Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Lesins, V. and Ruckenstein, E.(1984) 'Control of the Interaction Potential for Improved Resolution in Potential Barrier Chromatography', Separation Science and Technology, 19: 4, 219 — 240
To link to this Article: DOI: 10.1080/01496398408068580
URL: http://dx.doi.org/10.1080/01496398408068580

SEPARATION SCIENCE AND TECHNOLOGY, **19**(4 & 5), pp. 219-240, 1984

Control of the Interaction Potential for Improved Resolution in Potential Barrier Chromatography

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Abstract

Potential barrier chromatography (PBC) is a recently demonstrated protein separation technique which uses an isocratic elution procedure and exploits the differences in the interaction potentials between the proteins and the adsorbent. The interaction potential is determined by the van der Waals attraction, double-layer, Born, and hydration repulsions between the adsorbent and adsorbates and is very sensitive to the properties of the molecules, such as charge and size. A separation is feasible without any change in the composition of the mobile phase when the interaction potentials have surmountable potential barriers to adsorption and moderately deep adsorption energy wells. To ensure short analysis time and useful resolution, the total interaction potential must be controlled by suitably modifying the van der Waals attraction and the double-layer repulsion. The van der Waals attraction can be controlled by the introduction of small amounts of organic solvents in the aqueous mobile phase. The double-layer repulsion can be modified by changes in pH, ionic strength, or chemical nature of the ions of the mobile phase. Additionally, changes in temperature may be used to improve resolution. Here an updated high performance liquid chromatography version of PBC is reported. Using an isocratic elution procedure and an inexpensive ion-exchange column, the effect of changes in the pH, ionic strength, chemical nature of the ions, and organic solvent content of the mobile phase on the retention times and resolution of two model proteins (ovalbumin and bovine serum albumin) are demonstrated. Improved separations with high resolutions are achieved.

INTRODUCTION

In a recent publication (*1*) from this laboratory the initial development of a high pressure liquid chromatographic method for the separation of proteins

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0149-6395/84/1904-0219$3.50/0

was reported. This method, suggested by Ruckenstein and Prieve (*2*), was termed potential barrier chromatography. These authors recognized that a fine discrimination among similar particles or molecules was possible by properly tuning the operating conditions, such as pH and ionic strength, of a liquid chromatograph so that the potential energy between the adsorbate and the adsorbent possesses a moderate energy barrier to adsorption as well as a moderately deep adsorption energy well. Theoretical considerations indicated that small differences in the physicochemical properties of the adsorbates tremendously affect the depth of the energy well and the height of the potential barrier. For this reason, their chromatographic separation is possible. This contribution reports on the further development of PBC.

Although previous work (*1*) showed satisfactory resolution of the model proteins, a primary objective of the present work is to improve the resolution of the separation while keeping residence times short. This goal was accomplished through the modification of the double-layer and van der Waals interactions between the adsorbent and the adsorbates through changes in the operating conditions. These interactions along with the Born and hydration repulsions constitute the components of the total interaction potential which governs the adsorption–desorption process.

Initially, theoretical aspects of PBC are reviewed. Further details can be found elsewhere (*1, 2*). Subsequently, it is shown that the double-layer repulsion is easily modified through small changes in the ionic strength, pH, or chemical nature of the electrolyte of the mobile phase. Furthermore, it is shown that the van der Waals attraction can be modified by the addition of organic solvents to the mobile phase. Additionally, the effect of temperature on the resolution of the two model proteins is investigated. Finally, conditions for improved resolution are identified.

THEORY

As in any chromatographic separation, the separation process in PBC is based on the fact that the residence time of an adsorbate in a column filled with an adsorbent is directly related to the partition of the adsorbate between the stationary adsorbent and the mobile fluid. However, to achieve a useful separation, three general requirements must be satisfied. First, the different components of a mixture must travel through the column at different rates, thus forming discrete bands which disengage as time proceeds. However, since these bands broaden with time, the second requirement is that they disengage more rapidly than they broaden. Finally, the separation should occur in a relatively short period of time. In PBC these requirements are satisfied through the proper choice of the adsorbent and pH, ionic strength,

chemical nature of the electrolyte, organic additives, and temperature of the mobile phase for the given set of adsorbates. These parameters are important because they affect the interactions between the adsorbates and the adsorbent and hence, govern the adsorption–desorption process.

The interactions mentioned above consist primarily of the van der Waals interaction, the double-layer interaction, and the Born repulsion. The van der Waals interaction is always attractive between identical entities; however, under certain conditions, the van der Waals interaction between two dissimilar particles may become repulsive when separated by a medium (*3*). In PBC the van der Waals interaction is attractive and can be modified by the addition of organic solvents to the aqueous mobile phase.

The double-layer interaction arises from the charged surfaces of the adsorbates and the adsorbent. Associated with each charged surface is a diffuse cloud of counterions which extends into the solution surrounding the surface. Between identical nonzero charge surfaces, the double-layer interaction is necessarily repulsive; for dissimilar surfaces with like charges, it may become attractive in some special circumstances (*4, 5*). In PBC it is important that the double-layer interaction be repulsive which is, in general, a consequence of the adsorbate and adsorbent bearing like charges. Repulsive double-layer interactions are employed to moderate the depth of the adsorption energy well. The double-layer interaction is easily modified by changes in the ionic strength, pH, or chemical nature of the ions of the mobile phase. An increase in ionic strength will decrease the double-layer interaction. Alterations in the double-layer interaction resulting from changes in the pH or chemical nature of the electrolyte are dependent upon the nature of the adsorbent and adsorbates involved.

Other interactions involved include Born repulsion and hydration forces. Born repulsion results from the overlap of electron orbitals. Hydration forces arise from the removal, displacement, or rearrangement of water molecules in an interfacial region. It should be noted that without these short-range repulsive forces, the adsorption energy well would be infinitely deep and desorption would not occur.

