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Potential Barrier Chromatography: An HPLC Method for Protein Separations

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Abstract

Potential barrier chromatography (PBC) is a separation technique for proteins, colloidal sols, and possibly for viruses and cells; it is feasible when the potential energies of the van der Waals attraction, double-layer, Born, and hydration repulsions between adsorbate and adsorbent produce surmountable energy barriers to adsorption as well as moderately deep adsorption (energy) wells. The adsorbates can move over the potential barrier (for adsorption) when it is not too large and, if the adsorption well is not too deep, can also desorb without difficulty. The above-mentioned interaction potentials are very sensitive to small changes in adsorbate size, surface potential, Hamaker constant, ionic strength, etc. Thus two adsorbates which slightly differ in charge and/or size, etc., will require different lengths of time for one set of adsorption and desorption. Consequently, their residence times in the column (which involve repeated sets of adsorption and desorption) can differ enough to permit a separation. For a given adsorbent the depth of the adsorption well and the height of the potential barrier can be controlled by altering the double-layer repulsion via changes in the pH, ionic strength (as well as the nature of the electrolyte), and/or altering both the van der Waals attraction and the above repulsion via changes in the organic solvent-content of the mobile phase. Earlier calculations carried out for spherical particles have predicted that the method, under certain conditions, can separate even species which differ by only 5% in size, or by about 1% in surface potential, or by 1% in Hamaker constant. Here a high performance liquid chromatography version of PBC is reported. Using an isocratic elution procedure, an aqueous mobile phase, and an inexpensive ion exchange column, two model proteins (ovalbumin and bovine serum albumin) have been separated. The essence of the procedure is to prevent, by means of double-layer repulsion, the occurrence of a too deep adsorption energy well. This necessary repulsion, however, generates, in general, a potential barrier to adsorption. This barrier has to be moderate if adsorption is to occur.

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INTRODUCTION

Traditional separation procedures for proteins employ adsorbents that have polysaccharide matrices. Such adsorbents would crush under high pressures and hence, if small particle sizes are chosen for the adsorbent (in order to obtain efficient separations), the methods are limited to low flow rates of eluent and long analysis times. On the other hand, adsorbents based on silica can withstand high pressures. High performance liquid chromatography (HPLC) methods employ the latter type of adsorbents and in general involve short analysis times. HPLC methods are increasingly becoming available for protein separations (*1*); among these, size exclusion chromatography and reverse phase chromatography are well known. A complementary method is the recently proposed (*2, 3*) "potential barrier chromatography."

High performance size exclusion chromatography (HP SEC) requires the use of expensive adsorbents (*1*) and does not in general fully utilize properties other than the size of the adsorbates in effecting separations.

On reverse phase HPLC adsorbents, proteins adsorb strongly and require rather large amounts of organic solvents in the mobile phase for elution (*4*); such conditions may denature the proteins. In addition, for altering the organic solvent-content of the eluent, one uses "gradient-elution" procedures that require two solvent delivery pumps and possibly a solvent programmer as well and hence involve a larger capital expenditure than a method in which the proteins could be eluted without changing the composition of the "isocratic" mobile phase whence retention occurred.

This paper reports the development of a HPLC version of potential barrier chromatography (PBC) in which the above-mentioned shortcomings of the existing HPLC methods for protein separation are avoided.

In PBC, the operating variables (pH, ionic strength, etc.) are tuned such that the interaction potential (energy) between adsorbate and adsorbent possesses a moderately deep energy well separated from the bulk solution by a moderate potential barrier. Thus the adsorbates can undergo cycles of adsorption (by overcoming the potential barrier for adsorption) and desorption (by escaping from the not too deep energy well and overcoming the energy barrier to desorption) along the column. Since the interaction potential is very sensitively dependent on adsorbate size, surface potential, etc., two adsorbates that differ even slightly in such characteristics will reside in the column for appreciably different times and can thus be separated.

Initially, the physical basis of the method will be outlined. It uses concepts from colloid stability theory and essentially consists of viewing the chromatographic separations under consideration as resulting from an

adsorption–desorption process governed by physical interactions between the adsorbates and the adsorbent.

Subsequently, the experimental development of the method will be detailed. A conventional HPLC apparatus has been used in combination with a relatively inexpensive ion-exchange column and aqueous mobile phases. A mixture of model proteins has been separated by the method.

In all, a novel HPLC separation technique for globular proteins (and possibly for enzymes, viruses, cells, and colloidal sols in general) is demonstrated.

THEORY

When the adsorbate (be it a globular protein or a colloidal particle) approaches the adsorbent to distances of the order of 100 Å, certain electrostatic and nonelectrostatic physical interactions occur.

Most solid surfaces in contact with aqueous media become electrically charged by one or more of several possible mechanisms (5). Globular proteins (and polyelectrolytes, in general) and ion-exchange adsorbents acquire charges through surface-group ionization.

An electrically charged surface attracts counterions (ions of opposite charge relative to the surface) to its vicinity from the liquid. This effect of the charged surface on the counterions is opposed by the tendency of the latter to drift away to the bulk because of thermal motions. As a result of these opposing forces, the counterions attain a cloudlike distribution near the charged surface, akin to that of the earth's atmosphere. The charged surface together with the diffuse layer of counterions forms the electrical double layer. When two entities bearing electrical double layers approach each other, as the diffuse layers begin to overlap, the alterations in the distribution of counterions lead to a change in the free energy of the system which manifests itself in the double-layer interaction.

Between identical entities, the double-layer interaction is similar to Coulomb's law in that surfaces of opposite charges attract each other while surfaces of like charges repel. Between nonidentical surfaces, however, the nature of the double-layer interaction cannot be described so straightforwardly (*6, 7*).

