Chapter VIII

AFFINITY CHROMATOGRAPHY

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1 INTRODUCTION

Affinity chromatography is a separation technique in which a substance is selectivity adsorbed from solution onto an insoluble adsorbent through interactions with a particular group or molecule immobilized on the adsorbent. The selectivity that characterizes a well-designed affinity column is such that, in practice, its operation more closely resembles extraction than it does traditional organic solid-liquid partition chromatography. Affinity chromatography has proved a valuable isolation and purification technique in biochemistry [1-13]. In this application, its selectivity depends on the ability of biological polymers, especially proteins, to recognize and bind particular structures with great specificity.

High specificity in binding seems to require a high molecular weight in at least one of the associating molecules. Most of the compounds with which organic chemists work have molecular weight less than 1000 daltons, and it may be unrealistic to expect that methods used for separating biopolymers could be applicable to problems involving small organic molecules. Nonetheless, the rationality and simplicity possible in affinity purification methods are instructive, and their utility for certain types of separations is very great. This chapter outlines the affinity method and its current working tenets. Most of the illustrative examples concern enzymes. An effort is, however, made to explore the application of the affinity concept to the smaller molecules familiar to organic chemists.

2 THE AFFINITY CONCEPT

The characteristic ability of biopolymers to recognize and bind specific molecular structures is described as *affinity* binding; other adjectives such as "bioaffinity" or "bioselective" have also been used [1-4]. The insoluble moiety—the "*ligand*"—is chosen to resemble a substrate, inhibitor, or cofactor for the target protein—the "*ligate*". In an ideal separation, only this protein in a mixture of proteins associates strongly with the ligand, and its separation from the mixture can be achieved by a straightforward three-stage process (Fig. 8.1): (a) application of the protein mixture to a sorbent bearing the ligand, and specific binding of the ligate; (b) washing of the sorbent to remove nonbinding proteins; and (c) desorption of the adsorbed ligate by adding a soluble agent capable of displacing the ligand from the adsorbed ligate specifically. Under appropriate circumstances, the ligate may also be desorbed by changing the pH or other property of the solvent [13, 14].

Part of the enormous appeal of affinity chromatography is its conceptual chemical simplicity. A substance known to bind strongly to a target protein is chosen to be the ligand. This substance is immobilized on an inert carrier. The resulting sorbent is then used to adsorb the ligate or target protein selectively

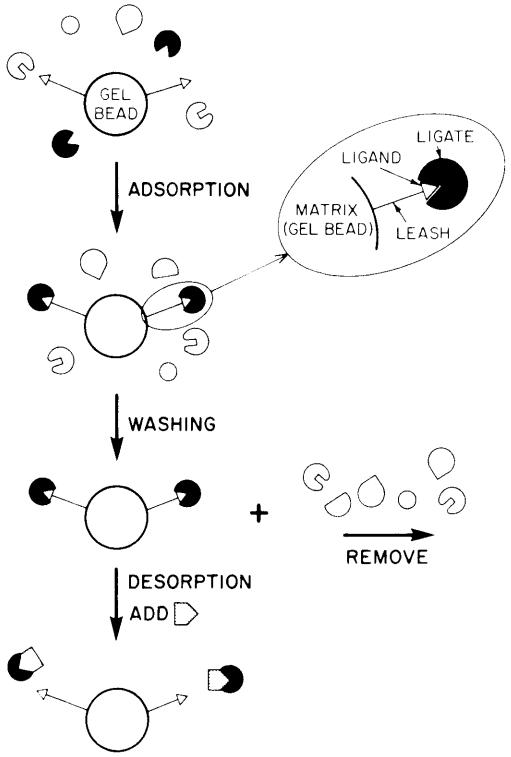


Fig. 8.1. Schematic outline of the course of an affinity chromatography.

from solution. In principle, this technique makes it possible to separate protein mixtures into their components strictly on the basis of their ability to recognize and bind specific structures. This basis for separation is markedly different from the conventional methods used with proteins, which rely on differences in gross physical properties such as molecular weight, solubility, and isoelectric point.

3 THE SELECTION OF LIGAND

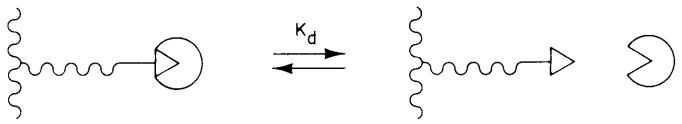
In the affinity purification of biopolymers, the selection of the ligand is the first item of business. For enzymes the choice is usually an inhibitor or cofactor, although the substrate or a substrate analog is occasionally used. The selection process is made easier if a fair amount is known about kinetic parameters characterizing the reaction(s) catalyzed by the enzyme being sought. For a substance to be useful as an affinity ligand three conditions must be met:

1. It should have a dissociation constant from the ligate of less than 10^{-3} M (or a binding constant greater than 1000).

2. It should be bifunctional; that is, it should offer a site that binds to the enzyme and a separate site through which immobilization may be possible.
3. It must be stable.

Importance of Dissociation Constant

To assess properly the importance of the dissociation constant, K_d (or the inhibition constant, K_i) of a ligand in affinity adsorption, it is useful to analyze a very simple model for this process. Consider the behavior of a dilute, ideal solution of two proteins on being applied to an affinity column to whose ligands the one protein binds with dissociation constant K_d while the second binds not at all. After the sample solution, having concentration $[E_0]$ in the protein that binds significantly, is applied, solvent is passed through the column. We will assume that the unbound protein migrates through the column at the same rate, V_s, as the solvent passes through it. How rapidly does the protein with dissociation constant K_d migrate?



$$K_d = \frac{[E]\,[L]_{eff}}{[EL]} \quad ; \quad \frac{[EL]}{[E]} = \frac{[L]_{eff}}{K_d} \tag{8.1}$$

$$\frac{[E]}{[E]+[EL]} = \frac{[E]}{[E_0]} = \frac{K_d}{K_d+[L]_{eff}} \tag{8.2}$$

To simplify the discussion, make several further assumptions: The concentration of the proteins is sufficiently low that the available binding sites on the gel are never saturated; equilibration of enzyme between bound and unbound forms is very rapid; the ligands are present in an "effective" concentration (see below) $[L]_{eff}$ in the gel; the protein does not interact in any way with the matrix; diffusional effects can be ignored. With these assumptions, the fraction $[E]/[E_0]$ of the protein of interest that is not bound on the column at any time is given by (8.2). Since bound enzyme does not migrate, since free enzyme migrates with velocity V_s, and since bound and unbound enzyme are in rapid equilibrium, the average rate, V_p, of migration of protein equilibrating between bound an unbound states is given by (8.3):

$$V_p = \frac{K_d V_s}{K_d + [L]_{eff}} = \frac{V_s}{1 + \dfrac{[L]_{eff}}{K_d}} \tag{8.3}$$

A plot of V_p/V_s versus $[L]_{eff}/K_d$ emphasizes a feature of affinity purification that must be understood to design and operate any affinity column successfully (Fig. 8.2). Affinity purification is an "on-off" phenomenon: If $[L]_{eff}/K_d$ is less

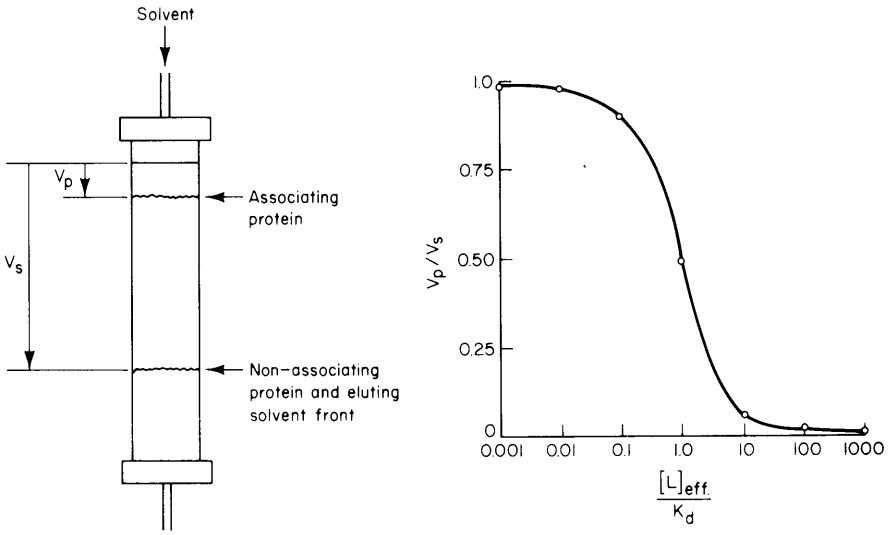


Fig. 8.2. Relationship between the V_p/V_s and $[L]_{eff}/K_d$; V_p is the velocity of migration of protein down the column under given conditions, V_s is the velocity of the solvent front, K_d is the dissociation constant of the ligand, L, from the protein, and $[L]_{eff}$ is the effective concentration of ligand in the gel.

than 0.1, the rate of migration of the binding protein along the column is retarded by less than 10%; if $[L]_{eff}/K_d$ is greater than 10, it is retarded by more than 90%. Since the objective of affinity adsorption is to achieve an easy separation of the desired from undesired proteins, it is preferable not to work with columns in which $[L]_{eff}/K_d < 0.1$, since no separation in this region will be "easy." Similarly, there is little to be gained (and possibly something to be lost) by designing $[L]_{eff}/K_d > 100$; the extent to which the ligate's rate of migration is retarded on increasing the strength of its binding is not significant, and dissociation of very tightly bound protein from the column may prove difficult (see below). Where $[L]_{eff}/K_d = 0.5 - 2.0$, differential migration of proteins should occur; that is, "chromatography" should be observed [15].