These individual interactions can be summed to form the total interaction potential ϕ between the adsorbate and the adsorbent. In general, a plot of the interaction potential ϕ versus h (the minimum distance between the adsorbent, idealized as a flat plate, and the adsorbate, idealized as a sphere) can display a variety of profiles. The profile relevant to PBC is shown in Fig. 1. At short distances a moderately deep adsorption energy well (primary minimum) occurs. At intermediate distances the double-layer repulsion makes the largest contribution to the interaction potential and hence a potential-barrier to adsorption occurs. At larger distances the exponential decay of the double-layer repulsion causes it to fall off more rapidly than the

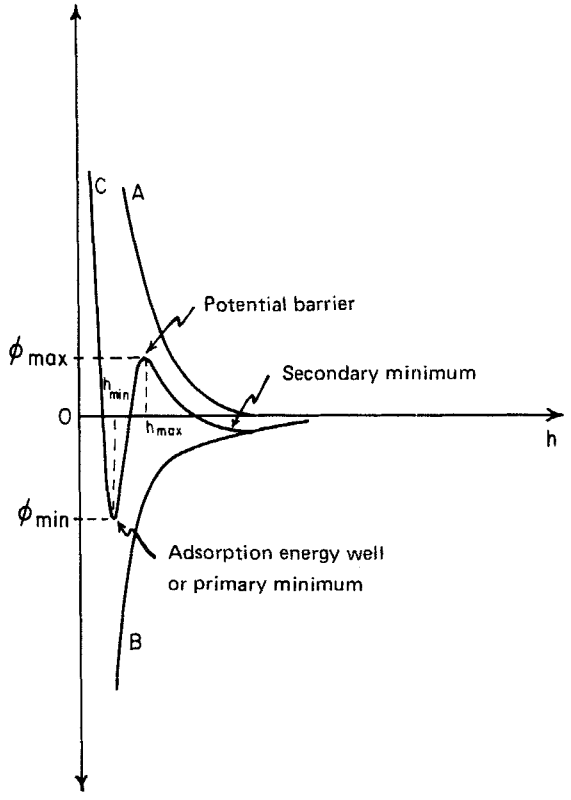


FIG. 1. Schematic potential energy profiles relevant to potential barrier chromatography. Curve A: Double layer interaction. Curve B: Van der Waals interaction. Curve C: Total interaction potential.

power law of the van der Waals attractive term and a second minimum occurs. In obtaining a profile as shown in Fig. 1, two points are especially noteworthy (*2*). First, there exists a narrow range of Hamaker's constant (*6*) (which is a measure of the strength of the van der Waals interaction) and surface potential (which is related to the double-layer interaction) for which a maximum and two minima exist. If the Hamaker constant is too small (large) or the surface potential is too large (small), the maximum and primary (secondary) minimum merge, leaving only one extremum. Second, depending on the ionic strength of the mobile phase, the primary minimum may be above (low ionic strength) or below (moderate ionic strength) the secondary minimum. However, in the former case, even if the potential barrier to

adsorption is surmountable, the amount of adsorbate in the primary minimum will be negligible since such an energy minimum is not energetically favorable. Thus it is seen that the choice of operating conditions must be made with care. General guidelines for modification of the interaction potential are as follows. The depth of the primary minimum is increased by increasing the van der Waals interaction (i.e., larger Hamaker constant) and/or reducing the double-layer interaction (i.e., decreased surface charge, higher ionic strength). On the other hand, the height of the potential barrier is raised by enhancing the double-layer interaction and/or reducing the van der Waals interaction.

Using an intuitive analysis (*7, 8*), the average retention time τ of an adsorbate, when adsorption-desorption equilibrium can be assumed between the bulk solution and the adsorbent, is given by

$$\tau = \{\varepsilon_p + S(2\pi kT/\delta_{\min})^{1/2} \exp(-\phi_{\min}/kT)\}v/Q \tag{1}$$

Obviously, useful resolution also requires that the peaks are not too broad. The variance, σ_L^2, which is a measure of peak broadening, is given by:

$$\sigma_L^2 = 2\left\{\frac{1}{1+\dfrac{\varepsilon_p}{KS}}\right\}^2\left\{\frac{QL^2}{Sv}\right\}\left\{\frac{a_p}{D_\infty h_{\max}}\right\}\left\{\frac{2\pi kT}{\delta_{\max}}\right\}^{1/2}\exp\frac{\phi_{\max}}{kT} \tag{2}$$

In the above equations ε_p is the void volume, S is the surface area per unit volume of adsorbent, k is the Boltzmann constant, T is the absolute temperature, v is the total column volume, Q is the volumetric flow rate of the mobile phase, a_p is the radius of the adsorbate, D_∞ is the bulk diffusion coefficient of the adsorbate,

$$\delta_{\max} = -d^2\phi/dh^2\,|_{h=h_{\max}} \qquad \text{and} \qquad \delta_{\min} = +d^2\phi/dh^2\,|_{h=h_{\min}}$$

The definitions of $h_{\max}$, $h_{\min}$, $\phi_{\max}$, and $\phi_{\min}$ are given in Fig. 1, and K, the equilibrium constant, is given by

$$K = \left(\frac{2\pi kT}{\delta_{\min}}\right)^{1/2}\exp\left(-\frac{\phi_{\min}}{kT}\right) \tag{3}$$

Note that the only parameters involved in the interaction potential that determine the retention time of the adsorbate are the depth ($\phi_{\min}$) and the shape ($\delta_{\min}$) of the adsorption energy well. On the other hand, peak broadening is also determined by the height ($\phi_{\max}$), shape ($\delta_{\max}$), and position

(h_{max}) of the potential barrier. Thus an unavoidable consequence of using double-layer repulsion to raise the adsorption energy well (to facilitate desorption) is the accompanying effect of peak broadening. In addition to peak broadening, the introduction of a potential barrier imposes some restrictions which are more easily understood on the basis of the following intuitive analysis. In most chromatographic procedures, a large number (10^3 to 10^4) of adsorptions and desorptions take place. In such cases the chromatographic process can be treated by equilibrium considerations (*7*). In PBC the characteristic time for adsorption τ_{a_i} for adsorbate i is given by

$$\tau_{a_i} = \frac{\varepsilon_p a_p}{h_{max} D_\infty} \left(\frac{2\pi kT}{\delta_{max}} \right)^{1/2} \exp \left(+ \frac{\phi_{max}}{kT} \right) \tag{4}$$

