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REVIEW

The Study of Ligand–Protein Interactions Utilizing Affinity Chromatography

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INTRODUCTION

From the inception of the affinity chromatographic method, it has been recognized that one of the critical parameters is the strength of interaction between the protein and the immobilized ligand. An interaction that is too weak will not give sufficient retardation for separation, while an interaction that is too strong could lead to essentially irreversible binding. Both situations have been all too frequently observed and most of the early workers in this field have their own "horror" stories about the failures of the method for their particular system.

In the early 1970s, several groups turned their attention to a more in depth study of affinity chromotography in an effort to learn more about the fundamental processes involved. It was only by ignoring the *preparative* uses of the method that more could be learned about the mechanisms of binding. This is perhaps a good example of the value of basic research. As this article will point out, much of the information derived from the study of affinity chromatography as an analytical tool is of value in predicting the successes or failures of the method in a purification scheme. However, the major emphasis of this article will be to describe the appli-

cation of affinity columns in an analytical method that yields information on binding of proteins to soluble and immobilized ligands. The basic message of this review is that the experiments and equations presented are quite general and can be more widely applied to derive valid binding data.

The literature reviewed will be divided into sections mainly on the basis of the experimental protocol utilized. These are analyses using:

A. Zonal elution chromatography
B. Frontal elution chromatography
C. Gradient elutions or batch absorption techniques
D. Theoretical studies and summary

To complete this introductory section, it is necessary to define the terms that will be employed throughout this review. Unfortunately, since these methods have been developed in several laboratories around the world, it is predictable that several systems of nomenclature have arisen. In an effort to inject some uniformity into the proceedings, I will propose the use of the following terms. We first define two equilibria for protein–ligand interactions.

$$\mathrm{P \cdot M\text{-}L} \overset{K_{\mathrm{M\text{-}L}}}{\leftrightharpoons} \mathrm{P + M\text{-}L} \tag{1}$$

$$\mathrm{P \cdot I\text{-}L} \overset{K_{\mathrm{I\text{-}L}}}{\leftrightharpoons} \mathrm{P + I\text{-}L} \tag{2}$$

In these equations, P is used to represent a protein species, or more generally, a polymeric species, such as a nucleic acid or polysaccharide. Ligands are represented by L, with M-L used to represent *mobile ligand*, and I-L used to represent *immobilized ligand*. This represents a departure from earlier work, where an overbar has been used to represent an immobilized species. $K_{\mathrm{M\text{-}L}}$ and $K_{\mathrm{I\text{-}L}}$ then represent the dissociation constants for the binary complexes of mobile ligand and protein (P · M-L) or immobilized ligand and protein (P · I-L), respectively. By eliminating the overbar, we hope to avoid errors due to typing or printing.

In describing the operating parameters of the columns, we will use V to represent the measured elution volume, V_o to represent the void volume of the porous column, and V' to represent the unretarded elution volume of the macromolecule under investigation. Again, these represent important departures from previous published work. (V_o has been used to represent V' and V_m has been used to represent V_o). Again my intention is to provide a consistent set of parameters that various workers can utilize. In this attempt I have abandonded the nomenclature that I have personally used. V_o is usually determined by the elution of a very large dye molecule such as Blue Dextran and V′ can be obtained by elution of a non-interacting analog of the protein or from the elution volume in the presence of an excess of mobile ligand.

ZONAL ELUTION CHROMATOGRAPHY

In the simplest case of competitive binding of a mobile ligand, M-L, and an immobilized ligand, I-L, to a monomeric protein, the following equation has been derived (*15*) to describe the response of elution volume to changes in the concentrations of the variable species and changes in the column parameters:

$$V = V' + (V' - V_o)\,([\text{I-L}]/K_{\text{I-L}})_i/\{1 + ([\text{M-L}]/K_{\text{M-L}})\} \tag{3}$$

By rearranging and taking reciprocals, the equation may be cast into a form that is quite useful.

$$\frac{1}{V - V'} = \frac{1}{(V' - V_o)}\frac{K_{\text{I-L}}}{[\text{I-L}]} + \frac{1}{(V' - V_o)}\frac{[\text{M-L}]}{K_{\text{M-L}}}\frac{K_{\text{I-L}}}{[\text{I-L}]} \tag{4}$$

In Eq. (4) it can be seen that a plot of the left side $1/(V - V')$ versus [M-L], the total concentration of soluble mobile ligand, will yield an intercept that contains the value of $K_{\text{I-L}}$ and a slope that contains the value of $K_{\text{M-L}}$. Thus, performing a series of experiments with different concentrations of the mobile ligand will permit the determination of these characteristic constants. $K_{\text{M-L}}$ can most readily be obtained by calculating the ratio of the intercept to the slope of a plot according to Eq. (4).

Plot $1/(V - V')$ versus [M-L]

$$\text{Intercept} = \frac{1}{(V' - V_o)}\frac{K_{\text{I-L}}}{[\text{I-L}]} \tag{5}$$

$$\text{Slope} = \frac{1}{(V' - V_o)}\frac{1}{K_{\text{M-L}}}\frac{K_{\text{I-L}}}{[\text{I-L}]} \tag{6}$$

Then

$$\frac{\text{Intercept}}{\text{Slope}} = \frac{1}{(V' - V_o)}\frac{K_{\text{I-L}}}{[\text{I-L}]} \Big/ \frac{1}{(V' - V_o)}\frac{1}{K_{\text{M-L}}}\frac{K_{\text{I-L}}}{[\text{I-L}]} \tag{7}$$

which simplifies to

$$\frac{\text{Intercept}}{\text{Slope}} = K_{\text{M-L}} \tag{8}$$

The value of $K_{\text{I-L}}$ can be calculated from the numerical value of the intercept and the known values of [I-L] and the column parameters V' and V_o.

The preceding equations are derived with the assumption that a small zone of protein is applied to a column such that [I-L] >> [P]. Thus these conditions are analogous to those in initial velocity studies in en-

zyme kinetics where [S] >> [E]. There are two major advantages to this procedure relative to the frontal elution techniques described later.

Firstly, the zonal elution method can be applied to very small amounts of protein. Since many investigators have utilized affinity methods to isolate small amounts of precious biomolecules, the zonal elution methods are perfect companions in providing quantitative binding information. Secondly, as we have seen, the equations describing the experiments are quite simple and are analogous to those of enzyme kinetic experiments. With terms defined as in the preceding, it is possible to relate the information obtained from chromatography directly to data obtained from enzyme kinetics or other methods.

Another advantage of the zonal elution method is that the passage of a small zone down a column does not cause any important changes in concentration of species, even though there will be zonal spreading during the experiment. A zonal experiment can be conducted under rigidly controlled conditions, unlike a batchwise elution or gradient procedure, where definition of concentrations becomes tenuous.