As the ionic strength of the medium is raised, the surface charges become increasingly screened and thus their effect on the counterions extends less and less into the solution. As a result, the interacting surfaces can approach each other closer than before without experiencing double-layer interactions. In addition, counterions can bind to the charged surface, thus reducing the

surface charge. In effect, by means of these two mechanisms, raising the ionic strength would reduce the double-layer interactions.

Another major interaction that should be taken into account is the van der Waals interaction (*8*) which originates from three effects: (a) the orientation or Keesom interaction between permanent dipoles, if any, of two molecules; (b) the induction or Debye interaction between the permanent dipole of a molecule and the induced dipole caused by it in another molecule; and (c) the dispersion or London interaction (in general, the most important among the three) that results from the existence of instantaneous dipole moments in each atom of any material due to the incessant motion of electrons. Such an instantaneous dipole moment generates an electric field which polarizes another atom, inducing in it a dipole moment. The interaction between these two dipoles results in the dispersion interaction.

Although the van der Waals interactions between atoms are short range (the potential falling off with the sixth power of the distance between their centers if the distances are not too large), between macroscopic or macromolecular species the interaction attains a long-range effectiveness due to the cooperation of numerous intermolecular interactions (*9–11*).

As has been reviewed at length elsewhere (*12*), the van der Waals interaction between adsorbate and adsorbent can be altered by coating the adsorbent with alkyl ligands or by means of additives to the eluent such as neutral salts at high concentrations (via lyotropic salt effects) and organic solvents.

At very small distances between the interacting entities, short-range repulsive forces develop. Born repulsion (*2*) results from the overlap of electron orbitals. Hydration repulsion (*5*) arises from the difficulty in eliminating water molecules that are held around charged or polar groups on the surfaces.

Approximate expressions for the potentials ϕ_{DL} of the double-layer interaction, ϕ_{vdW} of the van der Waals interaction, and ϕ_B of the Born repulsion, derived for the interaction between a sphere (the adsorbate) and a semi-infinite plate (the adsorbent), are summarized for illustrative purposes in Table 1. The sum of these interaction potentials is the total interaction potential ϕ:

$$\phi = \phi_{DL} + \phi_{vdW} + \phi_B \tag{1}$$

A plot of ϕ vs h (the distance between the center of the sphere and the surface of the plate minus the radius of the sphere) can have various shapes. Such profiles as are typical in chromatography are schematically shown in Fig. 1.

Curve A, with a monotonically decreasing potential energy, corresponds

TABLE 1

Approximate Expressions for the Potential Energies of the Interaction of a Sphere (Adsorbate) with a Semi-Infinite Plate (Adsorbent)

Electrostatic Double-Layer Interaction (2, 23, 24)

$$\phi_{DL}(h) = 16\varepsilon\left(\frac{kT}{e}\right)^2 a_p \tanh\left(\frac{e\psi_p}{4kT}\right)\tanh\left(\frac{e\psi_A}{4kT}\right)e^{-\chi h} \qquad \text{(T-1)}$$

where ε is the dielectric constant of the medium, k is the Boltzmann constant, T is the absolute temperature, e is the charge of a proton, a_p is the radius of the sphere (adsorbate), ψ_p and ψ_A are the surface potentials of the adsorbate and adsorbent, respectively, and χ is the reciprocal of the Debye length defined by

$$\frac{1}{\chi} = \left(\frac{8\pi n_0 e^2}{\varepsilon kT}\right)^{-1/2} \qquad \text{(T-2)}$$

where n_0 is the ionic strength of the medium.

Van der Waals Interaction (2, 10)

$$\phi_{vdW}(h) = \frac{A}{6}\left[\ln\left(\frac{h+2a_p}{h}\right) - 2\frac{a_p}{h}\frac{h+a_p}{h+2a_p}\right] \qquad \text{(T-3)}$$

where A is the Hamaker constant for the interaction between the adsorbent and the adsorbate across the medium. (See Ref. 12 for a detailed discussion.)

Born Repulsion (2)

$$\phi_B(h) = \frac{A\sigma^6}{7560}\left[\frac{8a_p + h}{(2a_p + h)^7} + \frac{6a_p - h}{h^7}\right] \qquad \text{(T-4)}$$

where σ is the collision diameter.

to the domination of double-layer repulsion at all distances. If this were the profile governing the interaction between adsorbate and adsorbent, adsorption would not occur. Besides, the macromolecular adsorbate would elute ahead of its uncharged version or a micromolecular tracer since the latter are not excluded from regions near the adsorbent's surface where the mobile phase velocity is smaller than that in the bulk. This phenomenon occurs in hydrodynamic chromatography (*13–16*) and it is also known in gel permeation chromatography (*17*) as the unwanted "ion exclusion" effect which leads to erroneous molecular weight calibration curves (that are constructed accounting only for steric and not for electrical effects).

