Journal of Chromatography, 443 (1988) 13–29
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1342

PREPARATIVE CHROMATOGRAPHY OF PROTEINS

ANALYSIS OF THE MULTIVALENT ION-EXCHANGE FORMALISM

AJOY VELAYUDHAN and CSABA HORVÁTH*
Department of Chemical Engineering, Yale University, P.O. Box 2159, New Haven, CT 06520 (U.S.A.)

SUMMARY

Multivalent ion exchange is proposed as a generally applicable formalism to describe the non-linear chromatographic adsorption of biopolymers. Single- and multi-component isotherms are calculated that explicitly account for the influence of the mobile phase modulator concentration. Three regions of binding (strong, intermediate, and weak) are distinguished on the basis of strength of interaction, and the potential advantages of operating in the strong region for preparative purposes are pointed out. The inapplicability of the Langmuir isotherm in this region is demonstrated, and separation schemes that take advantage of the characteristic features of biopolymeric adsorption are described. The rectangular single-component ion-exchange isotherms are shown to reduce in the presence of competition to reversible concave-down forms.

INTRODUCTION

The chromatographic separation of proteins is an area of great current interest. As pointed out in the literature[1], the primary purposes for carrying out a chromatographic experiment are either to extract information or to collect amounts of pure sample. Each purpose is best answered by a different method: the widely used technique of linear elution chromatography serves admirably to extract information, while non-linear techniques such as frontal and displacement (and some novel tandem schemes to be discussed later in this paper) are better suited to the preparative goal. Some confusion exists as to whether preparative techniques can be deemed to fall under the purview of chromatography. The governing equations underlying these different techniques are in fact identical, linear elution being essentially a degenerate case where each component is unaffected by the others. The difference between the various preparative modes is reflected only in different initial and boundary conditions. Further, the chemical interactions involved are identical and the same equipment can be used to carry out the various modes of separation. Hence, there is every justification, logical as well as operational, to consider all these techniques bona fide forms of chromatography.

In this paper, we will consider only the non-linear chromatography of proteins. On binding to surfaces, these proteins exhibit certain characteristic features. These experimentally well-documented features include the "irreversible" binding (that is,

0021-9673/88/$03.50

irreversible on the time scale of chromatographic interest) that many proteins show, for instance, on ion-exchange columns when the salt concentration in the mobile phase is low[2,3]. Again, it is frequently observed that such irreversibly bound proteins will be eluted from the column with practically no retention on changing the salt concentration somewhat, in agreement with the "all-or-nothing" principle enunciated by Tiselius, and Morris and Morris[4,5]. This sensitive dependence on the concentration of what we shall term mobile phase modulator (MPM) (which subsumes salt in electrostatic and hydrophobic interaction chromatography[6,7], and organic modifier in reversed-phase chromatography[8]) is characteristic of proteins. As we shall show, the Langmuirian formalism frequently used to describe adsorption behavior over a wide concentration range does not exhibit this property.

We are therefore led to examine other possible models for adsorption that will predict the two fundamental experimental features of protein chromatography described above: irreversible binding at low MPM concentrations and sensitive dependence of retention on MPM concentration in the mobile phase. We shall in what follows call these the characteristic features of biopolymer chromatography.

In order to motivate the formulation of such a model, we consider, as a physical analogy to the binding of a biopolymer to a surface, the Velcro fastener. We assume that a piece of Velcro consists of many identical hooks and eyes, each hook-and-eye pair having a strength of adhesion which could be quite small, and that the Velcro binds through the concerted but independent action of very many such pairs. While it is easy to separate a single hook and eye, the effort required to separate a small piece of Velcro consisting of, say, several hundred pairs is quite noticeable, even though the various hook-and-eye pairs are acting independently. The analogy with biopolymer binding to the stationary phase is quite fruitful: each functional group that binds individually to the surface of the chromatographic stationary phase represents a hook, having a resistance to separation (corresponding to its chromatographic retention, or Gibbs free energy change on binding) which may be quite small. However, the observed free energy change for the binding of the protein will be the sum of the free energy changes for all the individual interacting groups. Since the retention factor is exponentially related to the free energy change on binding, the retention factor for the protein is equal to the product of the individual retention factors for all the interacting groups, and therefore increases geometrically with the number of its groups that bind to the chromatographic surface. This model assumes no synergistic effects in the binding process: the possibility that the binding of one functional group facilitates the binding of others is not incorporated. Size effects are similarly not treated: it is clear that the binding of a very large protein will result in the shielding of some sites on the stationary phase that the protein is not directly interacting with, simply by virtue of its molecular dimensions. Since this screening effect is obviously a function of protein size and shape, the present formalism is restricted to proteins of roughly similar shapes and sizes. Further, a rigorous treatment of irreversible binding would seem to require a kinetic analysis, in terms of reaction rate constants, rather than a thermodynamic one, in terms of equilibrium constants; only a thermodynamic approach is presented here. In such an equilibrium approach, irreversible binding is reflected by a very strong affinity for the stationary phase. We shall show that even this simple model predicts the experimental features mentioned.

