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Design Parameters in Affinity Chromatography

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DESIGN PARAMETERS IN AFFINITY CHROMATOGRAPHY

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INTRODUCTION

Well over 400 papers have been published in the past few years which describe the application of the affinity method to the purification of biopolymers. It is fair to say that most are concerned only with solving a unique problem at hand, be it to purify a given enzyme, antigen, or whatever. Very likely the majority of the scientists with the isolation or purification problem at hand are not in a situation to consider more than a few variations on the several stock "recipes" in the literature. If only some of the tricks should happen work, then the results may be reported as a partial purification of the material. If the attempts do not work outright, of course they do not see print. It is not clear that the failures are understood or why only partial purification was achieved.

Yet as early as 1967 a set of guidelines was published which pointed out the criteria important to the preparation of affinity

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sorbents. In his book entitled Design of Active-site-directed Irreversible Enzyme Inhibitors, the late Baker [1] included a chapter (the last) on "Enzyme-Specific Columns." Here was spelled out the need for inert, hydrophilic insoluble carriers, as well as the importance of knowing what part of the ligand molecule could be used for immobilization ("bulk tolerance") without losing affinity for enzyme. Baker also suggested the need for obtaining an optimum density of functional groups in the carrier matrix so that efficient binding would result.

While Baker's suggestions went largely unnoticed, a paper from a group at the N.I.H. proclaimed "affinity chromatography" [2] and introduced the notion of a spacer-arm (we prefer the term "leash") molecule. This was recommended because, when the ligand was attached to the insoluble carrier via a leash or tether moiety, its interaction with active-site of an enzyme was optimized. Enzymes whose active-sites are located in deep clefts had steric problems in binding to ligands which were directly attached to insoluble surfaces. The leash is not a universal requirement however, and may even cause nonspecific binding problems [3], as we shall see.

Another important parameter is gel porosity. Cuatrecasas has observed that the affinity binding of an enzyme with a molecular weight of 17,000 daltons (staphylococcal nuclease) required the use of 4% agarose gel, which has a molecular weight exclusion limit of 15 million daltons [4]. The need for this high porosity is not yet clearly understood.

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Even with knowledge and application of the preceding criteria, success has not always presented itself in the preparation of an affinity sorbent. Thus there must be other factors which must be considered. One which is receiving more attention recently is the adventitiously introduced ionic groups in the affinity gel which result in nonspecific binding of proteins. A second is the nonspecific hydrophobic interactions due to leash (and ligand) groups introduced into gels. And a third is the efficacy of affinity binding as affected by the concentration of ligands which have been bonded into the sorbent [5-7]. These topics will be the central concern of this paper. By proper evaluation and understanding of these parameters, we should then be better able to tackle the yet uncharted affinity purification problems.

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IONIC EFFECTS

Because proteins are polyelectrolytes, one can expect ionic functional groups on any chromatographic sorbent to affect their elution behavior. These interactions are, of course, central to the more established technique of chromatography on ion-exchange celluloses. In affinity chromatography the presence of ionic groups in the ligand, leash, or matrix itself may give rise to problems of nonspecific binding.

Leftover Leashes

One source of extraneous ionic groups in the matrix can be the leftover charges due to incomplete attachment of ligands onto leashes. Such a situation is shown in FIG. 1. In the upper

FIG. 1

part of the diagram, a carboxylate anion due to unreacted caproic leash moiety is forming a salt with the cations on the surface of the enzyme. In the lower portion we see the expected active site interaction of the benzamidine ligand. The nonspecific interactions due to the caproic anions can be substantial if they are present in large amounts-perhaps as little as 10% of all of the functional groups. These problems were encountered by Hixson and Nishikawa [5] in their trypsin and thrombin studies.

They therefore used the percolation of chymotrypsin through affinity gel columns as a test for functional specificity. As can be seen in FIG. 2, chymotrypsin and trypsin share similar physical properties. Since they are both polycations at pH 8, we can expect both proteins to be bound or retarded in flow rate when passing through a gel containing many free caproate anions. However, a properly prepared benzamidine gel exhibits none of these anions. Thus, owing to the strict and different substrate specificities of the enzymes, the trypsin will be adsorbed to

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TRYPSIN		CHYMOTRYPSIN
23,600	M.W.	25,000
10.1	pI	8.6
	SPECIFICITY	
LYS, ARG		TRYP, TYR, PHE

FIG. 2

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the properly prepared gel while the chymotrypsin will not.

To avoid having these incompletely reacted leashes in the gel, Wilchek [8] has encouraged the strategy of attaching the ligand to the leash moiety first, then introducing the ensemble into the carrier gel. However, for many workers such a course requires much more skill and work in organic chemical synthesis compared to the effort of doing a sequence of reactions on an insoluble gel. FIG. 3 shows a comparison. The in vitro approach requires a protecting group on a bifunctional ligand (Cw) which is coupled to a protected leash moiety (yB). The protecting group (y) is removed from the leash which is then coupled to a second protected leash moiety (zA). The leash (z) and ligand (w) protecting groups are removed and the ensemble attached to the gel (G-x). Each of these steps (coupling as well as deprotecting) requires purification of the product before the next step is assumed. Depending on the complexity of the ligand-leash

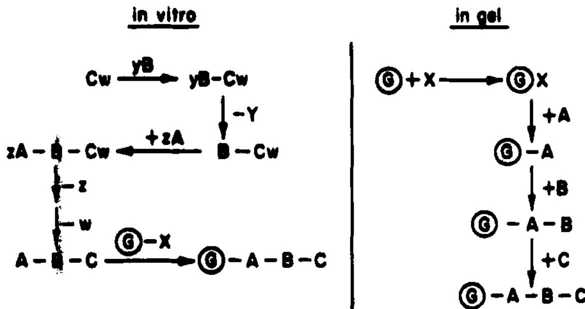

FIG. 3 Strategy for affinity gel preparation.

structure, the in vitro route may take several days to complete synthesis.

By comparison the in-gel approach is much simpler in that excess or unreacted reagents from a given reaction step are easily washed out of the gel particle and the following step is readily assumed. Such has been the attraction of Merrifield's solid-phase peptide synthesis (see J. M. Stewart's article elsewhere in this volume). Because covalent coupling to a gel results in the insolubilization of a compound, that compound may often be bifunctional and used without a protecting group for the second functionality. To avoid the problems of leaving unreacted leash moieties within a gel, care must be taken to drive each reaction step to 100% completion. This is usually done by using large excesses in reagent. Aliquots of gels should be taken after each step and tested by some appropriate means for completeness of reaction.

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Leash Linkage

Another source of unwanted charges may arise from the linkage connecting leash to gel. Here we focus our attention on the most frequently used reaction of cyanogen bromide on agarose gel.

