^2 = \sum (x_i y_i)^2/\sum (x_i)^2 \sum (y_i)^2$$

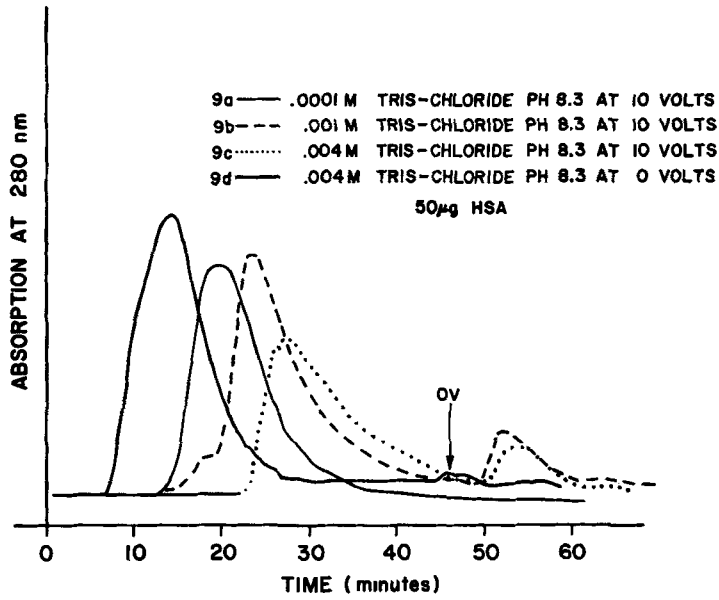

FIG. 9. Effect of buffer concentration on the retardation of HSA.

also been found to have quite significant effects on both retardation and electroretention. The first of these effects is shown qualitatively in Fig. 9 where effluent curves for HSA at 10 V in various concentrations of Tris acetate are compared with a corresponding zero-volt curve. Both retardation and electroretention are seen to increase markedly with buffer concentration over the whole range tested, 0.1 to 4 mM. A quantitative comparison of retardations is provided in Table 3.

However, it should be noted that proteins can significantly affect both the ionic strength and the pH of dilute buffers. Measurements of the pH

TABLE 3
Effect of Buffer Concentration on Retardation of HSA[a]

Buffer concentration (mM)	Retardation, r	pH, protein sample
0.1	2.15	6.40
1.0	2.83	7.95
4.0	3.3	8.28

[a] Other conditions: pH 8.3, Tris chloride buffer 40 μl/min flow rate, 10 V across electrodes.

of 0.5% HSA dialyzed against the buffers used for these four runs are given in Table 3. The pH is affected greatly except for the 4 mM buffer. The extremely low pH of the 0.1 mM buffer may reflect inaccuracies in the preparation of this buffer. We were not aware of the importance of electrostatic interference of pH measurements in such dilute solutions at the time these runs were made. Still further pH changes can be expected in EPC, but we have no reliable means of estimating these at present.

Qualitatively similar results have been obtained for other process conditions and will be submitted for publication shortly. To date, retardations have always increased with buffer concentration up to the highest concentration tested, 4 mM. Work with higher concentrations is planned.

Increases in electroretention with buffer concentration are also observed at all voltages studied for the two 50-μg curves of Fig. 8.

Near Isoelectric Electroretention

Contrary to what one might expect from a field-induced phenomenon, HSA was found to exhibit extremely high fractional electroretention in the neighborhood of its isoelectric point. Moreover, as shown in Fig. 10, this occurred even at high protein loadings and is quite sensitive to pH. The erratic behavior shown at pH 4.8 may simply reflect our inability to control pH with sufficient accuracy in this region. The γG, on the other hand, showed little electroretention at pH 8.3, near the isoelectric point of its major components.

DISCUSSION

It is clear from the results of this preliminary survey that EPC is a much more complex phenomenon than indicated by modeling efforts to date. Our previous predictions of retardation appear useful only as a qualitative guide outside the region of vanishingly small protein loads.

