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Electrical Field-Flow Fractionation in a Rigid Membrane Channel

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Abstract

Experimental problems associated with the previous flexible membrane system of electrical field-flow fractionation (EFFF) are first reviewed. The potential advantages of an effective EFFF system are then discussed and compared to those of electrophoresis. The general theory of EFFF is briefly stated, and the possible means for evaluating electrical field strength in the fractionating channel are discussed.

Results are presented for protein components showing that retention follows the predicted behavior with variations in field strength. Furthermore, retention becomes theoretically predictable in numerical magnitude with the aid of an internal standard. The determining parameter for this calculable retention is the ratio D/μ, where D is the diffusion coefficient and μ is mobility. The results show that D/μ becomes a measurable parameter using this system.

Preliminary efforts to induce the retention of denatured proteins and polystyrene beads are shown to be unsuccessful for entirely independent reasons.

INTRODUCTION

Electrical field-flow fractionation (EFFF) is the field-flow fractionation (FFF) technique that employs an electrical field for the retention of solute species (*1*, *2*). This method shows promise for the separation of any systems of charged species, but particularly for mixtures of charged macromolecules and for particles that commonly possess a surface charge.

In a previous study the performance characteristics of electrical field-flow fractionation (EFFF) in a flexible membrane channel were studied (*2*). There, two flexible reformed cellulose membranes were stretched over an intervening spacer in order to form a narrow-slit flow channel between them. These membranes allowed the passage of small ions and thus permitted the maintenance of an electrical field laterally across the channel. The retention and separation of various proteins were studied. A detailed description of the methodology is given elsewhere (*2*).

The flexible membrane system was originally envisioned as having both advantages and disadvantages in EFFF separations. An anticipated advantage was that the membrane, because of its flexibility, should become unusually flat and uniform when stretched across the face of the channel. This, it was reasoned, would help provide the extraordinary uniformity required of surfaces in FFF.

The major disadvantage anticipated for the flexible membrane column was the possible distortion of the channel by small pressure increments arising from various flow processes, including electroosmotic flow.

In its experimental realization, the flexible membrane channel appeared to offer serious practical limitations. Flow parameters were difficult to control and retention values were not entirely reproducible. Furthermore, there was a serious divergence from theory. As a result, this has been the only FFF system in which retention consistently failed to follow the theoretically predicted behavior with variations in field strength. Systems in which theoretical agreement has been found including those of thermal FFF (*3*), sedimentation FFF (*4*), and flow FFF (*5*).

One of the intrinsic advantages of FFF in characterizing complex materials is that the flow (space) is of a well-defined uniform cross section, containing no supporting medium. Therefore, the processes occurring in the channel are subject to a rather rigorous theoretical description. Because of this, retention can be related to fundamental parameters. In the case of EFFF, for example, retention is predicted to be a known function of the ratio D/μ, where D is the solute–solvent binary diffusion coefficient and μ is the solute mobility (*1*, *2*). Using the theoretical relationship, it should be possible to derive values of the D/μ ratio for various charged macromolecules from experimental data. In a completely analogous way, thermal diffusion factors have been obtained from the data of thermal FFF (*6*), and effective masses have been measured through the retention parameters of sedimentation FFF (*7*). In addition, diffusion coefficients have been measured through the application of flow FFF (*5*). In order for the equivalent procedures to be applicable to EFFF for the measurement

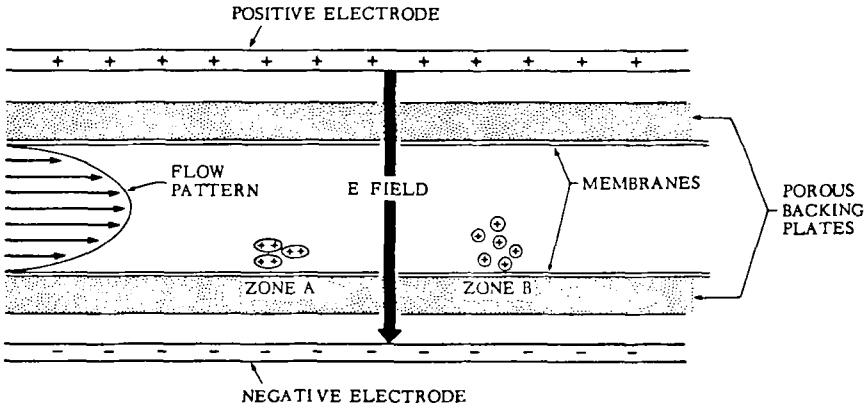

FIG. 1. Schematic side view of EFFF column with rigid membranes.

of D/μ, it is necessary to design a system of well-defined geometry in which the expected relationship between retention and field strength is realized.