This reaction, which was developed by Porath and co-workers [9, 10], has been used extensively to couple organic amines to polysaccharide carriers. Several structures have been proposed to describe the final mode of linking of the amine to the polysaccharide when cyanogen bromide is used. Of these, the isourea linkage has the potential for generating a positively charged group. The reaction pathway outlined in FIG. 4 indicates how this is possible. In agarose, the 1,3-propanediol structure is most likely involved in the primary reaction with BrCN. The formation of the cyclic imidocarbonate intermediate has been strongly supported by model compound studies using methyl 4,6-O-benzylidene-α-D-glucopyranoside [11] and trans-1,2-cyclohexanediol [12]. In our own studies (Nishikawa, unpublished observations) it appears that BrCN does not react readily with isolated hydroxyl groups to form even the unstable cyanate.

The formation of an isourea linkage is supported by the fact that ammonia is not released during reaction with the organic amine. Comparison of titration data with elemental nitrogen analysis shows that more nitrogen is obtained in the gel derivative than can be accounted for by titratable functional groups [13].

A complete titration curve also shows that the isourea linkage is associated with a high pK_a as seen in FIG. 5. There

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FIG. 4 Reaction of agarose with cyanogen bromide.

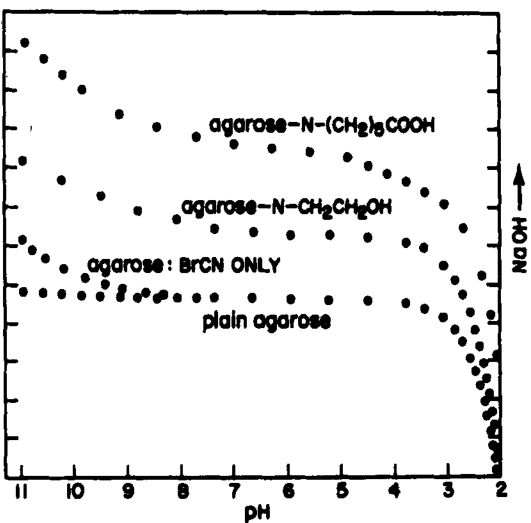

FIG. 5 Titrimetric analysis of agarose gels.

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is a distinct change in the uptake of sodium hydroxide at high pH in the gel samples bearing ethanolamine and 6-aminohexanoic acid adducts as compared to plain agarose or BrCN activated agarose. Wilchek recently also reported a similar observation and assigned a value of 10.4 for the pK_a of an isourea resulting from a primary alkylamine [8]. To avoid these adventitiously generated cationic groups, Wilchek found effective the use of acylhydrazides, such as adipic dihydrazide, instead of alkylamines. As indicated in TABLE 1, the pK_a for the acylhydrazide nitrogen is very low, and the pK_a for the resulting isourea linkage would be similarly low. Hence at physiological pH a leash structure derived from acylhydrazides would be nonprotonated.

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TABLE 1

Leash Functions in Cyanogen Bromide Coupling

Structure	pK_a
$-CH_2-CH_2-C(=O)-NH-NH_2$	~4.0
$-CH_2-NH-C(=O)-CH_2-NH_2$	~8.2
$-CH_2-CH_2-CH_2-NH_2$	~10.6

Work is in progress in our laboratories on evaluating the isourea linkages derived from these hydrazides as well as on leash structures which are derived without the use of cyanogen bromide.

It will be instructive to consider presently some preliminary findings in our laboratory which demonstrate the interfering effects of unwanted ionic groups on affinity chromatography. In our program to purify galactosidase from Aspergillus niger, we started with an examination of the work of Steers et al. [14] who purified the β-galactosidase from Escherichia coli on sorbents like those shown on FIG. 6. Gel B was found to retard the flow of a band of enzyme percolating through the column. Gel C in pH 7 buffer bound the enzyme so tightly that a substrate solution would not desorb it from the gel. However, the enzyme could be eluted with a pH 10 buffer. In view of the fact that the enzyme had in the past been purified on DEAE-cellulose [15] and that the

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GEL B

GEL C

FIG. 6 Affinity gels for β-galactosidase [14].

pK of isourea is near 10, it seemed to us that the binding of enzyme by Gel C could be explained by strong ionic interactions.

We have confirmed the observations of Steers and co-workers in our own lab. Furthermore we have examined appropriate control adsorbents and the results are summarized on Table 2. The number of net cationic charges per ligand or leash site has been calculated taking into account the contribution of the isourea linkage to agarose (A). It is evident from the adsorption pattern of enzyme onto the different gels that the polyanionic character of the protein dominates and directs the binding to sorbent sites. Of particular interest is the sorbent where the leash structure is attached to the agarose via the acylhydrazide (the fourth gel in Table 2). Where there is no neighboring

TABLE 2

Affinity Sorbents for β-Galactosidase

sorbent	charge/site	adsorbs
$A-O-C(=\overset{\oplus}{N}H_2)-NH-(CH_2)_6-\overset{\oplus}{N}H_3$	++	YES
$A-O-C(=\overset{\oplus}{N}H_2)-NH-(CH_2)_6-NH-C(=O)-CH_2CH_2-C(=O)-O^{\ominus}$	0	NO
$A-O-C(=\overset{\oplus}{N}H_2)-NH-(CH_2)_6-NH-C(=O)-CH_2CH_2-C(=O)-N$ (phenyl)–S–galactoside (HOCH₂, HO, OH, OH)	+	YES
$A-O-C(=NH)-NH-NH-C(=O)-(CH_2)_4-C(=O)-NH-$(phenyl)–S–galactoside (OH, HO, OH, CH_2OH)	0	NO

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cationic group, the phenyl-thiogalactopyranoside is indeed a weak ligand as is suggested by the inhibition constants, ~5 mM [8]. Full detailed studies on this problem will be published elsewhere [16].

Our studies on the use of the p-aminophenyl-thio-galactopyranoside as the ligand for β-galactosidase purification suggest that where solutions of high salt concentration or with markedly different pH are used to elute or desorb the enzyme, the process most likely involves nonspecific ionic binding. This is most strongly supported by the data in Table 3 where the K_i of the phenyl-thiogalactopyranoside is little affected by a tenfold change in salt concentration. In this connection the observations of Robinson et al. [17] on their scale-up study of

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TABLE 3

Inhibition Constants for β-Galactosidase (*E. coli*)

Compound		K_i
H_2N–S–O		1.2×10^{-3} M
S–O	0.02 M NaCl	0.4×10^{-3} M
S–O	0.25 M NaCl	0.3×10^{-3} M

the affinity system reported by Steers et al. [14] is particularly interesting. They observed 6000 units of enzyme to bind to their sorbent in 0.01 ionic strength buffer; however, this value fell to 350 units in 0.05 ionic strength buffer. Since the binding (inhibitor) characteristics of the ligand itself are not sensitive to salt in the medium, it would appear that Robinson et al. [17] as well as Steers et al. [14] were dealing largely with non-specific ionic adsorptions to the gel. O'Carra [3] and coworkers [18] have recently also come to the same conclusion regarding the β-galactosidase purification by affinity chromatography. Their work, however, led them to conclude that hydrophobic interactions were primarily responsible for the nonspecific binding. Recent findings in our lab suggest that hydrophobic effects are prominent in this system (as will be discussed in a following section of this paper). Full details are presented elsewhere [16].