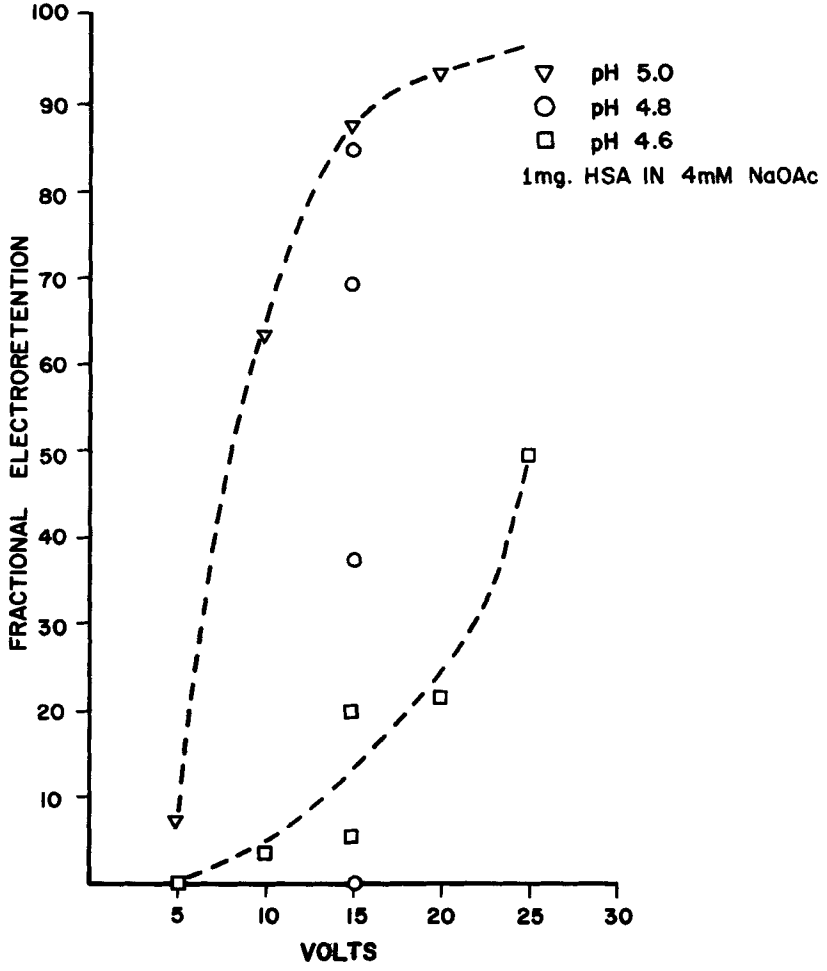

FIG. 10. Fractional electroretention as a function of voltage.

A nonlinear model by Shah et al. (*6*) does predict a decrease in retardation with an increase in protein loading as a result of the concentration dependence of viscosity and diffusivity. Quantitative comparison of our data with this model prediction will require quantitative characterization of membrane electrical characteristics.

However, even the Shah model is based on an oversimplified description of protein chemistry. Little is known of the physical chemistry of concentrated protein solutions, and a more detailed theory, which would include multiple point charge interactions, will be very difficult to construct. More physicochemical studies of single proteins are called for.

It is particularly important to note that strong interactions exist between the effects of the parameters represented in this study. Design of more definitive experiments is therefore a nontrivial undertaking.

However, even at our present limited understanding of the system, EPC is more rapid for separating the dissimilar proteins, HSA and γG, than either chromatography, conventional electrophoresis, or Cohn blood serum fractionation. The use of ultrafiltration fibers makes it relatively easy to scale up the large mass loadings, provided the manifolding problem can be solved, and the resulting geometry can be fairly compact to conserve cooling costs, etc.

For more difficult protein separations it is of first importance to obtain more reliable, if empirical, predictions for the degree of retardation and fractional electroretention.

From a theoretical and long-term practical standpoint, it appears most important to obtain at least a qualitative understanding of electroretention. Although this phenomenon is field induced, its nature is far from clear. Modeling efforts by Shah et al. (*6*) to describe electroretention in terms of the composition dependence of protein transport and equilibrium properties were unsuccessful. In part, this inability may be due to the use of protein properties applicable only to more dilute solutions than those encountered in EPC. The very large electroretention of HSA near its isoelectric point implies that protein association due to charge fluctuations as analyzed by Kirkwood and Shumaker (*7*) may be important, while the effect of ionic strength suggests electroosmotic flow may also be important.

Clearly, much also remains to be done in explaining the observed retardation behavior. However, for practical applications, it appears advisable to use lower electrical potentials so that the protein mixtures do not become as concentrated near the fiber walls. EPC behavior becomes more predictable and electroretention is eliminated under these conditions.

Longer fibers and slower flow rates would then be required to achieve a separation. Different fiber diameter to length ratios will certainly be examined more carefully for their ability to increase resolution.

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