In the earlier paper the disagreement with theory was attributed to the use of flexible membranes for the channel wall. In order to remedy this defect and to investigate another type of channel system, we have constructed and developed a rigid membrane channel for EFFF. This system works by layering a membrane on top of a porous backing plate. The principle is illustrated in Fig. 1.

This paper is intended to describe the characteristics of the rigid-membrane system. We have employed native proteins, denatured proteins, and polystyrene beads in an attempt to explore the potential and limitations of this approach to EFFF.

RATIONALE FOR EFFF

If a rigid, theoretically tractable EFFF column becomes available through the present approach, several important analytical advantages may result. First is the rigorous link between retention and D/μ, just mentioned, in which (a) retention is made a predictable function of physiochemical parameters D/μ, and (b) D/μ values can be determined for uncharacterized material through retention measurements.

For perspective, it is useful to examine some of the other potentially favorable characteristics of EFFF, and to make some comparisons with

electrophoresis which, of course, employs the same external field but in a different mode.

Electrophoretic separations, particularly in protein systems, are hindered by the difficulty of separating by size species with the same or similar surface characteristics (*8*). With equal surface charge density, different sizes acquire an equal or similar mobility and will, therefore, move at an equal or almost equal velocity in an applied electrical field. Efforts have been made to utilize the small mobility differences that do exist, but at best this provides only a 1.5 to 3.0-fold variation over a 1000-fold particle diameter range (*8*).

In the case of EFFF, by contrast, the velocity of the zone is determined by the D/μ ratio. (Over a wide range, zone velocity is proportional to D/μ.) Therefore, two solute particles with identical surfaces but different sizes will separate by virtue of unequal D values. In this way, EFFF should provide a valuable complementary tool to electrophoresis, making possible the separation of charged species that are inherently difficult to treat in electrophoresis because of surface similarities.

One major approach now used effectively to circumvent the equal-mobility problem in proteins is that of gel electrophoresis (*9*, *10*). By this technique the mobility of large molecules is selectively reduced by the presence of a gel network. Such gel systems provide outstanding resolution. However, the correlation of mobility with molecular size and other physical parameters is essentially an empirical matter. (It should be noted that gel filtration systems are likewise subject to the construction and interpretation of empirical calibration curves.) In FFF, by contrast, the well-defined geometry and flow profile, if experimentally achievable, should lead to zone velocities that are directly related by theory to D/μ.

Perhaps the most useful feature of EFFF relative to electrophoretic methods is its inherent elution nature. This advantage has been mentioned before (*1*, *11*, *12*). The separated zones are removed from the column by the continuous flow stream in the normal course of operation. This greatly facilitates the potential for detection and collection of fractionated samples.

It should be noted that both electrophoretic and EFFF separation are subject to manipulation by variations in pH, ionic strength, and similar parameters. These variations will affect mobilities and, to a lesser extent, diffusion coefficients. They will, therefore, allow one to shift the relative migration rates of different solutes. This potential was realized experimentally for EFFF in a previous paper (*1*).

It has been mentioned previously that EFFF allows the indefinite

cascading of a small applied voltage (*11*, *12*). Only a few volts across the channel are necessary to cause retention and separation. With this voltage fixed (but with increasing current), the length of the column can in theory be extended to any arbitrary limit, and the theoretical resolution can be correspondingly enhanced. Alternately, with a moderate increase in the voltage, the field intensity can be greatly increased and theoretical resolvability improved (*12*). Therefore, the possibility exists to apply a higher electrical field with a smaller overall voltage than in electrophoresis. Theoretical studies have shown that this further enhances the possible gain in resolution. A detailed theoretical comparison of the potential resolution obtainable in electrophoresis and EFFF has been presented elsewhere (*12*).

It is important to note that the low-voltage possibility has not yet been realized in that well over 90% of the voltage drop has always been lost in regions outside the channel.

