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Separation Techniques Based on the Opposition of Two Counteracting Forces to Produce a Dynamic Equilibrium

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Abstract

Useful compounds, whether produced by chemical synthesis or biological synthesis, often need to be purified from complex mixtures. Biochemists and chemists thus recognize the need for efficient new preparative purification techniques for product recovery. Such fractionation techniques must have high capacity and high resolution. In a novel group of separation methods suited to the preparative fractionation of proteins, antibiotics, and other classes of compounds, the chromatographic flow of a solute down the column is opposed by solute electrophoresis in the opposite direction. Useful separation is achieved when these two counteracting forces drive the solute to a unique equilibrium position within the separation chamber. The properties of chromatographic matrices, for example, gel-permeation matrices of various porosities, provide a means of establishing the unique equilibrium points. Extraordinary resolution and capacity are attainable by these methods.

In recent years, major improvements have been made in the development of analytical techniques for the separation of proteins and nucleic acids (1–3). However, methods for large-scale preparative fractionation have advanced more slowly and have relied heavily on the use of increased scale to adapt the analytical technologies (4). I describe here the results of initial investigations of a new group of separation methods that can provide the high capacity and high resolution needed for preparative fractionation. When solvent flow and electrophoresis directly oppose each other, their action on a particular solute can be precisely counterbalanced to achieve a steady state, but ordinarily there would be no restoring force moving the solute toward a particular equilibrium position. A chromatographic matrix can influence solute movement with a flowing solvent differently from the way it influences solute electrophoresis and thereby can bring about a balance between these opposing forces. With an appropriate configuration of different chromatographic matrices, movement driven by electrophoresis will dominate within part of the separation column while movement with the flowing solvent will dominate in another part of the column. These imbalances can produce a restoring force that concentrates the solute at a unique equilibrium position (5). The following example illustrates this principle and describes the approach used in the first experimental tests of this method.

A column (Fig. 1) was packed with a restrictive matrix, BioGel P-10 (Bio-Rad), on top of a bed of a much more porous matrix, BioGel A-50m. The colored protein ferritin is found to chromatograph rapidly downward through the upper part of this column (where it is excluded from the matrix beads) but more slowly through the lower part of the column (where it is included in the matrix beads). As illustrated in Fig. 1, at the appropriate applied voltage, the upward electrophoresis rate, R_E , will exceed the downward rate of movement with solvent flow, R_F , in the bottom matrix but will be insufficient to counteract the more rapid downward R_F in the top matrix. At this voltage, the net movement (R_N) of ferritin is downward in the top part of the column and upward in the bottom part of the column. The ferritin is thereby highly concentrated at an equilibrium position adjacent to the interface of the P-10 and A-50m gel beds.

This type of solute behavior provides the basis for a powerful separation method. Although I will illustrate the method with a specific example, the approach is very general and adaptable. I call this new group of separation methods counteracting chromatographic electrophoresis (CACE).

If CACE is to prove effective as a preparative procedure, it must have high capacity. In the two-component separation column described above, how much protein can be held in an equilibrium zone? A protein that is driven toward the interface of the two matrices is clearly not in equilibrium in either the upper or the lower matrix. Obviously, protein cannot accumulate indefinitely at the interface, yet, because the conditions in the upper and lower matrices trap the protein, it cannot escape. A concentration-dependent effect must cause R_E to equal R_F so that an equilibrium zone is established in an area of the column where the protein would otherwise (that is, at low concentration) still have a net mobility. One obvious way in which high protein concentration will alter electrophoretic mobility is by increasing the local electrical conductivity, thereby locally decreasing the voltage gradient and thus decreasing R_E . For example, as the ferritin begins to accumulate at the junction of the P-10 and A-50m matrices, its increasing concentration reduces its electrophoretic mobility until R_E exactly balances the slower of the two R_F values (that in the lower, A-50m, matrix). If the ferritin were to be further concentrated, then R_F would dominate and drive the protein down the column to dilute and enlarge the equilibrium zone until it again reaches exactly that concentration at which R_E and R_F balance. In sections of the A-50m matrix where the protein concentration is still low (outside the equilibrium zone), R_E will still dominate and move the ferritin upward to collect in the zone of high concentration that will form with its upper edge at the interface of the two matrices. The concentration of the ferritin in the equilibrium zone will thereby be regulated to a fixed value, and, as more protein accumulates, this zone will become proportionately broader.