A final example of nonspecific binding due to possible ionic groups in the leash moiety is the one observed by Blumberg et al. [19] in their study of the affinity purification of lectins for L-fucose. FIG. 7 shows the structure of the affinity sorbent containing L-fucosylamine which bound the lectins. The

FIG. 7 Affinity gel for fucose lectin [19].

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same sorbent was also observed to bind the β-galactosidase from *E. coli*. The lectins could be subsequently desorbed by solutions of L-fucose, but the enzyme could not. The latter could be eluted out by buffers containing high concentrations of salt.

Ionic Ligands

Ligands which are ionic can also give rise to ion-exchange problems in the sorbent. With the m-aminobenzamidine ligand for trypsin (see FIG. 1), Hixson and Nishikawa [5] found that the inclusion of 0.5 *M* KCl in the buffer medium enhanced the specificity of the binding. Another example is that reported by O'Carra and Barry [20], who studied the use of immobilized oxamate for affinity purification of lactate dehydrogenase. The inclusion of 0.5 *M* NaCl in the loading and washing buffer eliminated non-specific ionic binding to gel. However, high ionic strenth is not a universal cure as shown by acetylcholinesterase in its binding to sorbents containing quaternary ammonium ion ligands. Dudai et al. [21] and Schmidt and Raftery [22] have observed that high salt weakens the binding avidity of the enzyme to the ligand. Where ionic ligands must be used, experience suggests that the inclusion of salt (at least 0.15 *M*) in the buffering medium be investigated to optimize specificity of binding.

General Comments on Ionic Effects

Although it has been pointed out earlier by Porath [10] and others [23] that the cyanogen bromide coupling of amines to agarose can give rise to isourea linkages which are adventitiously accompanied by cationic charges, this as a problem source has

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been largely ignored. Recognizing this, one can, upon reviewing a number of affinity chromatography papers, understand why nonspecific binding problems have been encountered. Furthermore, it is apparent that the claims for specific affinity in a number of systems are invalid. At best, binding in these cases seems to be due to a combination of effects --ligand specificity reinforced by ionic binding.

In view of the several possibilities for nonspecific adsorption due to ionic effects, it is undesirable to effect elution (desorption) of an enzyme by increases in salt concentration in the buffer. Since only meager data are available on salt effects on affinity binding or on K_i of competitive inhibitors, it should be demonstrated for the enzyme at hand that high salt concentrations do indeed affect these parameters. As we have shown above for the phenyl-thiogalactopyranoside, there is no justification here to use salt gradients for elution of β-galactosidase from an affinity sorbent containing this ligand. Similarly, Barry and O'Carra have shown that the K_m of NADH to lactate dehydrogenase is little effected by KCl from 0 to 0.5 M [24]. Hence, where these enzymes are desorbed by salt gradients from gels containing immobilized NADH, one is not observing true affinity chromatography.

Similarly the use of pH-shifts in the eluting medium should be studiously avoided. Compared to salt gradients, this procedure provides for greater prospects of obtaining nonspecifically bound proteins. As Blumberg et al. [19] and others

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have pointed out, the only reliable indication of successful affinity chromatography should be the demonstration that desorption of an affinity bound enzyme can be effected by a solution of specific inhibitor or substrate. Once this has been verified for a particular system, it may then be simpler or more economical to use salt gradients or pH shifts to effect desorption provided that appropriate control tests are made for these conditions.

HYDROPHOBIC EFFECTS

In addition to being complex polyelectrolytes, proteins also possess many structural elements which arise from intra- as well as intermolecular hydrophobic interactions. Generally these hydrophobic elements are buried in the interior of the protein structure; however, there may be crevices and/or pockets near the surface which are important in the structural integrity and/or the functioning of the protein. It is possible that the interactions of a protein with certain hydrophobic surfaces result in an deleterious and irreversible adhesion.

A number of recent reports [25-27] have drawn attention to the significant effects of hydrophobic interactions in affinity chromatography. The term"hydrophobic chromatography" has been coined and a systematic method for taking advantage of this effect has been set forth by Shaltiel and Er-el [29]. But here we shall focus on the "side effects" or perhaps the unwanted aspects of hydrophobic interactions.

Matrix

With regard to the carrier gel or matrix, enzyme chemists have long observed that proteins are very frequently adsorbed

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nonspecifically and irreversibly to hydrophobic surfaces such as polystyrene beads. Ion-exchange resins of polyacrylic acid/ ethylene copolymers have seen some utility in the chromatographic purification of "compact" low molecular weight proteins such as RNAse A or cytochrome C. But the examples are few.

For affinity work, the hydrophilic structure of the polysaccharide, agarose has afforded a relatively inert, non-interactive support material and thus has been the carrier matrix of choice. Cellulose is another polysaccharide that has been used for affinity sorbents. While it has seen less service than agarose in recent years, immunochemists have used it widely for their immunoadsorbents, especially in the years preceeding 1968 (see review [30]).

While nonspecific adsorption problems due to hydrophobic effects have been largely avoided through the use of polysaccharide carriers, they have been exacerbated by the chemical functionalizations which have introduced the often hydrophobic leash and ligand structures.

Leash

The indiscriminate use of long hydrophobic leash structures in affinity gels have been called into question by O'Carra and co-workers [18]. In their study of the affinity purification of β-galactosidase as described by Steers et al. [14], they prepared a gel with the structure shown in FIG. 8. The length of the leash structure from the isourea linkage to the end of the phenyl ring was comparable to that shown for Gel C in FIG. 6.

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FIG. 8 Hydrophobic affinity leash [18].

Despite the absence of the galactopyranose ligand, the β-galactosidase is bound to the gel so firmly that even a 0.5 M KCl solution did not desorb the enzyme. Because of the aromatic structure involved, O'Carra [3] placed the nonspecific adsorption problems at the doorstep of hydrophobic forces. In view of the analysis of the isourea linkage in the first section of this paper, we lean to the interpretation that the cationic charge on the isourea linkage reinforces the hydrophobic interactions due to the N-phenylglycyl moiety in O'Carra's leash example.

A similar interpretation may be placed on the linear aliphatic structures developed by Shaltiel and co-workers [27, 29] for their hydrophobic chromatography. The fact that concentration gradients of salts (which were not necessarily chaotropic) could desorb proteins from these hydrophobic gels suggests a significant interplay of ionic forces here. By attaching hydrophobic structures to agarose gels by nonionic linkages, we have observed "true" hydrophobic binding. The results of these studies will be reported elsewhere.

Ligands

Hydrophobic ligands, especially aromatic ones, can also yield nonspecific binding of proteins. These ligands often are

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not very different from the hydrophobic leash structures discussed in the preceding section. The work of Stevenson and Landman [31] is an interesting case in point.