In the following, I will briefly discuss the results of several studies that have employed zonal elution methods. These will be given in roughly chronological order.

In one of the earliest publications utilizing the zonal method Andrews et al. describe the effect of glucose or *N*-acetyl-glucosamine on the migration of a zone of human galactosyltransferase through a column of Sepharose-α-lactalbumin (*2*). In this study, the monosaccharides caused *increased* retardation of the protein on the immobilized ligand in a linear fashion. These authors were among the first to point out the value of using zonal affinity chromatography to study weak interactions with materials available in microgram amounts.

In two papers, Dunn and Chaiken describe studies of the binding of staphylococcal nuclease to columns of Sepharose-immobilized thymidine-3′-(*p*-aminophenyl phosphate)-5′-phosphate (*15*, *16*). Strict adherence to Eq. (3) was demonstrated by linear plots of elution volume, V, versus the concentration of immobilized ligand, [I-L]. Using a series of soluble ligands, the value of Eq. (*4*) was shown by linear plots of $1/[V - V']$ versus [M-L] and values of $K_{M\text{-}L}$ and K_{I-L} were calculated. For five such mobile ligands, the agreement between the $K_{M\text{-}L}$ values obtained by chromatography and from classical enzyme kinetics was excellent. In addition, it was demonstrated that a zone of protein could be eluted in the absence of added mobile ligand (*16*). If the concentration of mobile ligand is set to zero in Eq. (3), the resulting simplified Eq. (9) results.

$$V = V' + (V' - V_o)\,([\text{I-L}]/K_{\text{I-L}}) \qquad (9)$$

Measuring V under these conditions would allow the calculation of $K_{I\text{-}L}$ if the effective [I-L] is already known. The difficulty inherent in this experiment is that the zone of protein will be spread out during its slow passage through the column and the peak position will be difficult to

measure accurately. Even so, this experiment illustrates the important principle that the binding of a protein to an affinity matrix is a dynamic process with many on and off steps per second.

Brinkworth et al. included a series of zonal elution experiments in their incisive study of the interaction of lactate dehydrogenase with reduced nicotinamide-adenine dinucleotide (*6*). These authors employed the formation of ternary complex between the enzyme NADH binary complex and oxamate immobilized on Sepharose (*53*). With columns preequilibrated with NADH at concentrations from 0 to 3.3 μ*M*, Brinkworth et al. demonstrated that the five isoenzymes of LDH, M_4, M_3H, M_2H_2, MH_3, and H_4 could be separated by a single column. Furthermore, their general quantitative treatment could be employed to evaluate the association of NADH with each isoenzyme species.

Chaiken and Taylor have explored the interactions of ribonuclease (*11*) and a catalytically inactive analog (*61*) with a column of uridine-5′-(Sepharose-4-aminophenylphosphoryl)-2′(3′)-phosphate. They first demonstrated the adherence of the results to Eq. (4) when 2′-CMP was used as the competing ligand at ionic strengths high enough to avoid nonbiospecific effects (*11*). The chromatographically determined values of $K_{M\text{-}L}$ were close to the values determined by inhibition kinetics. At low ionic strength, however, these authors observed retardation of protein species that could be attributed to ionic interactions, possibly with the isourea groups generated by cyanogen bromide coupling. Chaiken and Taylor also pointed out the possible complication arising at higher protein concentration where the [P] is no longer negligible. Nichol et al. have provided equations (*47*) to be discussed in the following section that can be utilized to account for this factor. For the moment, it is important to point out that Chaiken and Taylor have considered two sources of non-adherence to Eq. (4) and have successfully found conditions where these problems are not of importance.

In addition to their studies on native ribonuclease, Chaiken and Taylor also examined chromatography of the noncovalent complex, Ribonuclease-S, derived by subtilisin digestion. This consists of two peptides, the S-peptide, with residues 1–20 of the original protein, and the S-protein, with residues 21–124. The values of $K_{M\text{-}L}$ for 2′-CMP were very similar for native ribonuclease, ribonuclease-S, and for a semisynthetic ribonuclease-S complex containing *p*-F-Phe at position 8 in the S-peptide in place of the naturally occurring Phe (*11*). Since the semisynthetic complex was fully active, this is not surprising. In addition, the values of $K_{I\text{-}L}$ were also quite similar for all three species. This study established the value of such approaches for the characterization of semisynthetic materials. This was then exploited by Taylor and Chaiken to study the *inactive* semisynthetic analog [4-F-His^{12}, des 16-20] ribonuclease-S (*61*). A tritiated preparation was utilized to permit detection at concentrations low enough to avoid extra terms in the equation. The same affinity matrix as above was employed for these studies. In

general, the inactive analog bound active site ligands with the same specificity and nearly the same avidity as native ribonuclease-S. The reduced binding observed for some ligands could be attributed to a possible interaction of a positively charged histidine (at pH 5.2) in the native protein and a 2′-phosphate of the ligand. Of great importance was the observation that a good ribonuclease substrate, UpA, binds equally as well to the inactive analog as to the native enzyme. This, along with other data, allowed Taylor and Chaiken to conclude that the semisynthetic analog was inactive because of the chemical change of the catalytic His residue and not because of an alteration in binding affinity caused by conformational changes.

Malanikova and Turkova applied the zonal elution technique to the binding of trypsin to *p*-aminobenzamidine immobilized on hydroxyalkyl methacrylate gel (Spheron) as influenced by five mobile ligands (*43*). Again, a close agreement between chromatographic results and kinetic results for the mobile ligands was seen. This study also made an important comparison between the zonal elution method and the frontal elution method, described in the next section. The conclusion of Malanikova and Turkova was that identical results may be obtained with either method provided the experiments are done under the appropriate conditions (*43*). The value of $K_{I\text{-}L}$ obtained in this case was lower than the value of $K_{M\text{-}L}$ obtained for soluble *p*-aminobenzamidine. This has been interpreted to indicate that additional binding interactions are probably occurring in binding to the immobilized forms of the ligand. This is to be expected in most cases. As long as these are not ionic interactions, this can be advantageous.

Another study on ribonuclease binding was conducted by Smith et al. (*60*). A different immobilized ligand, 5′-uridine–triphosphate–hexane–agarose was shown to be superior in that nonbiospecific interactions were eliminated. Using a column with a bed volume of only 0.96 mL, they obtained excellent agreement between their calculated $K_{M\text{-}L}$ value and values reported from difference spectroscopy. This affinity matrix proved to be valuable in refolding studies, since reduced denatured ribonuclease was not retarded. This further illustrates the biospecificity already seen from the quantitative data on mobile ligand binding.