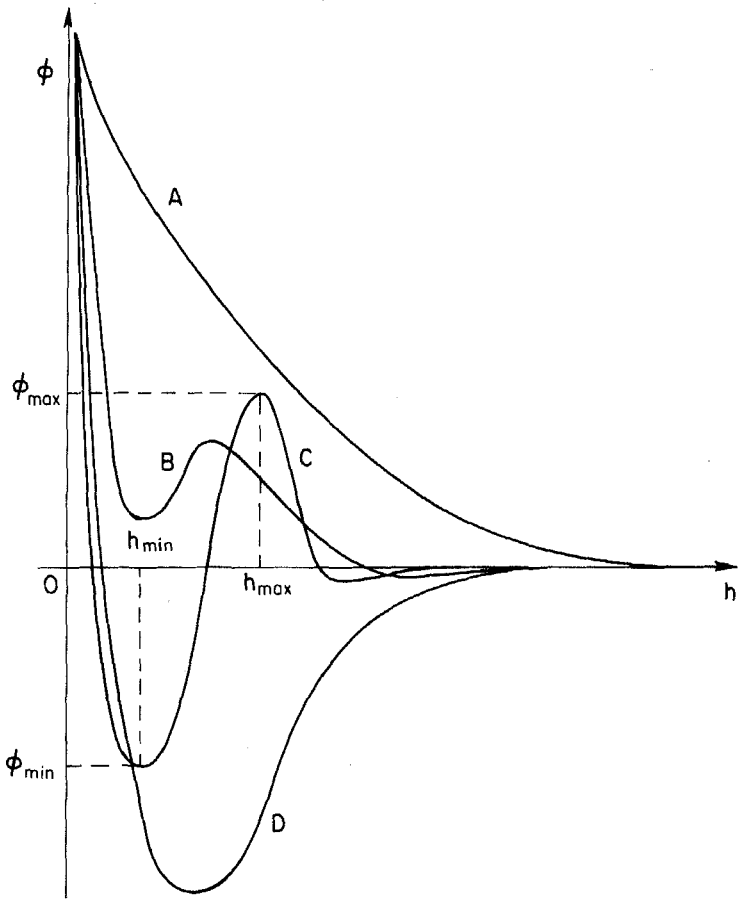

FIG. 1. Potential profiles revelant to the chromatography of hydrosols and globular proteins (schematic). While Curves A and D occur, respectively, in hydrodynamic chromatography and reverse phase protein chromatography, Curves B and C correspond to potential barrier chromatography.

Curve D is characterized by a single minimum and corresponds to the situation wherein the double-layer interaction is weakly repulsive, absent, or attractive, and acts in combination with the van der Waals attraction. As concluded in a recent review (*12*), such a profile is typical in hydrophobic interaction chromatography and reverse phase chromatography of proteins. When a protein pulse is introduced into a column of adsorbent with which it experiences such a profile, adsorption would be rapid and mass transfer to the adsorbent by convective diffusion would be the rate-determining step.

Prior to adsorption, the concentration gradient of adsorbate is from the bulk liquid to the adsorbent surface; subsequent to adsorption, this concentration gradient is in the opposite direction. If the potential minimum ("energy well") is shallow, desorption will be facile. If the minimum is very deep, however (a situation typical in reverse phase chromatography of proteins), desorption can be achieved only if the attractive forces are reduced such that the energy well is lifted to a level whence elution can occur by thermal motions. Such is usually brought about by the addition of organic solvents to the mobile phase.

In contrast to these two curves, Curves B and C display two minima and a maximum. By assuming a quasi-steady state transport of the adsorbate over the maximum and by parabolically expanding the potential energy function about the maximum and the minimum that is near the adsorbent surface, expressions have been obtained (*2, 3*) for the adsorption coefficient k_{ai} and the desorption coefficient k_{di} for adsorbate i as,

$$k_{ai} = D_\infty \frac{h_{\max}}{a_p} \left(\frac{\gamma_{\max}}{2\pi kT} \right)^{1/2} \exp\left(-\phi_{\max}/kT\right) \tag{2}$$

and

$$k_{di} = D_\infty \frac{h_{\max}}{a_p} \frac{(\gamma_{\max}\gamma_{\min})^{1/2}}{2\pi kT} \exp\left(- \frac{\phi_{\max} - \phi_{\min}}{kT} \right) \tag{3}$$

where D_∞ is the diffusion coefficient of the adsorbate in the bulk and $\gamma_{\max} = -d^2\phi/dh^2 \mid_{h=h_{\max}}$ and $\gamma_{\min} = +d^2\phi/dh^2 \mid_{h=\min}$, with the definitions of $h_{\max}$, $h_{\min}$, $\phi_{\max}$, and $\phi_{\min}$ being given in Fig. 1. The flux J of adsorbate toward the adsorbent under the action of the interaction potential is then given by

$$J = k_{ai} C_i^* (1 - x) - k_{di} n_i \tag{4}$$

C_i^* is the concentration of adsorbate i in the liquid very close to the packing surface, n_i is the number of entities of species i adsorbed per unit area of the adsorbent, and x is the fraction of the adsorbent surface already covered by adsorbates:

$$x = \sum \alpha_i n_i \tag{5}$$

where α_i is the surface area that a single entity of adsorbate i occupies on the adsorbent.

It is noteworthy that Eqs. (2) and (3) for the sorption coefficients are of the Arrhenius form with ϕ_{max} and $(\phi_{max} - \phi_{min})$ as the activation energies for adsorption and desorption, respectively. A parametric study (*2*) indicates that these potential barriers and adsorption (energy) wells depend very sensitivity on parameters such as ionic strength, surface potentials, and Hamaker constants. In other words, the sorption coefficients depend exponentially on potential barriers which in turn show extreme sensitivity to variations in the physicochemical properties of the adsorbate, adsorbent, and mobile phase. This sensitivity forms the basis of potential barrier chromatography in which a fine discrimination among very similar adsorbates could be achieved by tuning the column of a liquid chromatograph (say by changes in pH and ionic strength) such that a not too deep energy well is generated and surmountable potential barriers control adsorption and desorption.

By combining Eqs. (1)–(5), which are descriptive of the interaction potential, with the conventional equations of chromatographic mass transfer, theoretical results have been obtained (*3*) which are given here in Fig. 2. The significant message contained in these results is that for adsorbates smaller than 2500 Å in size (above which PBC becomes infeasible), situations have been identified in which separation is possible even when the adsorbates differ by only 5% in size (Fig. 2A), or by about 1% in surface potential (Fig. 2B), or by 1% in the Hamaker constant of the van der Waals interaction (Fig. 2C).

The factors which determine the residence times and the resolution of the adsorbates will become clearer from the following simple, intuitive analysis (*25, 26*).

The average time required for the particles to adsorb once, τ_a, the average time required for desorption, τ_d, and the residence time of the mobile phase (or an unretained tracer), τ_f, can be evaluated from

$$\tau_a = \varepsilon_p / k_a S \tag{6a}$$

$$\tau_d = 1/k_d \tag{6b}$$

and

$$\tau_f = \varepsilon_p v / Q \tag{6c}$$

where ε_p and v are the void fraction and total volume, respectively, of the chromatographic column, S is the surface area per unit volume of the adsorbent, and Q is the volumetric flow-rate of the mobile phase.