Empirically, it is a simple matter to determine the capacity of an equilibrium zone. In the example used in Fig. 1, a two-component P-10/A-50m column, 50 cm in length and 7 mm in diameter, was loaded with 10 mg of ferritin. At equilibrium this material concentrated into a narrow band about 2 mm wide. This corresponds to a protein concentration somewhat in excess of 100 mg ml^{-1} . As expected, this zone increased in thickness when more ferritin was added. The narrow glass column used in these experiments could have held more than 1 g of ferritin in an expanded equilibrium zone occupying roughly 10 ml (40 percent) of the column volume. For this size of apparatus, the CACE method therefore has a capacity about three orders of magnitude higher than electrophoresis and is roughly equivalent to the highest capacity chromatographic methods.

The efficacy of separation depends on the resolution of the method. As a first assessment of resolution, consider how many proteins might focus to the same position in the system described above. Virtually all proteins will be excluded from the P-10 gel and included in the A-50m gel. Thus, they will move with the solvent flow at similar rates, fast in the upper part of the column and slow in the lower part of the column. Nonetheless, at a particular applied voltage, only a very select group of proteins will be focused to the interface of the two matrices because proteins vary greatly in their electrophoretic mobility. Proteins will be swept off the column with the solvent flow or alternatively will migrate off the column by electrophoresis unless R_E falls within the boundary conditions established by the R_F values in the upper and lower matrices. Of course, by adjusting the sign and the magnitude of the applied potential one can change the direction and rate of electrophoresis of other proteins to bring them into equilibrium.

To define the resolving power of CACE, I sought to determine the minimum difference in properties that allows separation of two different proteins. By choosing two matrices with closer R_F values, one can form highly selective interfaces. Furthermore, mixtures of two

matrices should have intermediate properties and therefore can be used to establish interfaces with boundary conditions as closely spaced as desired. I prepared a column having a series of selective interfaces between its successive layers, each layer being composed of two matrices mixed in different proportions (Fig. 2). I analyzed R_E and R_F for myoglobin in the different compartments of the multilayered column. I measured R_E in the absence of flow and R_F in the absence of applied potential. The R_E values in the upper (P-300) and lower (A-5m) layers were indistinguishable, while R_F in the upper layer was almost twice as high as in the lower layer. Myoglobin moved at intermediate R_F values through those zones of the column made of mixed beds of the two matrices. In the simplest situation, R_F would be governed directly by the proportions of the two matrices. Thus, if R_F in the P-300 matrix is designated as 1 and in the A-5m matrix as 0.5, then, according to the relative proportions of the matrices (Fig. 2), the successive mixed-bed layers would be expected to have relative rates of 0.6, 0.7, 0.8, and 0.9 ($1 \times \text{fraction P-300} + 0.5 \times \text{fraction A-5m}$). If this expectation is correct, comparable changes in applied potential should shift the myoglobin equilibrium to successive interfaces. Consistent with this prediction, the equilibrium potentials when normalized to the equilibrium voltage of the upper interface were 0.61, 0.7, 0.79, 0.9, and 1. Thus, mixed beds provide a consistent variation in properties and can establish discontinuities with very closely spaced boundary conditions as required for high-resolution separations (6).

A pair of characterized proteins focusing to adjacent interfaces in the column in Fig. 2 would define the minimal resolvable difference for this column. The behavior of four colored proteins (hemoglobin, myoglobin, ferritin, and cytochrome c) was examined. By adjusting the voltage at a constant flow rate of solvent, I was able to focus each protein to any of the interfaces on this column. Small changes in the applied voltage caused the equilibrium position of a protein to shift from one interface to another. The conditions that gave equilibrium were unique for each protein; in fact, no two proteins could be forced to reside on the column at the same time. Since two of these proteins, hemoglobin and myoglobin, are electrophoretically similar, this result demonstrates that the method has a high resolving power. Table 1 gives a more quantitative indication of the differences in equilibrium conditions for these proteins.