The promise of zonal elution chromatography for the study of other types of macromolecule–ligand interactions has been demonstrated by Veronese et al. in their report on the binding of psychoactive drugs to bovine glutamate dehydrogenase (*65*). Perphenazine coupled Sepharose was prepared by several methods and, after evaluation for biospecific binding, the derivative prepared from epoxy-activated Sepharose was employed for quantitative studies. The usual adherence to Eq. (4) for six different drugs was observed and data was obtained to permit the calculation of $K_{M\text{-}L}$ values. The determined number are in the same relative

order as those obtained from kinetic assays, but since the kinetic assays include substrate binding at a second site, the actual values of $K_{M\text{-}L}$ from chromatography are not identical to the kinetic ones. Nonetheless, the values obtained by chromatography are relevant to the biological action of these drugs and support the role of drug—GDH interaction in the pharmacological effects. These authors also conducted some experiments with allosteric ligands, ADP, GTP, and NADH using alteration in elution volume to gauge the effects.

A quantitative analysis of the binding of lactate dehydrogenase to immobilized Cibacron-Blue has been carried out by Liu et al. (*38*). Initially, adherence to Eq. (9) was demonstrated and this was employed to calculate a $K_{I\text{-}L}$ of 12 μM. This can be contrasted to the value of $K_{M\text{-}L}$ of 0.2 μM obtained by measuring binding of free dye by spectroscopic methods. Liu et al. then consider the variation in binding with total concentration of protein. It would seem that these results can be rationalized by the fact that the higher protein concentrations used in these studies are equivalent to the concentration of immobilized dye. Thus, the results should more properly be described as a binding isotherm where the [P] becomes significant. Equations presented later as derived by Nichol et al. will adequately cover the case of very high protein concentration, relative to immobilized ligand concentration (*47*).

When Liu et al. used their dye column to measure NADH binding to LDH, they observed adherence with Eq. (4) and agreement between the calculated $K_{M\text{-}L}$ value, 3.4 μM, and the value derived from kinetic measurements. These experiments were performed with a concentration of protein that was 1% of the immobilized dye concentration. This is ideal for the application of Eqs. (3) and (4). The value of $K_{I\text{-}L}$ derived from this analysis is 13 μM, which agrees with that obtained from variation in [I-L], 12 μM.

Dunn and Gilbert have described the binding of chymotrypsin to 4-phenylbutyl-amine-succinyl-polyacrylic hydrazide agarose (*17*). In this study, three classes of soluble ligands were defined. The benzyloxycarbonyl derivatives of Phe, Tyr, and Trp caused an increased retention of the protein on the affinity column. This is direct evidence for the formation of a ternary complex of protein, immobilized ligand, and soluble ligand (P · M-L · I-L). The data of Andrews et al., noted earlier (*2*), was the first quantitative description of such an effect. In addition, Brinkworth et al. also utilized ternary complex formation to provide a biospecific separation (*6*). In those cases, the existence of a ternary complex was already suspected from the nature of the system studied. In fact, the separations were designed based on that premise. The results of Dunn and Gilbert (*17*) were unexpected, however, and therefore demonstrate the value of quantitative affinity chromatography in elucidating binding mechanism. A scheme describing this situation is as follows:

$$\begin{array}{ccc} P + I\text{-}L & \overset{K_{I\text{-}L}}{\rightleftharpoons} & P \cdot I\text{-}L \\ + & & \\ M\text{-}L & & \\ K_{M\text{-}L} \downarrow\uparrow & & \\ P \cdot M\text{-}L + I\text{-}L & \overset{K'_{I\text{-}L}}{\rightleftharpoons} & P \cdot M\text{-}L \cdot I\text{-}L \end{array}$$

where a new dissociation constant for the ternary complex, $K'_{I\text{-}L}$, is defined. An equation in the form of Eq. (3) can be defined (*17*) for this scheme:

$$V = V' + \frac{(V' - V_o)[I\text{-}L]\,[K_{M\text{-}L} + (K_{I\text{-}L}\,[M\text{-}L]/K'_{I\text{-}L})]}{K_{I\text{-}L}(K_{M\text{-}L} + [M\text{-}L])} \quad (10)$$

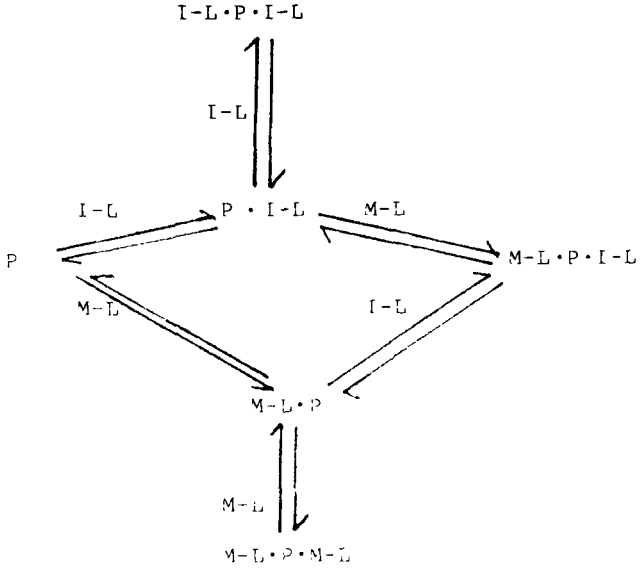
Again, this may be cast in a reciprocal form to yield an equation that is now linear in 1/[M-L] to permit the calculation of $K_{M\text{-}L}$ for the three CBZ-amino acids. Again, the values were in close agreement with data obtained from enzyme kinetic measurements. Since the strength of interactions of chymotrypsin with the immobilized ligand was quite weak (8.57 m*M*) it was possible to measure elution volume in the absence of soluble ligands quite accurately. This permitted the calculation of $K_{I\text{-}L}$ directly. This value could then be combined with the intercept value from double reciprocal plots to calculate $K'_{I\text{-}L}$ with a reasonable degree of accuracy. It is relevant to point out that if the interaction of chymotrypsin with the affinity matrix in the absence of added ligands had been stronger, the observation of the stimulatory effect of the CBZ-AA's would have been impossible. This illustrates that even a very weak affinity matrix can yield valuable data on biospecific binding.

The second class of ligands studied by Dunn and Gilbert did not affect the elution volume of the protein on the affinity column. These were *N*-acetyl-L-Phe, *N*-acetyl-L-Tyr, 4-phenylbutylamine, and phenylbutyric acid. These compounds were shown by enzyme kinetics to interact with the enzyme in a fashion that was sufficient to alter the reactions. It is therefore apparent that for those ligands $K_{I\text{-}L} = K'_{I\text{-}L}$.