It is well known that one limitation in high field strength separations in electrophoresis is the generation of excessive quantities of heat and the consequent distortion and spreading of the zone (*13*). In EFFF, disturbances of this kind are expected to be minimal. The superposition of a temperature gradient on top of the electrical field will not have significant effects unless convective currents are induced. (Convection could be specifically guarded against in this system by using a horizontal configuration with a selective cooling of the lower compartment.) However, the other effects of thermal gradients, such as flow profile distortion and thermal diffusion, have been well characterized in a completely analogous FFF system: thermal FFF. Here gradients up to 6000°C/cm have been intentionally induced to promote the retention and separation of polymer components (*14*). From this study it is clear that the presence of a gradient of lesser magnitude will have little effect on retention and zone dynamics providing the aforementioned convection is carefully avoided.

It should be noted that EFFF, like other FFF systems, is a one-phase system, free of interfaces, except those imposed by the channel wall. It is expected that one-phase systems will be inherently more gentle on sensitive macromolecules than will heterogeneous systems with a large interfacial area.

THEORY

The theory of the general FFF methodology has been detailed in many places; the application of this theory to the specific case of EFFF was

discussed in the previous paper (*2*). Very briefly, the electrical field compels charged solute particles to drift toward the lower membrane. At the steady state an exponential solute cloud forms at this membrane with a height governed by the characteristic length parameter, l. Quantity l is sometimes termed the "mean layer thickness" of the solute cloud, or layer. Mathematically, l is given by the equation

$$l = D/\mu E \tag{1}$$

This equation shows that the magnitude of l is determined by the electric field strength, E, and by the ratio D/μ of diffusion coefficient to mobility.

Components with small l values occupy the quiescent regions immediately adjacent to the lower membrane and are, therefore, significantly retarded with respect to the motion of solvent through the EFFF channel. The extent of retardation is measured by the retention ratio, R, which is defined identically to the retention parameter of chromatography: R = zone velocity/mean solvent velocity. Parameter R bears the following relationship to l:

$$R = 6\frac{l}{w}\left[\coth\frac{w}{2l} - \frac{2l}{w}\right] \tag{2}$$

where w is the width of the channel (the spacing between membranes). The ratio l/w is sometimes expressed as λ.

The combination of Eqs. (1) and (2) permits the calculation of retention values in terms of fundamental mobility and diffusion parameters. However, accurate E values must be available in order to utilize Eq. (1).

The acquisition of E is somewhat complicated in a rigid-membrane system because of the porous backing plate. With a thin, ion-permeable (flexible) membrane, by contrast, there is very little perturbation of the potential drop by the membrane, and E can be obtained approximately by neglecting the electrode potential and overvoltage from the ratio

$$E_0 = V/S_e \tag{3}$$

where V is the potential and S_e is the distance between electrodes. This method was used with the previously described flexible-membrane system.

The electrical field strength of the rigid-membrane system is disturbed by the backing plates supporting the membranes. These are ordinarily thick enough to occupy a substantial fraction of the space between the electrodes. Furthermore, their electrical resistance is higher than that of the free background solution due to tortuosity and constriction effects (*15*). Therefore, one has the twin problems of (a) measuring the true

potential in the channel, allowing for the disturbance of the backing plates, and (b) maintaining an adequate field strength despite the interference of the backing plates.

We have utilized two methods for obtaining E. A third method is outlined following our discussion of the two employed.

Conductivity Method. The field strength in our EFFF channel can be calculated from the equation

$$E = i/\kappa A \tag{4}$$

where i is the current, κ the buffer conductivity, and A the channel area. In theory, one can measure buffer conductivity κ in a standard cell. This can be combined with the A obtained from column geometry and the i value monitored continuously by the ammeter.

Certain limitations exist for this method that might conceivably introduce errors into the measured E value. First, the retained solute will alter the conductivity and thus the field strength to some degree. For the high dilutions we have used here, however, this is probably negligible.

More importantly, if the membrane is selectively permeable to ions of one charge, an ionic-strength gradient can become established, yielding a continuous range of E values from one side of the channel to the other. If the membranes are by chance not exactly equal in their ion selectivity, ion accumulation or depletion can take place in the flow space. This phenomenon, at its extreme, is found in the space between oppositely charged ion-exchange membranes, where total ion depletion can be induced (*16*).