When the potential barrier for adsorption is insurmountably high, adsorption will not occur. The characteristic adsorption time, τ_a, in this case

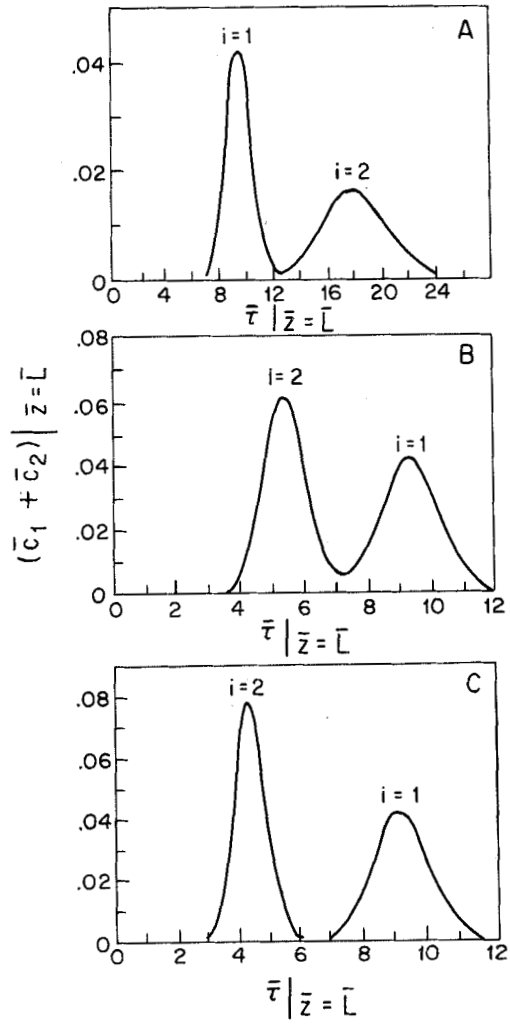

FIG. 2. Computed PBC chromatograms (from Ref. *3*) for mixtures of (A) particles of two sizes $a_{P1} = 850$ Å and $a_{P2} = 892.5$ Å, $A = 10^{-13}$ ergs, $\sigma = 6.5$ Å, $\psi_{P1} = \psi_{P2} = \psi_A = -23$ mV, $\varepsilon = 74.3$, $T = 300°$K, $n_0 = 0.05$ M, volume fraction of each type of particle in solution $= 10^{-3}$, inlet concentration of Particles 1 equals that of Particles 2 and adsorption is considered to have a much larger characteristic time than convective diffusion mass transfer. (B) Particles of two surface potentials, $\psi_{P1} = -23.0$ mV and $\psi_{P2} = -23.2$ mV, $a_{P1} = a_{P2} = 850$ Å. All other parameters are as in Case A. (C) Particles of two Hamaker coefficients, $A_1 = 10^{-13}$ ergs, $A_2 = 0.99 \times 10^{-13}$ ergs, $a_P = 850$ Å. All other parameters are as in Case A. Note: $\bar{c}_1 + \bar{c}_2$ and $\bar{\tau}$ are the nondimensionalized total particle concentration and the time respectively (for definitions, see Ref. *3*).

would be of the order of or larger than τ_f. Thus the "adsorbate" will elute along with an unretained tracer or even earlier (as discussed in the context of Fig. 1, Curve A). Even if the potential barrier is surmountable, the amount adsorbed will be negligible if the primary minimum in the potential profile is similar to the one in Curve B (i.e., $\phi_{\min} > 0$) since such an energy minimum is not energetically favorable. If the potential barrier for adsorption is moderate and if the energy well is moderately deep, with $\phi_{\min} < 0$, the adsorbates will make many exchanges between the sorbed and free states (provided, in addition $\tau_a \ll \tau_f$). Under these conditions the relative numbers of particles in the two states can be approximated by equilibrium considerations and the total time a particle spends adsorbed, τ_p, can be written as the product of the number of adsorption steps and the time required for one desorption, i.e.,

$$\tau_p = \frac{\tau_f}{\tau_a} \tau_d \tag{7}$$

Thus the total residence time of the particle in the column, τ, becomes,

$$\begin{aligned} \tau &= \tau_p + \tau_f \\ &= \tau_f(1 + \tau_d/\tau_a) \end{aligned}$$

or

$$= (\varepsilon_p + Sk_a/k_d)v/Q \tag{8}$$

It is important to note that the only parameter involving the interaction forces which affects the residence times of the adsorbates is the equilibrium constant k_a/k_d. Thus it becomes recognizable that (by substituting Eqs. 2 and 3 for the sorption coefficients in Eq. 8), when adsorption–desorption equilibrium can be assumed, adsorbate residence times are determined only by the depth and the shape of the adsorption energy well.

The essence of PBC consists in ensuring that the adsorption energy well is not so deep as to prohibit desorption. This is done by operating at a pH at which the double-layer forces between adsorbate and adsorbent are repulsive. By manipulating the ionic strength, this repulsion, and hence the depth of the energy well, can be controlled. If the double-layer repulsion is not weak, however, a potential barrier for adsorption usually exists. As discussed above, the height of this barrier has to be moderated so that adsorption is possible.

The maximum in the potential profile (in general a necessary consequence of using double-layer repulsion to control the energy minimum), while not affecting the residence times of adsorbates under adsorption–desorption

equilibrium, does influence peak broadening. By means of an intuitive analysis similar to the above (see Ref. *26*, Sect. 2.7), the contribution from adsorption–desorption to the variance σ_L^2, which is a measure of peak-broadening, can be evaluated from

$$\sigma_L^2 = 2\left(\frac{\tau_d}{\tau_a + \tau_d}\right)^2 \frac{\tau_a Q L^2}{\varepsilon_p v} \tag{9}$$

It is noteworthy that, unlike Eq. (8), this expression for σ_L^2 involves τ_a directly and not as a ratio with τ_d. Thus the introduction of a maximum in the potential profile actually increases peak broadening.