In their work to purify chymotrypsins from a variety of sources, these authors selected 4-phenylbutyl amine (PBA) as the ligand. Since chymotrypsins recognize the phenyl ring, such a ligand was a class-specific one. As a control test for the specificity of their gel preparation, Stevenson and Landman prepared a sample of active-site inhibited chymotrypsin as shown schematically in FIG. 9. In elution diagram A we see the behavior of native chymotrypsin on their affinity gel. The enzyme binds the phenylbutane structure and is desorbed by 0.1 M acetic

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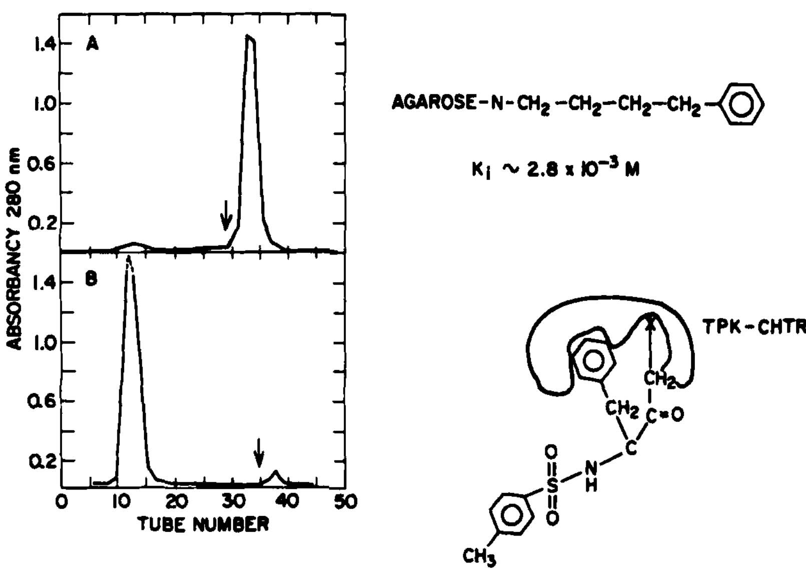

FIG. 9 [31].

acid (vertical arrow). In diagram B the active-site inhibited enzyme does not bind to the gel and is washed straight through the column. A change of the solvent to acetic acid yields a very small amount of protein [presumably due to incomplete reaction of the enzyme with tosylphenylalanylchloroketone (TPCK)]. Since chymotrypsin is a polycation, the binding of the enzyme to these gels containing 4-phenylbutylamine via the cyanogen bromide reaction most likely represented true affinity binding.

Even with seemingly unequivocal data indicating true affinity binding, the agarose gel containing PBA was shown later to exhibit nonspecific protein binding. Hofstee, as shown in FIG. 10, clearly demonstrated that such a gel has a strong avidity for a number of acidic proteins like ovalbumin or

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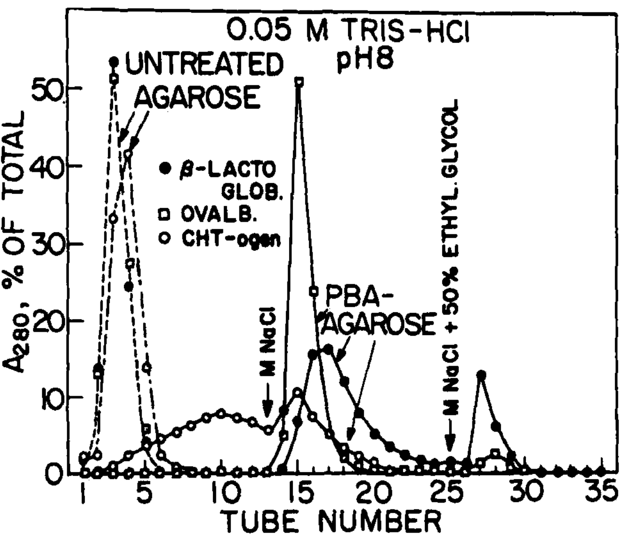

FIG. 10 [25].

β-lactoglobulin [24]. The retarded elution pattern for chymotrypsinogen (a polycation) suggested some ability for this zymogen to recognize the ligand.

We have also encountered hydrophobic problems. In our studies on the affinity purification of thrombin, the specific sorbent reported by Thompson and Davie [32] was studied and some preliminary results are shown on FIG. 11. Our gel contained p-chlorobenzylamine (pCBA) ligand at 13 μeq/gel compared to 21 μeq/ml gel reported by Thompson and Davie for their 4% agarose preparation. These relatively high ligand concentrations were necessitated by the relatively modest K_i (0.22 m$\underline{M}$) of the p-chlorobenzylamine to thrombin. In view of Hofstee's work we expected that a sorbent bearing this functional group would bind

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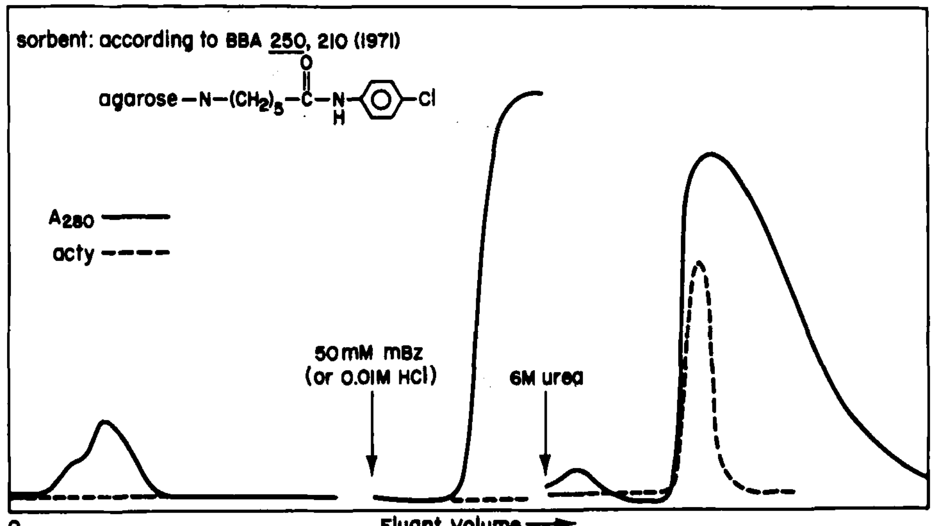

FIG. 11 Hydrophobic adsorption of trypsin on sorbent for thrombin.

proteins nonspecifically, and the experiments shown in FIG. 11 bear this out.

Both trypsin and chymotrypsin were bound tightly to this sorbent. Only degraded proteins were eluted directly from the column (as seen on the left portion of FIG. 11). A solution of 0.01 M HCl, which would normally suffice to remove these proteins (if they are bound primarily by ionic binding),was ineffective. Similarly ineffective was a solution of m-aminobenzamidine, the specific inhibitor for trypsin. Finally (as seen in the right portion of FIG. 11), a solution of 6 M urea was able to wash these proteins out of the gel. This, of course, strongly implicated hydrophobic binding as the predominant force in the adsorption.

In other experiments, egg white lysozyme was observed to bind to this gel at pH 8.1. However, 80% of this protein could be washed out with 10 mM HCl and the rest subsequently by 6 M urea. Bovine serum albumin, which is a polyanion at pH 7 and also very lipophilic, was bound tightly to the pCBA gel. It was not eluted with 0.01 M HCl, but 6 M urea effected its removal. These results also implicated strong hydrophobic binding of proteins to this gel.