Internal Standard Method. The problems cited above can be largely circumvented by injecting a probe substance—a well-characterized standard—to reflect the magnitude of the electrical field. The D/μ ratio of the probe must be known. Then the retention ratio, R, of the probe is experimentally measured; Eq. (2) is used to derive the l (or λ) value; and finally Eq. (1) is solved for E, all other parameters being known. Other variants of this principle exist, but they are based on the same principle. A useful variant simply relates the relative ratios, l/w and D/μ, that apply to some test substance and to the probe substance. Equation (1) gives

$$\frac{l/w}{(l/w)_{\text{probe}}} = \frac{D/\mu}{(D/\mu)_{\text{probe}}} \tag{5}$$

We have used lysozyme as an internal standard in this study. Values of D and μ are available in the literature (*17–19*).

The internal standard method may be imagined to have a few draw-

backs, too. First, D and μ values generally depend somewhat on ionic strength; if there is an ionic-strength gradient, there will be some variation in the D/μ ratio. However, this variation should ordinarily constitute only a second-order disturbance.

More serious would be interactions of the probe solute with the membrane. The validity of this method hinges on the correctness of Eq. (2). Unforseen effects like adsorption would render Eq. (2) inapplicable.

To its advantage, the internal probe would sample the same, thin layer next to the membrane that influences the retention of the uncharacterized solutes. Any unusual gradients or disturbances across the flow space in the field direction would be expected to influence the standard and the unknowns in much the same way.

Resistance Analysis Method. It is possible, using methods to be detailed later, to compare the resistances of different components (electrolyte, backing plates, free electrolyte) lying in series between the membranes. This approach could be used to calculate channel field strength. However, the method was not employed in this study.

EXPERIMENTAL

The basic configuration of the EFFF apparatus has been described in detail in Ref. *2*. The modification of the channel system is shown schematically in Fig. 1. The structure was made from a Plexiglas piece 20 in. × 3 in. × 1 in. (50.8 cm × 7.62 cm × 2.54 cm). A trough of dimensions 45 cm × 1.9 cm × 1.27 cm was cut in each Plexiglas piece to accommodate the platinum wire electrode. The trough was then covered with a porous backing plate to form a chamber. The backing material was a 1/8-in. (0.318 cm) thick high-density polyethylene frit (Porex Materials Corp.) with a 100-μm pore size. Two holes were drilled in the Plexiglas wall of each chamber, allowing the buffer to enter by the bottom hole and exit by the upper hole. The circulation of the buffer was performed by means of a micropump. The flow channel was formed by clamping a 0.356-mm thick Mylar spacer between two thin film cellulose acetate membranes which were cast on the porous backing plate. To prevent the possibility of leakage between spacer and membranes, silicone rubber was put on both sides of the spacer extending up to the channel boundary. The actual channel dimensions were 41.5 cm × 0.76 cm × 0.076 cm, and the channel volume was 2.4 cm^3.

The preparation and casting of the cellulose acetate membranes were carried out according to Manjikian's procedure (*20*). A solution of cellulose

acetate, acetone, and formamide (in the ratio 1/2.85/0.66) was cast on the porous polyethylene surface. This membrane should have low electrical resistance and small pore size permeable only to species of molecular weight less than 12,000.

The monodisperse spherical polystyrene beads (Dow Chemical Co.) had diameters of 907 ± 57, 1087 ± 27, 1756 ± 23, 3117 ± 22, 3570 ± 56, and 4808 ± 18 Å, respectively. The protein samples were obtained as follows: hemoglobin (bovine) from Mann Research Laboratories; cytochrome C (horse heart), albumin (bovine-serum), γ-globulin (bovine), and lysozyme (egg white) from Sigma Chemical Company. The denaturation of protein was done according to Weber and Osborn's procedures (*10*). The above proteins were incubated at 100°C for 2 min in 0.01 *M* sodium phosphate (Matheson Coleman and Bell Co.) buffer, pH 7.12, 1% sodium dodecyl sulfate (Eastman Kodak Co.), and 1% β-mercaptoethanol (J. T. Baker Chemical Co.), then the sample was cooled to room temperature. The sample was used directly without further dialyzing. The weight ratio of sodium dodecyl sulfate to protein was 3:1.

The two solutions used for the latex bead and denatured protein studies were, first, 0.1% by weight of sodium dodecyl sulfate (SDS), and, second, 0.01 *M* phosphate buffer at pH 7.12 containing 0.1% SDS. A 0.012 *M* sodium acetate buffer at pH 4.5 was used as the carrier in the native protein studies.