If the potential barrier (ϕ_{max}) is too high, equilibrium considerations are in question since the adsorption–desorption process will be too slow. In fact, if the potential barrier is excessively high, which is a consequence of low ionic strength and high surface charge, adsorption may not occur since the characteristic time for adsorption may be greater than the average residence time of the mobile phase or an unretained tracer. In this case the possibility exists for the adsorbate to elute ahead of the unretained tracer, since the adsorbate would be excluded from sampling the slower mobile phase velocities near the adsorbents surface due to the strong double-layer repulsion. However, for the case of a moderate potential barrier to adsorption and a moderately deep adsorption energy well, the adsorbates will make many exchanges between the adsorbed and desorbed state and hence, the relative number of molecules in the two states can be approximated by equilibrium considerations.

As previously stated, chromatographic zones must disengage more rapidly than broaden to obtain useful resolution. The resolution, R_S, can be defined as

$$R_S = \Delta Z_{ij} / (2(\sigma_i + \sigma_j)) \tag{5}$$

where ΔZ_{ij} is the distance between adjacent peak centers i and j, and σ_i and σ_j are the standard deviations of peaks i and j, respectively. In terms of the interaction potential, the resolution can be written as

$$R_S = \left(\frac{Sv}{8Q} \right)^{1/2} \frac{1}{\varepsilon_p} \frac{(1 - (K_i/K_j))(K_i S + \varepsilon_p)(K_j S + \varepsilon_p)}{(k_{a_i}^{-1/2} + k_{a_j}^{-1/2})(K_i S + \beta \varepsilon_p)} \tag{6}$$

where

$$\beta = \frac{\frac{K_i}{K_j} k_{a_i}^{-1/2} + k_{a_j}^{-1/2}}{k_{a_i}^{-1/2} + k_{a_j}^{-1/2}} \tag{7}$$

and

$$k_{a_n} = \varepsilon_p / S\tau_{a_n} \tag{8}$$

As expected, resolution cannot be obtained if $K_i = K_j$. For closely eluting peaks, $K_i \simeq K_j$ and resolution can be obtained by controlling peak broadening by decreasing the height of the potential barrier or perhaps through its elimination. In the latter case, although the term "potential barrier chromatography" may be a misnomer, the interactions which govern the separation are unchanged. Thus it is seen that the reduction of the potential barrier offers an alternate means of obtaining resolution for a given set of adsorbates as opposed to radically modifying the operating conditions to generate greater differences in the depth of the adsorption energy wells. Finally, if K_i and K_j differ significantly, peak broadening may be of secondary importance and the depth of the adsorption energy well must be raised in order to decrease analysis time.

To summarize, the essence of PBC is to identify operating conditions such that the interaction potentials of the individual adsorbates differ enough to allow for a separation. These differences in the total interaction potentials are accomplished through modifications of the component interactions, namely, the van der Waals attraction and the double-layer repulsion. However, the use of double-layer repulsion to raise the adsorption energy well to moderate levels, in general, leads to a potential barrier to adsorption. This barrier must be moderate for adsorption to occur and for the resolution of the adsorbates to be satisfactory.

EXPERIMENT

The experimental apparatus consisted of a solvent delivery system (Waters 6000A), a nonstop flow septumless injector (Waters U6K), a DuPont ZIPAX SCX column (2.1 mm i.d. × 100 cm), and a variable wavelength absorbance detector (Waters 450). The detector was interfaced with a strip chart recorder (Houston Instrument Omniscribe) and a peak timer and area integrator (Varian CDS 111). A digital pH meter (Orion

Research 601A) with a standard pH electrode (Orion 91-04) was used for mobile phase titrations. The pH meter was calibrated with certified buffer solutions obtained from Fisher Scientific Co. Experiments were performed at 25°C unless otherwise noted.

Mobile phases were prepared fresh daily using demineralized distilled water which was sonicated for 20 min prior to use. Mobile phases were allowed to equilibrate with the column for a minimum of 2 h before 100 μL injections of the proteins were introduced. The effluent was monitored at 280 nm.

The proteins were obtained from Sigma Chemical Co. and the buffer components and sulfate salts were obtained from Fisher Scientific Co. and were of analytical grade. Proteins, buffer components, and sulfate salts were used without further purification. Stock solutions of the buffer were refrigerated and used within 2 weeks.

The column showed a slight change in activity over the entire course of the experimental investigation. However, when comparison of results are made within the text, they were from experiments carried out over a time period in which the change in activity was negligible.

EXPERIMENTAL RESULTS AND DISCUSSION

The Effect of pH

Retention time data for bovine serum albumin (BSA) and ovalbumin (OV) injected as pure components at various values of pH and various ionic strength of K_2SO_4 are summarized in Table 1. However, before examining the experimental results, it is instructive to examine a typical protein titration curve as shown in Fig. 2. Proteins acquire a surface charge through the dissociation of numerous acidic and basic groups which include carboxyl, amino, imidazole, phenol, sulfhydryl, and guanidyl. The pH value for which the net charge on the protein molecule is zero is referred to as the pI. For pH values below the pI, proteins carry a net positive charge whereas for pH values above the pI, proteins carry a net negative charge. Thus for the proteins used in this study, OV (pI = 4.6) and BSA (pI = 4.8), the net surface charge was negative since operating conditions were always above the pI of both proteins. Similarly, the adsorbent used in this study, which consists of sulfonic acid residues in a polymer matrix, also has a negative surface charge. Since both adsorbate and adsorbent posses a surface charge of the same sign, it is anticipated that double-layer repulsion will occur. Additionally, by examination of Fig. 2, as the pH of the mobile phase is