Now consider the MPM as playing a role analogous to that of heat applied to the Velcro. The resistance to parting the hooks and eyes is drastically reduced, since the bond between each hook and eye is somewhat weakened. In the same fashion, a small change in MPM concentration attenuates somewhat the free energy of binding of each residue and the same geometric dependence described earlier causes the retention of the protein to be dramatically affected. Fig. 1 illustrates this sensitive dependence.

The characteristic features of protein adsorption are thus seen to rest on the single requirement of multipoint binding; the multipointed nature of protein adsorption has been shown experimentally by Jennissen[9], among others. Hence, any convenient formalism incorporating multipoint binding should yield results in agreement with experiments, even though its physical or mechanistic basis may be questionable. We adopt the classical ion-exchange formalism for multivalent ions, since it is simple and has a clear physical basis. As will be discussed later, analogues of the electroneutrality condition used in ion-exchange systems should be applicable to other modes of chromatography.

In the Theory section, the retention of a single protein in electrostatic interaction chromatography (EIC) is treated by the ion-exchange formalism as a function of salt concentration and pH. Three regions of binding (strong, intermediate, and weak) are distinguished on the basis of the strength of interaction. The above formalism is also believed to apply to other chromatographic modes in the strong and intermediate binding regions, which are the regions of primary interest in preparative separations. It is used to calculate multicomponent protein isotherms, which are required in the design of separation schemes for multicomponent mixtures. Since the multivalent nature of the ions makes the equations of this formalism highly nonlinear, computer solutions are necessary. The methods used are given in the section entitled Computational techniques.

THEORY

Single-component isotherms

We begin by considering EIC, for which the ion-exchange formalism could be considered a mechanistic model. The protein is drawn to the chromatographic surface

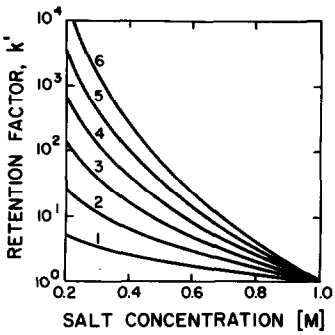

Fig. 1. Geometric dependence of the retention factor *versus* salt concentration curves on the characteristic charge, which is indicated next to each curve. The variation of retention factor with salt concentration becomes more pronounced as the characteristic charge increases.

by electrostatic attraction, and the number of residues that bind electrostatically to the surface will be termed the characteristic charge[10]. It should be emphasized that the characteristic charge has a definite physical significance: as the protein moves through the mobile phase under the influence of the electric field created by the fixed charges on the stationary phase surface, thermal motion causes it to rotate about its own axes, and it will therefore tend to bind in a preferred orientation (or orientations) that minimizes the free energy of its bound state[11,12]. The number of residues in contact or directly interacting with the fixed charges in this preferred orientation constitutes the characteristic charge of the protein under the given set of environmental conditions, such as pH and salt concentration in the mobile phase. At high protein concentrations, crowding effects at the surface of the stationary phase could cause a shift in the preferred orientation of the protein and thus a change in its characteristic charge, but we will not account for this possibility. However, to find the characteristic charge *a priori* in this fashion would involve detailed statistical mechanical calculations of considerable complexity. In this treatment, we forgo such calculations, and treat the characteristic charge as an empirical parameter of the same status as, for instance, the equilibrium constant in the Langmuir model of adsorption (we will later discuss how, in EIC, the characteristic charge may be found from experiments in the linear elution mode). This empirical treatment could have advantages in modelling other effects, as will be seen later.

Effect of salt concentration

Since, in ion-exchange, the stationary phase must always be electrically neutral, the binding of any molecule to the surface must be accompanied by the simultaneous expulsion of an equivalent number of counterions[13,14]. Thus, the salt concentration on the stationary phase must be explicitly accounted for, and this in turn implies that the easiest and most realistic way to represent the influence of salt concentration in the mobile phase is by direct competition for binding sites on the stationary phase. Thus, if the protein A has a characteristic charge m at a given pH and if the valence of the salt counterion S in n (since the MPM counterion is much smaller than the protein, its characteristic charge is simply equal to its valence), the stoichiometric exchange of salt from the stationary phase for protein is represented as

$$n\mathrm{A} + m\bar{\mathrm{S}} \rightleftharpoons n\bar{\mathrm{A}} + m\mathrm{S} \tag{1}$$

with an equilibrium constant K given by

$$K = \left(\frac{[\bar{\mathrm{A}}]}{[\mathrm{A}]}\right)^n \left(\frac{[\mathrm{S}]}{[\bar{\mathrm{S}}]}\right)^m \tag{2}$$

where overbars denote bound species, and terms enclosed in square brackets denote concentrations of the corresponding species. The process is considered as the exchange of counterions bound to the stationary phase, and such effects as Donnan exclusion, which account for co-ion concentrations, are not treated.

