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Affinity Chromatography

Darryl R. Absolom[abc]
[a] Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada [b] Department of Mechanical Engineering, University of Toronto, Toronto, Ontario, Canada [c] Department of Microbiology, State University of New York at Buffalo, Buffalo, New York, U.S.A.

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AFFINITY CHROMATOGRAPHY

Darryl R. Absolom

Research Institute, The Hospital for Sick Children,
Toronto, Ontario, Canada M5G 1X8;

Department of Mechanical Engineering, University of Toronto,
Toronto, Ontario, Canada M5S 1A4
and
Department of Microbiology, State University of New York
at Buffalo, Buffalo, New York, U.S.A. 14214

1 INTRODUCTION

Conventional procedures of protein purification generally rely on small differences in the physicochemical properties of proteins in the mixture, e.g. solubility, charge, molecular size and shape. Isolation on the basis of these differences, e.g. by means of ion-exchange chromatography, gel filtration or electrophoresis are often laborious with low yields. This is not surprising considering that the particular protein of interest may constitute less than 0.1% of the dry weight of the starting material. Considerable reduction in the activity of the macromolecules occurs during the isolation procedure owing to denaturation, chemical cleavage, enzymatic hydrolysis, etc. However, one of the most characteristic properties of these biological macromolecules is their ability to bind to other molecules in a reversible and highly specific manner. The formation of these stable, specific dissociable complexes forms the basis of a powerful separation technique generally called affinity chromatography. Several

attempts have been made to replace this name with more precise terms which place a special emphasis on the technique being a consequence of the specific affinity resulting from biological interactions and differentiating it from affinity caused through non-specific ion-exchange or hydrophobic interactions. Nomenclature such as biospecific adsorption chromatography[1], bioselective adsorption chromatography[2], ligand specific chromatography[3], and biospecific affinity chromatography[4] has thus been introduced. Indeed Landman and Pritchard[5] have suggested that unless genuine bioaffinity can be specifically demonstrated terms such as "activated gel column" are preferable. It should be noted however that the phenomenon of biospecific affinity may be the overall result of a combination of ionic, hydrogen-bond, charge-transfer and van der Waals interactions dominated by stereospecific factors. Apart from the variety of terms which have been used to describe the technique, the individual components have also been subject to various terms. The polymer to which one of the interacting species is covalently attached has been termed "solid support[6,7], carrier[6,7], matrix[3] or insoluble support[4]."

The interacting species which is linked to the polymer is called "the ligand[6,7], effector[8] or affinant[9,10]." The ligand is usually bound to the matrix through a third component. Porath and coworkers have suggested the name "connector substance" and for convenience the reactive chemical group involved in the attachment be called the "connector, active, or reactive group of the matrix[11]." The process of introducing these groups is generally referred to as "activation of the matrix." Steric hindrance considerations often make it desirable physically to separate the ligand from the matrix. This is generally achieved by introducing a hydrophilic chain between the matrix and ligand. This chain is referred to as "the spacer, the spacer arm, or extension arm." The actual process of covalently binding to the matrix through the spacer has been variously termed "coupling, insolubilization or immobilization." Generally the substance to be purified by

affinity chromatography has no particular terminology although the terms "affinity partner"[12], "ligate"[13], counter-ligand[10] or "substrate"[10] have been used. For the sake of clarity the terms to be used in this article are illustrated in Figure 1.

2 HISTORICAL DEVELOPMENT

The concept of separating substances on the basis of their specific interactions is not new. As early as 1907 Hedin had already described the purification and specific desorption of trypsin from charcoal[14-16]. The specificity of biological interactions was first employed as an isolation and purification method by Starkenstein in 1910[17] in which the isolation of α-amalyase was described. In this work an insoluble substrate (starch) was used. The first report in which the ligand itself was covalently attached to an insoluble cellulose matrix was by Campbell et al. in 1951[18]. Bovine serum albumin was linked to diazotized p-amino benzyl cellulose permitting the subsequent