TABLE 1
Experimental Results from Potential Barrier Chromatography[a]

Concentration of K_2SO_4 (mol/L)	pH	Retention times (in minutes) injected as pure components	
		OV	BSA
0.10	6.50	4.1	5.5
	6.25	4.7	7.5
	6.00	5.3	9.8
	5.75	6.0	13.3
	5.50	6.6	16.9
0.08	6.50	3.7	4.7
	6.25	4.1	6.0
	6.00	5.0	9.2
	5.75	5.9	12.6
	5.50	6.4	15.9
0.06	6.50	3.5	4.0
	6.25	3.9	5.2
	6.00	4.1	7.1
	5.75	5.3	10.8
	5.50	6.2	15.2
0.04	6.50	3.2	3.6
	6.25	3.4	4.2
	6.00	3.7	5.3
	5.75	4.2	7.4
	5.50	4.9	11.1

[a] Column: DuPont ZIPAX SCX (2.1 mm i.d. × 100 cm). Mobile phase: 0.01 *M* citrate buffer containing 0.003 *M* NaN_3. Flow rate: 0.5 mL/min. Sample volume per injection: 100 μL. Individual protein concentrations: 0.40 mg/mL. Detector sensitivity: 0.04 AUFS. Retention time is reported at maximum peak height.

progressively raised (at constant ionic strength) from values near the pI, the charge of the proteins increases. In other words, as the pH of the mobile phase is raised, the double-layer interaction imparts a greater influence on the total interaction potential (due to an increased surface charge on the proteins) until the double-layer interaction is dominant. In terms of the total interaction profile, as the pH of the mobile phase is raised, the adsorption energy well is continually raised, thereby decreasing the retention time of the adsorbates (see Eq. 1). This is indeed the experimentally observed behavior shown in Table 1. As the pH of the mobile phase is raised at constant ionic strength, the retention time of the adsorbates decreases, indicating that double-layer repulsion is indeed occurring. It is also noteworthy to point out

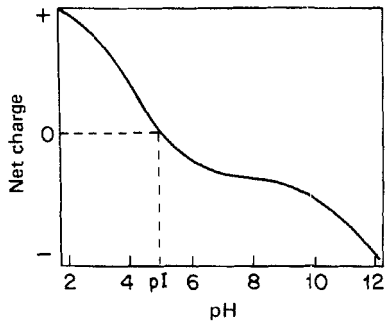


FIG. 2. Schematic of a hydrogen ion titration curve of a protein.

that the changes in the retention time are exclusively due to the changes in the net charge of the proteins, since any change in the dissociation of the strongly acidic sulfonic acid groups of the adsorbent will be negligible in the range of pH studied. Representative chromatograms illustrating the effect of pH on the separation of OV and BSA are shown in Fig. 3. These results are typical of other ionic strengths in that an optimum pH for resolution occurs at each ionic strength investigated. Note the different degrees of separation indicating the sensitivity of PBC to changes in pH. Thus for amphoteric molecules (such as proteins), it is seen that double-layer repulsion and thereby the total interaction potential are easily modified through changes in pH. Finally, it should be mentioned that in addition to raising the adsorption energy well, the potential barrier to adsorption is increased by increasing the surface charge of the proteins. However, only if the characteristic time for adsorption remains small in comparison to the residence time of an unretained tracer, retention will occur.

Specific Ion Effects

Although the double-layer interaction can be effectively controlled through changes in pH, it is also conveniently controlled by varying the ionic strength or chemical nature of the electrolyte of the mobile phase. Retention time data for BSA and OV injected as pure components at pH 6.00 and various ionic strengths of several sulfate salts are summarized in Fig. 4. Note that the general trend is an increase in the retention time of the adsorbates as the ionic strength of the mobile phase is raised. This result is intuitively expected if double-layer repulsion is occurring. As the ionic strength of the mobile phase is raised, the surface charge of the adsorbent as well as that of the adsorbate

becomes increasingly screened and their effect on counterions extends less and less into the bulk solution. This allows the adsorbate to approach the adsorbent to increasingly shorter distances without experiencing significant double-layer repulsion. In terms of the total interaction potential, as the ionic strength of the mobile phase is raised, the double-layer contribution decays more rapidly and hence the potential barrier to adsorption is decreased whereas the depth of the energy well is increased. This results in an increase in retention time. Note that in contrast to ion-exchange chromatography, elution in PBC is aided by decreasing the ionic strength of the mobile phase and/or increasing the surface charge of the protein.