The range of values of K_d that will result in useful binding of ligate to ligand is limited, in practice, by the values of $[L]_{eff}$ that can be obtained experimentally. Upper limits in this parameter are determined by two factors. First, the chemical modification of the gel that is required to introduce the ligand changes its properties: in particular, extensive modification introduces additional cross-linking into the gel, and may seriously decrease its porosity. Second, for reasons

that are not entirely understood, only a small fraction (about 1%) of the ligands physically present in the gel are available for binding [15]. For cyanogen bromide-activated agarose, measured ligand concentrations for typical gels are given in Table 8.1 [15]. Thus, the useful accessible range of $[L]_{eff}$ is 0.05-0.5

Table 8.1 Maximum Ligand Concentrations Accessible in Cyanogen Bromide-Activated Agarose [15]

Agarose Density (%)[a]	*MW Exclusion Limits (10^6 daltons)*	*Ligand conc. (μeq/ml)*
2	50	~5
4	15	15-20
6	5	50-60

[a]The "density" of the gel is the concentration of agarose in the gel in units of grams of dry agarose per 100 ml of gel. The higher the density of the agarose, the lower the porosity of the gel.

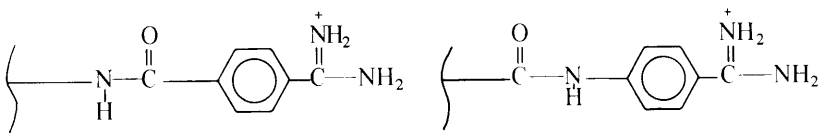
m*M*. For affinity binding, a useful K_d in 2% agarose would therefore be 2×10^{-5} *M*; in 6% agarose, K_d would be about 2×10^{-4} *M*.

Bifunctionality

To be useful, the potential ligand must have two functional groups: one, a "binding site," which is recognized by the ligate, and a second "coupling site," which permits the ligand to be chemically bonded to the matrix, either with or without an intermediary leash or tether group (see below). The coupling site must be located in such a way that it does not impair recognition by, and binding to, the ligate. Information concerning the "bulk tolerance" [16] of an enzyme, that is, its sensitivity to changes in the structure of a potential ligand, may be available from studies of inhibition kinetics. If data are not available, candidates for ligands should be tested before immobilizing them in gels.

Stability

The chemical lability of the ligand is occasionally a problem, especially with ligands involved in oxidation-reduction reactions. A more common problem is the stability of the coupling site on the ligand: for instance, phosphodiester linkages can be attacked by the nonspecific phosphodiesterases often found in crude tissue extracts. The functional group orientation at the coupling site may be important in stability. For example, in developing an affinity ligand for trypsin, it was observed that if *p*-amidinobenzoic acid (**1**) was used as a ligand it could be cleaved from the sorbent by the enzyme [14]. By contrast, *p*-aminobenzamide (**2**) yielded a stable sorbent [14].



1 2

Sources of Ligands

Inhibitors and cofactors are the most frequent candidates as ligands for enzymes. Since ligands must have low dissociation constants from the enzyme to be effective, it is possible that "transition-state analogs" might also be useful. These substances, which are designed to resemble the transition state of the enzyme-catalyzed reaction, have been shown in certain instances to bind at the enzyme active sites more strongly than either substrates or products [17]; in addition, they are usually not transformed chemically by the enzyme, and are thus more stable than either substrates or products.

Having successfully chosen, modified, and immobilized a known inhibitor, cofactor, or binding factor for the target protein, it may still be difficult to establish whether or not the modified, immobilized material binds to this protein. It is sometimes possible to test soluble analogs for their ability to complex in solution, but usually it is simpler to determine directly the ability of the gel to effect the desired separation than to study the behavior of soluble models. For quantitative work, it may be desirable to determine the binding constant between ligand and ligate in the gel: several strategies have been proposed for this purpose [15, 18].

4 SELECTION OF CARRIER OR MATRIX

The insoluble polymer that provides the support for an affinity column should have a number of properties. First, it should not itself interact with proteins, that is, it should be hydrophilic and have no intrinsic charged groups [19]. The more hydrophobic the matrix, the more probable would be protein adsorption by interactions between the gel and hydrophobic regions of the protein (see below). Charged groups would certainly interact with the charged amino acid side chains. The extent to which either hydrophobic or ion-exchange interactions would determine the adsorption of a protein on the gel would obviously depend upon the composition of the protein and of the solvent; equally obviously, a support that minimized the interactions would be more generally useful than one that did not. Second, the gel should have good porosity [20]. It appears that efficient equilibration and reasonable capacity require gels whose molecular weight exclusion limit is approximately 100 times that of the target protein. Third, the gel should have good mechanical properties. Efficient opera-

tion of an affinity chromatography column, as any chromatography column, requires that it be possible to pack the support into the column easily in such a way that the liquid phase flows through the column bed evenly, without channeling, and as rapidly as possible. Columns with good flow characteristics are most easily prepared if the column material has the physical form of uniformly sized, mechanically strong, resilient spheres. Delivery of the protein molecules to the interior of the bead is achieved by a combination of two types of flow: movement of bulk liquid through the voids between the beads carries protein to the individual beads, and diffusive motion through the (approximately) stationary liquid in the pores of the beads distributes the protein to the immobilized ligands in the bead interior. A uniform, narrow distribution of bead sizes is necessary to achieve uniform flow through the void space of the column (Fig. 8.3*a*); a distribution of beads having many that are small results in particularly poor flow characteristics, because the small beads tend to plug the channels between their larger partners (Fig. 8.3*b*) [21]. The admixture of non-

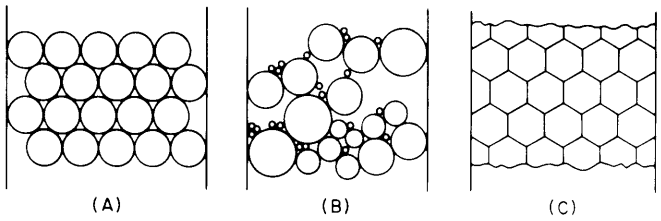


Fig. 8.3. Schematic representations of (**A**) a column packed with uniform spheres, (**B**) a column containing a mixture of spheres of different sizes, and (**C**) a column in which the spheres have been fused by pressure.

porous, siliconized glass beads with porous Sephadex to resist compression and fusing is reported to be a useful technique [22]; this technique should also work with other column materials. Strong, resilient beads are required to resist (and recover from) deformation under pressure. When a column composed of weak beads is compressed (either intentionally in trying to increase the flow rate through the column, or accidentally), the beads are squeezed together and deform (Fig. 8.3*c*). This deformation, if carried far enough, eliminates the column void volume and fuses the beads. A column subjected to this type of pressure becomes, essentially, a solid gel block, and it is impossible in practice to achieve a useful flow rate through it. Mechanically strong beads resist deformation, and resilience helps the bead recover its spherical shape after a transient deformation. Fourth, the gel should be amenable to the chemical functionalization and modification required in covalently linking ligand to gel (see below).

Four types of hydrophilic polymers have been used for matrix materials: agarose, Sephadex, polyacrylamide, and cellulose. Agarose is a polygalactan

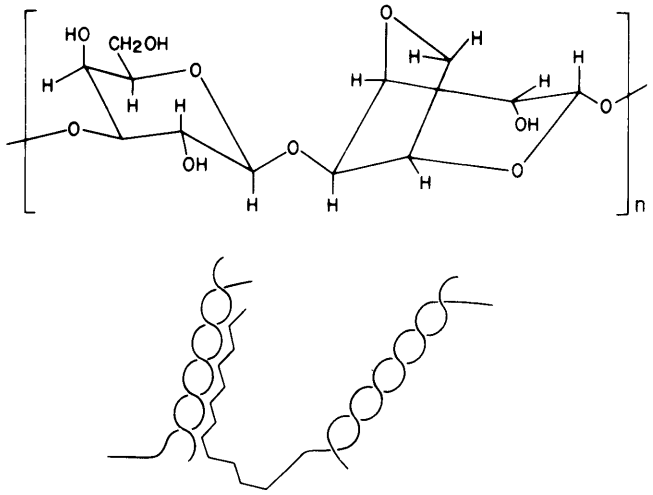


Fig. 8.4. The structure of agarose.

(Fig. 8.4) obtained from seaweed [23, 24]. It is commercially available in beaded form (Sepharose, Bio-Gel A), which is obtained by dispersing a hot aqueous solution of the polymer into a cold immiscible solvent [25]. The relatively firm gel structure arises from intra- and interchain hydrogen bonding; no covalent cross links are present.

Sephadex is a proprietary gel material available in bead form, made by cross linking dextran, a 1,6-glucose polymer made by certain microorganisms, with epichlorohydrin (Fig. 8.5) [26]. The gel is available in various molecular pore sizes, but at high porosity it is mechanically inferior to agarose.

Polyacrylamide is commercially available in beaded form (Bio-Gel P) with covalent cross links derived from *N,N'*-methylene bisacrylamide [27]. It also is available in a variety of molecular pore sizes, and like Sephadex, the higher-porosity gels are somewhat soft and mechanically unstable. Of all the matrices it is the most resistant to biodegradation.

Cellulose is commercially available in fibrous or granular powders. The latter (e.g., Whatman CC-31) is composed of microscopic cigar-shaped particles of uniform size (about 35 μ). Cellulose is microcrystalline because of interchain hydrogen bonding of the 1,4-glucose polymer, and, as a result, is insoluble in water. It has been used frequently by immunochemists in preparing affinity sorbents [28, 29]. Enzymologists have used it less often, because it has a relatively low capacity for derivatization and because the derivatizing reactions are slow [29, 30].

While none of these materials suffers from incompatibility with proteins, some of the other functional requirements of affinity purification set limits on their

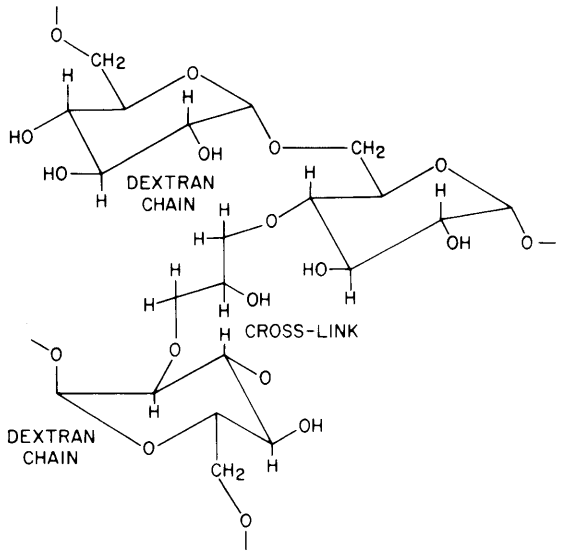


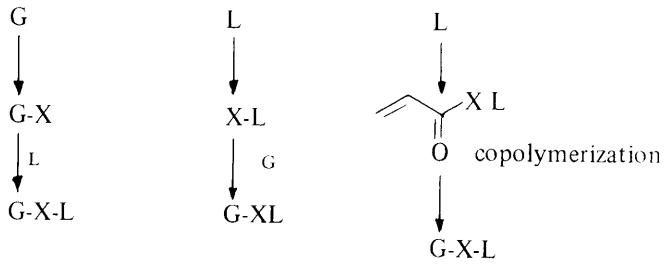
Fig. 8.5. The structure of Sephadex. The position of the cross links is not unique.

use. At present, everything considered, agarose appears to be the matrix material of choice for affinity supports. To improve its thermal and solvent stability, Porath and co-workers have developed procedures for chemically cross linking the gel [31].