The binding of CBZ-Ala-Ala gave a unique result in this study. The addition of this mobile ligand caused decreased retardation of chymotrypsin on immobilized phenylbutylamine (*17*). The effect was not directly competitive, however, since even at saturating concentrations of CBZ-Ala-Ala, the zone of protein did not elute at a volume equal to V'. A sample of chymotrypsin inactivated by TPCK was used to define V'. This was also measured by the emergence of autolysis products and by the passage of a sample of trypsin through the same column. Since the complex of chymotrypsin and CBZ-Ala-Ala eluted later than V' defined by three experiments, we may conclude that again a ternary complex is formed with $K_{I\text{-}L} < K'_{I\text{-}L}$ for this case (8.57 vs 15.6 m*M*).

Positive and negative effects were also observed by Danner et al. in the chromatography of carboxypeptidase B on agarose-D-phenylalanine (*13*). The mobile ligands D-Phe and *N*-acetyl-D-Phe gave straightforward competitive elution of the enzyme with $K_{M\text{-}L}$ of 9.7 and 7.8 m*M*, respectively. The mobile ligands β-phenylpropionic acid and ϵ-aminohexanoic acid produced enhanced retardation of the protein, analogous to the effect of CBZ-aromatic amino acids on chromatography of chymotrypsin discussed above. Again, the expanded binding scheme [Eq. (10)] was able to describe the data and yielded dissociation constants for the binary complexes of 1.8 m*M* for CPase B · β-phenylpropionic acid and 0.65 m*M* for CPase B · ϵ-aminohexanoic acid.

Zonal elution chromatography has also proved of value in the study of systems where higher order interactions are possible (*9*). Eilat and Chaiken have described the binding of the IgA myeloma protein of BALB/c plastocytoma TEPC 15 to columns of phosphorylcholine-Sepharose (*18*). The bivalent immunoglobulin A monomer interacted with a column containing a low density of immobilized ligand in a fashion consistent with Eq. (4). However, when the immobilized ligand was present at higher density, so that the bivalent antibody could interact with two immobilized species at once, deviations from linearity could be observed, indicating that bivalent reactions were occurring. The practical result was that binding appeared to be tighter when the density of I-L was higher. Chaiken et al. derived an expression (*10*) to describe these interactions based on the following scheme:



where the possibility of binding two mobile ligands, M-L, or two immobile ligands, I-L, is allowed. To simplify the derivation, Chaiken et al., made the assumption (*10*) that the equilibrium constants for binding im-

mobilized ligand were equivalent for binding to free protein, protein-mobile ligand complex, or protein-immobilized ligand complex. Likewise, the equilibrium constants for binding immobilized ligand to free protein, protein-mobile ligand complex, or protein immobilized-ligand complex are assumed to be identical. This yields the expression:

$$1/(V - V') = \{1 + 2([M\text{-}L]/K_{M\text{-}L}) + [[M\text{-}L]/K_{M\text{-}L}]^2/(V' - V_o)\,[2([I\text{-}L]/K_{I\text{-}L}) + [[I\text{-}L]/K_{I\text{-}L}]^2 + 2[M\text{-}L][I\text{-}L]/K_{M\text{-}L}\,K_{I\text{-}L}]^2\}$$

that fit the experimental data at higher density of immobilized ligand and permitted extraction of the macroscopic constants $K_{I\text{-}L}$ and $K_{M\text{-}L}$.

A further expansion of the application of zonal affinity chromatography to more complex systems was achieved by Angal and Chaiken in their study of neurophysin binding to Met-Tyr-Phe immobilized on agarose (*3, 8*). Initially, competition between immobilized ligand and mobile lysine–vasopressin was demonstrated. However, at [M-L] near zero, some deviation from linearity was observed. In addition, the elution volume of the protein was shown to increase dramatically as the concentration applied was increased. Furthermore, these authors prepared an affinity matrix in which neurophysin was immobilized and then observed the retardation of a zone of soluble protein. In this case, the addition of soluble lysine–vasopressin increased the retardation of the soluble neurophysin. These observations were rationalized by a scheme in which neurophysin is able to undergo a reversible monomer–dimer equilibrium. The experimental observations permitted the evaluation of the monomer–dimer dissociation constant and a consideration of the effect of added mobile ligands upon that equilibrium.

FRONTAL ELUTION CHROMATOGRAPHY

In this review, the discussion of experimental achievements has been arbitrarily divided into sections based on the experimental approach taken. As will be seen below, several investigators have, in fact, applied a variety of techniques to a single system. In those cases where a comparison has been made, the results are independent of the methodology employed, thus implying that all approaches have validity. It is important, however, to recognize the differences between the methods and to stress the special advantages offered by each technique. In this section frontal elution methods will be described. In this approach, a solution containing the protein of interest is applied continuously to an equilibrated column until the activity of the protein emerging from the column matches the activity applied at the top of the column. The major advantages of this procedure are twofold. Firstly, the concentration of protein is usually sufficiently high so that it is quite easy to measure the activity or concentration in the effluent. Thus the problem of very shallow peaks

seen in zonal elution experiments is avoided. Also, definition of a plateau of activity is easier than definition of a peak of activity from a zone and so the elution volume can normally be measured with more accuracy. Secondly, the experimental setup is optimal for measuring the dependence of elution volume on the concentration of protein (or macromolecule) species. Thus, the evaluation of $K_{I\text{-}L}$ can be based on a greater number of data points.

Despite these advantages, the frontal elution procedure does suffer from the disadvantage that it requires large amounts of sample in most cases. This will make this method less valuable for many investigators who have isolated small amounts of biomolecules. Nevertheless, valuable results can be obtained using this procedure.

A most valuable contribution to the literature of quantitative affinity chromatography was provided by Nichol et al. in 1974 (*47*). They considered the various possible equilibria between two soluble components and an immobilized species. Combining expressions for the total concentrations of the macromolecule with contributions for partitioning in a gel matrix permitted the derivation of a general expression for column elution. Nichol et al. then considered various special cases within their general derivation that correspond to the types of binary and ternary complexations that are observed (*47*).