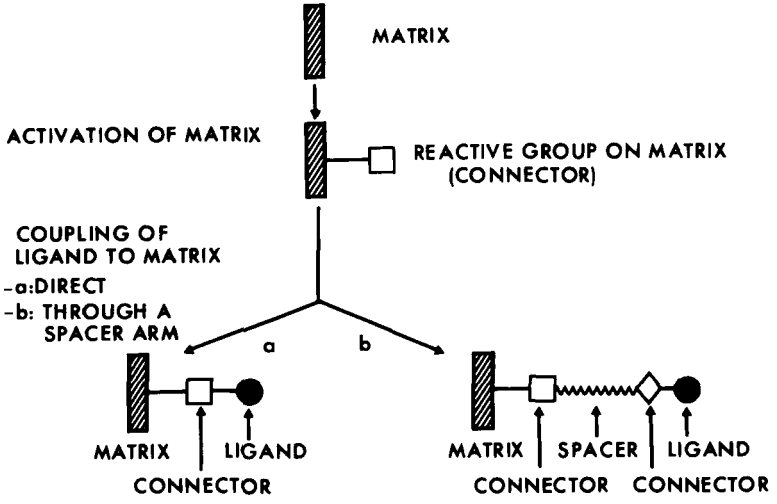


Figure 1

selective purification of rabbit anti-albumin antibodies. For the next decade very little real progress was made in the development of affinity chromatography. This was probably due to the nature of the then available insoluble matrices which did not allow complex formation between the ligand and material to be isolated. Non-specific adsorption of contaminants was generally observed when polymers with hydrophobic or ionogenic groups were used. However in 1967 the reports by Porath and coworkers in which the ligand was directly attached to agarose which had been preactivated with cyanogen bromide[19-21] led to a rapid and extensive development of the technique. Another major advance was achieved through the insertion of hydrophilic spacer arms between the ligand and the matrix[22-25] which serve to reduce greatly the non-specific adsorption of other proteins present in crude preparations.

Affinity chromatography is thus a type of adsorption chromatography in which the molecule which is to be purified is selectively, specifically and reversibly bound onto a specific structurally complementary ligand which is itself irreversibly, and usually covalently, attached to an insoluble, immobile matrix. Instead of being coupled to an insoluble matrix the ligand itself may be rendered insoluble and immobile through crosslinking with agents such as ethylchloroformate or glutaraldehyde. This technique is particularly useful in the production of water insoluble and stable protein polymers. Affinity chromatography readily lends itself to application in the following general areas: (1) Purification of a particular substance from complex starting mixtures. (2) Rapid and complete separation of native and denatured forms of the same molecular species. (3) Removal and concentration of small amounts of the specific biological entity of interest from large volumes of contaminating material. (4) Selective fractionation of various blood cells or cellular components.

The popularity of affinity chromatography is largely due to its success in achieving rapid and complete separations which are

difficult or impossible and time consuming when more conventional procedures are employed. Other advantages of the technique include: (1) Separation and isolation is often achieved in a single purification step. (2) Enrichment or concentration of the selected material is considerable. (3) Recovery yield of non-denatured, fully active material is high. (4) As a consequence of its rapid and specific concentrating capacity, the method allows the processing of large volumes of crude material. However, unlike the more conventional separation procedures, owing to the selectivity of the specific interactions involved on which it is based, affinity chromatography does require considerable prior knowledge of the nature of the interacting components before it can be successfully applied to a particular purification procedure.

3 PRINCIPLE OF AFFINITY CHROMATOGRAPHY

A successful separation and purification of a substance by means of affinity chromatography requires the following: (1) That a specific and complementary ligand is available and that it can be attached covalently to a stable, preferably hydrophilic, matrix to form the chromatographic bed. (2) That the immobilized ligand once attached retains its specific binding affinity for the material of interest, i.e. the counter-ligand. (3) That a selective, non-denaturing method for desorbing the bound counter-ligand, after removal of the unbound, non-specific material should exist.

A schematic representation of the principle of affinity chromatography is given in Figure 2.

4 ESSENTIALS OF AFFINITY CHROMATOGRAPHY

The matrix, spacer and ligand constitute the three main components of an affinity column.

4.1 Insoluble Matrix Supports

The selection of a suitable matrix and the most appropriate method of ligand attachment will depend on which system best responds at a certain time to the experimental conditions. After briefly discussing the characteristics of the "ideal" support, the

most common solid supports and the methods for their activation and subsequent ligand attachment will be described. At the present time agarose is undoubtedly the most commonly employed matrix material. Accordingly a large section is allocated to it and the other commonly employed matrix materials are described in much less detail. The use of spacers, blocking of unreacted chemical groups, coupling procedures and the splitting of attached ligands will be discussed separately.

Characteristics of the Ideal Matrix.

Porath[1,4] has suggested that the required properties are as follows:

(1) It should exhibit chemical and mechanical stability and be insoluble over a range of experimental conditions such as high and low pH, detergents, varying ionic strength conditions, exposure to various organic solvents, various pressure conditions.