5 GEL FUNCTIONALIZATION AND LEASH SELECTION

Derivatization

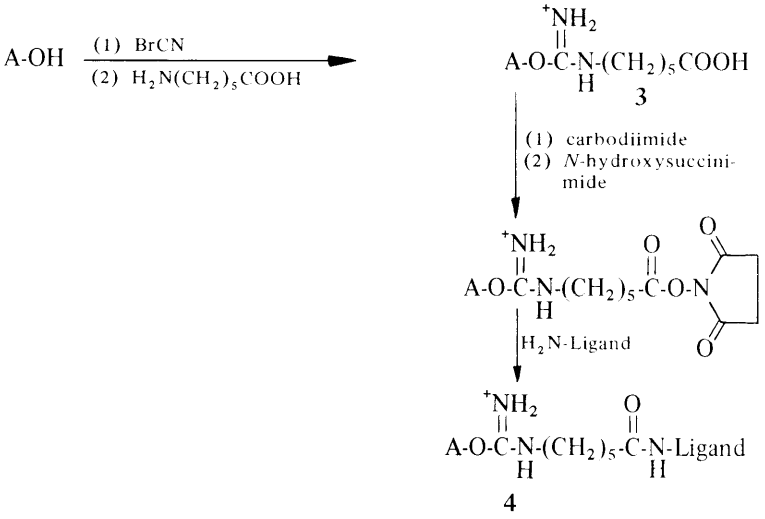
In principle, there are at least three different synthetic strategies for covalently linking a ligand to the gel (Scheme 1). The first, which is synthetically the



Scheme 1. Schematic synthetic strategies for preparing affinity gels: G is the organic polymer portion of a gel, X is a leash, and L is a ligand.

simplest and by far the most commonly used, is to treat the gel with a reagent (or sequence of reagents) that generates active sites, and then to allow the ligand (or leash) to react with this activated gel. A second, which is more difficult synthetically but which is potentially capable of reducing some of the unwanted functionality introduced into the gel by the first, is to functionalize and activate the ligand, and then to allow the activated ligand to react with the gel. A third, which is only really applicable to polyacrylamide gels, is to include the ligand or the activating group in the form of a reactive monomer in the polymerization that forms the gel. In view of the need to have uniform spherical beads, this strategy suffers from serious technical difficulties.

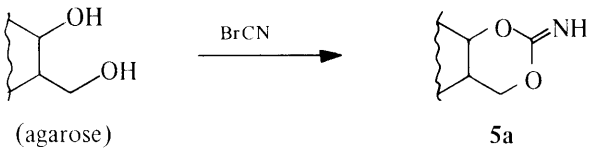
The first of these procedures has been widely exploited, and the model provided by Cuatrecasas and Parikh [32] has been modified to permit the immobilization of a number of ligands to agarose using a variety of leashes. The procedure for coupling amines to the activated agarose has been examined in some detail, and empirically optimized [33, 34]. A representative procedure for immobilizing an amine-contining ligand via a leash structure is shown in Scheme 2.



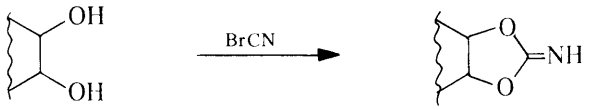
Scheme 2. A representative derivatization procedure for agarose (= A-OH).

Affinity chromatography has been developed as a procedure for purifying biochemicals. It was developed by individuals who were concerned with avoiding organic synthesis whenever possible. Consequently, the procedure most widely used in initial activation of agarose and Sephadex, that is, treatment with cyanogen bromide, is versatile and extremely simple, but not particularly clean from

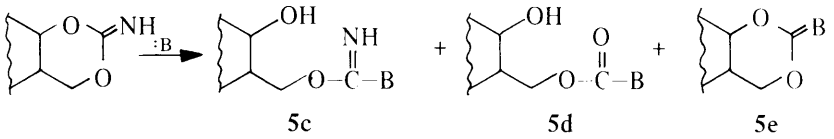
a chemical viewpoint. Initial reaction with a gel bearing proximate hydroxyl groups is believed to generate an imidocarbonate group [35]. Reaction of this functionality with the nucleophilic moiety (:B) of a leash or ligand appears



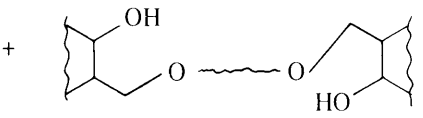
(agarose) **5a**



(Sephadex) **5b**

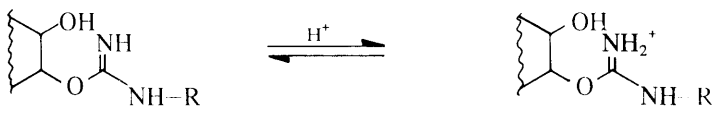


5c **5d** **5e**



5f

to generate several groups: the predominant one when (:B) is an aliphatic amine is believed to be the isourea linkage **6**. This group is a strong base: the pK_a of **6b** is approximately 10.4 [36, 37]. At solution pH values typical of those used

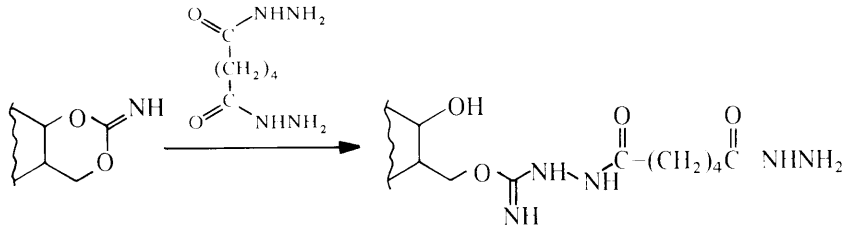


6a **6b**

in manipulating proteins, it will exist predominantly in the cationic, protonated form. Thus the functional groups generated during coupling to cyanogen bromide-activated agarose are usually cationic, and they introduce elements of ion-exchange adsorption into subsequent interactions with proteins [15, 19, 37-39]. Although these charged centers may either strengthen or weaken the adsorption of the target protein, and are not necessarily undesirable from that perspective, they can encourage unwanted unselective adsorption of anionic

proteins.

Several modifications of the standard coupling procedure have been proposed that eliminate the cationic isourea group generated by reaction of an amine with **5a**. One is to effect the linkage with the cyanogen bromide-activated agarose using an acid hydrazide [37-40]. The resulting isourea analog (7) is sufficiently less basic than **6b** that it is not protonated at pH 7. Approaches based on entirely different functionalization approaches also appear useful. Porath and



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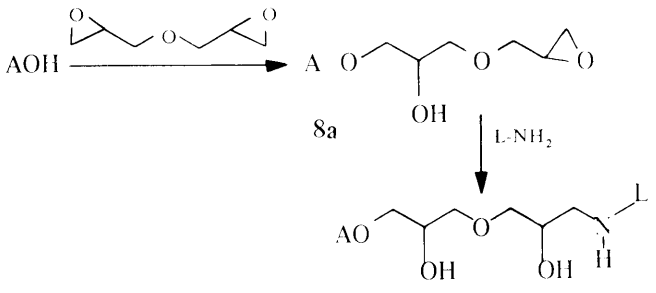
co-workers have reported the use of bis-diglycyl ethers that immobilize ligands via stable, noncharged leash structures (8) [41].



8a

8b

Two other potential problems with cyanogen bromide-activated agarose deserve mention. First, the stability of the isourea linkage toward hydrolysis has not been carefully explored. Although this group has appeared to be stable at neutral pH in water for extended periods of time, recent work by Schwyzer and co-workers [42] indicates that appreciable breakdown can occur for pH > 8. Second, it appears that the functionalized gel itself may slowly hydrolyze [43]. Both factors might result in short operating lifetimes for affinity sorbents under certain circumstances.

While sharing the same coupling chemistries as agarose, Sephadex has received much less attention as an affinity support because its lower mechanical stability at high gel porosity and its lower overall molecular porosity, compared to

agarose, make it a less attractive support. The affinity adsorption process seems to require rather porous gel structures for optimum efficiency [20]. Polyacrylamide has also been used much less frequently even though a systematic approach to its derivatization has been reported [27]. While a wider range of derivatizing approaches is possible with this polymer, its inferior mechanical stability and low porosity presently make it less attractive than agarose.

In summary, cyanogen bromide-activated agarose is presently the gel that provides the best general starting material for affinity chromatography. Its drawbacks—a propensity to include the positively charged isourea groups in coupling with amines, good but not outstanding mechanical properties, susceptibility to microbial attack, and relatively high cost—are offset by the convenience with which it can be generated and utilized, and by the procedures and experience accumulated in its previous use.