The rigorous form of the mass-action representation used above would use activities instead of concentrations. In order to use concentrations, the corresponding activity coefficients must be introduced. However, these need not be constant, es-

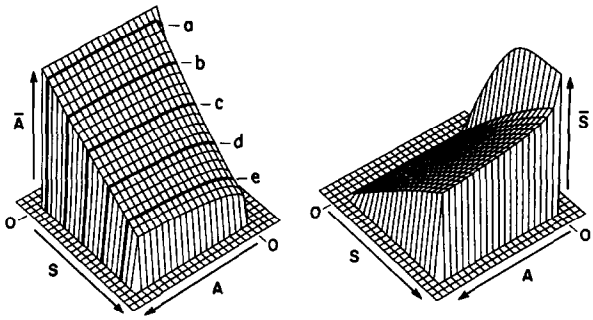

Fig. 2. Single-protein isotherm in the presence of salt. Two-dimensional surface representations of the stationary phase concentrations of the protein A and the salt S as a function of their mobile phase concentrations; the figure on the left is the protein isotherm surface, while the salt surface is shown on the right. Symbols a through e denote the line isotherms shown in Fig. 3.

pecially at high concentrations, and incorporating this effect would change the functional form of the model. For simplicity, we shall not account for possible variations in activity coefficients, and shall treat K, which is usually termed the selectivity coefficient in the non-ideal theory of multivalent ion-exchange[15], as an equilibrium constant. If such non-idealities are in fact significant, this would be reflected in the experimental data used to fit the characteristic charge m, and should result in a non-integral value for m (which does not affect the formalism). Treating the characteristic charge as an empirical parameter thus allows the concentration-averaged forms of these complex non-idealities to be "lumped" into it, while preserving a simple formalism.

The electroneutrality condition applied to the stationary phase yields

$$m[\bar{A}] + n[\bar{S}] = \Lambda \tag{3}$$

where Λ is the concentration of binding sites on the surface.

Eqns. 2 and 3 allow the calculation of the two unknown stationary phase concentrations, assuming that Λ and the mobile phase concentrations of the salt and protein are known. Solving these equations gives the adsorption isotherm of the protein A in the presence of the salt as a two-dimensional surface (Fig. 2). An important feature of the single-component ion-exchange isotherm is that the depen-

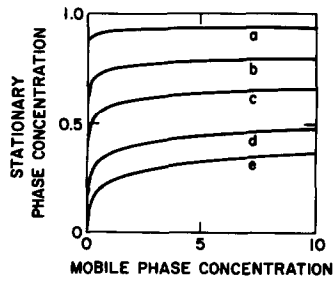

Fig. 3. Single-protein isotherm in the presence of salt. Line representations of the single-protein isotherm at fixed salt concentration. Curves a through e represent the sections shown in Fig. 2.

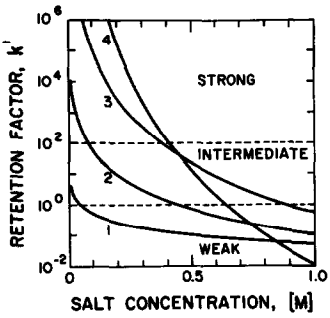

Fig. 4. Domains of binding. The weak binding region is characterized by retention factors < 1, and the strong binding region by retentions > 100. Curve 1 represents a "small molecule", while curves 2, 3, and 4 represent biopolymers with decreasing characteristic charges and equilibrium constants. The graph depicts the tendency of proteins to remain in the region of strong binding (or the upper portion of the intermediate region) at low MPM concentrations.

dence of protein binding on the mobile phase concentration of salt is explicitly given. Fig. 3 shows the line isotherms corresponding to the surface isotherms given in Fig. 2. It is convenient to define domains of binding based on the retention factor in the linear region of the adsorption isotherm, and these are shown in Fig. 4, which also depicts some representative trends in the variation of retention with MPM.

Comparison with Langmuir isotherms

The form of the Langmuir isotherm applicable to one protein in the presence of salt (this will later be called an explicit isotherm) is given by

$$q_p = \frac{a_p c_p}{1 + b_p c_p + b_s c_s} \tag{4}$$

where c and q are the mobile phase and stationary phase concentrations respectively, and a and b are empirically fitted parameters; the subscripts p and s refer to protein and salt. It is well known that such multicomponent Langmuirian isotherms can be converted into equivalent stoichiometric forms corresponding to univalent ion-exchange isotherms by introducing a dummy variable[16]. Our purpose here is simply to compare the Langmuirian and the multivalent ion-exchange formalisms in order to show that the Langmuirian form does not predict the sensitive dependence of retention to MPM concentration that was described earlier as one of the characteristics of protein adsorption. It has been shown[17,18] that if in analytical ion-exchange chromatography the logarithm of the retention factor of a multivalent ion is plotted against the logarithm of the salt concentration, a straight line results, with an absolute value of the slope equal to the ratio of the valences (or characteristic charges, for proteins) of the protein and the salt. It should be noted that, in our previous derivation, we accounted for the co-ion concentration explicitly[18]. If the process is genuinely one of counterion exchange, the co-ion concentration can be ignored, and the simplified form of the described relation is

$$\log k' = \log(\varphi K^{1/n}) + \frac{m}{n} \log\left(\frac{\Lambda}{n}\right) - \frac{m}{n} \log[S] \tag{5}$$

in the notation of this paper, where φ is the phase ratio; we note that the equilibrium constant used in ref. 18, K_b, is related to K by $(K_b)^n = K$. This result can be used to find the characteristic charge m and the equilibrium constant K from experimental linear elution data. The salt valence n is known; Λ can be found by titration, which also gives φ, since φ is the ratio of Λ to the mobile phase volume V_0. Thus, if ES and EI represent the slope and intercept, respectively, of the straight line of best fit to the experimentally obtained retention factor *versus* salt concentration data, it follows that

$$m = -n(\mathrm{ES}) \tag{6}$$

and

$$K = \left(\frac{e^{\mathrm{EI}}\Lambda^{\mathrm{ES}}}{\varphi}\right)^n \tag{7}$$