(2) It should permit rapid separation of large volumes of starting materials which implies the high rigidity and suitable

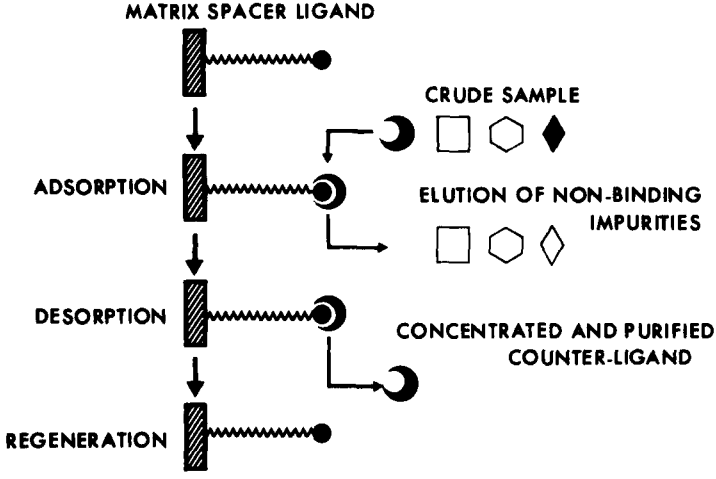


Figure 2

form of the chromatographic particles to ensure high flow rates. Particles should be larger than 5 μm and smaller than 200 μm in diameter.

(3) It should contain functional, chemically active groups which can be easily derivatized for the covalent attachment of ligands. Activation and coupling should be achieved under conditions which do not alter the characteristics of the support. In order to assure easy accessibility of the coupled ligands and a large effective surface area, a high degree of permeability is desirable.

(4) It should be hydrophilic and free of ionic or hydrophobic groups to avoid nonspecific adsorption and possible denaturation.

(5) It should be resistant to microbial or enzymatic degradation. This is necessary in view of the repeated use and storage of the chromatographic material and the usual application of crude biological samples.

(6) An additional desirable property is that it should be possible easily and reproducibly to synthesize the the matrix material. Commercial availability of the matrix is an attractive alternate.

Unfortunately a matrix possessing all of these characteristics does not as yet exist. In the pioneering days of this technique cellulose, polyacrylamide, poly-p-aminostyrene and EMA (a 1:1 copolymer of ethylene and maleic anhydride) were used.

Cellulose (formed from linear β-1,4 linked D-glucose units) is commercially available, crosslinked with bifunctional reagents such as 1-chloro-2,3-epoxypropane. However, cellulose matrices frequently exhibit considerable non-specific adsorption. This problem is enhanced when cellulose is used together with oxidative agents such as potassium or sodium periodate, since aldehyde and carboxyl groups are generated thereby increasing the extent of non-specific adsorption. Also, the glycosidic bonds are sensitive to acid hydrolysis. In addition the fibrous and non-uniform geometry of cellulose reduces flow rates and penetration of large

macromolecules. Coupling procedures of binding ligands of a proteinaceous nature to cellulose have been described in several reviews[26-28]. At the present time the use of cellulose and its derivatives as an affinity matrix is not widespread. The coupling of amino ligands to EMA simultaneously introduces reactive carboxyl groups. The matrix therefore acts as a weak ion-exchange resin which results in very considerable non-specific adsorption. The properties of enzymes bound to this matrix have been extensively evaluated[28,29].

Of the original matrix supports polyacrylamide still is the most widely employed and whilst it possesses many good qualities it has one major disadvantage. The various derivatization procedures, which introduce reactive carboxyl groups into the matrix, cause considerable shrinkage of the beads[24,25]. Advantages of this matrix however include the stability of polyacrylamide in a wide pH range (pH 1-10) and their stability in all commonly used organic eluents. In addition this matrix does not contain ionized groups and is biologically inert. Since the matrix is a synthetic polymer it is extremely resistant to microbial and enzymatic attack. Procedures for the coupling of ligands to the commercially available forms of this matrix have been described in detail elsewhere[30].

The major disadvantage of polystyrene supports is their hydrophobic character, which results in considerable non-specific adsorption, coupled with very low porosity which results in low flow rates.

More recently a considerably improved matrix was found in the form of dextran gels. Dextran gels are formed by crosslinking long-chain polysaccharides consisting largely of glucose subunits linked mainly through α-1,6 glucosidic linkages. Soluble dextran can be rendered insoluble for use as a suitable matrix by crosslinking with 1-chloro-2,3-epoxypropane under alkaline conditions. The resulting three dimensional gel has a relatively hydrophilic nature and consequently a fairly low non-specific adsorption. Dextran gels are thermally relatively stable; they

can withstand heating up to 110°C. Unfortunately, however, dextran gels with the required permeability characteristics become too soft and collapse under the operating pressures of most chromatographic procedures. In addition, in the presence of oxidizing agents (used to activate the gel) both aldehyde and carboxyl groups are formed. Other disadvantages of this matrix system include its susceptibility to microbial degradation and the tendency to swell markedly in organic solvents. Nevertheless, stabilized dextran gels have been employed without any further modification as the specific affinity adsorbent for the isolation and purification of a series of lectins. Coupling procedures for dextran are similar to the methods which will be described for agarose and its derivatives.