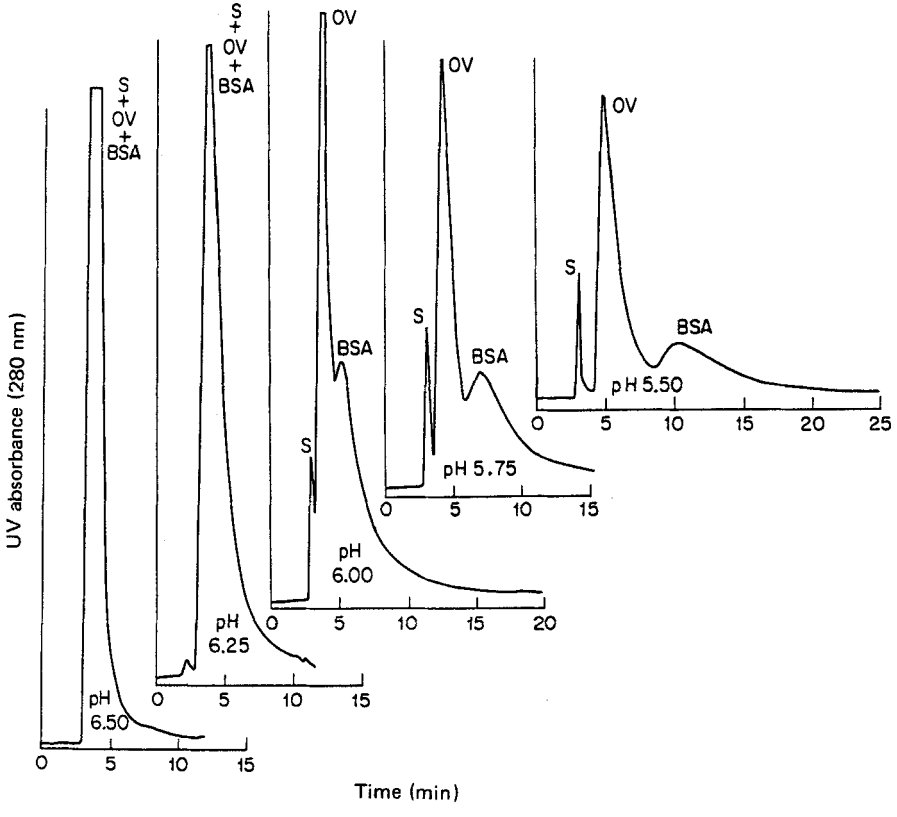


FIG. 3. Separation of ovalbumin (OV) and bovin serum albumin (BSA) by potential barrier chromatography as a function of pH (given in figure) and 0.04 M K_2SO_4. S denotes the solvent peak. All other details as in Table 1.

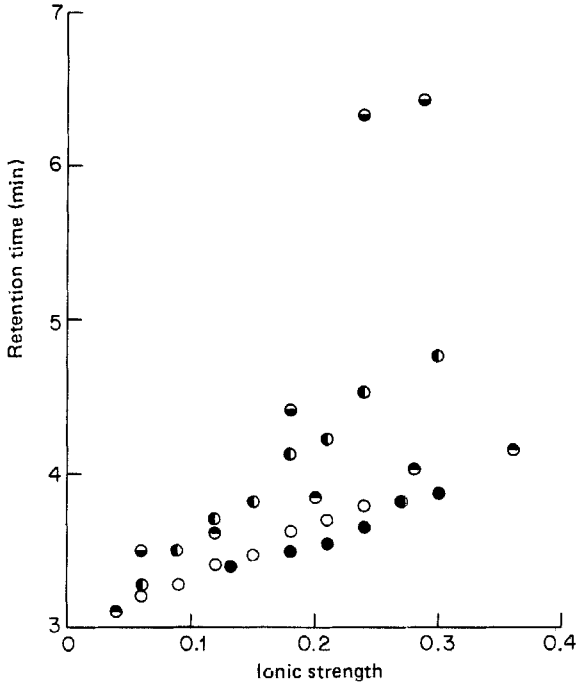


FIG. 4a. Retention times of ovalbumin (OV) at pH = 6.00 and various ionic strengths of several sulfate salts (●, Li_2SO_4; ○, Na_2SO_4; ◐ $(NH_4)_3SO_4$; ◒, K_2SO_4; ◓, $MgSO_4$). All other details as in Table 1.

In addition to screening the surface charge, counterions can bind to the charged surface of the adsorbent and the adsorbate in the Stern layer (*9*), thereby reducing their effective surface charge and hence reducing the double-layer interaction. In fact, as the electrolyte concentration increases, increasing amounts of potential drop occur in the Stern layer, allowing for a closer distance of approach between the adsorbate and adsorbent before significant double-layer interactions occur. However, not all ions bind to the charged surface to the same degree. Since the sulfonated fluorocarbon polymer-coated packing used in the present study is largely hydrophobic, specific adsorption in the Stern layer is expected to be enhanced by larger size and thus larger polarizability, and by lower hydration, which itself is a function of ion size. Therefore the selectivity of this packing, based on the size of the ion, is expected to occur in the order

$$NH_4^+ > K^+ > Na^+ > Li^+ \qquad (9)$$

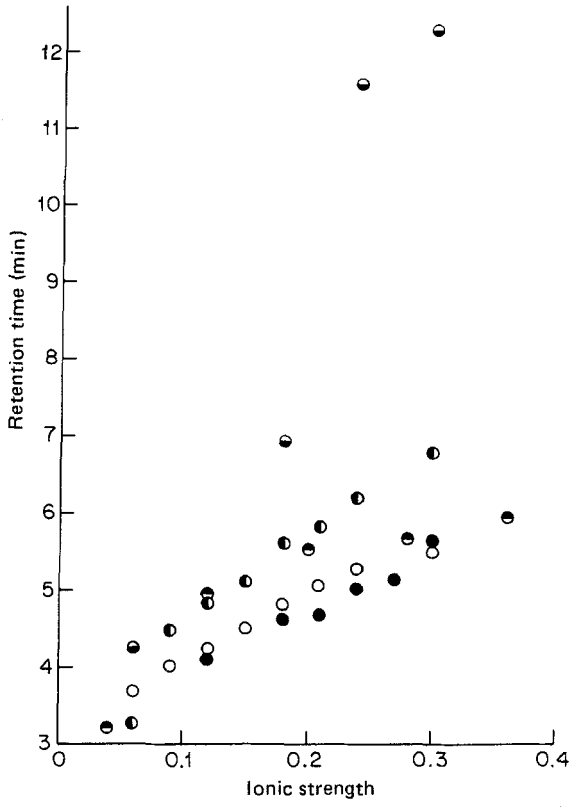


FIG. 4b. Retention times of bovine serum albumin (BSA). All other details as in Fig. 4a.

It should be noted, however, that experimental evidence on the adsorption of various ions on hydrophobic surfaces is not always consistent (*10*).

The selectivity of the packing used in the present study for various ions is not available in the literature. However, other manufacturers (*11*) offer strongly acidic cation exchangers, composed of sulfonic acid functional groups attached to a styrene divinylbenzene copolymer lattice, which are similar to the present packing in that both contain sulfonic acid groups attached to a hydrophobic polymer lattice. The selectivity for adsorption is reported (*11*) to be in the order

$$K^+ > NH_4^+ > Na^+ > Li^+ \qquad (10)$$

Although the alkali metals follow the same order, the ammonium ion shows anomolous behavior with regard to the selectivity of adsorption expected from the size of the ion. However, it seems likely that this behavior of the ammonium ion, with its four hydrogen atoms, is due to its unique hydrogen bonding ability with water. Our data (Fig. 4) show that the retention times of both adsorbates, at the same ionic strengths of various electrolytes, follow the latter inequality, indicating that adsorption in the Stern layer also follows the latter selectivity. Finally note that differences in retention times are accentuated at the higher ionic strengths because the amount of ions adsorbed is higher and because the shielding of the electric field is greater.