There has been some discussion in the literature[17,19] as to whether eqn. 6 should contain an additional factor of 2, which enters when the co-ions are explicitly accounted for. Theoretically, our neglect of the co-ion concentration in the ion-exchange process is based on regarding the co-ions associated with a charged protein under given set of environmental conditions, such as pH, as being of two types. The co-ions of the first type are stripped away from the protein when it binds to the surface of the stationary phase, while those of the second kind remain associated with the protein when it is bound. The protein molecule along with the co-ions of the second kind can then be treated as a polyion. The protein molecule can now be regarded as the equivalent of a neutral salt which, in solution, fully dissociates into the polyion and the co-ions of the first kind. Since these co-ions of the first kind are thermodynamically unchanged during the ion-exchange process, they cancel out of the equilibrium relation. Support for this approach is seen in ref. 19, where the characteristic charges of oligodeoxyadenylates were experimentally determined (since these were small molecules, their characteristic charges were identical to their net charges, at least for the oligomers of less than ten bases). The experimental characteristic charges were found to agree with a version of the present model, in which the above-mentioned factor of 2 is not introduced.

Returning to the analogy between the Langmuirian and the univalent ion-exchange formalisms, it can be seen that the maximum value of the slope of the straight line that eqn. 5 describes is unity, corresponding to a salt counterion of unit valence. Thus, it would be necessary to change the salt concentration by a factor of 100 to cause the retention factor to change by a factor of 100. Experimentally, however, it has been found[20,21] that changing the MPM concentration by a few percent can cause a protein that was previously irreversibly bound on the experimental time scale to be eluted near t_0. This supports our contention that the Langmuirian formalism is incapable of predicting the kind of sensitive dependence on MPM concentration that proteins routinely exhibit. The domain in which this sensitive dependence holds, and its significance for chromatographic separation, will be examined in the Results and discussion section.

The above demonstration was limited to concentrations in the analytical range,

i.e. in the linear range of the protein isotherm; similar considerations apply to the non-linear region as well.

We note in passing that the Langmuirian formalism could not have been ruled out simply because it considers all molecules to have a characteristic charge of unity, which is flagrantly unrealistic. A formalism need not have direct physical significance, as long as it conforms well to experimental results. In this instance, it is conceivable that the Langmuir isotherm could fit protein adsorption data by using a very large value for the binding constant; in fact, this has been done, for a fixed pH and salt concentration[22,23]. The analysis above showed that the Langmuirian formalism is incapable of exhibiting the drastic changes caused by changing the salt concentration or the pH, changes that are a consequence of multipoint binding. It is precisely because the Langmuirian equations for protein adsorption in the presence of salt do not incorporate such multipointedness that they fail in the region of strong binding. In summary, the single-component Langmuir formalism is adequate when the mobile phase conditions, such as pH and MPM concentration, are fixed; the Langmuir formalism that accounts for such changes in mobile phase conditions falls short of concordance with experiments.

Effect of pH

We will for simplicity consider a protein of characteristic charge m having a charge distribution that is spatially uniform and of equal strength, *i.e.*, the protein consists of identical charges, placed uniformly on its surface. If the ionization constant for each charged functional group is K', an analogue of the Henderson–Hasselbalch equation gives us

$$\mathrm{pH} = \mathrm{p}K' + \log \frac{\alpha}{1 - \alpha} \tag{8}$$

where α, the extent of ionization, is a fraction varying from 0, corresponding to the group being completely non-ionized, to 1, corresponding to complete ionization. If we now take the equilibrium constant for one charge, K_1, to vary linearly with the extent of ionization, the geometric dependence of the equilibrium constant on the number of charges yields

$$K_m = (K_1)^m = (u\alpha)^m \tag{9}$$

where K_1 and K_m are the equilibrium constants for one group and for the protein, respectively, and u is a constant of proportionality.

Using eqn. 8, it follows that

$$K_m = V\left[\frac{\exp(\mathrm{pH} - \mathrm{p}K')}{1 + \exp(\mathrm{pH} - \mathrm{p}K')}\right]^m \tag{10}$$

where the constant V is given by $V = u^m$. Eqn. 10 shows that the equilibrium constant for the protein has an exponential dependence on the pH. It must be emphasized that this is purely a formal calculation, intended to extract an estimate of how the retention factor (or equilibrium constant) would vary with the pH. Therefore, we do not deal with the oppositely charged residues of different charges that can be expected

in reality: the literature is replete with such descriptions of pH effects in ion-exchange (*e.g.*, ref. 24). The restricted analysis presented above does serve, however, to emphasize the sensitive dependence of retention on pH in EIC, which can be exploited in certain separation schemes to be discussed later. It should be noted that while the sensitive dependence on pH is a characteristic of EIC, such a dependence on MPM is expected to apply to multipoint binding in the other chromatographic modes as well.