I used another approach to quantitatively define the resolution. I measured the minimal fractional change in R_E required to alter the equilibrium position, by determining the fractional change in applied voltage required to shift a protein from one interface to the next. Because proteins will differ in R_E in proportion to their intrinsic electrophoretic mobilities, this measurement, carried out with one protein, can be equated to the minimum difference between two proteins that would permit their resolution.

Very small changes in applied voltage do not shift R_E outside the boundary conditions; thus I found that, over a narrow range of voltages, myoglobin remains stable at one equilibrium position. For example, at a flow rate of about 3.6 ml hour^{-1} and applied voltages between 562.5 and 544 V, myoglobin focused to interface B in Fig. 2 (7). As the voltage is further decreased, the myoglobin band becomes less distinct and a slight accumulation of myoglobin appears at the next lower interface (interface C in Fig. 2); a small additional voltage decrease (to 525 V) causes the band to move to a new equilibrium position at the next lower interface. The smallest change in voltage required to move myoglobin from one equilibrium zone to another was 4 percent. Thus, for the column tested, the minimal difference in the R_E values of two proteins required to separate them is 4 percent. To give this measure of resolution some context, note that proteins have a wide range of electrophoretic mobilities (8) and that for many proteins the alteration of a single charged amino acid can change the mobility by several percent (9).

In addition to its high capacity and resolution shown here, CACE has a number of additional features that indicate its diverse potential applications. For example, the system has a continuous-flow capability because CACE effectively concentrates the purified material at a

single equilibrium position. Thus, in a simple type of continuous-flow apparatus, the purified material could be continuously withdrawn from a port at the equilibrium position.

Ordinarily the use of electrophoresis for large-scale operations is restricted because of problems of heat dissipation. In formats of CACE with short column beds and high flow rates, the heat removed with the flowing solvent avoids this problem.

Definition of the equilibrium conditions is greatly simplified if the protein is visible. The development of colored markers (or the development of other monitoring techniques) should similarly simplify the process of identifying equilibrium conditions for noncolored solutes.

The range of applications of CACE can be greatly extended if different types of matrices are used in the column. Although I have discussed only gel-permeation matrices here, the method is by no means limited to this class of matrices. Any pair of matrices that create the described imbalances in R_F and R_E can be used. If one uses different types of chromatographic matrices and conditions, it should be possible to purify proteins, antibiotics, or other classes of compounds, and to execute the separations on the basis of different properties such as size, charge, hydrophobicity, or chemical affinity.

Another important feature of these methods is that good separation can be obtained even when multiple components focus on the same column. As a consequence of the conditions establishing an equilibrium zone, such multiple components will actually form distinct equilibrium zones stacked one on top of the other. Furthermore, a column that contains a continuous gradient (that is, two matrices mixed in continuously varying proportions) can be used to generate many distinct equilibrium positions.

At present, I project the potential of this technology as follows. Because of the experimental tests that are required to define the equilibrium conditions for any particular solute, the most likely use of CACE will be for the purification of materials that have already been characterized, and analytical applications will, at first, be limited. Although practical preparative purifications by CACE have not yet been examined, the measurements reported here indicate that the capacity and resolution of this method are as good as or better than those obtained in many of the established separation methods. Thus, with refinement, this technology could have major applications in a wide range of preparative fractionations.

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6. Despite the consistent behaviors of the matrices described here, it is not clear that one can make a priori predictions of what types of matrices will work. Thus, I did not get myoglobin to come to equilibrium between layers of P-10 and P-300.

7. The very low equilibrium flow rates for myoglobin are expected because the *pH* (7.4) is near the myoglobin isoelectric point so that its charge and electrophoretic mobility will be low.
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R_F - Rate of movement with fluid flow

R_E - Rate of movement by electrophoresis

R_N - Net rate: balance of R_F and R_E

⊕ Applied voltage drives components upward

Fig. 1.