All experiments were carried out at room temperature (22 ± 1°C). A Chromatronix IV pump supplied carrier flow. Peaks were detected with a Laboratory Data Control Ultraviolet Detector. A voltage regulated power supply (fabricated in our technical shops) was used to apply the electric field. The applied current and conductance of the solvent were measured with a Simpson volt-ohm-milliamperemeter and conductivity bridge (Model PH-70CB, Barnstead Still and Sterilizer Co.), respectively. The retention ratio, *R*, was measured by comparing the retention volume to the channel volume (2.4 ml), the latter obtained by injecting proteins into the system without applying an electrical field.

RESULTS AND DISCUSSION

Loss of Field Strength in Channel. The porous backing plates provide an extra resistance to current flow, and may, therefore, be expected to "sap" a large percentage of the total electrical potential, as noted earlier. In order to study this effect, current vs voltage measurements were made in a special EFFF unit having an electrode spacing of 2.44 cm under four

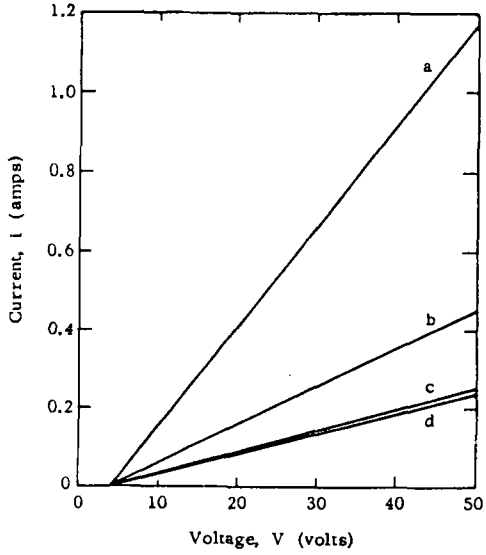

FIG. 2. Current–voltage curves for EFFF system with (a) pure electrolyte, (b) one backing plate, (c) two backing plates, and (d) two backing plates coated with membrane. Electrode spacing = 2.44 cm.

different conditions: and (a) pure electrolyte between the electrodes; (b) a single backing plate without a membrane; (c) two plates without membranes; and (d) two backing plates with coated membranes in place.

The results are shown in Fig. 2. The presence of backing plates is seen to reduce the current significantly; the membranes are a lesser disturbance. The resistances calculated from these results are 39.1 Ω for the pure sodium acetate buffer; 140.6 Ω from the two uncoated backing plates (saturated with buffer); and 14.6 Ω from the two membrane coatings. Thus of the total potential drop (less electrode potential and overvoltage of 4.06 V) in this particular system, roughly 72% occurs across the backing plates, 8% across the membranes, and about 20% across the electrolyte. The thin sheet of electrolyte composing the 0.356-mm wide fractionating channel receives only about 0.3% of the total potential drop. This design is, therefore, very inefficient insofar as the utilization of electrical potential is concerned. Future work should have as one objective the improvement of efficiency.

Retention and Separation of Native Proteins. Because of the large

potential loss through the porous backing plate, it became necessary as a practical matter to work at higher voltages with this system than were employed with the flexible-membrane column. However, this necessity was reduced to a small degree by decreasing the gap between electrodes from 5.08 to 1.66 cm in our working column.

Retention of native proteins was successfully induced by the applied electrical field, and the level of retention was found in all cases to increase with field strength, as expected.

In order to test the retention relationship quantitatively, a plot of l/w (derived from measured retention by means of Eq. 2) versus $1/E$ (where E is the lysozyme value noted earlier) was made for each protein. As Fig. 3 shows, the data form straight lines intercepting the origin, as demanded by theory (*2*).

These results represent a considerable improvement over the results for the flexible-membrane column. The latter led to nonzero intercepts, presumably because of membrane flexing and channel distortion. These