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General Comments on Ionic and Hydrophobic Interactions

A most significant combination of effects on affinity binding is that resulting from simultaneous ionic and hydrophobic interactions. As Hofstee [25], Hjerten [28], and others have pointed out, the evidence so far on "hydrophobic chromatography"

suggests that it is complicated by ionic binding as well. Since hydrophobic interactions are entropy driven and dependent on the presence of water (which primarily participates in hydrogen bonding), it is not surprising that ionic and hydrophobic interactions can mutually reinforce each other so that the resultant effect is much greater than the sum of the two. In FIG. 12 we symbolize the overlapping relationships of hydrophobic with both hydrogen bonding and ionic interactions. From this perspective we would expect that whenever there is immobilized a discrete combination of functional groups bearing these interactive capabilities, the result will be a strongly adsorptive surface. The increase or decrease in specificity will depend on the proximity to each other of the fixed groups as well as the differences in chemical topology of the proteins in the mixture exposed to the sorbent. In a recent example, Chu and Chaykin found significant improvement in the affinity purification of aldehyde oxidase if the sorbent bearing the ligand also included a positively charged nitrogen atom in the leash structure approximately 7 Å from the ligand [33].

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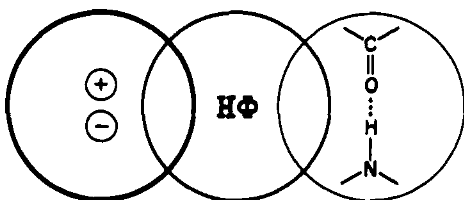

FIG. 12 Interacting forces in affinity adsorption.

This deliberate manipulation of the binding (or inhibition) constant of a ligand-leash structure, of course, is reminiscent of the attempts by enzyme chemists to improve substrate or inhibitor binding by chemical alterations. In addition to increasing knowledge of structure-activity relationships, such efforts should enhance the probabilities for converting ligands with marginal binding capabilities into more useful ones. Much work lies ahead in this area.

LEASH AND LIGAND CONCENTRATION EFFECTS

Perhaps the most neglected of the important parameters in the design of affinity sorbents is the concentration of ligand (with or without leash) in the gel. Relatively few reports have included data of sufficient quality to document its importance [5-7]. Furthermore, until recently a convenient means for preparing absorbent gels with defined amounts of functional groups has not been available [13].

While Baker had earlier suggested a need to determine the optimum density of functional groups in an affinity sorbent [1], in 1971 Hixson and Nishikawa [34] presented a model with equations for predicting useful affinity binding conditions. This was used to predict the ligand density needed to bind thrombin, which was verified by experimental work [5]. More recently Graves and co-workers [35] have reported a mathematical model which might be used to predict success or failure of affinity binding. Wankat has also proposed an analytical model [36], as have O'Carra [3] and Dean and co-workers [37].

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Limits of Binding

Binding capacities and ligand concentrations might be expected to have a significant relationship to each other, and knowing this relationship should facilitate sorbent gel preparation. One approach is to study the adsorption limits of affinity gels. Nishikawa et al. [38] recently presented a Langmuir-isotherm description for affinity adsorption which included a derivation of

$$[EL] = \frac{K_b[E][L_o]}{1 + K_b[E]} \quad (1)$$

The complex [EL] is in units of moles of enzyme per unit volume of gel, and is clearly limited by the concentration of binding site, $[L_o]$, built into the gel. [E] is the concentration of enzyme in contact with sorbent gel at equilibrium and is different from $[E_{tot}]$ which is the concentration of enzyme placed in contact with the gel at the beginning of the experiment. K_b is the binding constant in units of liters/mole.

The hyperbolic function represented by Eq. (1) generates the expected sigmoidal curve when [EL] is plotted against the log of the enzyme concentration. Figure 13 shows a family of curves for a model system in which a binding constant of 1000 is assumed. As the $[L_o]$ is increased with the different gel preparations, we see a corresponding increase in the saturation limits of the bound enzyme complex [El]. However, the 50%

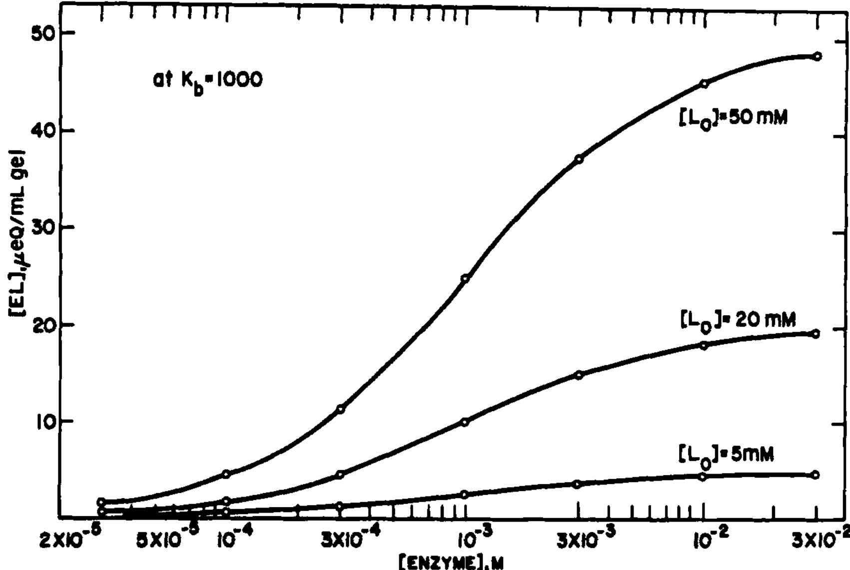

FIG. 13 Enzyme binding to affinity sorbent.

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binding concentration for the enzyme is fixed at 1 mM in all cases. We would expect that this value is determined by the K_b. In terms of K_d (which is $1/K_b$) equal to 1 mM, it would appear that saturation binding would be seen at 100 mM in [E]. It is interesting to compare the model curves with that observed for a real system. FIG. 14 shows data obtained from trypsin absorption to agarose gel containing m-aminobenzamidine [38]. In contrast to the ideal model, the concentration of enzyme-ligand complex at saturation is much smaller than that expected from the concentration of $[L_o]$. The apparent or "effective" ligand concentration within the gel matrix is roughly 1/100th of the chemically determined value. Outside of mentioning

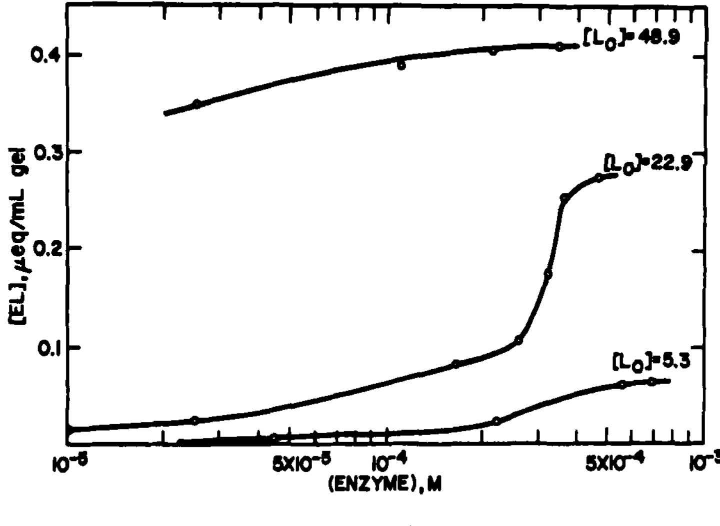

FIG. 14

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possible steric problems posed by the agarose polymer to the accessibility of ligands, we presently have no explanation for this low availability. Previously Hixson and Nishikawa [5] also found similarly low values from pulse-loading chromatographic experiments. Low effective ligand concentrations have been observed by Dean and co-workers [39] in sorbents for NAD^+-dependent dehydrogenases. Similarly, Whitney has reported that the effective ligand concentration in affinity sorbents for carbonic anhydrase is about 1% of the chemically determined concentration [40].