Leash Structure

Affinity adsorption requires the association of two polymers: the ligate and a portion of the gel polymer bearing the attached ligand moiety. This type of association might be expected to be subject to important steric constraints. As suggested in Fig. 8.6, the binding site of the enzyme might be sufficiently buried

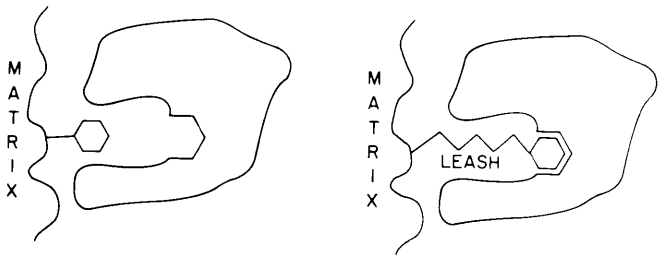


Fig. 8.6. Schematic illustration of possible interactions of a protein having its binding site in a deep crevice with ligands on short (left) and long (right) leashes.

in a cleft that it would not be accessible to a ligand on a short leash. Furthermore, the fact that only about 1% of the ligand groups present in a gel are available for binding can be rationalized as reflecting steric constraints on the ligand-ligate interaction. While these considerations suggest that a flexible leash (or "spacer-arm," as originally called) separating the ligand from the gel backbone would lead to improved binding, and although the structure of the leash *does* influence the strength of the binding in an important way (see below), there is no solid evidence establishing that the *length* of the leash is an important parameter. A number of enzymes have been adsorbed to ligands mounted virtually directly to the carrier [44, 45]. Perhaps more strikingly, recent studies suggest that the leash structures and coupling conditions reported in the early work by

Cuatrecasas et al. [1] can give rise to nonspecific binding [19, 46, 47].

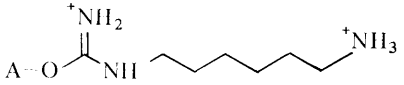
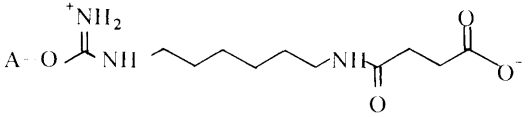
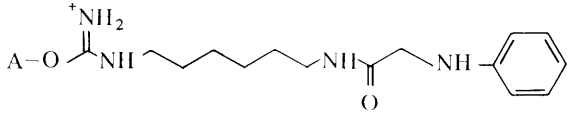
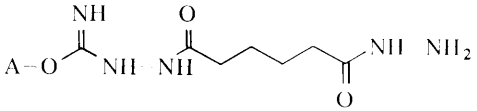
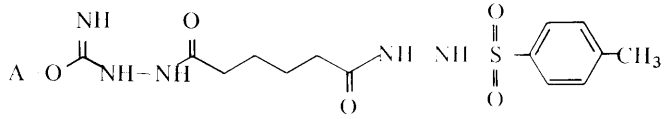
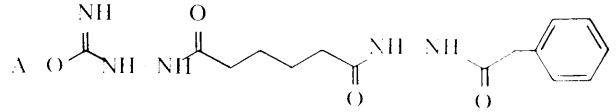
Clearly the minimum function of a leash moiety is that of a connector. Originally the apparently improved binding of enzymes to ligands that were immobilized through leash structures was interpreted as resulting from increased translational freedom of the ligand. Examination of the binding of β-galactosidase to a variety of gel derivatives sheds light on functional utility of leash structures. This enzyme was originally purified from extracts of *Escherichia coli* by Steers et al., by affinity chromatography. Table 8.2 lists a number of sorbents subsequently tested with this enzyme. Where possible, the ligand concentration in the gel has been indicated so that useful comparisons of binding could be made. Perhaps the most striking feature of these data is the binding of the enzyme to a wide variety of structures (Gel-1, -3, -5, -8, -9, -11, -12). Gel-8 was the one reported by Steers et al. [48], but the results show that the enzyme binds whether the correct ligand (-thio-galactoside) is present or not (Gels-3, -11, -12). All structures except one (Gel 5) that bind the enzyme bear cationic isourea linkages, and all contain some hydrophobic structures. These observations suggest that the enzyme could be purified on the basis of relatively nonspecific hydrophobic adsorptions, and this type of purification has in fact been demonstrated by Rimerman and Hatfield [49].

The importance of these and related studies to the practice of affinity chromatography is clear. The central objective of affinity chromatography is to construct gels whose binding characteristics are determined solely by the specific interactions between the immobilized ligands and protein binding sites. These studies make it evident that strength of nonspecific ionic and hydrophobic binding of proteins to the leash and ligand may be as strong or stronger than the specific affinity adsorption. Since the selectivity of binding by these nonspecific interactions would not be expected to show a correlation with that shown by affinity binding, they provide a mechanism for a potentially serious limitation to the selectivity of the technique.

Two approaches have been taken to reduce the problem of nonspecific adsorption. One has been to use nucleophiles that generate functional groups having low p*K*a's (e.g., hydrazines, Gel-10) in coupling with the cyanogen bromide-activated agarose [37-40]. While the epoxide coupling reagents described by Porath and co-workers [41] have not been specifically tested in this connection, they also should generate innocuous functional groups in appropriate couplings. The second is to construct leashes that are designed to be as hydrophilic as possible [51].

Many of the present problems in preparing affinity gels are intrinsic to the strategy used in the immobilization: procedures that build outward from a preformed gel, using amines and carboxylic acid derivatives as reagents, are fast and convenient, but do not permit removal of incompletely or incorrectly reacted immoblized materials from the gel (Scheme 1). Three possibilities to

Table 8.2. Binding of β-Galactosidase to Various Affinity Gels

Gel	*Sorbent Structure*	*[L] (μeq/ml)*	*Binding of β-galacto-sidase*	*Desorbed by*	*Ref.*
1		13	Bound	4*M* Gu Cl[b]	40
2		13	Flow retarded	1*M* NaCl	40
3		?	Bound		46
4		10	Not bound		40
5		10	Bound	4*M* Gu–Cl[b]	40
6		10	Flow retarded		40

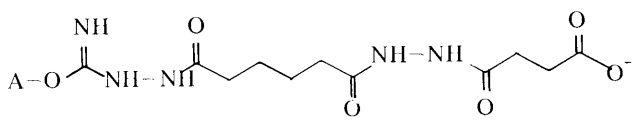
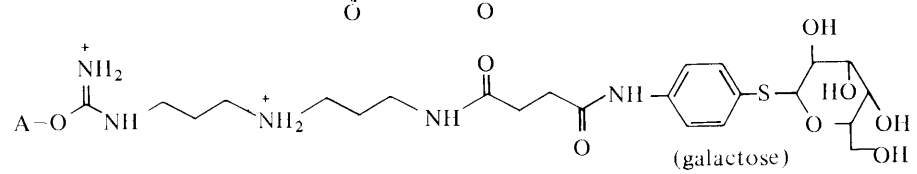
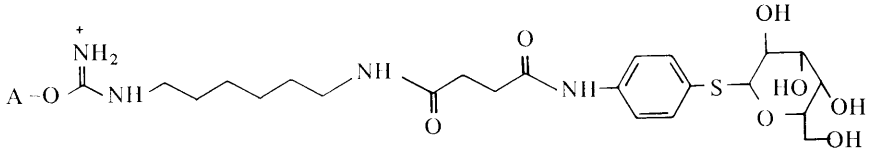
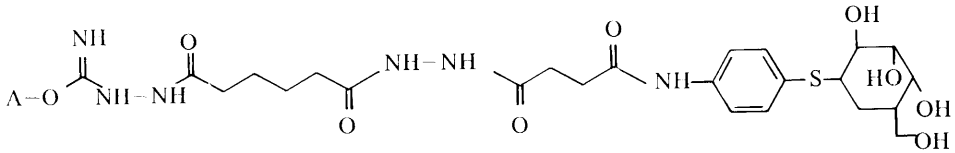
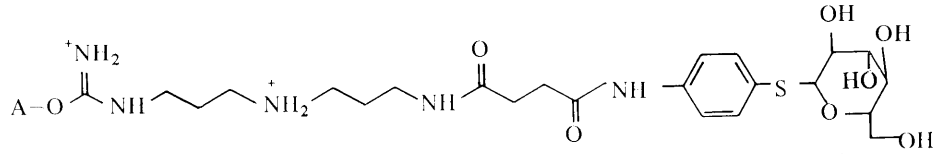
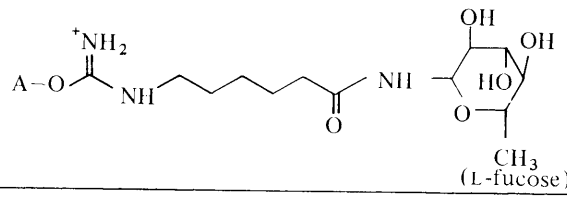
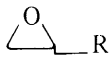
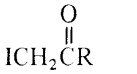
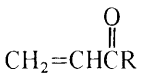
7		10	Not bound		40
8	 (galactose)	6	Bound	pH 10	48
9		4.8	Bound		40
10		9	Not bound		40
11	 (glucose)	?	Bound	pH 10	13

Table 8.2. cont. Binding of β-Galactosidase to Various Affinity Gels

Gel	*Sorbent Structure*	*[L] (μeq/ml)*	*Binding of β-galacto-sidase*	*Desorbed by*	*Ref.*
12		2	Bound	High salt	50

[a] All experiments were carried out using 4% agarose (A) as the gel.
[b] GuCl, guanidine hydrochloride.

correct the deficiencies of this procedure exist. One would maintain the same synthetic strategy, but would place less reliance on linking groups by formation of amides from amines and carboxylic acid active esters—reactions that are certain to generate ionic residues in the gel—and develop instead alternative coupling procedures that would still generate hydrophilic groups but which would produce only nonionic products and byproducts. Possible procedures for this purpose might include appropriate combinations from these nucleophilic and electrophilic groups:

Nucleophile	Electrophile
ROH	
RSH	O=C=N–R
	
	

A second would modify the existing procedure by preparing pure ligand with leash attached, and linking the preformed unit to the gel (*b* of Scheme 1). A third, and chemically best-defined procedure (*c* of Scheme 1), would prepare a ligand-leash combination functionalized in such a way that it could be copolymerized with an appropriate monomer (acylamide, 2-hydroxyethyl methacrylate) to form a porous gel.