A major breakthrough in the development of affinity chromatography was achieved with the introduction of beaded agarose as the insoluble matrix; this presently is the most widely used carrier. In its natural state agarose occurs as part of a complex mixture of charged and uncharged polysaccharides referred to as agar which is isolated from red seaweed (Rhodophyceae). The number of charged sulphate groups can be reduced under alkaline conditions by treatment with sodium borohydride. Agarose consists of repeating subunits of D-galactose and 3-6 anhydro L-galactose linked alternatively through the 1-3 and 1-4 positions (Figure 3). Hjertén[31] developed methods for the transformation of molten agarose into agarose spheres for chromatographic purposes. One way of achieving this is to inject hot, molten agarose through a fine nozzel into ice-cold ethanol or distilled water. Agarose appears to be the candidate which best fits the requirements of an ideal matrix for affinity chromatography. The desirable properties which beaded agarose possesses include:

(1) The hydroxyl groups on the sugar residues can be easily derivatized by a variety of methods for the covalent attachment of ligands.

(2) The macroporosity of the gel matrix[32] also makes the interior of the matrix available, thereby increasing the effective

surface area for ligand attachment, resulting in enhanced binding capacities.

(3) Beaded agarose is mechanically and chemically stable under most experimental conditions. The support is stable in the pH range 4-9 and at temperatures above 0°C and below 40°C. The matrix is not damaged by high salt, urea or guanidine hydrochloride concentrations, or by 50% solutions of organic solvents such as ethylene glycol or dimethylformamide.

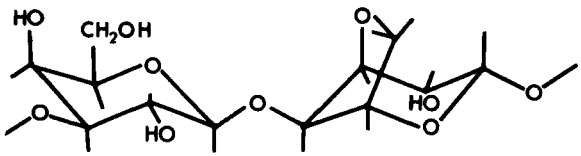
(4) The presence of the unusual sugar unit, 3,6-anhydro-L-galactose, makes agarose exceptionally resistant to microbial degradation.

(5) The porous matrix structure of beaded agarose extends the fractionation range of separations possible to include substances with molecular masses as large as 40 million daltons. The matrix can be formed from various concentrations of agarose which gives rise to different bead sizes suitable for different molecular weight separations, cf. Table 1.

Thus separation is achieved on the basis of selective bioaffinity as well as on the basis of molecular sieving.

(6) As a consequence of the chemical treatment of the molten agarose before gelling, the matrix contains very few sulphate, sulphate-half ester and carboxyl groups and thus non-specific

STRUCTURE OF AGAROSE



D-galactose 3-6 anhydro L-galactose

Figure 3

adsorption is low. (However, these groups may cause considerable non-specific adsorption of basic proteins dissolved in low ionic strength buffers, $\mu < 0.02$).

(7) The spherical geometry of agarose ensures good flow properties with a minimum of bed channeling, thus providing rapid separation.

(8) Beaded agarose is readily available from several commercial sources, e.g. Pharmacia, Bio-Rad, Serva, P-L Biochemicals).

In addition for those applications which require the matrix to be subjected to long periods in dissociating media such as guanidine hydrochloride, chaotropic salts, urea, organic solvents, or high temperatures, agarose is also available in a tightly crosslinked form with considerably increased thermal, chemical and mechanical properties and which nevertheless has the same porosity as the parent gel. Crosslinking of agarose[33,34] has been achieved using epichlorohydrin or 2,3-dibromopropan-1-ol. This treatment reduces the number of hydroxyl groups (theoretically by ~ 50%) available for the covalent coupling of ligands. Several workers[35-37] however have reported that this loss of effective binding sites can be compensated for through the addition of sorbitol or phloroglucinol during the crosslinking reaction.