It should be noted that the use of specific ion effects can be a useful tool in PBC. For example, if a given ion is known to bind selectively to a certain component of a complex mixture, the retention time characteristics of that component can be exaggerated through the use of that specific ion rather than some nonspecific ion. Although the specific ion effects reported here are not large, they certainly do exist. The effect of various ions on the separation of OV and BSA is illustrated in Fig. 5. Regarding retention time and resolution, specific ion effects are more pronounced at higher ionic strength and less dramatic at lower ionic strengths than those shown in Fig. 5.

The Use of Organic Solvents

In order to use PBC to its maximum potential, it is necessary to control the van der Waals interaction. This is accomplished through the introduction of organic solvents. (For reviews on how organic solvents affect the van der Waals interaction, see Ref. *12*.) In general, the addition of organic solvents to an aqueous mobile phase decreases the van der Waals interaction between the adsorbates and a hydrophobic adsorbent and leads to a decrease in the retention time of the adsorbates. This result is easily explained in terms of a hydrophobic effect. Although globular proteins are hydrophilic, a large part of the nonpolar residues of the constituent amino acids in proteins are exposed to the water interface as opposed to the expected preferential location of these hydrophobic residues in the interior of the molecule (*13*). These nonpolar residues on the protein surface form patches of distinct hydrophobic character. Thus, the introduction of small amounts of an organic solvent to an aqueous mobile phase can create a more favorable atmosphere for the proteins. Since the organically modified mobile phase has become more favorable, the fraction of time the protein resides in the mobile phase before one adsorption occurs will increase. Additionally, the fraction of time the protein stays adsorbed on grains of the packing will decrease.

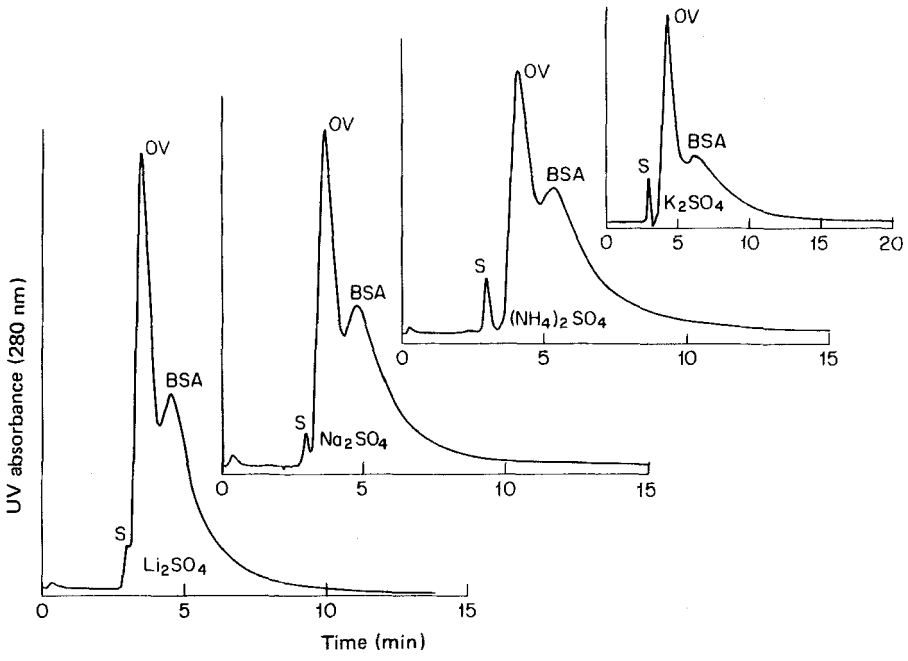


FIG. 5. Separation of ovalbumin (OV) and bovin serum albumin (BSA) by potential barrier chromatography at pH = 6.00 and 0.06 *M* concentrations of several salts (given in the figure). AUFS 0.1. All other details as in Table 1.

Thus a decrease in the retention times of the proteins is expected. In this context it is interesting to note that OV and BSA elute in the order of hydrophobicity (BSA > OV) (*14, 15*). Since BSA is more hydrophobic than OV, it will spend a greater fraction of time in the adsorbed state before desorption and a smaller fraction of time in the mobile phase before adsorption as compared to OV. This results in a larger retention time for BSA. However, it should be noted that elution will not always occur in increasing order for hydrophobicity. Hydrophobicity only yields qualitative insight to the magnitude of the van der Waals interaction; it does not yield information regarding double-layer interactions, which may alter the order of elution. However, for the proteins under consideration, since their surface charges (*16, 17*) and, hence, their double-layer interaction are similar, hydrophobicity does in fact predict the order of elution.