In comparing the curve shapes in FIG. 14 to those in FIG. 13, we see only very rough resemblances. Even after examining several other such curves, we are not able to discern a coherent pattern to these shapes. No doubt subtle variations in the chemical

reactions on these gels are a contributing factor to the variety of absorption curves.

Extent of Binding

The avidity of binding to a sorbent can be evaluated by the use of Eq. (2), which can be derived by a route analogous to that for Eq. (1) [38]:

$$\frac{[EL]}{[E_o]} = \frac{K_b[L]}{1 + K_b[L]} = \theta \qquad (2)$$

(fraction of enzyme bond to sorbent). Here we treat the ligand concentration as the variable for some fixed amount of enzyme input, $[E_o]$. If we assume the same system considered in FIG. 13 at [L] = 50 mM, we find θ = 0.98. That is, 98% of the enzyme will be bound to a gel which contains 50 μeq ligand/ml gel. At [L] = 10 mM, we find that 91% of the enzyme is bound. These calculations suggest that if a ligand has K_i = 5 mM, then a gel must contain at least 50 μeq ligand/ml gel to effect good binding of enzyme. Note that with this model we have not even taken into account the markedly low availability of a ligand that is immobilized into a gel.

With ligands exhibiting K_i larger than 1 mM, the problem is that of coupling ligands into a gel to high densities. Here there may be a practical limit. We have observed, as shown in Table 4, that the coupling of organic amines to agarose gel beads with cyanogen bromide appears to have distinct limits [13]. We have calculated the maximum hydroxyl concentration in the various gels

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from the density and the mean residue weight of the fundamental disaccharide unit. If we assume that only the 1,3-diol structure (see FIG. 4) is involved in the coupling, then the maximum possible functional group concentrations would be 0.073, 0.146, and 0.219 M, respectively, for the 2, 4, and 6% gels. However, the observed maxima for covalent bonding of leash functions in these gels are considerably lower. While the reasons for this are not clear, one might expect that the gel structure of agarose, which arises from inter- and intrachain double helices involving hydrogen-bonding [41], lowers the availability of hydroxyl groups for chemical reaction.

While the exclusion molecular weights for each of the gels listed in Table 4 relate to gel porosity, we have no idea of the distribution of hole sizes in any of the gels. This information would be useful in the preparation of affinity gels for enzymes of

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TABLE 4

Agarose Parameters

Gel density (%)	Max[ROH] (M)	MW exclusion limit (daltons)	$[L]_{limiting}$ (μeq/ml)
2	0.292	50,000,000	~5
4	0.583	15,000,000	15-20
6	0.875	5,000,000	50-60

very high molecular weight. Implicit in the requirement for high porosity [4] is the notion that the apparent ligand concentration in a gel would vary according to the hydrodynamic volume of the enzyme being tested. For a gel with a given $[L_o]$, the $[L]^{eff}$ would be higher when measured by a low molecular weight enzyme than when tested by one with a high molecular weight. The lack of quantitative data on gel porosity presently precludes answers to questions such as: Can an enzyme of 300,000 daltons and possessing a single active-site be expected to bind to a 2% agarose gel containing 5 μeq ligand/ml with K_i = 100 μ$\underline{M}$?

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In-gel parameters by Klotz plots

In preceding sections we have dealt with values for K_i (or K_d) which have been determined for the soluble inhibitor or ligand. The values for [L] have been determined chemically on the bulk of the gel at hand. For better understanding of the affinity process as well as to better predict by the design the utility of a particular sorbent, it would be useful to determine the two parameters in situ or in gel. Where a given sorbent does not seem to bind an enzyme very well, such an analysis might help to explain why. With affinity gels that work, the in-gel analysis of K_i and [L] is a worthwhile documentation that the system is functioning properly.

Our analytical approach was suggested by the work of Hirose and Kano [42] who analyzed the partitioning of soluble ligands between adsorption to a protein and permeation of gel beads. FIG. 15 shows the manipulation of the equation describing enzyme

$$R = \frac{[EL]}{[E]} = \frac{K_b[L_o]}{1+K_b[E]}$$

as reciprocals

$$\frac{1}{R} = \frac{1+K_b[E]}{K_b[L_o]} = \frac{1}{K_b[L_o]} + \frac{[E]}{[L_o]}$$

COMPARE TO KLOTZ PLOT:

$$\frac{1}{R} = \frac{K}{N}\frac{1}{[D_o]} + \frac{1}{N}$$

FIG. 15 [42, 43].

adsorption to affinity gel. We define the ratio R = [EL]/[E]. A plot of 1/R vs [E] should give us a straight line with slope = $1/[L_o]$, and the ordinate intercept should be $1/K_b[L_o]$. In form, our equations and plots closely resemble those originated by Klotz in 1946 [43]. FIG. 16 is an example of the analysis applied to a trypsin affinity gel where $[L_o]$ = 19.2 μeq/ml gel of 4% agarose. Because most enzymologists usually deal with dissociation constants, we have handled our calculations in terms of K_d which is equal to the reciprocal of K_b and has units of mole/liter. The results show an affinity gel dissociation constant of 1.4×10^{-5} M and an effective in-gel ligand concentration of 0.44 μeq/ml gel. As in the saturation binding experiments (see the section entitled "Limits of Binding"), we again observe the effective ligand concentration to be but a small fraction of the chemically determined values.