In the study of Brinkworth et al. (*6*) already described in the preceding section, the chromatography of lactate dehydrogenase on Sepharose–oxamate was examined by frontal elution methods. Addition of NADH increases the retardation of the protein implying that the binary complex P · NADH, has an affinity for the immobilized oxamate. The expression utilized to evaluate the data, recast in the terminology utilized in this review is:

$$1/(V - V') = \{1/(V_o\,[\text{I-L}]\,[\text{M-L}]/K'_{I\text{-}L}K_{M\text{-}L})\} + \{1 + ([\text{P}]/K_{I\text{-}L})\}/(V_o\,[\text{I-L}]/K'_{I\text{-}L}) \quad (11)$$

In this expression $K'_{I\text{-}L}$ refers to the binding equilibrium between the LDH–NADH binary complex and immobilized oxamate. (Since the free enzyme did not bind, $K_{I\text{-}L} = \infty$). The main difference between this expression and the previous ones given earlier is the presence of the [P] term in the numerator of the right-hand term. In zonal elution experiments, this concentration is negligible and this term drops out. The data of Brinkworth et. al. allowed the evaluation of the binding constant between enzyme and NADH when iteration was used to fit a plot of $1/(V - V')$ versus 1/[M-L] (*6*). This was necessary since the equation is defined for the free concentration of NADH. In situations where [M-L] >> [P] there will be no difficulty, but in limiting cases where these concentrations are similar, the procedures of Brinkworth et. al. can be used to define the limiting tangent in the double reciprocal plot (*6*).

Beginning in 1975, Kasai and Ishii have published a series of papers (*31–34, 37, 50, 68*) dealing with the quantitative affinity chromatography

of trypsin. In a preliminary communication they gave a derivation for frontal elution chromatography in the presence of a mobile phase containing competitive inhibitors (*32*). They demonstrated that a linear relationship existed according to the following equation:

$$V_i = V_o + K_i(V - V_i)/[I_o]$$

where V_i is elution in the presence of inhibitor, V is the elution in the absence of inhibitor, K_i is the dissociation constant for mobile ligand–protein complex, and $[I_o]$ is the total mobile ligand concentration. Kasai and Ishii plotted V_i versus $(V - V_i)/[I_o]$ for a series of concentrations of mobile ligand from frontal elution patterns. This derivation is equivalent to others we have already described, but the plotting format is awkward for two reasons. First, the measured elution volume appears on both sides of the equation. Second, their method requires knowledge of the elution volume in the absence of mobile ligand, V. This cannot be readily measured for systems where there is a large amount of retardation, i.e., the ratio $[I\text{-}L]/K_{I\text{-}L}$ is large, but could be readily measured in this case. The equation can be rewritten using our definitions as follows:

$$V = V' + (K_{M\text{-}L}/[M\text{-}L])[V' + (V' - V_o)([I\text{-}L]/K_{I\text{-}L}) - V]$$

which can be rearranged to be equivalent to Eq. [3].

The affinity matrices prepared by Kasai and Ishii were derived by coupling agarose to a mixture of di- and tri-peptides obtained from trypsin digestion of protamine (*31*). These peptides terminate in arginine, thus providing a biospecific ligand for trypsin from cow and from *Streptomyces griseus* (*68*). In later studies, Kumazaki et al. prepared a matrix using Gly-Gly-Arg as the unique ligand and demonstrated that the properties were virtually identical to those of the mixture (*37*). They explicitly stated the point that a system of low affinity can be advantageous in an analytical study. They have also conducted experiments at negligibly low concentrations of enzyme to permit use of their expression in frontal elutions.

In 1977 Nishikata et al. published an important paper on the interaction of trypsin with oligopeptides resembling substrates or product as studied by quantitative affinity chromatography (*50*). One conclusion they obtained was based on a comparison of Gly-Ala-Arg, Gly-Gly-Arg, and Gly-Val-Arg as affinity ligands. Gly-Ala-Arg gave the lowest $K_{I\text{-}L}$ value (0.048 m*M*) about three times better than Gly-Gly-Arg (0.13 m*M*) or Gly-Val-Arg (0.14 m*M*). This order was confirmed by a study of inhibition of trypsin by the soluble *N*-acyl derivatives of these tripeptides. This careful analysis points out the sensitivity of the chromatography method to structural change. In addition, the length of the immobilized ligand affected the interaction with trypsin with a tripeptide being more effective than a dipeptide.

These authors also used a very clever method to ascertain how much of the immobilized ligand is actually accessible to the enzyme. The

ligands were immobilized as methyl esters and the amount of the methanol released on exposure to enzyme was quantitated. In this case, almost all the immobilized ligands were accessible.

In later publications, where several other conditions were varied (pH, temperature, ionic strength, dielectric constant), Kasai and Ishii made a number of excellent points about the value of quantitative affinity chromatography as a method for studying ligand–protein interaction (*34*). The method can give good data at pHs far from the pH optimum for activity and absolutely pure protein is not required. Furthermore, unlike difference spectroscopy, no special chromophores are required.

Finally, Kasai and Ishii have described a series of experiments in which the protein component, trypsin, is immobilized and the ligands are then passed through in a frontal elution experiment (*33*). Again, equations can be derived to permit the calculation of binding constants from elution volume data. These studies allow the evaluation of the integrity of the immobilized protein and these authors report that trypsin was not affected by the immobilization process. These authors also note that nonspecific interactions can limit the value of this technique, but these are easily detected in this system.

Frontal elution methods were also employed by Katoh et al. in a study of the binding of trypsin to Sepharose-immobilized soybean trypsin inhibitor or arginine peptides (*35*) prepared according to the methods of Kasai and Ishii (*31*). Ishiwata and Yoshida have also employed the procedures of Kasai and Ishii in their study of ribonuclease T binding to guanyl-(2′-5′)-guanosine coupled to aminohexyl-Separose (*29*).

At this point we can refer to three studies in which frontal elution techniques were compared (*43*) with zonal elution methods. In these systems agreement was seen between the two sets of results. Malanikova and Turkov directly compared trypsin binding to immobilized benzamidine (*43*), Dunn and Gilbert utilized both methods (*17*) for analysis of chymotrypsin binding to 4-phenylbutylamine-Sepharose (*17*), and Liu et al. used these methods (*38*) for their study of lactate dehydrogenase binding to immobilized dyes.

Next, we will consider several studies where systems involving multivalent solutes have been studied by frontal elution methods. Nichol et al. have provided the most rigorous approach to this type of system in their study of aldolase-phosphate interactions with cellulose phosphate as the affinity matrix (*49*). The main value of their approach is to systems where there is weak binding between the components and large amounts of protein are available for study.

Oda et al. expanded their studies of frontal chromatography to concanavalin A binding to immobilized *p*-aminophenyl-β-D-glucopyranoside (*55*). These authors have derived expressions for binding of multivalent species to ligands for the specific case where only one binding site can interact with the affinity matrix and where the binding sites are identical and independent. This is a normal assumption in all studies

of multivalent systems. A further condition in this approach is that the concentration of protein must be less than the value of the intrinsic dissociation constant, $K_{I\text{-}L}$. The $K_{I\text{-}L}$ values were measured for the dimeric form of concanavalin A and for the β-form of the protein (where there is a single split between residues 118 and 119) to be 0.12 and 0.26 m*M*, respectively. Again, the affinity for the immobilized matrix is not high thus the frontal elution method works quite well. Competitive effects were then studied for ligands known to interact with the binding sites and a linear relationship was observed when the data was plotted according to equations derived by Oda et al. Fifteen different ligands were analyzed by their methods. The data give reasonable agreement with information available from other methods and establish the validity of their approach.