Domains of binding

It has been shown that three regions of binding may be demarcated in the plot of the dependence of retention (or retention factor) of a representative protein on MPM concentration. These regions were illustrated in Fig. 4, where they were described as domains of strong, intermediate, and weak interactions.

The region of strong interaction is characterized by irreversible binding over chromatographic time scales, and this is reflected in the rectangular shapes of single-component isotherms under conditions of low MPM concentration[25,26]. As will be discussed later, if the structural integrity of the protein is unaffected upon binding or release, this region has several advantages for preparative chromatographic separation. The main gain is much higher column capacities, as can be seen from the isotherm figures (Fig. 2), but it has thus far been very rarely employed. The multivalent ion-exchange formalism implies that the characteristic features of the adsorption of proteins can be used to good purpose in their separation, and it turns out that, in many cases of practical interest, not only is the separation facilitated, but the yield and throughput are materially augmented. These advantages enjoyed by nonlinear chromatographic modes operating in the strong binding domain may be attributed to two factors: at high concentrations, the sorbent surface in the column is being utilized efficiently, in marked contrast to linear or quasi-linear elution chromatography; in addition, the selectivity changes with concentration, and in favorable cases it can be much higher in the non-linear range of concentration than in the linear region used in elution. At the same time, the possibility of denaturation or low recovery calls for the use of stationary phases with the appropriate surface properties.

The intermediate region of binding is where the multipoint nature of the binding has been attenuated, so that the protein begins to behave as a "small molecule". (It might be noted that, in EIC, a small molecule with, say, five fully ionized charges will exhibit the same geometric variation of retention at sufficiently low salt levels; the usage of the terms small and large molecules is therefore not strictly accurate. We retain this usage because it is physically evocative.) It is in this region that linear and quasi-linear elution are carried out as preparative techniques. As a consequence, many studies on protein adsorption have also been in this region[27,28].

The region of low binding (the domain in which the retention factor is less than unity) is of little significance *per se*, being the region where the binding has been attenuated to the point where the molecule is practically not retained. That this region can be reached rapidly from one of strong binding on the application of a suitable change in pH or MPM concentration is useful, however, to the stepwise elution techniques of protein separation described later.

Multicomponent isotherms

The foregoing discussion of binding domains makes it clear that, everything else being equal, we would wish to conduct our preparative separations in the region of strong binding. It is clear that this involves high protein concentrations, which implies that all the protein molecules cannot be accommodated on the limited number of binding sites on the stationary phase. They will have to compete for the sites, and their success will be dependent both on their relative concentrations and their intrinsic affinities for the stationary phase. Their binding in the presence of other proteins is therefore suppressed in comparison to their single-component isotherms, which show their binding characteristics in the absence of such competition. A tabulation of protein concentrations on the stationary phase in the presence of such interference effects constitutes a multicomponent isotherm. In order to calculate such isotherms theoretically, a formalism is required. We adopt the ion-exchange model that has just been developed as a formalism. Since we intend it to apply to all modes of chromatography (*e.g.*, reversed-phase and hydrophobic interaction chromatography) it must be emphasized that this is a purely formalistic approach. We shall attempt to provide a physical motivation for the equations that follow, but it must be noted that mechanistic interpretations based on the ion-exchange process are not applicable. In the strong region of binding, it is reasonable to think of the stationary phase as saturated: at such high concentrations, almost all the sites must be occupied by proteins. Any protein molecule that binds must consequently expel another, already bound, molecule. By the same token, the sum of all the molecules found on the stationary phase (weighted by their degree of multipointedness) should be constant. The applicability of such an approach, which considers the binding of one species to be accompanied by the simultaneous expulsion of already bound molecules, has been pointed out by Regnier and Maszaroff[29].

These considerations allow us to invoke the multicomponent ion-exchange formalism, but some notation is first required. In this formalism, MPM plays the role of salt in the actual ion-exchange process, as has been described earlier. Similarly, the index of multipointedness (IMP) replaces the characteristic charge, and the electroneutrality condition becomes a pseudo-mass balance, expressing the extreme degree to which binding to the stationary phase is thermodynamically favored. Finally, we refer to systems as *multicomponent* only when there is more than one protein involved, since our interest lies in separating mixtures of proteins, and not in separating a protein from an MPM solution; in keeping with this convention, our previous discussion of the ion exchange of a single protein yielded single-component isotherms, even though the salt counterions were explicitly accounted for. The corresoponding Langmuir isotherms which account for MPM will be called explicit, and those obtained at fixed pH and MPM concentration will be called implicit, since the effects of pH and salt are implicit in the isotherm parameters.