Table 5 lists the results of this analysis applied to several different gel preparations. Generally there is a correlation between the $[L_o]$ and $[L_o]^{eff}$. However, the values for the $K_d{}^{app}$

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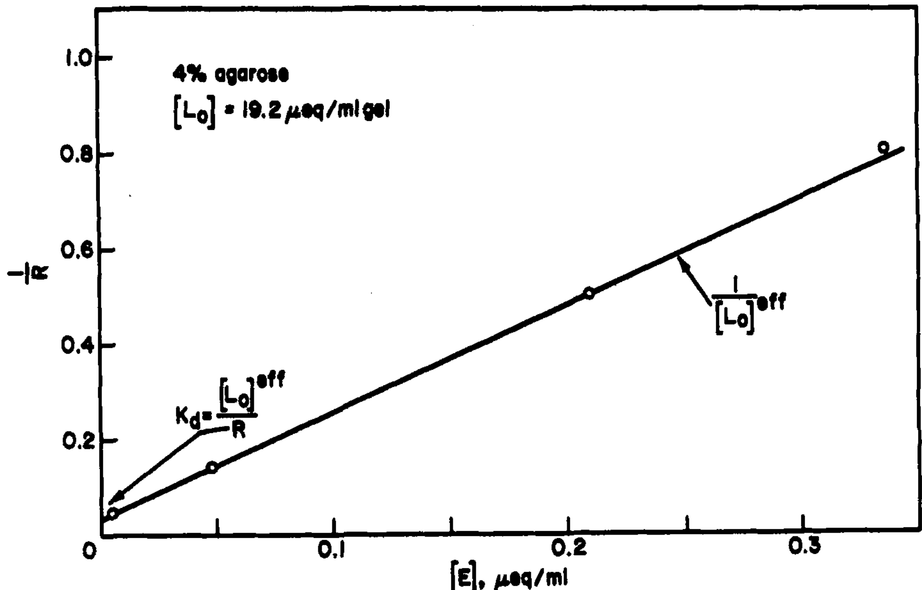

FIG. 16 Trypsin binding to mBz gel.

appear to be somewhat erratic when comparing different gel preparations. We have no ready explanations at present for this large variation. The best binding gel binding gel ([L] = 48.9) shows a K_d^{app} value approaching that of the free solution inhibition constant (= 8 X 10^{-6} M) for m-aminobenzamidine. But mostly the values are in the range of 50 to 100 μM. Clearly, more preparations must be examined by this technique to ascertain the significance of these variations.

Elution Ratio

In addition to static adsorption methods for measuring affinity processes, it is also of interest to have chromatographic parameters which are measured under dynamic (operating) conditions

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TABLE 5

In-gel Binding Parameters for Trypsin Sorbents

Agarose (%)	$[L_o]$ (μeq/ml)	$[L_o]^{eff}$ (μeq/ml)	K_d^{app} (m$\underline{M}$)
6	48.9	0.41	.0049
6	22.9	0.19	.086
6	11.1	0.095	.143
6	4.9	0.125	.36
4	19.2	0.44	.014

in a packed column. One attempt to relate enzyme binding to chromatographic parameters was reported in 1972 by Hixson and Nishikawa [44]. Another approach is outlined below starting from an expression used in adsorption chromatography [46] for the retention volume, which we will call the elution volume:

$$V_e = V_o + K_p V_g$$

The partition coefficient, Kp, is the ratio (adsorbed enzyme)/(unadsorbed enzyme); V_o is the volume of the mobile phase; and V_g is the volume of the sorbent. For affinity sorbents derived from agarose, there is present a gel-filtration effect on the enzyme in addition to affinity sorption. Thus

$$V_e = V_o + K_{av} V_i + K_p V_g$$

where K_{av} and V_i are distribution constant and imbibed volume, respectively (standard terms in gel-filtration theory). With 4%

agarose gel, $K_{av} = 1$ for an enzyme-like trypsin. Thus the "straight through" elution volume of the protein (without any affinity sorption) is $V_s = V_o + V_i$. Now $K_p = [L]/K_d$, terms which are defined in the adsorption isotherm model. On making the substitutions and factoring, we obtain the expression for the elution ratio:

$$\frac{V_e}{V_s} = 1 + \frac{[L]}{K_d} \frac{V_g}{V_s} \tag{3}$$

Experimentally, we have observed that the gel bed volume, V_g, is approximately equal to V_s. The effective ligand concentration, however, is only about 1% of the chemically determined value, as we noted in a preceding section. Thus the selection of V_g as well as [L] presents a problem. For the sake of simplicity, the calculation of the elution ratio in the experiments described in FIGS. 17 through 20 have assumed that V_g and V_s are equal. Furthermore, the calculations in FIGS. 18 through 20 have used affinity parameters which have been obtained from Klotz plots.

Since the elution ratio is readily measured experimentally, we have a feasible way of assessing the ratio of affinity parameters, [L]/Kd, the value of which can be also obtained by the Klotz plot. In FIG. 17 is shown the pH dependence of the elution ratio as well as the K_d and the $[L_o]^{eff}$ for a single affinity gel preparation. This sorbent for trypsin was made from 6% agarose and contained 19.1 μeq of m-aminobenzamidine/ml gel.

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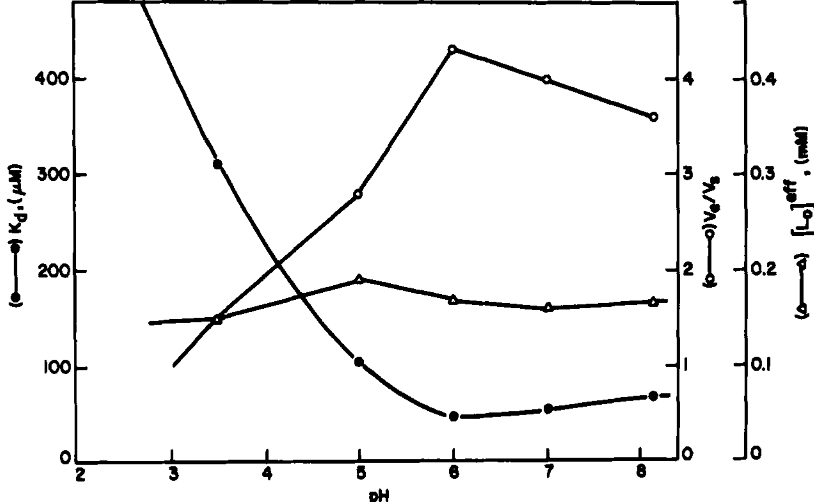

FIG. 17 Trypsin sorbent: In-gel binding parameters vs pH.