Balancing attractive and repulsive forces to control adsorption and desorption, as described above, is worthwhile despite the appearance of the potential maximum and the accompanying effects; double-layer repulsion constitutes an excellent means of raising the adsorption energy well from depths that may prohibit desorption (without recourse to the use of organic solvents).

Thus PBC forms a sensitive separation method that uses differences in both adsorption and desorption characteristics to effect separations. Hence it is complementary to:

(1) Size exclusion chromatography which does not involve adsorption.
(2) Reverse phase chromatography of proteins that in general makes use of differences only in the desorption characteristics to effect separations (e.g., at what points along a linear increase in the organic solvent-content of the mobile phase that different proteins are elutable).

Another noteworthy item is that since the characteristic times for adsorption and desorption in PBC are much larger than the characteristic time for convective diffusion, the *rate* of transport to (or from) the adsorbent is controlled by the sorption processes. In reverse phase chromatography of proteins, on the other hand, both adsorption (which occurs spontaneously) and desorption (brought about by the addition of organic solvents) occur rapidly such that convective diffusion is likely to be the rate-determining step for transport to (and from) the adsorbent.

In addition, since the rates of adsorption and desorption in the presence of a particular mobile phase are comparable in PBC, the method possesses an operational advantage in that it lends itself to an isocratic elution procedure in which elution is achievable without changing the composition of the mobile phase whence retention occurred.

EXPERIMENT

The experimental apparatus comprises (connected in series) a solvent delivery pump (Waters 6000 A), a nonstop flow septumless injector (Waters U6K), a microparticulate stainless steel column (DuPont ZIPAX SCX), a variable wavelength UV detector (Waters UV450), a peak timer and peak-area integrator (Varian CDS 111), and a strip-chart recorder (Houston Instruments, Omniscribe). The column (2.1 mm i.d. $\times$ 100 cm length) is quite long and thus permits sufficient residence time of the adsorbates for the separation to manifest itself. The adsorbent (DuPont ZIPAX SCX), schematically shown in Fig. 3, consists of an impermeable glass core onto which are attached silica microspheres which are in turn coated with a fluorocarbon polymer containing sulfonic acid groups. The interstices between the microspheres have an average size of about 1000 Å (*18*). Thus the macromolecular solutes would not be sterically excluded from physico-chemical interactions with the microspheres. In this context, it should be mentioned that while this adsorbent is used in general for separating small molecules, its use for protein separations has been previously attempted (*19*). The adsorbent will be negatively charged for pH $>$ 2. The requirement for repulsive double-layer forces is fulfilled since, at the pH values used in this study (pH = 6 and 7.5), the model proteins chosen as adsorbates, bovine serum albumin (pI = 4.8) and ovalbumin (pI = 4.6), will carry net negative charges. (Proteins carry a net negative charge for pH values above the isoelectric point pI.) The double-layer repulsion can be decreased by raising the ionic strength.

The experimental procedure is straightforward. The mobile phase (of a chosen pH and salt concentration) is passed through the column for about 2 h, an interval sufficient for equilibration. Subsequently, this flow is continued and pulses (100 to 200 μL/injection) containing either a single protein component or a protein mixture, all dissolved in the mobile phase, are injected and the effluent is monitored by UV absorbance at 280 nm.

The mobile phases and stock solutions were prepared using deionized, double-distilled water. All the salts and buffer components were purchased from Fisher Chemical Co. (analytical grade) and the proteins were purchased from Sigma Chemical Co. The stock solutions were refrigerated and the mobile phases were prepared fresh daily and sonicated for 30 min prior to usage.

Experimental Results and Discussion

Retention time data for bovine serum albumin (BSA) and ovalbumin (OV) (injected as pure components) obtained at pH 7.5 with various concentra-

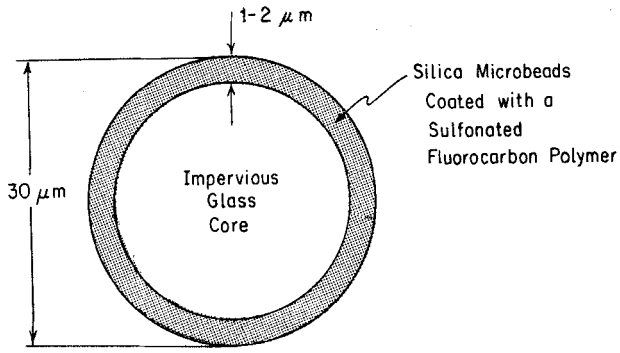

FIG. 3. Schematic of the pellicular DuPont ZIPAX SCX adsorbent; the porous outer structure has an average pore size of 1000 Å (*18*).

tions of $(NH_4)_2SO_4$ in the mobile phase are summarized in Table 2. The electrostatic double-layer forces between adsorbate(s) and adsorbent are repulsive at this pH. One would expect, hence, that the proteins would be eluted around the void volume peak at very low ionic strength, and as the salt concentration of the mobile phase is raised, the retention time of each protein would be larger. Such is indeed the behavior experimentally observed. When the mobile phase is merely the pH 7.5 citrate buffer with no added $(NH_4)_2SO_4$, OV and BSA elute at 2.75 and 2.84 min, respectively (at a flow rate of 0.5 mL/min), retention times little different from the void volume peak which occurs at 2.75 min; in the presence of 0.16 M $(NH_4)_2SO_4$ in the same buffer, however, OV and BSA elute at 3.88 and 4.43 min, respectively (the void volume peak occurring in this case at 2.92 min). Although the elution of OV and BSA at this pH occurs without requiring changes in the composition of the mobile phase, the retention times are not very different. Hence a separation of the two proteins from a mixture was not attempted.