These approaches to the preparation of affinity gels that contain few unplanned hydrophobic or ionic sites are more complex synthetically than presently used procedures. Before beginning one, it is important to decide what characteristics are required of the product. Eliminating ionic effects is straightforward in principle: the product gel should contain no groups that are charged at the pH at which the gel is to be used. Eliminating hydrophobic sites is less obvious: how does one best measure the hydrophobicity or hydrophilicity of a particular leash structure? Studies of the origin of hydrophobic effects [52-54], or their importance in determining protein structure [55], and of their application in various forms of chromatography [56] have been extensive. These studies have established that the hydrophobic effect originates in major part in an increase in structure (and decrease in entropy) of water in the vicinity of the hydrophobic group, and correlated the magnitude of this effect with the surface area of the hydrophobic moiety [57, 58]. These studies are not, however, practically very useful in the task of designing leashes having desirable hydrophilic (or hydrophobic) character. Perhaps the most useful numbers for this purpose are

two sets of group hydrophobicity parameters, one assembled empirically by Hansch and co-workers through examination of the partitioning of a variety of substances between water and 1-octanol [59], and the second—the so-called HLB (hydrophilic lipophilic balance) numbers [59, 60, 61]—gathered to correlate structure with surface activity. A more elaborate treatment of group solubility parameters is also available [62], and a variety of useful solubility data are available from Hildebrand's cohesive energy density compliations [63]. The former treatment is, however, concerned primarily with hydrophobic moieties, and the latter is not sensitive to small differences in hydrophilicities.

The Hansch π_X values are defined for a particular substituent X by the change in the partition coefficient P of some test substance H-R between water and 1-octanol when a hydrogen atom of H-R is replaced by the substituent X.

$$\underset{(H_2O)}{X\text{-}R} \rightleftharpoons \underset{(1\text{-octanol})}{X\text{-}R} \quad P_X$$

$$\underset{(H_2O)}{H\text{-}R} \rightleftharpoons \underset{(1\text{-octanol})}{H\text{-}R} \quad P_H$$

$$\pi_X = \log P_X - \log P_H$$

The values have been successfully correlated with the binding of small molecules to bovine serum albumin [64]. HLB numbers are measured in a variety of ways, and are subject to a number of interpretations [60, 61]. Briefly, the magnitude of the group HLB numbers, $(HLB)_X$, can be related approximately to the influence of the group concerned on the partitioning of some test substance between water and a hydrocarbon using the equation

$$\log \frac{C_H}{C_W} \cong 1.20\,(7 - \Sigma(HLB)_X)$$

where C_H and C_W are, respectively, concentrations of the test material in the hydrocarbon and water phases, and $\Sigma(HLB)_X$ is the HLB number of this material, obtained by summing contributions from its constituent parts. Clearly π_X and $(HLB)_X$ cannot be directly compared, but each separately gives a useful scale of hydrophilicities. Values of π_X and of $(HLB)_X$ are listed in Table 8.3 for several groups. Where these series overlap, there is general agreement in the ordering of groups according to hydrophilic character, with the exception of the HLB number for $N(CH_3)_2$. This value is sufficiently high that it suggests that the species actually contributing to the partitioning is $[N(CH_3)_2H]^+$.

Utilizing these values to design optimally hydrophilic polymer-leash-ligand combinations for affinity chromatography will, in general, require more information about the details of interactions of particular proteins and hydrophobic groups than is presently available. One elementary question that can, however,

Table 8.3 Values of π_X and $(HLB)_X$ for Representative Organic Moieties [a]

X	π_X	$(HLB)_X$
SO_4^-		38.7
$CO_2^-K^+$		21.1
$CO_2^-Na^+$		19.1
$SO_3^-Na^+$		11.0
$CONH_2$	-1.71	
OH (tert)	-1.49[b]	
OH (sec)	-1.39[b]	
C=O	-1.21.	
NH_2	-1.19	
OH (prim)	-1.16	1.9
O (ether)	-0.98	1.3
NO_2	-0.85	
CN	-0.84	
$COCH_3$	-0.71	
$NHCH_3$	-0.67[b]	
CO_2H	-0.67	2.1
OCH_3	-0.47	
$N(CH_3)_2$	-0.30	(8.45)
CO_2CH_3, $OCOCH_3$	-0.27	2.4
OCH_3	-0.26[c]	0.83
CH_2NHCH_2	-0.17[b]	
F	-0.17	
$CH_3N(CH_3)CH_2$	0.18	
CH_2CH_2O	0.34	0.33
Cl	0.39	
SCH_3	0.45	
Br	0.60	
CH_3, CH_2	0.66[d] 0.50[b]	-0.475
CH, C		-0.475
I	1.00	
OC_6H_5	1.61-2.08[c]	
$(CH_3)_2CH$	1.81[c]	
C_6H_5	2.1[c]	-1.66
$(CH_3)_3C$	2.26[c]	
Cyclohexyl	3.18[c]	

[a] Values of π_X are taken from or derived from Refs. 60 and 65, and those for $(HLB)_X$ from Ref. 59, unless indicated otherwise.
[b] Ref. 66.
[c] Ref. 59. This reference contains π values for several other alkyl groups.
[d] Ref. 67.

be approached using available data is that of the relative hydrophilicities of groups that might be used as leashes. Several representative structural types are

summarized in Table 8.4 in terms of π group values calculated from Table 8.3.

Table 8.4 Approximate Relative Hydrophilicites (π) Derived from Table 8.3 of Representative Structures That Might be Incorporated Into Leash Moieties

Group	π	$100 \times \pi$/e.w.
$-CH(CH_2OH)-CONH-$	-1.55	-1.8
$-CH_2CONH-$	-1.05	-1.8
$-CHOHCHOH-$	-1.0	-1.7
$-CH_2CH(CONH_2)-$	-0.39	-0.55
$-CH_2CH(CH_2OH)CH_2O-$		
$-CH_2CH(OH)-$	+0.16	+0.36
$-CH_2CH_2O-$	+0.34	+0.77
$-CH_2CH(COCH_2CH_2OH)-$	+1.21	+0.85
$-CH_2CH(C\equiv N)-$	+0.48	+0.91

For comparison, these values are normalized to a common weight basis by dividing by the group equivalent weight and multiplying by 100 ($100 \times \pi$/e.w.). The $-CHOHCHOH-$ moiety is assumed to approximate agarose and Sephadex; more detailed estimates are probably not worthwhile, since internal hydrogen bonding of unknown magnitude will significantly influence the actual value. With this assumption, it would appear that polyserine or polyglycine should be the most hydrophilic neutral leash structure that would be conveniently available. Of the leashes lacking amide moieties, polysaccharides of one or another type would seem most suitable. Polyvinyl alcohol, polyethylene oxide, and poly(hydroxyethyl methacrylate) (HEMA) leashes would appear significantly more hydrophobic.

6 SOME OPERATIONAL CONSIDERATIONS

Assuming now that one has at hand an affinity sorbent prepared in accordance

with the guidelines outlined in preceding sections, some general procedures for its use can be considered.

Adsorption

To prevent mechanical fouling of the packed affinity column, it is necessary to remove cellular debris or other insolubles from cell extracts by filtration or centrifugation before introduction to the column. Often a prefractionation of the extract is also in order to diminish nonspecific binding or interference with the affinity adsorption process. Many proteins and high-molecular-weight nucleic acids can interfere with column operation. These problems may be particularly significant if the target enzyme is present only at low concentrations in the crude extract. The binding of a dilute enzyme solution may be inefficient if the binding constant to the ligand is only moderate. It may be helpful in this case to concentrate the enzyme solution before introducing it into the affinity column.

If possible, moderate concentrations of sodium or potassium chloride should be included in the loading buffer: 0.5 *M* KCl can help suppress nonspecific adsorption due to ionic groups in the leash and/or ligand or left over from incomplete reactions in the immobilization process [14, 15].

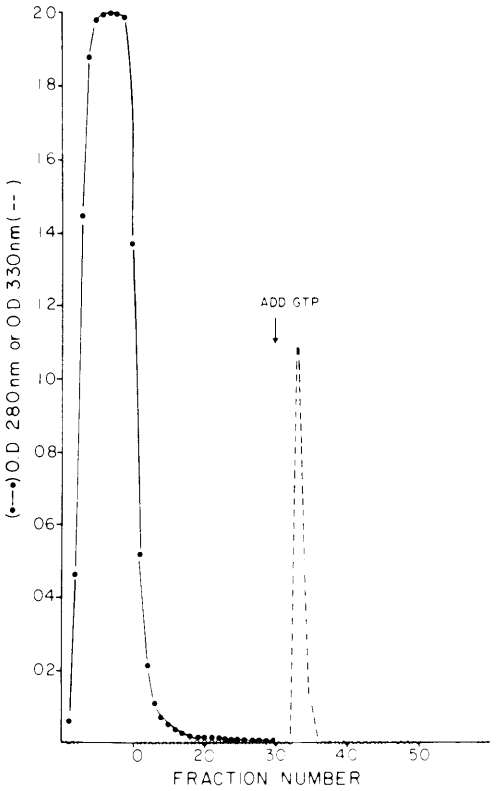
Washing

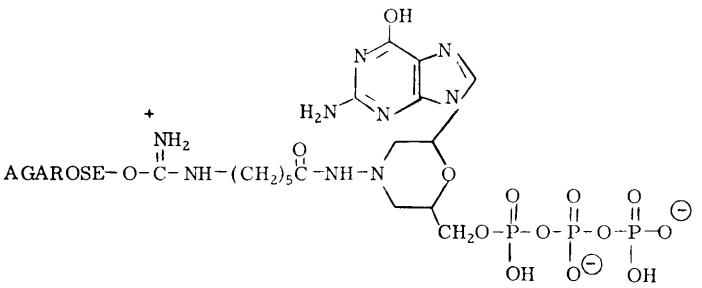
Nonbinding components in a crude mixture are best removed by washing with the loading buffer. Unless there are other indications, usually an eluant volume equivalent to 2-3 bed volumes is sufficient.

Desorption

Desorbing the specifically bound ligate is best accomplished by washing with a solution of a specific desorbing agent in the loading buffer. A representative elution diagram is given in Fig. 8.7. This agent can be a soluble form of the ligand, a competitive inhibitor, a substrate, or a substrate analog. The concentration necessary for effective desorption will depend on the binding constant of the ligand, its concentration in the gel, and the binding constant of the desorbing agent itself. Salt or pH shifts or gradients to desorb ligates usually desorb nonspecifically bound contaminants and lower the selectivity of the affinity process. If, however, an affinity sorbent has been rigorously proved to be very specific for the desired target protein, then a pH shift may be simpler and more economical than desorption using a specific desorbing agent. There is little known about the effects of ionic strength on enzyme-ligand binding. In the few reported studies, there is no effect [68, 69]. Thus, if a salt gradient (e.g., between 0.01 and 1 *M*) causes a ligate to desorb from an affinity sorbent, one has prima facie indication that the binding involves ion-exchange interactions and is probably nonselective.