One important advantage of the ion-exchange formalism is that the multicomponent isotherms are conceptually on as sound a footing as the single-component forms. This contrasts with the multicomponent Langmuir forms[30,31], which, as is well known, are not thermodynamically rigorous[32,33].

The isotherms for two proteins A and B can be calculated in a fashion entirely analogous to the previous single protein calculation. The expulsion of MPM coun-

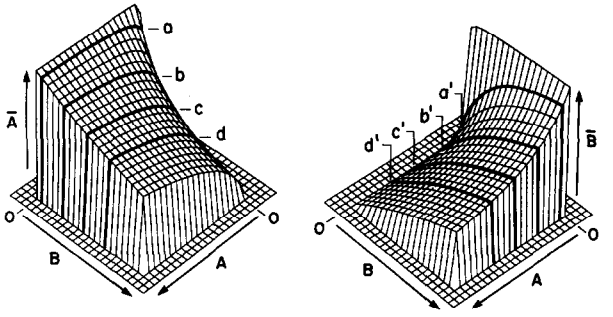

Fig. 5. Multicomponent protein isotherms. Two-dimensional surface representations of the stationary phase concentrations of two proteins A and B as a function of their mobile phase concentrations, at fixed MPM. A has an IMP of 10, while the IMP of B is 6. Curves a through d and a' through d' denote the line isotherms shown in Fig. 6.

terions from the stationary phase by each protein, acting individually, can be represented as

$$n\mathrm{A} + a\bar{\mathrm{S}} \rightleftharpoons n\bar{\mathrm{A}} + a\mathrm{S} \tag{11}$$

and

$$n\mathrm{B} + b\bar{\mathrm{S}} \rightleftharpoons n\bar{\mathrm{B}} + b\mathrm{S} \tag{12}$$

where a and b are the corresponding IMP values, and S is the MPM of "IMP" n, and the notation is similar to that used earlier. The equilibrium constants are given by

$$K_{\mathrm{A}} = \left(\frac{[\bar{\mathrm{A}}]}{[\mathrm{A}]}\right)^{n} \left(\frac{[\mathrm{S}]}{[\bar{\mathrm{S}}]}\right)^{a} \tag{13}$$

and

$$K_{\mathrm{B}} = \left(\frac{[\bar{\mathrm{B}}]}{[\mathrm{B}]}\right)^{n} \left(\frac{[\mathrm{S}]}{[\bar{\mathrm{S}}]}\right)^{b} \tag{14}$$

The pseudo-mass balance condition becomes

$$a[\bar{\mathrm{A}}] + b[\bar{\mathrm{B}}] + n[\bar{\mathrm{S}}] = \Lambda \tag{15}$$

There are three equations in the three unknown stationary phase concentrations, and these can be solved if the corresponding mobile phase concentrations and the concentration of binding sites on the stationary phase are known. These multicomponent isotherms are shown as two-dimensional surfaces in Fig. 5 where the MPM concentration in the mobile phase has been fixed. We note that to each component can be attributed a surface that consists of all the stationary phase concentrations it takes as all the independent variables (the mobile phase concentrations) are varied. On comparing Figs. 2 and 5, it is seen that the suppression of binding

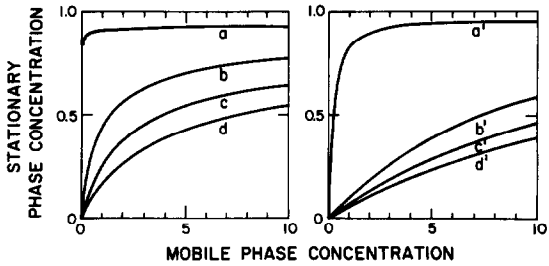

Fig. 6. Multicomponent protein isotherms. Line representations of the sections shown by symbols a through d and a′ through d′ of the isotherm surfaces in Fig. 5.

caused by MPM is greater than that due to the other protein. This is simply because the MPM is present in much higher concentration than that of the competing proteins. The line isotherms corresponding to the isotherm surface depicted in Fig. 5 are shown in Figs. 6 and 7. The line isotherms in Fig. 7 cross even though the mobile phase concentrations of the two proteins are equal. This is because while the more strongly bound protein has a higher initial slope, its greater characteristic charge causes its saturation concentration to be lower than that of the other protein. The stationary phase concentrations in all the other figures have been scaled to saturation concentrations of unity. It should be noted that while the single component ion-exchange isotherms are rectangular, the corresponding multicomponent forms are hyperbolic. Irreversibility in this equilibrium formalism is represented as a limiting case, in which the equilibrium constants are very high and the line isotherms are rectangular. The hyperbolic nature of the multicomponent line isotherms indicates that competition causes a departure from the irreversible limit to the more common reversible forms found in the regions of intermediate and weak binding. This has important consequences for such preparative modes as displacement, and will be discussed later.

The isotherms for n-component protein mixtures can be similarly calculated.