At pH 6 on the other hand, separation is achieved; the chromatograms obtained by injecting mixtures of OV and BSA at various salt concentrations are plotted in Fig. 4 and the retention time data are summarized in Table 3. It is instructive to peruse these data along with theoretical results (*2*) on the effect of raising the ionic strength on the total interaction potential profile, given here in Fig. 5. (The theoretical calculations were done earlier (*2*) for a different situation involving colloidal particles and the results are relevant to the present discussion, if only in the qualitative sense.) At low ionic strengths the potential profile is dominated by double-layer repulsion which would prohibit adsorption. As the ionic strength is raised, this repulsion is moderated and, with the aid of the van der Waals attraction and Born and

TABLE 2

Experimental Results from Potential Barrier Chromatography
[pH 7.5 citrate buffer (0.01 *M*)][a]

Concentration of $(NH_4)_2SO_4$ in eluent (mol/L)	Retention times (in min) injected as pure components		
	Solvent peak	OV	BSA
0.00 (20 μL)	2.75	2.75	2.85
0.06	2.98	3.10	3.22
0.10	3.00	3.37	3.58
0.12	2.95	3.52	3.82
0.14	3.01	3.63	3.83
0.16	2.92	3.88	4.43

[a]Flow rate 0.5 mL/min; chart speed 0.1 in./min; wavelength 280 nm; recorder attenuation 16; detector sensitivity 0.4 AUF; sample volume per injection 200 μL (unless mentioned otherwise); for other details, see text.

hydration repulsions, surmountable potential barriers to adsorption and desorption appear (Fig. 5). (It is important to note that without the short-range Born and hydration repulsions, the minimum in the potential profile will be infinitely deep and desorption will never occur.)

As the cascade of potential profiles of Fig. 5 would qualitatively indicate, the experimental data (Fig. 4) show that with no added $(NH_4)_2SO_4$ the proteins are eluted in the void volume peak and, as the salt concentration is raised, elute at appreciably longer times, governed by the mechanism of PBC. At pH 6 the proteins show a significant separation as the chromatograms (Fig. 4) illustrate. These data demonstrate that PBC is able to separate proteins, at retention times under 30 min, without requiring (for achieving elution of the proteins) changes in the composition of the mobile phase from which retention occurred.

It is worthwhile to reiterate the proposed explanation for the behavior experimentally observed and examine if there is an alternative one. The proposed mechanism rests on the existence of double-layer repulsion between adsorbate and adsorbent. With no added salt, this repulsion dominates and causes elution of the adsorbates in the void volume peak or earlier; as the salt concentration is raised, it gives rise (in combination with van der Waals attraction and Born and hydration repulsions) to surmountable potential barriers and to adsorption wells which result in larger residence times. Through all such changes in the salt concentration, the

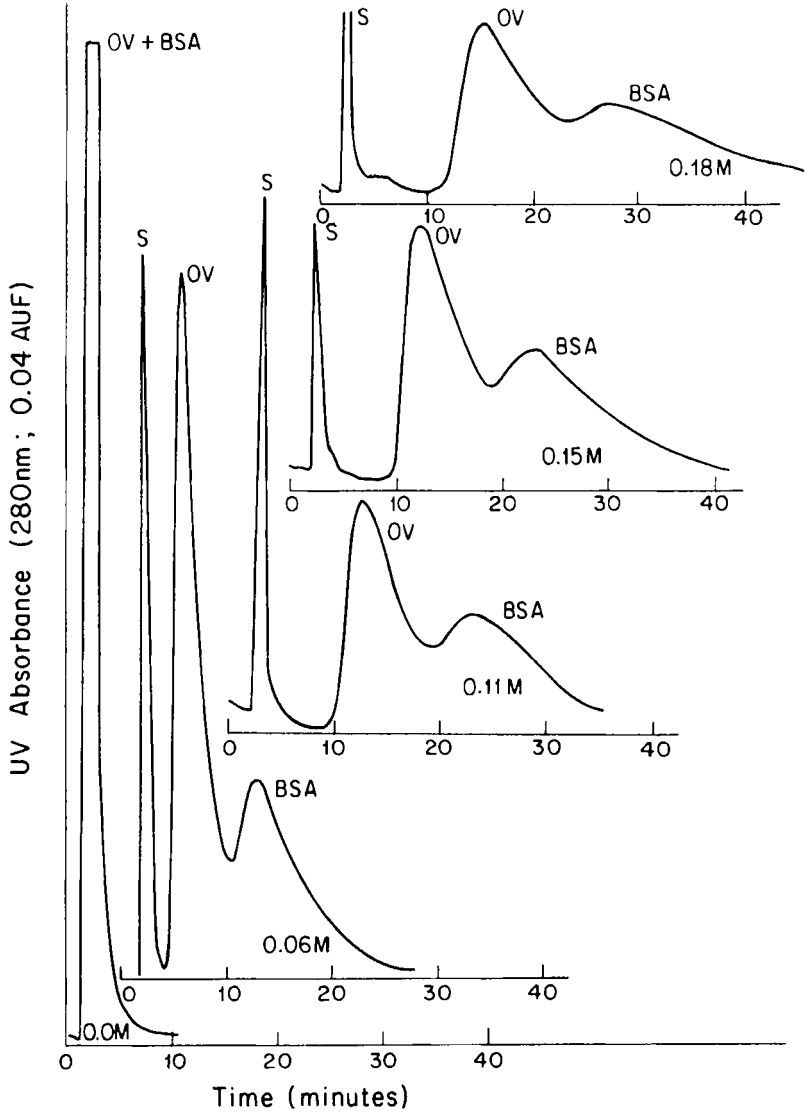

FIG. 4. Separation of ovalbumin (OV) and bovine serum albumin (BSA) by potential barrier chromatography as a function of salt concentration. Column: DuPont ZIPAX SCX (2.1 mm i.d. × 100 cm length). Mobile phase: 0.01 *M* citrate buffer, pH 6 containing 0.003 *M* NaN_3 and various concentrations of $(NH_4)_2SO_4$ (given in the figure). Flow rate: 0.5 mL/min. Sample volume per injection: 100 μL. For other details, see Table 3. S denotes the solvent peak.