It is useful to appreciate several potential practical problems in desorption procedures. First, a number of qualitative observations suggest that, for reasons that have not been explored, adsorption of a protein on a gel may not be entirely reversible: the longer the protein is allowed to remain adsorbed, the more difficult it may be to desorb it. Second, even when adsorption is fully reversible, desorption may be a very slow process. Desorption of the ligand from the protein binding site requires an initial first-order dissociation of the complex, followed by trapping of the free protein by some species capable of occupying





its binding site. Even assuming that the rate of formation of a protein-ligand complex is very fast ($\sim 10^8\ M^{-1}\ sec^{-1}$), the half-life for dissociation of a complex with $K_d = 10^{-(5+X)}\ M$ is $\tau_{1/2} \sim 10^{-(5-X)}$ min. Thus, for a strong complex ($K_d = 10^{-10}\ M$), the half-life for dissociation becomes significant ($\tau_{1/2} \sim 1$ min). Since the rate constant for formation of the ligand-protein complex may in fact be orders of magnitude slower than $10^8\ M^{-1}\ sec^{-1}$, and since many dissociation events may be required before the protein is trapped in soluble form, the overall process of eluting adsorbed protein from gel may take hours. Third, elution by displacement requires quantities of the competitive ligand that may not be available. Finally, and in some instances most seriously, it leaves unresolved the problem of generating protein that does *not* have its binding site occupied. Replacement of an insoluble binding group (the insoluble ligand) with a soluble one may complicate the problem of separating protein and ligand: it is, in many ways, ideal to have the ligand insoluble when attempting to free protein from ligand. Fortunately, although these considerations are of real importance, in practice it is usually possible to find conditions that result in release of adsorbed protein from the gel, or dissociation of a soluble ligand-protein complex. In particular, with very strongly bound agents, a shift in pH often significantly decreases the strength of the binding and increases both the extent and rate of dissociation.

The preceding points are largely directed toward enzyme purification. With affinity sorbents for other biopolymers, other desorption methods have been used. For example, with antibodies bound to immunosorbents, pH shifts or chaotropic salt solutions [71] are routinely used. These conditions are required because a more specific desorbing agent is usually not available and because the binding constants for these systems are quite large (about 10^8).

7 BIOPOLYMERS OTHER THAN ENZYMES

Except for occasional references to immunosorbents, our deliberations here on

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Fig. 8.7. Affinity purification of D-erythrodihydroneopterin triosphosphate synthetase. A partially purified enzyme preparation (13.2 ml) was applied to the gel column (1 × 8.5 cm), containing the following sorbent:

The flow rate was about 0.5 ml/min and 2-ml fractions were collected. The column was washed with 0.05 *M* phosphate buffer (pH 6.8) containing 5 m*M* EDTA. The large peak of nonadsorbing material contained no enzyme activity. The low level of 330-nm absorbance seen here is due to the large amount of protein being eluted. Desorption of the enzyme was accomplished with 4 ml of a solution of guanosine-5′-triphosphate (GTP, 0.5 mg/ml) in the buffer followed by additional washing with buffer. The 330-nm absorbance in fractions 32-35 is due to the enzyme product, D-*erythro*dihydroneopterin triphosphate, which is used to detect the presence of enzyme. Protein monitoring at 280 nm in these fractions is precluded because of high background absorbance by GTP. [Reproduced by permission from R. J. Jackson, R. M. Wolcott, and T. Shiota, *Biochem. Biophys. Res. Commun.*, **51**, 428 (1973)].

the affinity approach to purifying biological molecules have centered on enzymes. We note, however, that this methodolgy is potentially applicable to the purification of practically all types of biopolymers and biopolymeric aggregates, including materials that may consist of very large numbers of molecules, namely, microsomes, membrane fragments, viruses, whole bacterial cells, and so on. Antibodies (immunoglobulin G), lectins (previously called hemagglutinins), transport proteins (serum albumin), nucleic acids, lipo-proteins, and glycoproteins have also been purified by affinity chromatography. Examples are reviewed elsewhere [2-5]. Furthermore, very similar considerations apply, in principle, to the immobilization of a wide variety of other types of biologically active materials, and to the study of their interactions with other soluble or surface-bound species. Subjects to whose examination these techniques are pertinent include biocompatible and particularly nonthrombogenic materials [73], cellular adhesion and recognition [74], and mechanism of action of cellular membrane hormone receptors [5, 10, 43].

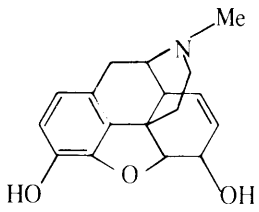
8 EXTENSIONS OF THE AFFINITY CONCEPT TO SMALL MOLECULES

Outside of synthetic polymer chemistry, organic chemistry ordinarily deals with molecules that are less than 1000 daltons in molecular weight. Thus the organic chemist seldom resorts to the physical methods for characterization commonly employed by biochemists and polymer chemists: viscometry, sedimentation (ultracentrifugation), light-scattering, osmometry, gel permeation chromatography, and so on. Affinity adsorption methods offer to the biochemist yet another technique that takes advantage of the macromolecular nature of biopolymers. The extension of affinity adsorption to low-molecular-weight substances is not trivial. The concept underlying affinity adsorption can, however, have heuristic value to organic chemists because it exploits structural specificity in a singularly effective way. It may be possible that some of the procedures used in the affinity purification of biopolymers will suggest solutions to organic problems involving small molecules.

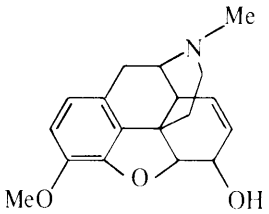
Hapten-Antibody Systems

Immunochemists early discovered that relatively small molecular structures (called *haptens*) were recognized by antibodies [75]. Application of this phenomenon has progressed to the point that today dozens of commercially available clinical tests use a specific antibody reagent to recognize and measure a drug or compound. These immunoassay (radioimmunoassy, RIA, if the reagent uses a radioactive isotope as tracer) tests are used, for example, in forensic situations to detect heroin, barbituates, and other substances in serum or urine [76]. Thus it is possible for organic chemists to use macromolecules to deter-

mine the structure of a small molecule. It is fairly easy to prepare immunoreagents that distinguish between morphinans and barbiturates; more refined methods are now available that can distinguish morphine (**9**) from codeine (**10**) [77].



Morphine, **9**



Codeine, **10**

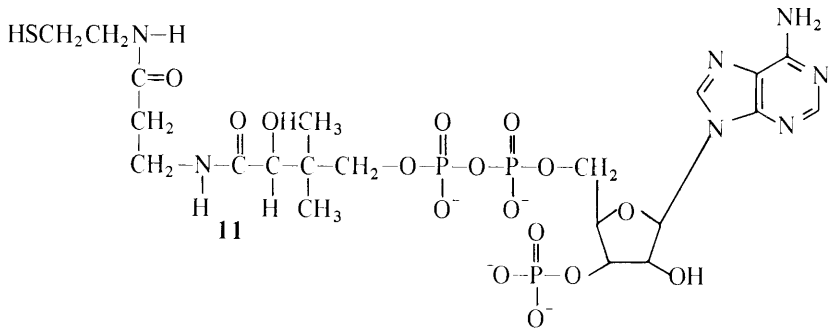
The early work of Landsteiner contains scores of examples of high structure specificity exhibited by antibodies [78]. This type of specificity could, in principle, be used by organic chemists in other types of structure determination. The following is but a partial list of structures distinguishable by specific antibodies:

Aniline
o-Aminobenzoic acid
m-Aminobenzoic acid
4-Methyl-3-amino benzoic acid
o-Aminocinnamic acid
p-Aminocinnamic acid
p-Aminophenylarsenic acid

Although this ability to distinguish between similar structures is exciting, the complexity of the procedures used to generate and isolate specific antibodies limits the use of this approach in routine structural analysis. For those who may have sufficient reasons for pursuing immunological assay procedures, Campbell et al. provides a good introduction [29].

Coenzyme A purification

The purification of coenzyme A (**11**) by Chibata and co-workers [79] illustrates a possible procedure that might be used for isolating other natural products. A reduced form of coenzyme A (CoA) was immobilized on agarose and used to screen dialyzed extracts of various bacteria, which were known to accumulate CoA, for a protein that bound it specificially. The highest concentration of an appropriate binding protein was found in extracts of *Sarcina lutea*, and this substance was isolated by affinity chromatography using the immobilized CoA. The isolated binding protein was in turn immobilized on agarose and used to



11

isolate soluble CoA from other sources. This sorbent had the capacity to bind 85 μg/ml of CoA.

The capacity of the gel incorporating this binding protein points to a problem that is expected also with immobilized antibodies for specific organic compounds: these sorbents have very low molar capacities per volume of sorbent. This low capacity could be predicted from the fact that macromolecular ligands occupy much space in the carrier sorbent. Thus while it is technically possible to turn tables on the enzymologist and use immobilized macromolecules to isolate low-molecular-weight substances, the low capacity of gels containing the macromolecular ligands makes the productivity of a given volume of gel very low. For the isolation of small quantities of natural product, however, the approach of finding a specific binding protein or developing a specific antibody may be the most practical one available. With plant-derived materials (e.g., alkaloids) one might search for the binding protein in extracts of the soft tissues of the plant itself.

D,L-Tryptophan Resolution

An analytical use of an affinity sorbent containing a macromolecular ligand is suggested by the work of Stewart and Doherty [80]. Based on the fact that serum albumin preferentially binds L-tryptophan, a sorbent was prepared by immobilizing this protein to agarose. Figure 8.8 shows a separation of 500 nmol of D,L-tryptophan into its enantiomers on this sorbent. As in the preceding examples, the capacity of a gel containing immobilized serum albumin for binding L-tryptophan is very low. It is, however, suitable for analytical purposes.