TABLE I

Properties of Beaded Agarose

Approx. Agarose Concentr.%	Wet Bead Diameter μm	Fractionation range (Molecular Mass) proteins	polysaccharides
2	60 - 200	$7x10^4$ - $4x10^7$	$1x10^5$ - $2x10^7$
4	60 - 140	$6x10^4$ - $2x10^7$	$3x10^4$ - $5x10^6$
6	60 - 165	$1x10^4$ - $4x10^6$	$1x10^4$ - $1x10^6$

The combination of polyacrylamide and agarose as a matrix has been introduced by Uriel et al.[38]. This support has a polyacrylamide network and an interstitial agarose gel. Rigidity and uniformity of bead size as well the presence of different types of functional groups are the reported advantages of this system[39,40]. Limited thermal and acid stability are its major drawbacks.

Inorganic carriers such as porous glass beads have also been used. Glass derivatives have an outstanding rigidity and are extremely resistant to microbial degradation. Glass chromatographic beds have the advantage that the column bed volumes do not change when the aqueous environment is altered. The acid stability of glass is good but degradation in an alkaline environment occurs. Non-specific adsorption to glass is rather high but may be greatly reduced by covalently coating the beads with a monomolecular layer of dextran, glycerol or γ-aminopropyltriethyoxy-silane[41,42]. Porous glass beads and derivatives have been used extensively for the immobilization of enzymes. This matrix is commercially available from several suppliers in controlled bead sizes to allow effective molecular sieving (e.g. Bio-Rad, Corning, Serva, Waters Associates). Other synthetic polymers which have fairly recently been introduced include: a copolymer of hydroxyethyl methacrylate and ethylene dimethacrylate[43,44], spherical nylon beads covalently coated with glycerol[45] and copolymers of amino acids[46].

4.2 Ligands

The choice of the ligand for coupling to the insoluble, inert carrier matrix is mainly influenced by two factors:

(1) The ligand exhibits a specific and reversible binding affinity for the substance to be purified. Elution profiles will vary depending on the strength of the interaction between the immobilized ligand and the substance to be purified. This binding strength is generally characterized by the association constant (K_{ass}, l/mole) or the dissociation constant (K_{diss}, mole/l) of the interaction:

$$\text{Ligand} + \text{Substrate} \underset{K_{diss}}{\overset{K_{ass}}{\rightleftharpoons}} \text{Ligand - Substrate} + X \text{ Calories} \quad [1]$$

It is usually recommended that the ligand should have a binding affinity for its substrate in free solution in the range of: 10^{-4} moles/litre > K_{diss} > 10^{-8} moles/litre (10^{4} litres/mole < K_{ass} < 10^{8} litres/mole)
For interactions having high values of the affinity constant (K_{diss} < 10^{-5} moles/litre, K_{ass} > 10^{5} litres/mole) the substrate is likely to be very tightly bound to the ligand and desorption will tend to be difficult generally requiring harsh or denaturing elution conditions. Conversely, if the binding constant for the interaction is low (K_{diss} > 10^{-3} moles/litre) then it is probable that there will be only partial or incomplete separation of the substrate from the other contaminating macromolecules. Such interactions are too weak to permit successful affinity chromatography. It should be noted that the affinity constant for a ligand in solution is generally higher than that for an immobilized ligand.

(2) In addition to a reasonable affinity, the ligand should also possess other functional groups which can be chemically modified in order to permit covalent attachment to the matrix. It is important to consider the region of the ligand that is to be attached to the matrix. If more than one functional group is available the ligand should be coupled via the group least likely to be involved in the specific interaction with the molecule to be isolated. (Specific chemical modification procedures will be discussed later.) The main principle of specific interactions between biological macromolecules is the complementarity of the binding sites. The high reactivity of substrates follows from the close interaction of conformationally and configurationally oriented groups of the molecule with complementarily positioned groups or sites within the specific enzyme. Thus immobilization of the ligand should not induce conformational changes or alter the nature of the binding sites. The effectiveness of the

chromatographic procedure will depend largely on this aspect of the technique.

As the technique relies on the specificity of the interaction between the ligand and the substance to be isolated, the purity of the isolate depends on the initial quality of the coupled ligand. It is generally not necessary to attach large concentrations of the ligand in order to prepare an efficient adsorbent. Indeed the available evidence suggests that a high concentration of coupled ligand is likely to increase the binding affinity (making subsequent elution more difficult), steric hindrance and non-specific adsorption. For most systems it is generally sufficient to couple 1-20 μ moles ligand per ml of swollen matrix. However, in the case of very low affinity systems ($K_{diss} > 10^{-4}$ moles/litre) the maximum possible ligand concentration should be employed as this will tend to increase the binding affinity thus permitting effective separation from the other components. High molecular weight ligands usually offer more possibilities for the preparation of an adsorbent than low molecular weight species owing to the increased availability of functional groups.