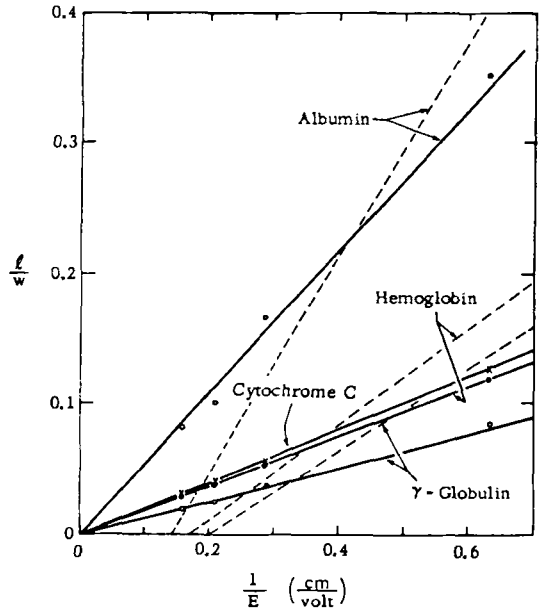

FIG. 3. Plot of l/w vs $1/E$ for native proteins retained in this system (solid line) and the earlier flexible membrane column (dashed line). Only the present system gives a proper intercept at the origin.

earlier results are shown for comparison in Fig. 2. The buffers for the two systems are nearly identical (both at pH 4.5, but ionic strength at 0.0124 instead of 0.02), and therefore the curves from the different systems should almost superimpose one another for a given protein. The same general trends are, in fact, observed, but the lines fail to coincide, indicating a serious failing in the flexible-membrane system.

The uniform straight lines through the origin indicate that a basic problem of the flexible-membrane system can be remedied through the present approach. However, a further evaluation is necessary to determine if the slopes of the lines are consistent with theory. Figure 4 is directed toward this question. In this figure, theoretical lines are compared with the experimental lines. The theoretical values of l/w are derived from Eq. (5) and the known mobility and diffusion parameters for albumin, hemoglobin, and the lysozyme probe in the pH 4.5 buffer: $\mu = 1.3 \times 10^{-5}$ and $D = 5.9 \times 10^{-7}$ for albumin (*18*, *21*, *22*); $\mu = 4.9 \times 10^{-5}$ and $D = 8.2 \times 10^{-7}$ for hemoglobin (*18*, *23*, *24*); and $\mu = 6.35 \times 10^{-5}$ and

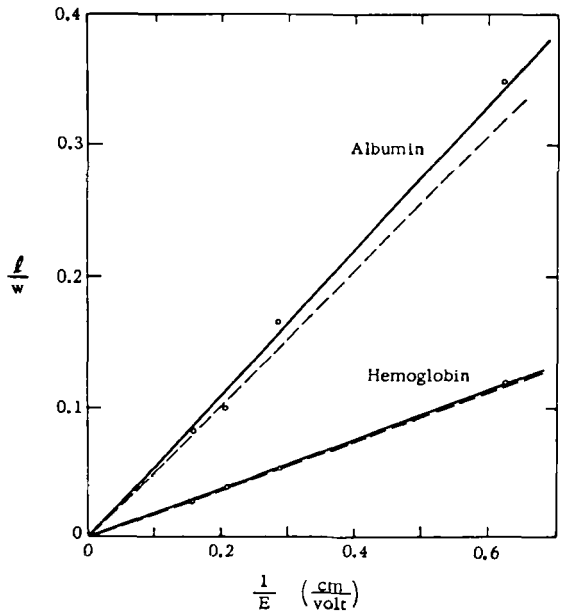

FIG. 4 Experimental (solid line) vs theoretical (dashed line) retention parameters for albumin and hemoglobin. Lysozyme was the internal standard.

$D = 10.4 \times 10^{-7}$ for lysozyme (*17–19*). All μ's reported here are in units of cm^2/V-sec and all D's are in cm^2/sec.

The agreement of Fig. 4 is very satisfactory. It confirms the utility of the rigid-membrane channel in providing a constant environment and a uniform cross section for the theoretically predictable retention of charged solutes.

The width of solute peaks, as reflected in the plate height, has always been a less predictable property of FFF systems than retention. Figure 5 shows the results of plate height measurements made at different linear velocities. The plots are almost linear, as predicted by theory, but in only one case do they extrapolate to the origin, as also predicted by theory when longitudinal diffusion is assumed negligible. All of the experimental values of plate height are larger than the theoretical values, as shown by the comparison of the two lines for each solute in the figure. However, the lysozyme plot is remarkable among the various FFF systems so far tested in having plate height values unusually close to the theoretical values.