9 LIMITATIONS OF THE MACROMOLECULAR LIGAND

Although the ideas underlying affinity chromatography are best illustrated with examples involving a macromolecule as one partner, macromolecules may not, in fact, offer *practical* binding reagents for many problems involving separations of small molecules. Antibodies are expensive; naturally occurring binding

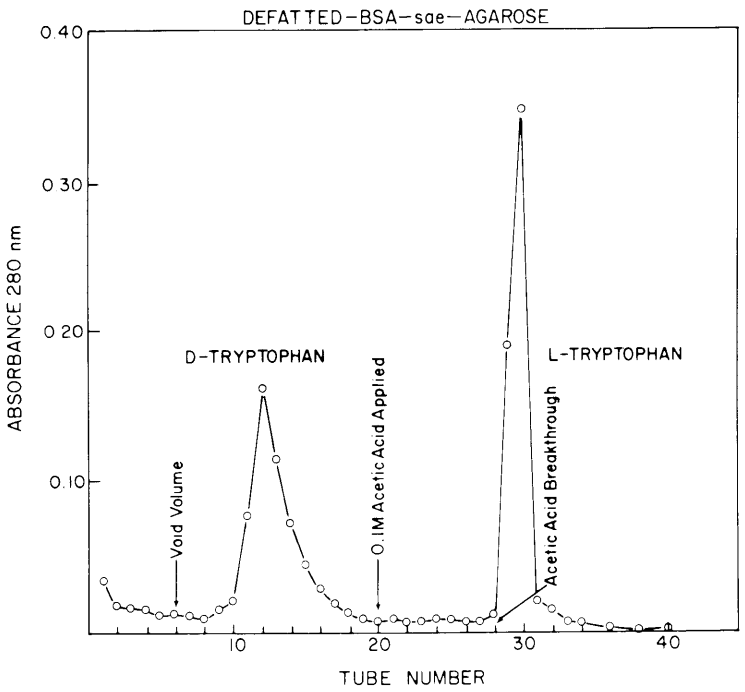


Fig. 8.8. Separation of D,L-tryptophan on defatted bovine serum albumin-succinoylaminoethyl-agarose. D,L-tryptophan dissolved in 0.1 ml of 0.1 *M* borate buffer (pH 9.2) containing 1% (v/v) dimethylsulfoxide, was applied to a 0.9 × 25-cm column. The column contained 630 nmol of immobilized bovine serum albumin. Elution was at 30 ml/hr with borate buffer (no dimethylsulfoxide) for 20 tubes followed by 0.1 *N* acetic acid. The void volume was determined from the elution volume of dimethylsulfoxide. [Reproduced by permission, from K. K. Stewart and R. F. Doherty, *Proc. Nat. Acad. Sci. U.S.*, **70**, 2850 (1973).]

proteins may not be available in useful quantities at any price; the capacity of columns containing immobilized macromolecules is low. Efforts to increase capacity by working with smaller ligands encounter a familiar problem: binding of small-molecule ligates to small-molecule ligands usually shows much lower selectivity than binding to macromolecular ligands.

10 NEEDS OF SMALL-MOLECULE AFFINITY SYSTEMS

To see more clearly the possible extension of affinity methods to organic chemistry, the progression of systems shown in Fig. 8.9 is useful. System A is the one preoccupying enzymologists. The potential and limitations of B for organic chemistry has been sugested in the preceding section. System C is one

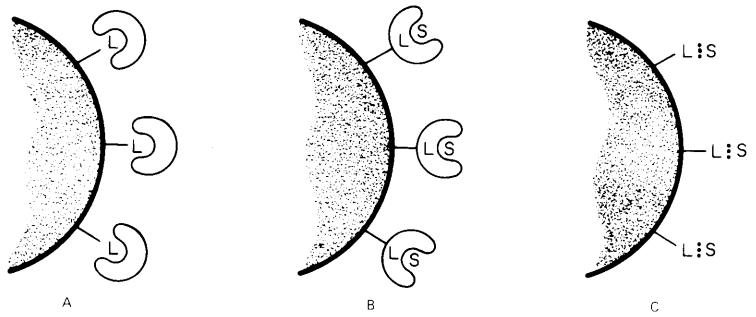


Fig. 8.9. Three interactions between free and immobilized macromolecules, low-molecular-weight ligands (L), and small molecules (S) that might be exploited for affinity separations.

that could, in principle, be most useful to organic chemists and will be explored in this last portion of the chapter.

The structural feature that allows globular proteins to achieve high specificity in binding—their rigid structures, which permit the existence of stable, three-dimensional cavities containing arrays of ionic, polar, and hydrophobic groups is well understood in principle, although detailed understanding of the thermodynamics of binding to, and catalysis by, proteins is still incomplete [52, 81]. Low-molecular-weight ligands normally are not large enough to contain a structurally defined cavity of sufficient size to enclose even small organic groups, but instead rely on a limited number (usually one or two) of strong, specific interactions for their association.

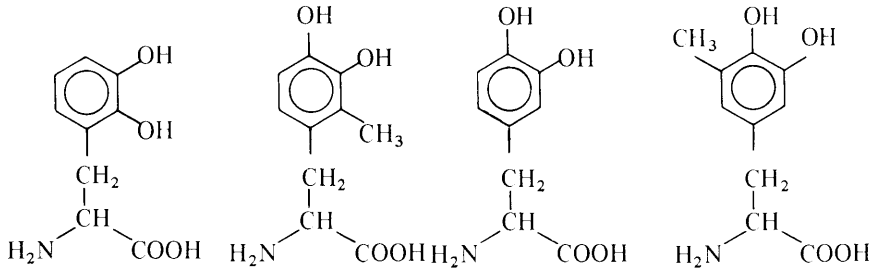
Despite the relatively nonspecific interaction in small-molecule affinity systems, useful separation methods utilizing them should still be possible. In particular, since the ligands are small, it should be possible to introduce them in high concentration into gel supports. In chromatographic use, such supports will have low values for the HETP (height equivalent of the theoretical plate), and thus provide more theoretical plates per linear unit of column than will immobilized macromolecular supports.

11 SOME EXAMPLES OF SMALL-MOLECULE AFFINITY SYSTEMS

Chiral resolutions

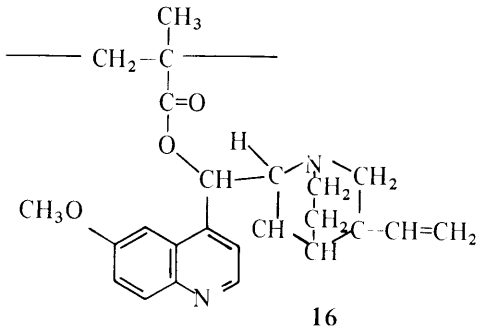
Separations of racemic compounds into their stereoisomers have been treated more extensively elsewhere in this volume [82], so our discussion here will be brief. As it happens, most of the examples of small-molecule affinity systems deal with chiral resolutions, and we are obliged to illustrate the concept with a few examples selected from this area of chemistry.

In 1939 Henderson and Rule [83] reported that solid lactose could serve as a sorbent for the chiral resolution of *d,l*-phenylenebisiminocamphor. The racemic compound (50 mg) dissolved in 8 liters of petroleum/benzene (8:1) was passed through a column containing about 10 kg of lactose. Only a low enantiomeric excess was observed in the best fractions, and the recovery was low. Further, the dimensional requirements of the column were impractical. Poor solubility of the camphor derivative and its weak interaction with lactose were blamed for the incomplete resolution. Cellulose filter paper resolves certain D,L-amino acids [84]. Complete separation of isomers was seen with 2,3-dihydroxyphenyl-D,L-alanine (**12**) and 2-methyl-3,4-dihydroxyphenyl-D,L-alanine (2-methyl-DOPA) (**13**) but not with DOPA (**14**) or 5-methyl-DOPA (**15**). Curiously, variations in the solvent composition (butanol-acetic acid-water) were not found to affect separation of stereoisomers but only the rate of migration. These resolutions were attributed to the asymmetric surface provided by the microcrystalline domains of the cellulose fiber.



12 **13** **14** **15**

These examples use naturally occurring materials as resolving sorbents and are limited in application. Synthetic polymeric carriers incorporating optically active groups broadened the scope of chromatographic resolution. One of the earlier examples was that of Grubhoffer and Schleith [85], who converted polymethacrylate (Amberlite XE-64) to the acid chloride form, then allowed it to react with quinine to obtain an optically resolving sorbent (**16**). This material was capable of retaining *d*-mandelic acid while allowing *l*-mandelic acid to pass. The two enantiomers did not, however, yield well-separated elution bands. A more recent example of preparation and use of synthetic resolving sorbents comes from the work of Davankov and co-workers [86]. Chloromethylated polystyrene was coupled with L-proline (or L-hydroxyproline) to obtain an optically active sorbent (**17**). These materials were used with divalent metal ions (Cu^{2+} or Ni^{2+}) to complex other α-amino acids from racemic mixtures stereoselectively. The extent of resolution obtained with these resins depends on the nature and concentraion of the immobilized ligand, of the complexed metal ion, and of the mobile ligate [87]. Using L-proline as the ligand, a 30-ml column



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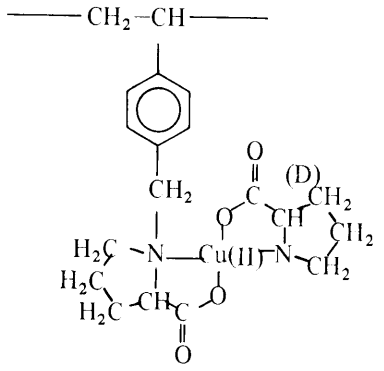


17

was capable of resolving 0.1-0.2 g of D,L-proline completely in a single pass. Similarly, complete resolution D,L-threonine was observed with a sorbent incorporating L-hydroxypyroline.

The results of these workers emphasize the variables that determine stereoselectivity and reveal a substantial improvement in the technology of optical resolution of amino acids. Buss and Vermulen have reviewed related resolution systems for organic compounds [88]. A review of resolutions with cyclic polyethers (crown ethers) is presented by Cram elsewhere in this volume [89].