Although it is clear that the addition of organic solvents will modify the van der Waals interaction, modification of the double-layer interaction may also occur. In general, such additives lower the dielectric constant of the

TABLE 2
Experimental Results from Potential Barrier Chromatography[a]

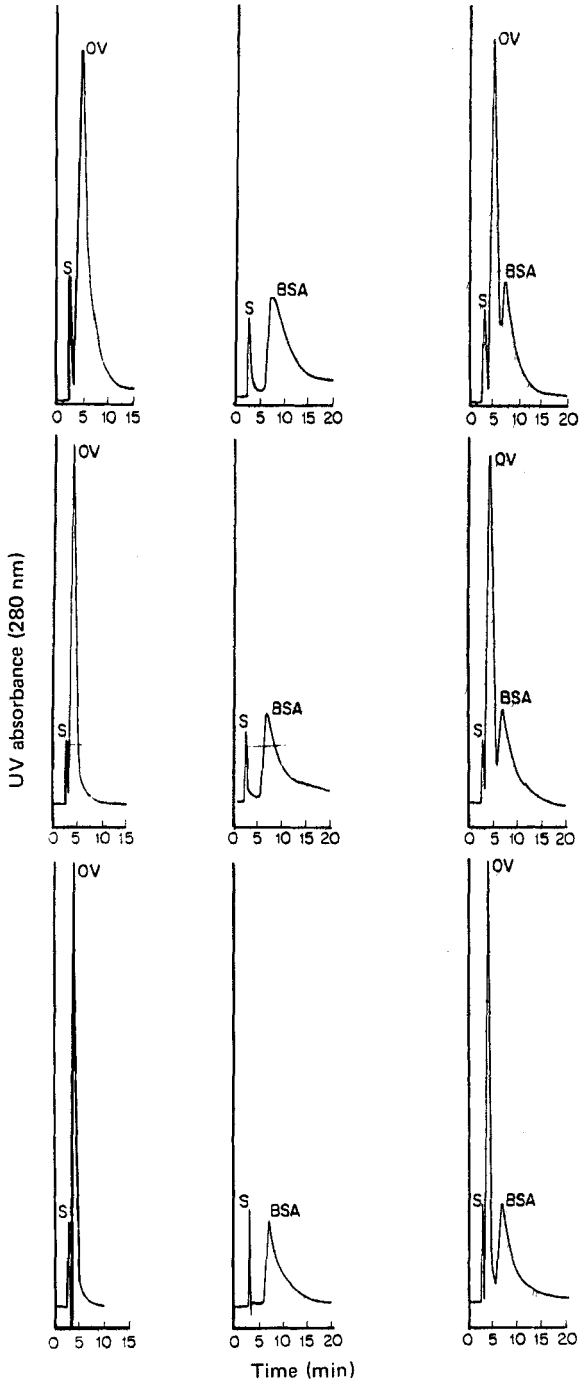
Mobile phase conditions: Concentration of K_2SO_4 (mol/L)	pH	Retention times (in minutes) injected as pure components			
		1 mL CH_3CN/250 mL		10 mL CH_3CN/250 mL	
		OV	BSA	OV	BSA
0.01	6.50	3.0	3.1	3.0	3.1
	6.00	3.1	3.5	3.1	3.6
0.06	6.50	3.5 (3.5)	4.3 (4.0)	3.3 (3.5)	4.3 (4.0)
	6.00	4.1 (4.1)	7.0 (7.1)	3.8 (4.1)	7.1 (7.1)
0.10	6.50	3.9 (4.1)	5.3 (5.5)	3.9 (4.1)	5.5 (5.5)
	6.00	5.0 (5.3)	9.5 (9.8)	4.5 (5.3)	9.8 (9.8)

[a]Shown in parentheses are results for acetonitrile-free conditions. All other details as in Table 1.

mobile phase; thus double-layer interactions may be enhanced through the use of such additives. However, with the addition of such agents, the degree to which surfaces are charged may be reduced since a reduction in the dielectric constant of the mobile phase will decrease the dissociation of weak acidic groups as well as causing pairing between counterions and surface charges. However, it is likely that the above enhancement and the latter attenuation of the double-layer interaction effectively cancel one another at low volume fractions of organic additives. Thus, any variation in the total interaction potential at low volume fractions of the organic solvent is likely to be due to the alteration of the van der Waals interaction.

Retention time data for OV and BSA, injected as pure components, for two volume fractions of acetonitrile (CH_3CN) at various ionic strengths and pH values of 6.50 and 6.00 are summarized in Table 2. Note that the retention times are, in general, less than those for the corresponding acetonitrile-free mobile phases. The introduction of acetonitrile also decreases peak broadening as illustrated for a typical case in Fig. 6, leading to improved resolution.

FIG. 6. Typical chromatograms illustrating the decrease in peak broadening and improved resolution obtained through the introduction of acetonitrile at pH = 6.00 and 0.06 M K_2SO_4. Top row: 0 mL CH_3CN/250 mL. Middle row: 1 mL CH_3CN/250 mL. Bottom row: 10 mL CH_3CN/250 mL. Left column: Ovalbumin (OV) as a single component. Center column: Bovine serum albumin (BSA) as a single component. Right column: Mixture of OV and BSA. All other details as in Table 1.



At these small volume fractions of acetonitrile, any alteration in the double-layer interaction is likely to be negligible and, therefore, the observed behavior is due to a decrease in the van der Waals interaction. The decrease in retention time is easily explained with reference to Eq. (1). As the van der Waals interaction decreases, the adsorption energy well is raised, leading to a decrease in retention time. The decrease in peak broadening can also be explained in terms of the decrease in the van der Waals interaction. With reference to Eq. (2), note that the first term in brackets on the right-hand side contains the equilibrium constant K, which is exponentially dependent upon ϕ_{min}. Thus a decrease in the depth of ϕ_{min}, which is reflected by a decrease in retention time, leads to a reduction in peak broadening. Although the reduction of the van der Waals interaction also increases the potential barrier, the effect is probably less important than the change in the depth of the primary minimum because the potential barrier is probably small under these operating conditions.

Referring to Table 2, note that pH-induced changes in the retention times of the proteins are nearly the same for both acetonitrile-free and acetonitrile-modified mobile phases. This implies that changes in the retention times of the proteins with small volume fractions of acetonitrile are primarily due to changes in the van der Waals interaction and not in the double-layer interactions.