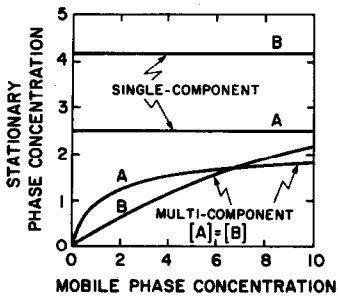

Fig. 7. Single- and multicomponent isotherms of proteins A and B, which have IMPs of 10 and 6 respectively, at constant mobile phase concentration of MPM. The multicomponent isotherms are shown here for equal mobile phase concentrations of A and B.

COMPUTATIONAL TECHNIQUES

The calculation of the single- and multi-component isotherms described above were performed numerically on a MicroVAX computer in the Yale Chemical Engineering Department, since analytical solutions to algebraic equations of higher than fourth degree are known not to exist. It is, in fact, simpler to calculate solutions numerically even for cubic and quartic equations, since the analytical solutions are somewhat cumbersome. In the instance of a single protein, it is possible to solve only for the stationary phase protein concentration (the MPM stationary phase concentration can then be separately found from the electroneutrality condition), but this process yields several extraneous roots. While it is not difficult to discard these spurious roots, the simultaneous solution of both protein and MPM stationary phase concentrations is not much more difficult, and spurious roots are obviated. In the case of several proteins, of course, only the second method is applicable. Several FORTRAN subroutines available through the VAX 8600 at the Yale Computer Center were used to solve the algebraic system of equations. Such calculations, in a somewhat different approach, have been reported in the literature[34]. The two- and three-dimensional graphs were generated using the Td3 graphics package, also accessed at the Yale Computer Center.

RESULTS AND DISCUSSION

Validity of Langmuirian isotherms

In order to put the present model in perspective, it would seem worthwhile to explain how and where the Langmuir model fails, especially in light of its wide acceptance hitherto. The fundamental feature of protein adsorption that the explicit Langmuirian isotherms do not predict is the sensitive dependence of retention on MPM concentration, as shown earlier. This feature becomes important only if the MPM concentration is low enough to ensure that the protein is initially in its domain of strong binding. However, most experimental data available in the chromatographic literature to date are limited to the intermediate binding region, and this is understandable on several counts. Most preparative separations are still carried out in the mildly overloaded elution mode, where the proteins are at low concentrations, corresponding to the quasi-linear regions of their binding isotherms. Further, the natural method of applying separation techniques that have worked well on small molecules to protein mixtures is to find conditions under which the proteins behave like small molecules, and this again leads to the intermediate binding region. It therefore comes as no surprise that the implicit Langmuirian isotherm provides a reasonable fit to most data currently available[22,23].

It is, of course, possible to choose a suitably high equilibrium constant for the Langmuir form, so that a good approximation to the rectangular isotherm is obtained for a single protein at low MPM concentrations; rectangular isotherms are merely extreme forms of monotonic concave-down functions, and the Langmuirian form should fit such functions well. However, the calculation carried out in the Theory section indicates that even if such an equilibrium constant is chosen, the variation of the corresponding isotherm with MPM will not show the precipitous decrease of the ion-exchange model. Thus only the isotherms resulting from the multivalent ion-

exchange formalism exhibit the characteristic features of protein binding under strong binding conditions on the one hand, and reduce to concave-down forms at intermediate and low binding.

Separation schemes

Since the ultimate goal is to achieve successful separations of mixtures of practical interest, an important aspect of a realistic model is the framework it affords on which the rational design of efficient separation processes may be based. The multicomponent isotherms that can be calculated from the model, in addition to the species mass balances, provide a complete description of the chromatographic process, as long as the IMP values and equilibrium constants can be calculated from experimental data.

A useful preparative chromatographic mode is displacement, in which the sample mixture is fed into the column and is immediately followed by a continuous flow of a substance, termed the displacer, having an affinity for the stationary phase that is greater than that of any component in the mixture. The displacer forms a sharp front that pushes all the other components ahead of it, by virtue of its greater affinity. In this mixed region ahead of the displacer front, the lesser retained substances are similarly kept from binding and pushed ahead by the more strongly retained substances. This eventually results in a final pattern consisting of a series of adjacent pure bands ahead of the displacer front[35]. The applicability of displacement chromatography to the separation of proteins may be questioned, given the irreversible nature of the single-component protein isotherms in the strong binding region. Work in our laboratory has shown that displacement can be very effective in protein separation[36], and the multicomponent isotherm surfaces shown previously in Fig. 4 provide the necessary reconciliation. Even though the single-component isotherms are rectangular, the competitive forms are reversible and much less steep, and can in fact appear to be hyperbolic. The mixture should consequently separate into pure bands.