The extent of ligand coupling to an activated matrix is influenced by several factors: number of reactive groups on the activated matrix, the ratio of the quantity of ligand present, quantity of activated matrix present, concentration of the ligand, accessibility of the reactive groups, ionic strength, pH and temperature of the coupling solution and reaction time.

As mentioned previously a ligand is suitable for the isolation of biologically active macromolecules if it binds to these products in a specific and reversible manner. The particular purification problem in question will therefore decide the nature of ligand which is to be employed. Some possible combinations are given in Table II.

Classification of ligands is therefore on the basis of their biochemical function rather than on their structural or chemical properties. Generally ligands have a very narrow specificity

which is selective for only one type of counter-ligand. This requires the production of separate and specific adsorbents for each purification procedure. A useful modification is to employ the so-called "general ligand" which as its name implies is a ligand which displays an affinity for a larger group of biological macromolecules. For example, several different dehydrogenases can be isolated by employing oxidized nicotinamide-adenine dinucleotide (NAD^+) as the ligand and which is required as a cofactor by this group of enzymes. Within each group or class of adsorbed macromolecules there is either structural or functional similarity. Partial purification and specificity in general or group adsorbents is determined by both the selectivity of the general ligand as well as the use of selective elution conditions. Examples of typical general ligands are given in Table 3.

4.3 Spacer Arms

The active or binding site of a biological substance often is located deep within the molecule and direct coupling of a ligand

TABLE II

	LIGAND	COUNTER-LIGAND (and vice-versa)
a.	enzyme:	substrate inhibitor cofactor (co-enzyme)
b.	receptor:	lectin hormone vitamin F_c part of immunoglobulin
c.	antibody:	antigen hapten
d.	lectin:	glycoprotein polysaccharide
e.	DNA/RNA:	complementary nucleotides

TABLE III
Some Typical General Ligands

General Ligand	Specificity	Applications	Eluents
Protein A-	F_c region of IgG	IgG (many species), IgG subclasses and fragments immune complexes antigens	1 M HAC pH 3, 0.1 M glycltryrosin pH 7
Concanavalin-A	α-D-glucosyl, α-D-mannosyl residues	Glycoproteins, membrane proteins glycolipids, polysaccharides	free sugar (methyl-α-D-glucoside):pulse or gradiant (0-0.5 M). Decreased pH (not below 3) Borate buffer 0.1 M, pH 6.5)
Lentil Lectin-	Similar to Con A (weaker affinity)	Membrane proteins, glycoproteins	Free sugar (methyl-α-D-glucoside):pulse or gradient (0-0.1 M)
Wheat germ Lectin	N-acetyl-β-D-glucosaminyl residues	Glycoproteins, polysaccharides cells (esp. T-lymphocytes)	Free sugar (N-acetyl-β-D-glucosamine) pulse or gradient
Helix pomatia Lectin	N-acetyl-α-D-galactos-aminyl residues	cells (esp. T-lymphocytes) glycoproteins	Free sugar (N-acetyl-α-D-galactosamine)
Poly(U)-	Poly(A), oligo (A) sequences	mRNA plant nucleic acids interferon	Formamide (90%) Increased temperature
Poly(A)-	Poly(U), oligo (U) sequences	hnRNA, viral RNA, mRNA-binding proteins, RNA polymerase, antinucelic acid antibodies	Formamide (90% for RNA) Increased ionic strength (for proteins)
Lysine	Acidic proteins	Plasminogen plasminogen activator	Specific agent (0.2 M amino-caproic acid)
		ribosomal RNA	Increased ionic strength (0.05-0.3 M NaCl)
Cibacron Blue	see section 9.1	Wide range (> 50) of nucleotide-requiring enzymes, albumin, α_2-macroglobulin, interferon	Free cofactor eg NAD^+; pulse or gradient(0-0.2M) Increased ionic strength (up to 1 M NaCl) Decreased or increased pH
5' AMP or 2'5' ADP	NAD^+ analogue $NADP^+$ analogue	NAD^+ dependent dehydrogenases $NADP^+$ dependent dehydrogenases	Free cofactor e.g. NAD^+, $NADP^+$ gradient (0-0.2 M) increased ionic strength (up to 1 M) NaCl). Decreased or increased pH

to a matrix may result in a low purification capacity of the adsorbent due to steric hindrance between the matrix and the counter-ligand. To overcome this problem "spacer-arms" are often inserted in between the matrix and the ligand to facilitate more effective exposure of the ligand and to increase the capacity of the prepared adsorbent. The length of a spacer arm is critical. If it is too short, the arm will not be effective and there will be no improvement in counter-ligand binding. If the arm is too long there is a marked danger of non-specific adsorption reducing the specificity of the separation procedure.