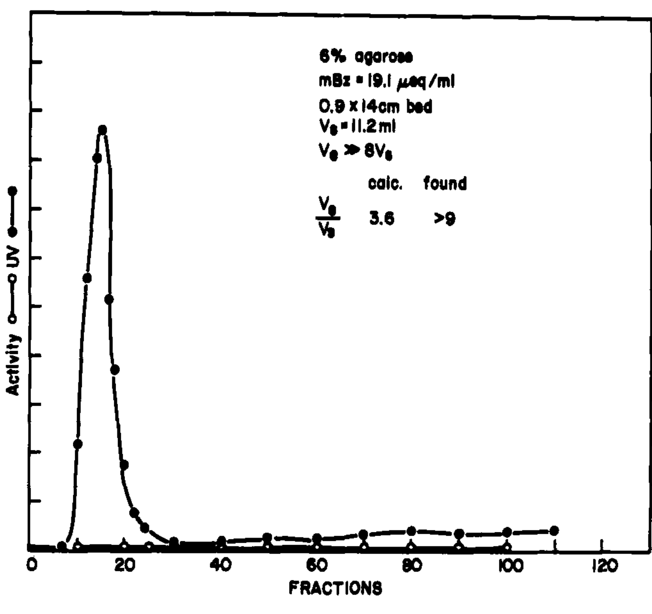

FIG. 18

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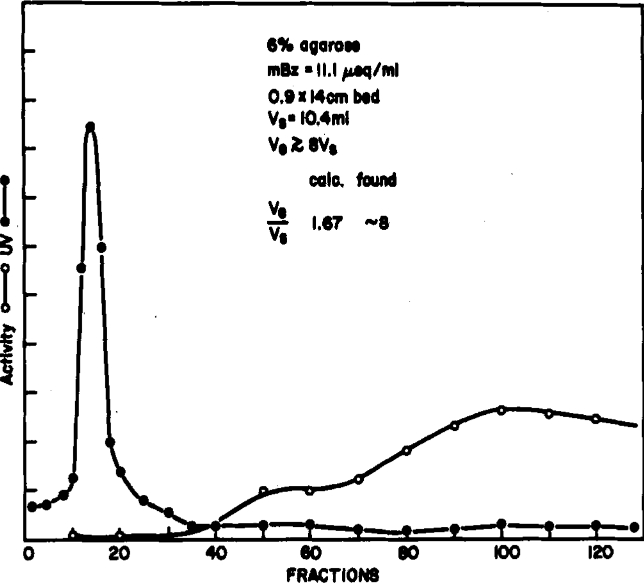

FIG. 19

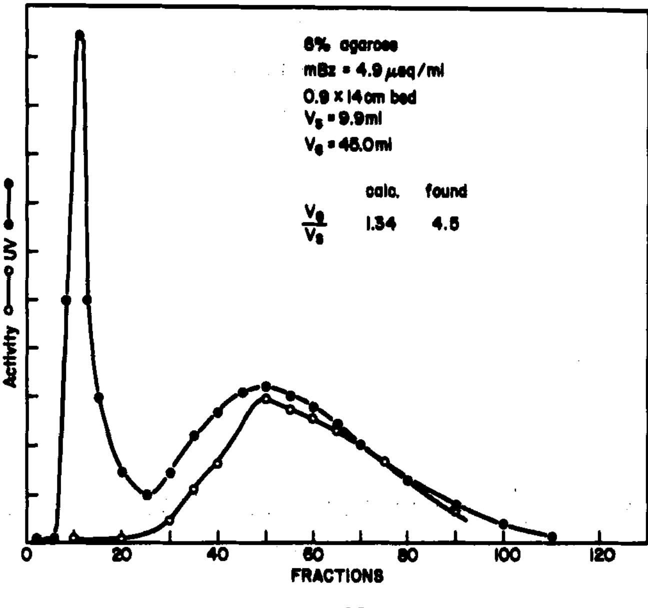

FIG. 20

At lower pH the elution ratio declines due to poorer binding of the ligand by the enzyme, which is also indicated by an increase in the K_d at low pH. And as it should be, the effective ligand concentration remains the same throughout the pH range tested. Thus even though a coherent pattern in the variation of these parameters is not evident when comparing a variety of sorbent preparations, such as in Table 5 when the analyses are applied to a single gel preparation, meaningful information is revealed.

The elution ratio is further examined in a series of chromatograms presented in FIGS. 18 through 20, where each was obtained with a different gel preparation. At the higher ligand concentration the enzyme binds well, and correspondingly the elution ratio is very large. At lower ligand concentrations the enzyme binds more weakly to the sorbent and this is accompanied by a correspondingly lower elution ratio. In these examples there is a striking difference between calculated values and the experimentally observed data. The observed values are roughly 3.5 to 4.5 times greater than the calculated ones. While not completely ruled out, the high ionic strength (0.5 M) of the buffers used should diminish the possible Donnan-equilibrium effects [45] due to the immobilized benzamidine. Regardless of the present reasons for the quantitative discrepancies, the determination of the elution ratio seems to be a useful measure for predicting chromatographic utility of an affinity gel. The ratio value is fairly revealing for a very poorly binding gel as well as for the very strongly binding one. Certainly more work

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is needed to assess its usefulness on gels with marginal performance characteristics.

General Comments

It is interesting to note that the Langmuir adsorption isotherm model is a reasonable description of the affinity process. The chromatogram in FIG. 20 agrees with the shape predicted for a convex isotherm from adsorption theory [46] as shown in FIG. 21.

To summarize the various considerations that we have given to the quantitative parameters in affinity chromatography, we offer some calculated predictions in Table 6. The values for the limiting K_d's have been obtained using Eq. (2) and (3). The effective ligand concentration has been estimated as 1% of the chemically determined ligand concentration. The apparent ligand concentration has been estimated as 3.5X the effective [L]. The K_d values are somewhat lower than 1 mM and differ between static and dynamic binding experiments. These values are predicted limits for "true" affinity binding. Adventitious ionic and

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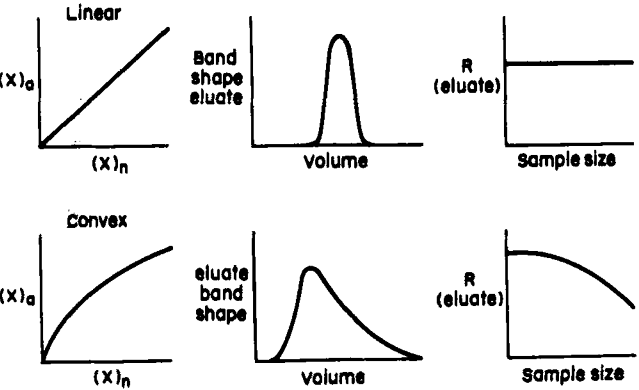

FIG. 21 Isotherm type and chromatographic parameters [46].

TABLE 6

Calculated Limits for K_d of Useful Ligands

Agarose (%)	$[L]^{eff}$ a (m$\underline{M}$)	$[L]^{app}$ b (m$\underline{M}$)	K_d^{stat} c (μ$\underline{M}$)	K_d^{dyn} d (μ$\underline{M}$)
6	0.6	2.1	67	230
4	0.2	0.7	22	77
2	0.05	0.175	5.6	19

a $[L]^{lim}$ X 0.01 (see Table 4 data).
b $[L]^{eff}$ X 3.5.
c Calculated where $\theta > 0.9$.
d Calculated where $\frac{Ve}{Vs} > 10$.

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hydrophobic effects could raise or lower these values. With ligands whose K_d values are greater than those predicted in Table 6, useful sorbents may still be obtained. We expect that differential migrations on these sorbents could still offer a practical isolation of the desired protein.

For the near future in affinity chromatography we expect that more quantitative data will be forthcoming so that the design of affinity sorbents can be put on a more rational basis. We anticipate that new hydrophobic gel derivatives which are free of undesired ionic and hydrophobic groups will become available. The synthesis of new hydrophobic polymers, such as polyhydroxy ethyl methacrylate, will be welcome, especially in macroreticular form, since the need continues for mechanically stable carriers. These developments then should make large-scale and commercial applications of affinity chromatography a practical endeavor.

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