The characteristic features of protein binding described above lend themselves to a form of separation process that is conceptually trivial and operationally facile, but which may yet be very efficient in realistic separation tasks. This method is stepwise elution. If we define the elution window of a protein as the smallest range of MPM concentration in the mobile phase that would cause an analytical sample of the protein to pass from its region of strong binding to that of weak binding, the multipoint character of protein binding causes these windows to be quite narrow; this is simply a restatement of the "all-or-nothing" principle. It is therefore quite likely that a protein mixture has elution windows that do not overlap. The mixture is first loaded onto the column in the frontal mode, where it is passed continuously into the column until the second front begins to emerge. (The region between the first and second fronts consists of the least retained compound in pure form. All the other regions contain mixtures, which is why frontal analysis cannot be used as a stand-alone multicomponent preparative technique, in contrast to elution and displacement.) We can then change either the incoming MPM concentration or the pH in a series of steps, each step resulting in the elution of one protein from the column[20]. Two schematic drawings of how this technique might be used are shown in Fig. 8. Stepwise elution as the separation step may be replaced by either gradient elution or

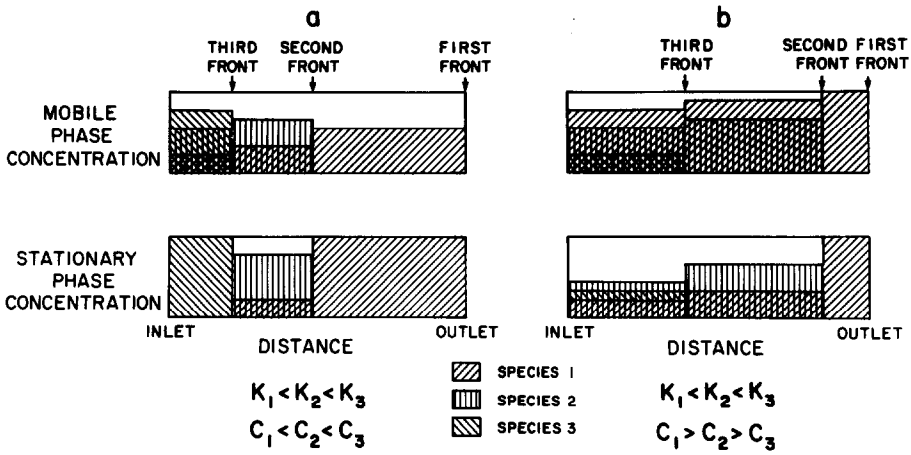

Fig. 8. Schematic representation of frontal chromatography. In (a) the stated conditions tend to enhance selectivities; the third component is taken to suppress the adsorption of the other two completely. In (b) the conditions are in opposition; consequently, the separation factors are not as large and the separation not as good as in (a).

displacement. Experimentally, gradient elution might be the method of choice, since it is necessary to ascertain the elution windows for stepwise elution, and a suitable displacer must be found to achieve a separation by displacement. Against this must be set the fact that stepwise and gradient elution techniques require that the separation factors of the sample components be sufficiently large (a more precise condition is that the smallest separation factor be larger than a fixed number which is determined by such factors as the column efficiency), while displacement should be effective even when the separation factors are close to unity.

This method also lends itself to the measurement of multicomponent isotherms: if a mixture of known concentration is fed into a column until a steady-state is reached (when the effluent concentration is equal to that of the influent), the material bound to the column can again be washed off and collected by suitably increasing the MPM concentration. (After stopping the flow of protein mixture, it is necessary to purge the mobile phase contents of the column prior to introducing the MPM or pH step.) Analysis of the effluent gives the stationary phase concentrations of the various proteins, and consequently one point on the multicomponent isotherm surfaces.

Preparative separation strategy could be summarized, somewhat paradoxically, in the statement that the key to separation is competition: in modes like displacement, competition between proteins causes their isotherms to become reversible and hence allows separation, while in stepwise elution, competition with MPM causes the traversal of the elution window. Of course, the statement is only true of non-linear chromatographic modes.

Applicability to other forms of chromatography

While the formalism used is based on ion exchange, its range of applicability is significantly wider. It is well adapted to describe such forms as affinity chromato-

graphy, since strong specific binding is characteristic of affinity, and the binding sites will tend to be saturated, thus validating an analogue of the electroneutrality condition. Even reversed-phase chromatography, which can frequently involve a two-step binding process[37] (the first step is binding to the surface, the second involves conformational changes in the protein so that it might better accommodate itself to the surface), may be represented by the ion-exchange formalism through the expedient of lumping the two processes. This should be reasonable when the time constants for the two processes are not too different.

CONCLUSIONS

The formalism presented here offers an alternative approach to the computation of multicomponent isotherms, and hence to the rational design of separation schemes tailored to the special features of a given protein mixture. By allowing for a two-stage binding process, the formalism could be extended to cover most realistic reversed-phase separations (as mentioned in the Results and discussion section, at present it will apply only to a restricted class of two-step processes.) It should be noted that, while the formalism is presented for protein mixtures, there is no reason why it should not apply, in the absence of specific binding effects, to other biological and synthetic polymers. Work is underway in our laboratory to apply the formalism quantitatively to the analysis of various chromatographic modes, and thereby allow the detailed comparison of various schemes in terms of such parameters as yield, throughput, and cycle time. It must be re-iterated that separation schemes that involve the domain of strong binding can only be useful if the proteins that are subjected to such conditions retain their structural integrity. In this regard, the constant improvement in the homogeneity of the stationary phases being synthesized today is an important factor, and should help to make non-linear separation modes the methods of choice in the future.

ACKNOWLEDGEMENTS

The present work was supported by Grants Nos. GM20993 and CA21948 from the National Institutes of Health, U.S. Department of Health and Human Resources.

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