Two approaches are available for the inclusion of spacer arms in the formation of an adsorbent:

(1) Covalent attachment of the spacer to the matrix followed by coupling of the ligand to the spacer arm.

(2) Prior formation of the ligand-spacer arm complex followed by attachment of this product to the matrix.

Both techniques are currently in use although the former is more common as it is technically less demanding and several matrix-spacer arm conjugates are commercially available. Barry et al. have compared both methods and have concluded that the former has the most advantages[47].

Several different types of spacer arms have been introduced. Hydrocarbon chains (α,ω-diaminoalkanes, α,ω-amino carboxylic acids), polyamino acids (poly-DL-alanine, poly-DL-lysine, poly-L-lysysl-DL-alanine, poly-glycine), and even natural proteins (eg. albumin) have been employed as spacer arms. The properties of a spacer arm should include the following:

(1) They should form stable covalent linkages with both the matrix and the ligand. These bonds should be stable under a variety of experimental conditions.

(2) The spacer should be uncharged and hydrophilic in nature in order to minimize non-specific adsorption. Coupling of the spacer arm to the matrix should not result in the formation of charged groups as these will themselves result in non-specific sorption.

(3) The spacer arm should be resistant to chemical and microbial degradation.

A variety of commercial, pre-synthesized spacer-matrix derivatives are available. Some of these are listed in Table 4.

5 COUPLING PROCEDURES

The use of certain coupling procedures depends on the nature of the functional group(s) present in the matrix and the ligand, the mechanical and chemical stability of both the matrix and ligand. The specificity of the binding interaction between the ligand and the substance to be purified, will indicate which groups on the ligand are least critical for the reaction with the binding substance and may thus be used for coupling to the matrix. In virtually all cases prior to the coupling of a spacer arm or ligand, the matrix has to be activated. The specific procedure involved will depend on the type of matrix and ligand to be used. It is beyond the scope of this chapter to discuss the large number of individual methods available for the various matrices. These techniques have been reviewed in depth elsewhere (e.g. see ref. 10). At the present time, however, agarose is undoubtedly the most commonly used material. A review of the chemically reactive derivatives of polysaccharides has been published by Kennedy[48]. A range of activated agarose derivatives are available from a number of commercial suppliers.

The chemically reactive groups which are generally used for attachment are amino, hydroxyl, or carboxyl functions, or the aromatic residues of histidine and tyrosine. A partial listing of the functional groups often employed for coupling ligands to commercial agarose derivatives is given in Table 5. This lists matrix material from a single supplier and it should be emphasized that equivalent and different matrices are available from other commercial sources.

A more general review of binding procedures is given in Table 6. Some of the more commonly employed reaction mechanisms used to couple ligands to agarose are schematically displayed in Fig. 4. (Modified from Fig. 3.6, Affinity Chromatography, Pharmacia Fine Chemicals, with permission).

TABLE IV

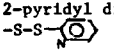
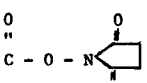
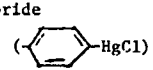
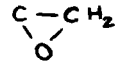
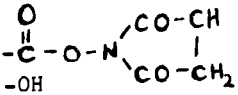
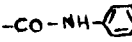
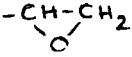
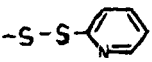
MATRIX (Agarose derivative).	SPACER ARM	TERMINAL REACTIVE GROUP ON SPACER	LIGAND BONDING Reactive Group	Type of Bond
a. PHARMACIA				
AH-Sepharose-4 B	1,6-diaminohexane	Primary amine groups ($-NH_2$)	Carboxyl (-COOH)	Peptide
CH-Sepharose-4 B	6-aminohexanoic acid	Primary carboxyl groups (-COOH)	Amine ($-NH_2$)	Peptide
Epoxy-activated Sepharose-6 B	1,4-bis-(2,2-epoxy-propoxy-)butane	Oxirane groups 	Hydroxyl (-OH)	Ether
			Amino ($-NH_2$)	Alkylamine
			Thiol(-SH)	Thioether
Thiopropyl-Sepharose-6 B	2-pyridyl disulphide hydroxypropyl	2-pyridyl disulphide 	Disulphide	Thiol-disulphide exchange
			Heavy Metal Ions	Mercaptides
			Alkyl or Aryl Halides	Thioether
			C = O N = N C = O	Addition Reactions
b. BIO-RAD				
Affi - Gel 10	-	Activated carboxyl N-hydroxyl succinimide ester 	Primary Amino or Aliphatic	Peptide
Affi - Gel 102	-	Amino ($-NH_2$)	Alkyl halide	C-N covalent
Affi - Gel 202	-	Carboxyl (-COOH)	Sulphydryl Hydroxyl	Disulphide Ester
Affi - Gel 401	-	Sulphydryl $(-CH_2)_3SH$	Sulphydryl Free Carboxyl	Disulphide Thioester
Affi - Gel 501	-	Organomercury Chloride 	Active-Sulphydryl	Mercaptide