The Origin of Tailing

In a previous publication (*1*) it was reported that tailing in the chromatograms was possibly due to a nonlinear adsorption isotherm and/or a physicochemical effect due to the heterogeneity of the adsorbent surface due to the adsorbates themselves. Recent experiments have shown that tailing exists, and remains essentially unchanged, with protein loadings as small as 10 μg. Thus it seems plausible that some other mechanism for tailing is involved. It is also important to note that tailing is not an artifact of PBC. Tailing of BSA has been observed on hydroxylapatite (*18*), diethylaminoethyl cellulose (*19*), and DEAE-Sephadex (*20*). The tailing of OV has been observed on hydroxylapatite (*18*). In these cases, tailing has been attributed to the heterogeneity of the proteins themselves. It has been reported (*19*) that most BSA samples are composed of at least 5 or 6 components. Similarly, OV has been shown to consist of 3 components (*20, 21*). Furthermore, results have shown that the components of BSA present isoelectric heterogeneity with the two major components differing by 1 unit charge (*22*). Similarly, OV exhibits charge heterogeneity as indicated by its electrophoretic behavior (*21*). Although these heterogeneities may be small, they

will lead to minor changes in the total interaction potentials of the individual components of the protein samples and, hence, minor differences in retention time and peak variance. These minor differences in the total interaction potential can manifest in the form of tailing when the individual components of the protein elute very close to one another but still form a single peak.

The Effect of Temperature

The last easily adjustable experimental parameter is temperature. Examination of Eqs. (1) and (2) show that both retention time and peak variance

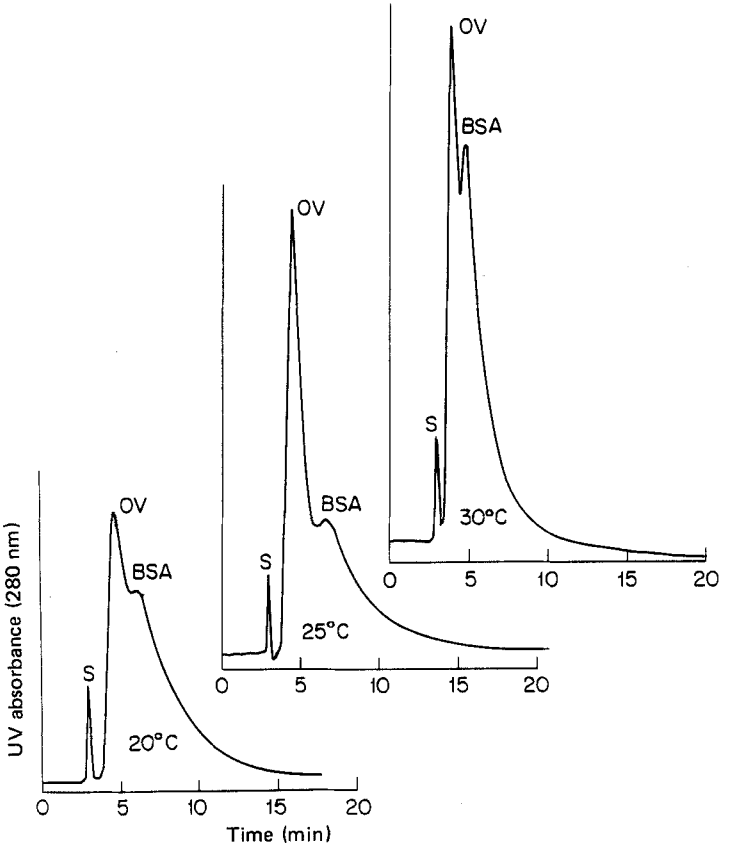


FIG. 7. Chromatograms illustrating the effect of temperature (shown in figure) on the separation of ovalbumin (OV) and bovine serum albumin (BSA) at pH = 6.00 and 0.06 M K_2SO_4. All other details as in Table 1.

have an Arrhenius-type temperature dependency. However, the physicochemical properties of the system (e.g., dielectric constant, surface tension, dissociation constants, etc.), which determine the total interaction potential, and hence ϕ_{min} and ϕ_{max}, are themselves functions of temperature. Thus the effect of temperature on the total interaction potential and, consequently, on the retention time and peak variance is not easily predictable. The results of a typical temperature study are shown in Fig. 7. Although a change in temperature does not in itself achieve separation, changing the temperature of the system may be employed to optimize a separation. Thus, by increasing the organic content of the mobile phase in addition to increasing the temperature, separation with resolution near 1 is achieved as shown in Fig. 8. It should be noted, however, that a modification of the double-layer

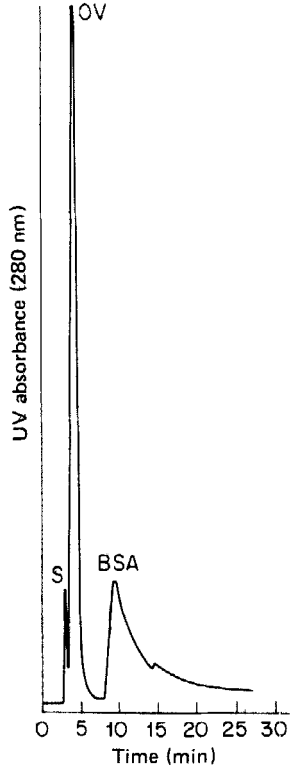


FIG. 8. Improved separation of ovalbumin (OV) and bovine serum albumin (BSA) through modification of the interaction potential at pH = 6.00 and 0.1 M K_2SO_4. 20 mL CH_3CN/250 mL. T = 30°C. All other details as in Table 1.

interaction, due to the higher volume fraction of acetonitrile, in addition to the modification of the van der Waals interaction already discussed, may have also occurred.

SUMMARY

By properly tuning the operating conditions of a high pressure liquid chromatograph, the separation of two model proteins with improved resolution has been achieved. General procedures for controlling the interaction potential have been discussed to yield basic operating guidelines. It has been shown that the double-layer repulsion is conveniently modified via changes in pH, ionic strength, or chemical nature of the ions of the mobile phase. Furthermore, by the addition of an organic solvent to the mobile phase, the van der Waals interaction is also easily modified. Additionally, through changes in temperature, improved resolution may also be achieved. Thus it is seen that interactions which comprise the total interaction potential, and govern the adsorption-desorption process, are easily modified to allow a chromatographic separation of high resolution in a relatively short time.

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Received by editor July 7, 1983