TABLE 3

Experimental Results from Potential Barrier Chromatography [pH 6 (see also Fig. 4)][a]

Concentration of $(NH_4)_2SO_4$ in the mobile phase (mol/L)	Retention times (in min[b]) (injected in a mixture)		
	Solvent peak	OV	BSA
0.00	3.26	3.26 (0.562)	3.26 (0.434)
0.06	3.13	6.49 (0.436)	13.91 (0.428)
0.11	3.28	13.14 (0.848)	23.71 (0.458)
0.15	3.00	13.03 (0.608)	23.59 (0.394)
0.18	3.09	16.27 (0.458)	28.27 (0.422)

[a]Sample volume per injection 100 μL; detector sensitivity 0.04 AUF. All other details as in Table 2.

[b]Shown in parentheses are concentrations of proteins in the sample (mg/mL).

double-layer repulsion, while reduced by the rise in the ionic strength, is significant enough (to moderate the adsorption energy well and to cause moderate potential barriers to adsorption); at high salt concentrations, however, it may be reduced to such an extent that no potential barrier for adsorption may exist (e.g., the potential profiles for 0.2 and 0.4 *M* in Fig. 5). In such an event, similar to reverse phase chromatography, adsorption would occur directly into an energy well without impedance from a potential barrier. If the energy well is not too deep, elution can occur without requiring any modification of the mobile phase. If the well is very deep, elution may be achieved by reducing the ionic strength (compare the potential profiles corresponding to 0.4 and 0.2 *M* in Fig. 5).

In the present study, due to a lack of detailed information on the parameters of the interaction potential, it is not possible to identify the salt concentration at which the potential profile changes from one with a potential barrier (for adsorption) to one without. Nevertheless, it is very likely that at the lower salt concentrations (e.g., 0.06 and 0.11 *M* in Fig. 5) the profile with a potential barrier exists.

Insight about the resolution in PBC may be gained by comparing the elution behavior of the proteins injected as single components with that when the proteins are injected as a mixture. Figure 6(A) shows such a comparison of chromatograms [obtained at pH 6 with 0.11 *M* $(NH_4)_2SO_4$ in the mobile phase].

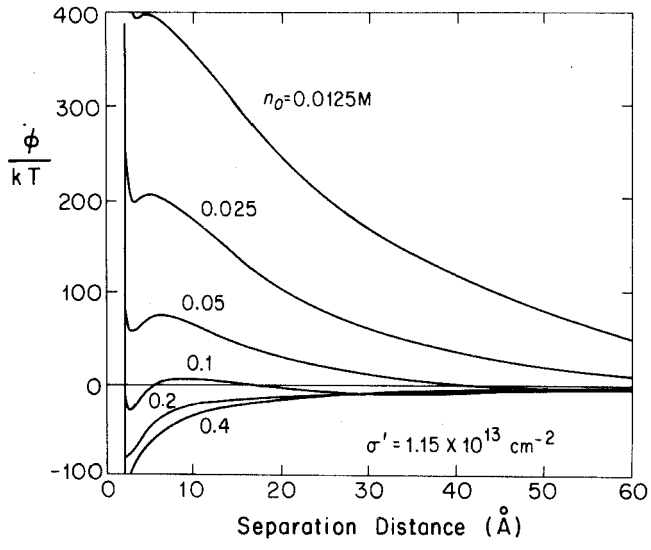

FIG. 5. Theoretical results (*2*) on the influence of ionic strength on the interaction potential at constant surface charge density. $a_P = 0.1$ μm, $\sigma = 5$ Å, $A = 10^{-13}$ ergs, surface charge density of adsorbate or adsorbent, $\sigma' = 1.15 \times 10^{13}$ protonic charges per cm^2 ($\psi_P = \psi_A = 59.26$, 45.66, 34.17, 25.00, 18.02, and 12.87 mV for ionic strength; $n_0 = 0.0125$, 0.025, 0.05, 0.1, 0.2, and 0.4 *M*, respectively).

Both the proteins, when injected as single components, result in peaks which are skewed on the trailing edge (solid lines in Fig. 6A). This indicates that bands having a higher concentration of protein travel faster along the column than the ones of a lower protein concentration (*20*). This in turn leads one to infer (*21*) that if one constructs an adsorption isotherm for this case, it would be nonlinear and have a "negative curvature" as shown schematically in Fig. 6(B). That is, the adsorption of the protein(s) in question is self-impeding.

Nonlinearity of the adsorption isotherm is usually caused by competition between the adsorbing entities (whether from a single component or from a multicomponent mixture) for sites on the adsorbent. Although this is taken into account in the cited theory of PBC (Eq. 4), it is not noticeable for the small absorbate loadings considered in the calculations (see the symmetric Gaussian peaks of Fig. 2) since the effect is significant only at high fractional coverages of the adsorbent.

Another possible cause of nonlinearity is a physicochemical effect that can be significant even at relatively low fractional coverages of the adsorbent.