Kumagai et al. have utilized tubulin-Sepharose to study calcium dependence of calmodulin binding (*36*). A value of 2.1 μ*M* at 55 m*M* KCl and 4.0 μ*M* at 200 m*M* KCl was obtained for the dissociation constant for the calmodulin–tubulin complex in the presence of calcium. The Hummel-Dryer method of gel filtration was also employed to study this association and a value of 3.5 μ*M* was obtained.

A thoughtful analysis of the binding of proteins to immobilized residues has been provided by Jennissen (*30*). In this study he has considered the transition from a ligand density where univalent binding is obtained to a density where multivalent adsorption will occur. Equations are developed to describe cooperative, multivalent adsorption–desorption loops. In attempting to apply this analysis to zonal elution of phosphorylase b, on butyl-Sepharose, Jennissen has noted that nonsymmetrical peaks are obtained (*30*). This indicates that, even at a low degree of ligand substitution, some adsorption hysteresis is present. Since the nature of the interaction of this protein with butyl-Sepharose is undoubtedly of the hydrophobic type, it is perhaps not surprising that multiple interactions are required for retention which can then lead to the hysteresis observed.

GRADIENT ELUTION AND BATCH ADSORPTION TECHNIQUES

In this section we will describe a variety of studies that have employed other techniques to study binding. Although much of the data described cannot readily be analyzed by the equations presented in preceding sections, many of these studies provide important practical results as well as critical theoretical background.

A specific case in this regard is the seminal study of Akanuma et al. dealing with the binding of carboxypeptidase B to D-phenylalanine coupled to carboxymethyl-Sephadex (*1*). This was one of the earliest studies to note the power of affinity chromatographic methods for providing di-

rect evidence for ternary complex formation. They predicted that soluble ligands that do not directly compete for the same binding site as the immobilized ligand could produce three types of effects on elution volume, positive, negative, or neutral. The effect would depend on the relative strength of interaction for immobilized ligand of the free enzyme and the enzyme–mobile ligand complex. Such a positive effect was shown in the case of carboxypeptidase B binding to immobilized phenylalanine. No significant binding was seen in the pH range 7–9 unless ϵ-aminocaproic acid was added. Therefore, it is apparent that the carboxypeptidase ϵ-aminocaproic acid binary complex binds well to immobilized Phe to make a ternary complex. If the concentration of ϵ-aminocaproic acid in the eluting buffer was decreased exponentially after carboxypeptidase was absorbed, the enzyme eluted when the concentration decreased to about $10^{-7}M$. On the other hand, the *addition* of β-phenylpropionic acid to a similar column of carboxypeptidase·ϵ-aminocaproic acid bound to immobilized Phe caused the elution of the enzyme, presumably as a carboxypeptidase·ϵ-aminocaproic acid·β-phenylpropionic acid ternary complex. These results were later confirmed and extended by Danner et al. using slightly different matrices and employing zonal elution techniques (*13*). Also, the positive, negative, and neutral effects predicted by Akanuma et al. (*1*) were observed by Dunn and Gilbert in their study of chymotrypsin affinity chromatography (*17*).

O'Carra and Barry were also among the first to recognize the complexities as well as the advantages of affinity chromatography of multisubstrate enzymes (*4, 53, 54*). In their studies direct evidence was accumulated for the compulsory-ordered mechanism for lactate dehydrogenase. They also demonstrated that it is the nicotinamide end of NADH that induces a site for binding of pyruvate-type compounds wheras the other end of NADH is involved in the major interactions with the enzyme surface (*53*).

O'Carra and Barry utilized a column of immobilized oxamate for binding LDH and described conditions to eliminate troublesome ion exchange effects (*53*). LDH was shown to bind to the affinity matrix only in the presence of NADH, which established that the binary LDH–NADH complex is the first species formed. An advantage to the use of this ternary complex formation principle for isolation is that nonbinding proteins may be eliminated by washing the column with NADH present and then elution by termination of NADH addition would leave behind any protein adsorbed to the affinity matrix by nonspecific effects. Although NAD^+ provided some retardation of LDH, this could be competitively abolished by adding pyruvate to the mobile phase. These observations are consistent with the quantitative differences in NADH and NAD^+ binding.

O'Carra and Barry have also utilized considerations of binding affinities in their extensive study on various immobilized dinucleotide derivatives for the dehydrogenases (*4*). These authors emphasized the

vital point that the method of immobilization can have an important effect on mechanistic studies. Unambiguous chemistry is essential for reliable mechanistic study.

In a later study, O'Carra et al. utilized differences in binding of the H and M forms of LDH to immobilized oxamate in the presence of NAD^+ to obtain a separation of these isoenzymes (*54*). This is a perfect example of the application of quantitative differences to a practical separation problem and is especially notable since it appeared at a very early stage.

Mosbach and his colleagues have pioneered in the application of "general-ligand" affinity chromatography, where a cofactor such as NAD^+ is immobilized to form an affinity matrix for several proteins (*56*). Specific elution conditions are then found to provide separation of various enzymes in a mixture. One such method is the application of a gradient of increasing NADH concentration. Differences in the *two* affinity constants that are operational, $K_{I\text{-}L}$ and $K_{M\text{-}L}$, provide further opportunities for separation of proteins. These initial studies were followed by publication of a general method for determination of dissociation constants for dehydrogenase–coenzyme complexes (*7*). A linear relationship was observed between the eluting concentration of nucleotide and the dissociation constants measured by other techniques. Objections to this approach include the difficulty inherent in accurately assessing the precise concentration when elution begins and the empirical nature of the linear relationship. Despite these recognized problems, excellent agreement has been obtained for known cases, and the new cases where the $K_{M\text{-}L}$ values are interpolated are in agreement with subsequent analyses. These authors point out again the advantage that one can begin with a crude enzyme preparation and obtain purification as well as quantitative data in a single experiment.

Yokosawa and Ishii have compared anhydrotrypsin (with Ser 183 converted to dehydroalanine) to native trypsin with respect to its binding to immobilized arginine containing peptides (*69*). Their main conclusion was that the derivative bound product-like species more tightly. In this study, Yokosawa and Ishii utilized a gradient elution procedure and have reported the concentration of mobile ligands that allowed elution without equating these values to $K_{M\text{-}L}$ constants.