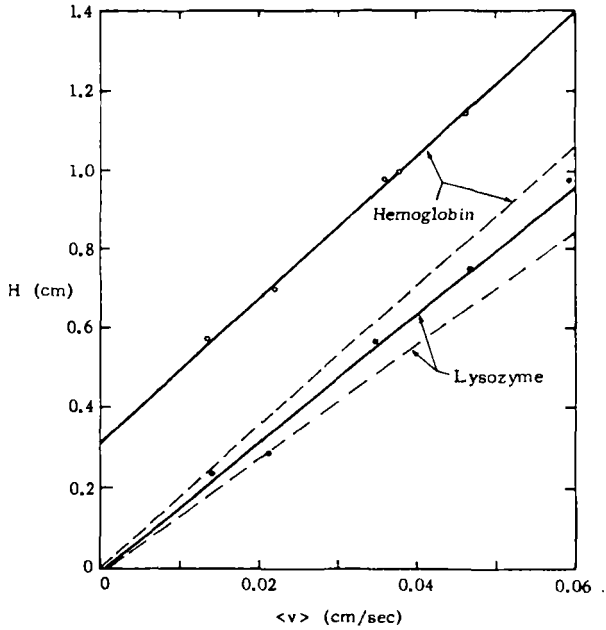

FIG. 5. Plate height vs linear mean flow velocity for hemoglobin and lysozyme. Experimental least square plots (solid lines) lie above theoretical (dashed) lines, but the difference is unusually small for lysozyme.

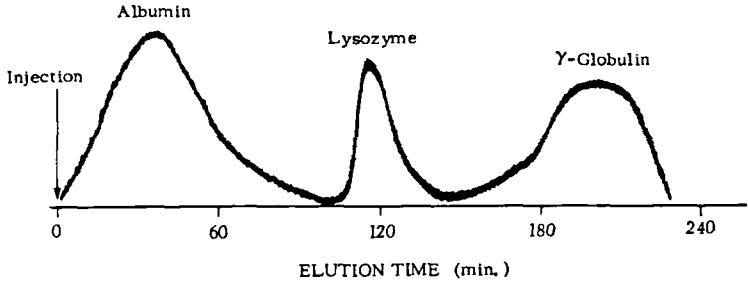

FIG. 6. Separation of albumin, lysozyme, and γ-globulin at 30 V, pH = 4.5, and a flow rate of 5.3 ml/hr. Column void volume, 2.4 ml.

Finally, several fractionations of protein mixtures were accomplished in this work. An example is shown in Fig. 6, where albumin, lysozyme, and γ-globulin are separated from one another. Of especial note is the narrowness of the lysozyme peak, in accord with the unique plate height characteristic of this substance just mentioned. Also of note is the overall tendency of peaks to get no wider with increases in elution volume. The latter characteristic is one of the most promising aspects of FFF generally (*12*).

Denatured Proteins. EFFF applied to denatured proteins could become a tool for measuring protein molecular weights. With a constant mobility, μ, induced by an SDS coating (*10*), retention would hinge solely on diffusion coefficient D. For the random coils of denatured proteins, D increases in a predictable manner with molecular weight, thus permitting the acquisitions of molecular weight values from retention.

Several preliminary attempts were made to retain denatured proteins in the SDS and SDS–phosphate buffers. However, no significant retention was observed even at potentials up to 160 V, which gave E values as large as 5.5 V/cm in the fractionating channel. However, the total potential drop across the channel, evaluated from conductance measurements, was only of the order of 0.4 V. Although the problems with this system are not yet entirely clear, it is possible that the latter potential is simply not high enough to induce any important degree of separations in this system.

Polystyrene Latex Beads. Attempts were also made to separate polystyrene latex beads ranging in diameter from 907 to 4808 Å in the same buffers as employed with the denatured proteins. Here, however, the problems encountered were of totally different nature. The peak eluted without an applied field rapidly decreased in area as the field was applied

and increased. Before significant retention could be induced, the peak had disappeared. Our conclusion was that some form of adsorptive interaction existed between the beads and the membrane. Increasing field strength would force the beads toward the membrane surface and enhance the rate of adsorption, until finally no measurable quantities remained in solution. The adsorption was apparently irreversible because the peak failed to reappear following the elimination of the field.

Conclusion. The present column system represents an important advance in EFFF because of its capability of yielding predictable retention. However, the inefficient use of the applied potential ranges in seriousness from mildly annoying to perhaps the total loss of retention, depending on the solute–buffer system. Also the membrane does not appear to be inert to all solutes. Serious work aimed at remedying these defects could yield a much more versatile EFFF system.

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