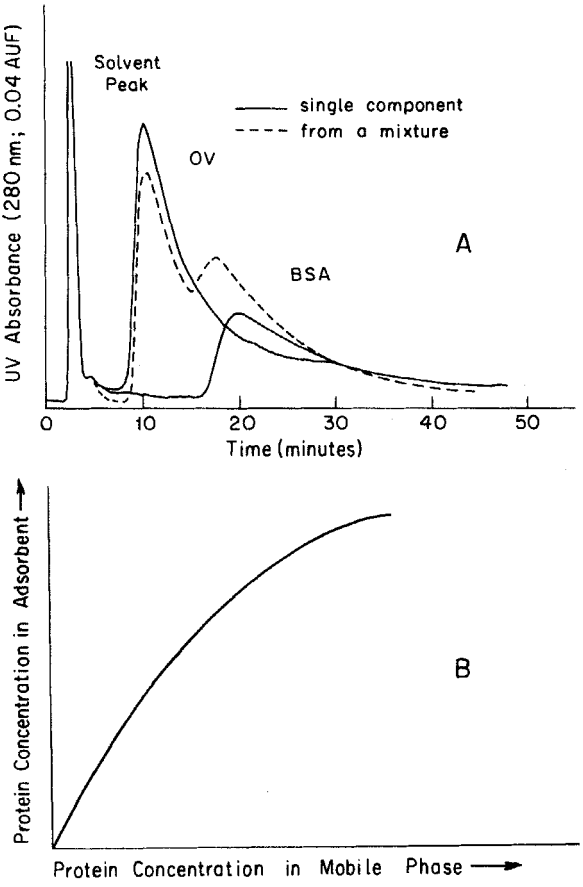

FIG. 6. (A) Interaction of the PBC adsorbent with OV and BSA injected as single components (solid lines) or in a mixture (dotted lines); concentration of $(NH_4)_2SO_4$ in the mobile phase, 0.11 *M*; sample volume per injection 200 μL. All other details are as in Fig. 4. (B) Adsorption isotherm with a negative curvature (schematic).

The interaction potential is extremely sensitive to its parameters and hence would be affected by any heterogeneity of the adsorbent surface. The effect of the heterogeneities of the "clean" adsorbent itself on peak assymetry has been examined in Giddings's monograph (*26*). Nevertheless, adsorption itself introduces a heterogeneity in that a partially protein-covered adsorbent would differ from the initial "clean" adsorbent in characteristics that govern further adsorption. Although such coverage-induced heterogeneity is too complex to be rigorously analyzed, insight may be gained from qualitative

considerations. (See Ref. *22* for such an analysis of cell deposition.) For example, let us suppose that the adsorbates have surface potentials (of the same size as, but) larger in magnitude than the adsorbent surface potential. As mentioned earlier, the double-layer repulsion is greatly affected even by very small changes (e.g., 1%) in its parameters such as the surface potential. By assuming a coverage-weighted surface potential of the adsorbent, it can be seen that, as the number of adsorbed entities increases, the electrostatic double-layer repulsion between the adsorbate in the liquid and the partially (adsorbate-) covered adsorbent would increase leading to larger potential barriers (for adsorption) as well as shallower energy wells. As a corollary, when the number of adsorbed entities decreases (i.e., in the trailing part of the peaks), the double-layer repulsion becomes smaller and renders the energy well deeper. Thus the adsorbates tend to stay longer in the column.

If the rise in the repulsion is significant when the column is loaded with larger amounts of adsorbate(s), a plausible explanation emerges as to why the proteins elute closer from a mixture (dotted lines in Fig. 6A) than as single components (solid lines in Fig. 6A). BSA possibly experiences a larger double-layer repulsion with a partially OV-covered adsorbent than with a "clean" adsorbent and consequently elutes earlier when injected in a mixture with OV than when injected by itself.

This reduction in resolution (when the elution of the mixture is compared with that of the single components) is observed at all the salt concentrations used, as shown in Fig. 7 in which the capacity factors of the proteins (again, alone and from a mixture) are plotted as functions of the salt concentration in the mobile phase.

SUMMARY

Drawing parallels between flocculation–repeptization phenomena at the colloidal level and adsorption–desorption phenomena at the macromolecular level, chromatographic separations for globular proteins have been interpreted using concepts from colloid stability theory (*2, 12*). From such an approach a proposal had emerged (*2, 3*), potential barrier chromatography (PBC), a novel method that would separate globular proteins and colloidal sols by using repulsive double-layer forces to moderate the adsorption energy wells. The present paper reports an attempt to develop, experimentally, a high performance liquid chromatography version of PBC. The theoretical basis of the method is outlined. Data are reported which show that, using an isocratic elution procedure, aqueous mobile phases (nondenaturing to proteins), and a relatively inexpensive ion-exchange column, two model proteins (ovalbumin and bovine serum albumin) have been separated.

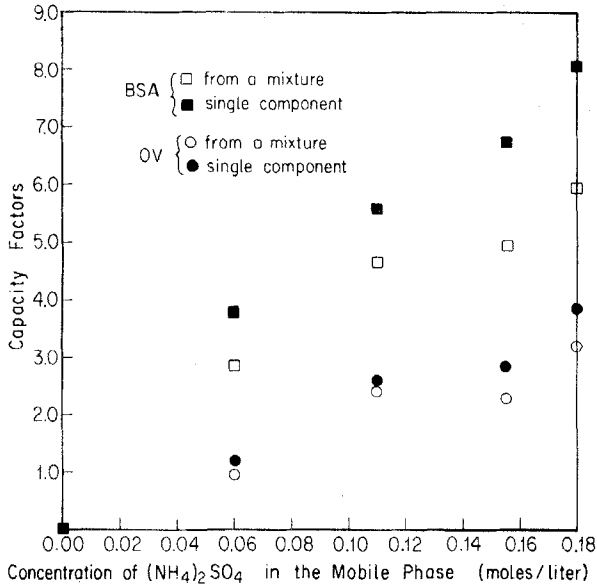

FIG. 7. Capacity factors for OV and BSA in potential barrier chromatography, injected as single components (Filled circles = OV, Filled squares = BSA) and as a mixture (open circles = OV, open squares = BSA). Capacity factor $\equiv (V_{protein} - V_{solvent})/V_{solvent}$, where V refers to the retention time of the peak in question. Sample volume per injection 200 μL. All other details as in Fig. 4.

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