Rosenmeyer and Seela employed a linear gradient approach to measure the binding of adenosine deaminase to polymer-bound inosine (*58*). By assuming that the value of $K_{I\text{-}L}$ is equivalent to the value of $K_{M\text{-}L}$, they have derived an expression that allows calculation of the dissociation constant from a single experiment in which bound enzyme is eluted with a linear gradient. Although this approach appears to give valid results for this sytem, there is no reason why the operative dissociation constants should be so similar in other systems. Therefore, their simplification is not recommended for most other studies. Rosenmeyer and Seela also have measured the concentration of mobile ligand required for biospecific elution at a variety of temperatures and used this

data to calculate enthalpy and entropy of interaction (*58*). These results were consistent with the suggestion that hydrogen bonding is of fundamental importance to the binding process.

The remaining studies described in this section employ batch wise methodology to study protein ligand binding. I have included studies where protein is bound and then flushed off by a stepwise change in elutant. I wish to emphasize again that, although quantitation is difficult because of the change in conditions, many of these studies provided the impetus to attempt the more precise analyses described earlier.

Gawronski and Wold published two papers in 1972 dealing with the S-peptide S-protein system of ribonuclease described earlier (*22, 23*). The S-peptide was covalently attached to agarose and the association of the S-protein was studied by quantitation of the amount of free protein as a function of concentration. Because of problems with aggregation of S-protein at lower pH values, the reverse system of immobilized S-protein and soluble S-peptide was also studied. Both methods gave $K_{I\text{-}L}$ values of 1–2.5 $\times$ $10^{-6}M$. It is also possible to demonstrate that the resulting complex, RNase-S'-agarose, was enzymatically active, thus establishing that the complex is formed in a biospecific fashion. In the second paper, Gawronski and Wold extended their studies to measure the thermodynamic parameters for the dissociation (*23*). This data permitted the conclusion that the binding process is entirely entropy driven to be made.

The studies of Mosbach and his collaborators on general ligands in affinity chromatography were quoted earlier in this section (*7, 56*). In one of their earlier publications, they discussed the application of selective elution with different mobile ligands to the separation of several related enzymes (*46*). This study makes use of the general principal that differences in values of $K_{I\text{-}L}$ or $K_{M\text{-}L}$ will lead to different elution volumes. Thus, although no quantitation was attempted, the experiments provided the rationale for later quantitative studies.

Another early critical paper was the study of Nishikawa et al. that specifically discussed the issues of affinity of the interactions between a protein and an immobilized ligand and the concentration of that immobilized species (*51, 52*). They employed the trypsin-immobilized benzamidine system for their practical study and used equilibrium-binding experiments in a batchwise fashion to measure the amount of protein bound. Plots were then made of the measured concentration of bound enzyme versus total enzyme added and compared to ideal Langmuir adsorption isotherms. This study pointed out several practical problems, including inaccessible binding sites and unequal distribution of immobilized ligands when diluted with underivatized gel.

Dean and his colleagues have conducted a thorough study of binding of dehydrogenases and kinases to immobilized cofactors (*26, 27, 39–42*). In an early study using immobilized NAD^+, quantitation of bound protein indicated that only 1% or less of the immobilized cofactor

was available for binding although the undefined nature of the linkage is problematic (*41*). A very important observation was that the amount of immobilized ligand was more critical than the amount of soluble macromolecule. In later studies, this group has utilized chemically defined adsorbents to permit more critical analysis (*26*). They made the point that a group of enzymes may interact in different ways with a general ligand and, thus, affinity differences are to be expected. The binding strength of three proteins was shown to be linearly dependent on the concentration of immobilized ligand, although it should be noted that the binding strength was ascertained by measuring the KCl concentration required to displace bound protein. In a study of glycerokinase and LDH, the influence of column length and incubation time was determined (*42*). Practical suggestions were derived for the optimal arrangements for successful isolations. In an extension of this work, temperature changes were shown to influence the binding in such a fashion that selective elution can result (*27*). Again these results are derived from salt elutions and the temperature variation in ionic strength is a complicating parameter. Using the finding that longer incubation times gave greater binding, a final study was carried out in which incubation times of 16 h were used (*41*). The resulting binding was measured and the data analyzed to yield an apparent dissociation constant of 1.1 μM. Based on the extremely long time required for equilibrium to be achieved, there are two possible interpretations. The diffusion of enzyme through the matrix could be severely limited, or the successful binding could require multivalent attachment. They also demonstrated inhibition of binding by soluble NADH and derived a simple relationship to permit calculation of $K_{I\text{-}L}$ assuming 22 μM for $K_{M\text{-}L}$. Differences were also noted between batchwise and column procedures.

McLoughlin et al. have more recently employed immobilized NAD with different modes of attachment and various elution procedures in an effort to optimize separation of glycerol-3-phosphate dehydrogenase (*45*).

Tsapis et al. investigated the binding of human hemoglobin and its isolated α- and β-chains to agarose-immobilized haptoglobin by column and batchwise techniques (*63*). They concluded that immobilized haptoglobin retained its affinity for hemoglobin chains. This data was analyzed by traditional Scatchard relationship.

Bottomley et al. have developed a method for studying protein–ligand binding that is dependent upon measurements of the quantity of bound species and not upon elution volumes (*5*). This procedure depends upon equilibration of a column with a binding component in the mobile phase, followed by perturbation with an added ligand and measurement of the released binding component. This methodology and the resulting equations were applied to insoluble myosin (mixed with Sephadex G-50) or to myosin coupled to Sepharose. The binding of

nucleotides was quantitated and the results were shown to agree with literature values (*67*).

Fex et al. utilized affinity chromatography to study the interaction between prealbumin and apo- or holo-retinol-binding proteins (*19*). The apo form showed a higher affinity for immobilized prealbumin.

Several studies have been published in which proteins are eluted from ion-exchange columns by addition of substrates or inhibitors. An example of this principle of affinity elution has recently been described by Scopes (*59*).

Turkova et al. have attempted to utilize the amount of binding of pepsin to a matrix of immobilized L-Phe- D-Phe-OCH_3 to calculate the equilibrium constant for the complex (*64*). They employed the relationship derived by Graves and Wu (*25*). Although an acceptable fit was obtained for data from three different enzyme species, these results are clouded by the fact that ion exchange effects are operative in this system. Turkova et al. utilized elution by 1*M* NaCl to remove bound enzyme. Chernaya et al. have described problems that arise in the affinity chromatography of the very acidic aspartyl proteases (*12*). The immobilized L-Phe-D-Phe-OCH_3 employed by Turkova et al. (*64*), also bound a sample of pepsin that was totally inhibited by pepstatin, thus indicating that the matrix binding does not involve the active site.