Schematic representation of the apparatus. A 50-cm glass column (0.7 cm internal diameter) was packed to a height of 25 cm with BioGel A-50m (Bio-Rad), and the remainder of the column was filled with BioGel P-10. The column was connected to electrode reservoirs by short sections (1 cm) of large-bore (1.2 cm internal diameter) tubing plugged with 15 percent polyacrylamide gel. Additional ports allowed the column to be connected to a peristaltic pump to give a regulatable flow of the carrier solvent (10 mM tris acetate, pH 7.4) through the column. Ferritin loaded on this column was brought to equilibrium with the flow rate set at about 0.17 ml min^{-1} and the applied voltage adjusted to 600 V. The flow of the carrier solution is indicated by the group of arrows at the top. Vectors to the right indicate the magnitudes and directions

of the electrophoretic rate (R_E), the rate of solvent flow (R_F), and net (R_N) solute mobilities. In the equilibrium zone, R_E and R_F are equalized by concentration-dependent effects (see text).

Fig. 2.

Use of a multilayered column to measure resolution. A column (20 cm by 0.7 cm internal diameter) was packed with BioGel A-5m, BioGel P-300, and mixtures of these matrices to produce a series of layered beds as indicated. This layering gives rise to subtle but discontinuous changes in properties so that the rate of movement of myoglobin with solvent (50 mM tris acetate, pH 7.4) flow (R_F) differs in each layer. A protein will be focused at the interface between any of these pairs of layers if R_E is between the R_F values of the two bounding layers. R_E is determined by the product of the electrophoretic mobility (M) of the solute (myoglobin, in the example in the text) and the voltage gradient [dV/dD], where D is the distance]. The fractional change in dV/dD required to change R_E to a value, R_E' , that will cause myoglobin to move from one interface to another provides a direct measure of the resolving power of the method. That is, as shown by the formulas at the right, R_E and R_E' can be related, respectively, to M and M' , the mobilities of two proteins that differ in electrophoretic mobility by the smallest amount that will permit complete resolution.

Table 1

Relative equilibrium voltages measured for different proteins in the separation chamber depicted in Fig. 2. Since R_E must be the same for the different proteins when they come to equilibrium and since R_E is proportional to the product of the voltage gradient and the electrophoretic mobility of a protein, the voltage at which each protein attains equilibrium will be inversely proportional to its electrophoretic mobility in the column. Ferritin, hemoglobin, myoglobin, and cytochrome c were brought to equilibrium at a particular interface in the segmented column described in Fig. 2. The large differences in observed equilibrium voltages indicate the sensitivity of the separation technique to differences in the electrophoretic mobility of the different proteins. Obviously the charge of the very basic protein cytochrome c is reversed.

Protein	Applied potential* at equilibrium	Molecular weight	Isoelectric point	Estimated [§] electrophoretic mobility at pH 7.4 (cm ² V ⁻¹ sec ⁻¹)	Estimated charge at pH 7.4//
Ferritin	6.7	460,000	4.5 [†]		
Hemoglobin (human)	32	64,000	6.87 [‡]	-1.2×10^{-5}	-6
Myoglobin (horse)	56	16,900	6.58 [‡]	-1.05×10^{-5}	-1.2
Cytochrome c	Polarity reversed	12,000	10-11		+ 1.5

*The potential is given as a dimensionless value, because I normalized the applied voltage to a constant flow rate by dividing by the pump speed at equilibrium.

[†]Taken from isoelectric focusing data.

[‡]Taken from electrophoresis data (9,10).

[§]Measured free electrophoretic rates were extrapolated to pH 7.4. No corrections were made for ionic strength or temperature (9,10).

//The charge for normal hemoglobin was estimated by comparison of the electrophoretic mobilities of normal and sickle cell hemoglobin. It is based on the known difference of two charges between normal hemoglobin and sickle cell hemoglobin and an assumed linear change in electrophoretic mobility with charge (9). The charge on horse myoglobin was estimated from the slope of charge versus pH measured for whale myoglobin (10) and the isoelectric point for horse myoglobin (11). The charge on cytochrome c is an estimate based on the amino acid composition and normal pK_a values of side chains.