Cis-Trans separations

The separation of *cis*- and *trans*-isomers is a classical problem in organic chemistry (Fig. 8.10). Kundu and Maenza [90] observed that the commercially available cross-linked dextran gel, Sephadex G-10, easily separates many *cis-trans*-isomer mixtures. While it is not yet clear whether pK or hydrogen-bonding differences are responsible for these separations, it appears that the *trans*-compound is usually bound more tightly than the *cis*-compound. The cross-linked lattice of Sephadex also presents a molecular sieving matrix that may contribute to the separation. This separation technique may be useful for com-

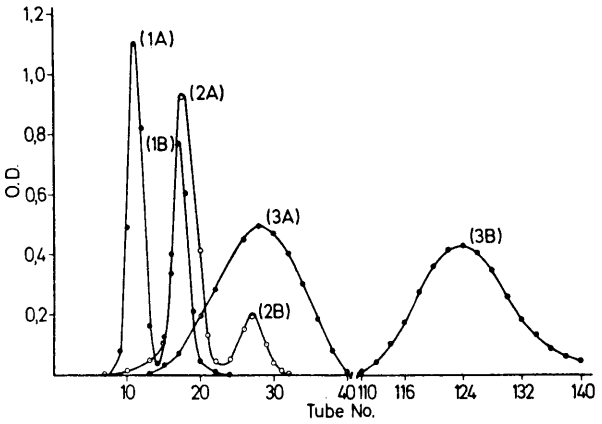


Fig. 8.10. Cis-trans isomer separation on Sephadex G-10. Separations were performed with isomeric pair mixtures containing 500 μg of each compound dissolved in 2 ml of 0.5% NH_4HCO_3 solution (pH 9.0). The column bed was 1.8 × 120 cm. Effluent was monitored at 250 nm; 2.5 ml fractions were taken. •–•–• Fumaric (1A) and maleic (1B) acid mixture. Θ–Θ–Θ Crotonic (2A) and iso-crotonic (2A) acid mixture. Θ Θ Θ Coumaric (3A) and coumarinic (3B) acid mixture. [Reproduced by permission from H. Kundu and F. Maenza, *Naturwissenschaften*, 57, 544 (1970).]

pounds sensitive to distillation.

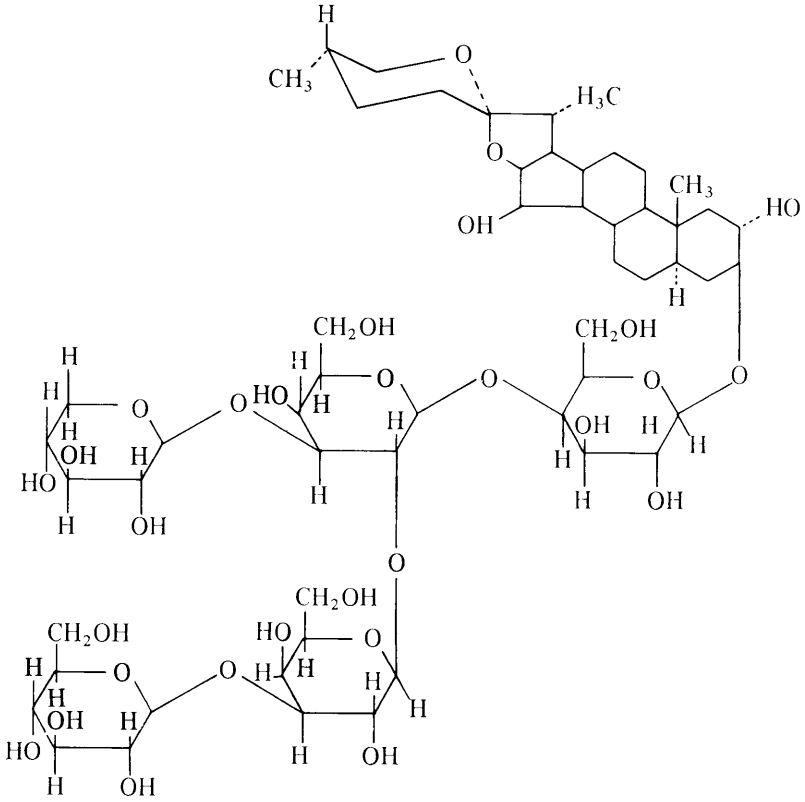
Steroid Separations

The precipitation of 3β-hydroxysteroids with digitonin has been long known and developed over the years as a useful separation method. Recently, Taylor expanded its use in a thin-layer chromatographic system for group separations of Δ^4-3-oxosteroids and Δ^5-3β-hydroxysteroids [91]. The thin-layer substrate was composed of 10% digitonin (**18**) in silica gel containing 13% (w/w) $CaSO_4 \cdot \frac{1}{2}H_2O$.

12 OTHER EXTENSIONS OF THE AFFINITY CONCEPT

This survey of the spectrum of affinity systems from high to low molecular weights suggests, as a generalization, that macromolecular systems are characterized by high selectivity and low capacity, and small-molecule systems by low selectivity and high capacity. Systems incorporating biological macromolecules present further disadvantages: they are restricted to predominantly aqueous systems, and their experimental aspects present significant technical and emotional hurdles for most organic chemists.

It would be useful to develop an effective compromise between the selectivity of macromolecular affinity methods and the convenience of *non*-biological macromolecular complexing agents. For maximum versatility, it should



digitonin **18**

be possible to construct such materials by using a sample of the material that is to be retained in the chromatography to make a molecular "impression" on some suitable support.

Such an approach was first described by Dickey, who showed that silica gel formed in the presence of methyl orange or some of its homologs (ethyl, *n*-propyl, or *n*-butyl orange) would exhibit a significantly enhanced adsorptivity for that particular dye [92]. Bernhard confirmed the essential aspects of Dickey's findings a few years later [93]; see Fig. 8.11. In the meantime Curti and Columbo reported that the methods could be used to make sorbents that were selective for optical antipodes of camphorsulfonic and mandelic acids. A single passage of a 200-ml solution of 0.01 *M d,l*-camphorsulfonic acid through a column containing silica gel specific for *d*-camphorsulfonic resulted in a 30% enrichment of *l*-camphorsulfonic acid [94]. Dickey published a more extensive study in 1955 [94] that revealed refinements in the preparation of the specific gels as well as data indicating the structural selectivity. With gels prepared using

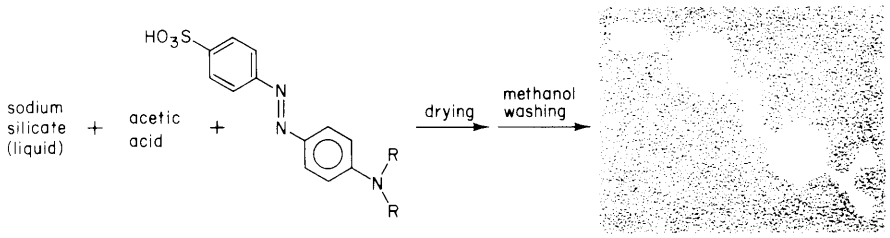


Fig. 8.11. Outline of the formation of a shape-selective silica gel.

methyl orange, the adsorptivity was greatest for the methyl dye and lowest for the butyl dye. The converse was observed for gels prepared with butyl orange. As interesting as these results were, Dickey himself noted severe limitations to the preparation of these sorbents. Chief among these was the requirement for some degree of ordinary adsorption of the target compound onto silica gel before a specific adsorbent could be formed. He noted that gel formation could only be carried out in aqueous media, and in the presence of water many compounds such as amino acids bind poorly to silica gel [96].

A more recent attempt to synthesize a specific adsorbent for small molecules involved synthetic organic polymers. Wulff, Sarhan, and Zabrocki [97] approached sorbent preparation in such a way that the result simulated an "active site" composed of "discontinuate" words rather than "continuate" words [98]. (Linear polymers that bear pertinent information in the near-neighbor sequence of functional groups are described as having *continuate* words; stuctures that arise from segments of a linear polymer that are removed from each other are described as being composed of *discontinuate* words. The terms are illustrated in Fig. 8.12). To obtain the desired structure, a vinyl derivative of D-glyceric acid was first prepared. This monomer was mixed with ethyl vinyl benzene and

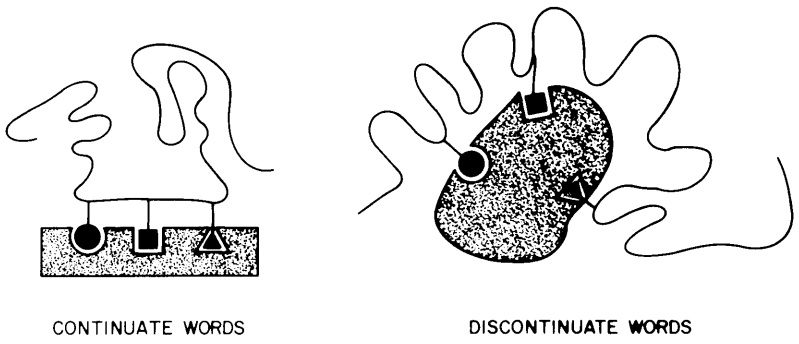


Fig. 8.12. Continuate and discontinuate words.

divinylbenzene and polymerized. After grinding and sizing the polymer particles, the template (D-glyceric acid) was removed by acid hydrolysis. The resulting sorbent preferentially adsorbed D-glyceric acid from a D,L-mixture. The resolving factor α (the ratio of distribution coefficients between the sorbent and solutions of the D- and L-forms) in these experiments was small (1.034), and it seems probable that the internal mobility of groups in these gels is too high to retain the retain the rigid arrangements required for high selectivity. This same sorbent had an α value of 1.036 for D,L-glyceraldehyde, and 1.012 for D,L-glyceric acid methyl ester. See Fig. 8.13.

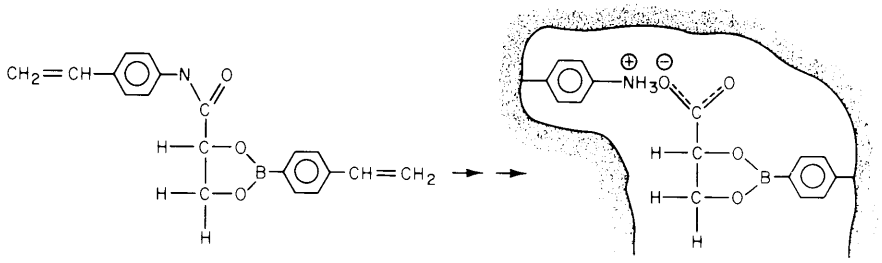


Fig. 8.13. Preparation of a cavity selective for D-glyceric acid.

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