THEORETICAL STUDIES AND SUMMARY

In this final section, I will refer to several general treatments of the theoretical basis of affinity chromatography that have provided a foundation for the practical studies described in the preceding sections.

Freeman has considered the influence of chemical interactions on separations in a gel matrix as a mechanism for increasing resolution in column chromatography (*21*). He derived equations in terms of distribution coefficients for solute-gel interaction, for solvent–gel interaction, and for solvent–solute interaction that he termed masking. Following this theoretical work he prepared a copolymer of 2-methyl-5-vinylpyridine with divinyl benzene and demonstrated that acidic solutes interacted with this gel according to the equation he had provided (*20*).

Graves and Wu employed an equilibrium model in their analysis of affinity chromatography, although they began with a major emphasis on batch adsorption methods (*25*). In their derivation they also found it convenient to consider the concentration of protein species to be much less than the concentration of immobilized ligand and, therefore, negligible. They presented an expression for the determination of $K_{I\text{-}L}$ and the capacity of a given gel from measurements of bound enzyme as a function of total enzyme concentration. Making some simplifications, Graves and Wu obtained a simple relationship that is equivalent to Eq. (3). The hy-

perbolic dependence of the fraction of enzyme bound on the concentration of immobilized ligand was then intuitively analyzed to allow predictive statements to be made. Of particular interest is the statement that, at low values of protein concentration and relatively weak binding ($K_{I\text{-}L}$ = $10^{-3}M$), leakage of protein will occur long before saturation of the capacity of the column.

By considering the addition of a solution containing a competitive inhibitor, Graves and Wu have calculated the redistribution of protein from the gel-bound phase to the soluble P·M-L complex (*25*). They were then able to analyze the influence of volume and $K_{M\text{-}L}$ and $K_{I\text{-}L}$ on the recovery of protein from a batchwise elution experiment. This permitted prediction of the concentration of soluble inhibitor necessary to effect elution.

Next, these authors used the approximate equation to simulate a column washing experiment by assuming that the column consisted of only one well-stirred compartment. Although this is an approximation, it provides a very useful way to predict the success of an affinity chromatographic purification. They consider a binding step, followed by washing with 10 volumes and elution with 20 volumes. For weak binding to immobilized ligand, a large portion will be lost during washing. If a change in $K_{I\text{-}L}$ from 10^{-5} to 10^{-1} can be achieved by changing conditions, the equations of these authors predict a 98.9% recovery of applied activity. It is reassuring to note that much of the experimental work discussed in this review demonstrates that these predictions are quite accurate.

Porath and Kristiansen considered biospecific separations in terms of the fundamental equilibria involved (*57*). Based on these considerations they were able to make suggestions concerning the most efficient way to achieve separation of a particular molecule. They recommend the use of gradient elution to provide a gentle change in conditions without denaturing the protein of interest. They also considered situations in which a third component is required for biospecific complex formation. This review is notable because it bridges the gap between the considerations of binding that we have been describing and the practical side of how the knowledge of these constants can be useful in designing an efficient affinity separation.

Denizot and Delaage employed a statistical approach (*24*) to a consideration of binding and releasing steps during an affinity chromatographic experiment (*14*). Equations were derived to permit the evaluation of the rate constants for binding and dissociation process from the position and width of the elution peaks. The ratio of these two rate constants would yield the equilibrium constant for the process. However, in practice, the rate-limiting steps in affinity chromatography are usually diffusion through the pores of the matrix. Thus, the formulations of these authors will only be valid for low capacity, low porosity systems in which surface binding is permitted.

Hethcote and DeLisi have conducted a thoughtful analysis of affinity chromatography in term of chemical kinetics and mass transfer kinetics (*28*). They state the important point that binding molecules to a surface has important similarities to events that occur on cell surfaces or membrane surfaces. Again, since the width of a zone of protein eluting from a column contains important information about the distribution of that protein, and, hence, about rate processes, Hethcote and DeLisi confined their analysis to zonal elution chromatography. Equations were derived to cover many different situations, including fast and slow chemical steps and penetrable as well as impenetrable beads. These authors discuss the limitations of various formulations and show that most are similar under the appropriate conditions, which must be appreciated by each investigator. In an effort to avoid some of the limitations of slow mass transfer, Hethcote and DeLisi are now employing reversed-role chromatography where the protein is immobile and the small ligands are in the mobile phase. There are usually no limitations on mass transfer of small ligands.

Winzor et al. have expanded their consideration (*2, 6, 44, 47–49, 62*) of expressions to describe the equilibrium composition during affinity chromatography of a multivalent protein on a multivalent matrix with a univalent ligand present (*66*). With this background, Winzor et al. discuss plotting techniques and indicate diagnostic features of specific cases that can be readily observed.

Finally, I would like to close this review with some suggestions based on our experiences with affinity chromatography. We have pointed out that the elution of a zone of protein from an affinity column will be governed by Eq. (9). It should be clear that the larger the ratio of immobilized ligand to the dissociation constant for immobilized ligand–protein complex $[\text{I-L}]/K_{\text{I-L}}$, the larger the elution volume will be. This could make our determination of V in that case impossible. Therefore, if one wished to use such an experiment to determine a value for $K_{\text{I-L}}$, it is advisable to begin with a matrix with a lower concentration of immobilized ligand. In addition, Eq. (9) can also provide an explanation for certain "failures" of affinity chromatography. If an extract is applied to a column and then exhaustively washed with starting buffer, it is conceivable that the protein of interest could be eluted as a broad peak with a large elution volume. When the column is subsequently eluted with a buffer designed to disrupt the association and elute the protein, nothing would be obtained. The operator might then incorrectly conclude that the protein was irreversibly bound to the matrix.

Another result from our analyses concerns the use of the reciprocal Eq. (4) to derive values of $K_{\text{M-L}}$ from the intercept/slope method, Eq. (4–7). In certain cases, the value of the intercept we obtained was extremely small, such that the numerical value was unreliable. An examination of Eq. (5) for the intercept of plots of $1/(V' - V_o)$ versus [M-L] shows that the value of the intercept will be smaller as the size of the

column gets larger, $(V' - V_o)$ and as the ratio of $K_{I\text{-}L}$/[I-L] gets smaller. Adjusting the column size is straightforward, although one must be careful not to use such a small column that the amount of protein approaches the amount of immobilized ligand. The ratio $K_{I\text{-}L}$/[I-L] can most easily be altered by reducing the concentration of [I-L] that will increase the ratio and, hence, increase the value of the intercept. If this is not practical, then it may be necessary to utilize a different immobilized ligand (different value of $K_{I\text{-}L}$). With all these parameters as potentially adjustable factors, it should be appreciated that there is great flexibility in experimental setup to achieve the objectives of deriving quantitative binding data.

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