TABLE V

Some Commercial Agarose Derivatives

Type of ligand	Functional group	Derivative of Sepharose	Comments
Proteins, peptides, amino acids.	$-NH_2$	CNBr-activated Sepharose 4B	Method of choice for proteins and nucleic. Safe, easy coupling in mild conditions. Very well documented. Use CNBr activated Sepharose 6MB ("Macrobeads") for cell separations.
		CH-Sepharose 4B	Coupling via 6-carbon spacer arm. Organic solvents can be used for water-insoluble ligands. Carbodimide coupling method used.
		Activated CH-Sepharose 4B	Activated gel for spontaneous coupling via spacer arm. Mild conditions. Especially useful for small, sensitive ligands.
		Epoxy-activated Sepharose 6B	Activated gel for coupling amino acids and peptides. Hydrophilic spacer arm. Organic solvent can be used.
Amino acids, keto acids, carboxylic acids	-COOH	AH-Sepharose 4B	Coupling via 6-carbon spacer arm. Organic solvents can be used for water-insoluble ligands. Carbodiimide coupling method used
Sugars, other hydroxyl compounds	-OH	Epoxy-activated Sepharose 6B	Activated gel for coupling via extremely stable ether bond. Hydrophobic spacer arm. Organic solvents can be used
Proteins	-SH	Thiopropyl-Sepharose 6B Activated Thiol-Sepharose 4B	For covalent chromotography of -SH containing substances. Coupling reactions are reversible
Amino acids, other low MW compounds		Epoxy-activated Sepharose 6B	For a stable irreversibly coupled product.
Low MW thiol	-SH	Thiopropyl-Sepharose 6B	e.g. Coenzyme A; ligand-matrix bond cleavable
Heavy metal derivatives	heavy metal		Reversible, immobilization of mercurated polynucleotides (RNA,DNA) etc use reduced form of gel.
	other		Alkyl and aryl halides can be coupled; also substnaces containing C=O, N=N, C=C bonds in certain conditions.

TABLE VI
General Binding Procedures

Spacer arm (connector) group used	Optimum pH of the coupling reaction	Active group on the spacer arm for ligand attachment	Activation Reagent
a. AMINO GROUPS			
$-NH_2$	7.5-8	$-CO-NH-NH_2$	$NaNO_2$+HCI
$-NH_2$	4.5-6.0	-COOH	Carbodiimide
$-NH_2$	8.7	-OH	2-Amino-4,6-dichloro-s-triazine
$-NH_2$	8.5	-OH	Bromoacetyl
$-NH_2$	8	-OH	Oxidation with $NAIO_4$
$-NH_2$	8-10(down to 6)	-OH	BrCN
$-NH_2$	8.5-13		Yields stable amide-linked derivative in aqueous solution
$-NH_2$	5-10		
$-NH_2$	8-10	-OH	Benzoquinone
$-NH_2$	7-5		-
$-NH_2$	8-5		$Cl_2C=S$
$-NH_2$	7.7	$-NH_2$	Glutaraldehyde
$-NH_2$	9-12		-
b. CARBOXYYL			
-COOH	4.5-6	$-NH_2$	Carbodiimide
-COOH	4.5-6.0	-SH	Carbodiimide
c. HYDROXYL			
-OH	Alkaline	$-NH_2$	Oxidation with $NaIO_4$
-OH	9-13		-
	7.5-8.5		$NaNO_2$ + HCL
d. SULPHYDRYL			
-SH	9-13		-
-SH	wide range of of conditions		-
			-
-SH	4.5-6.0	-COOH	Carbodiimide
	Alkaline solution	$-NH_2$	-

6 ADSORPTION

The conditions which favor optimum complex formation between the coupled ligand and the compound to be purified will determine the conditions used in the adsorption of the crude sample to the affinity matrix. Factors such as ionic strength, pH, temperature, the presence of certain metal ions, concentration of the substance to be purified, flow rate and steric accessibility of the binding sites on the bound ligand all play an important role. The impurities are washed away by means of the coupling buffer, or by

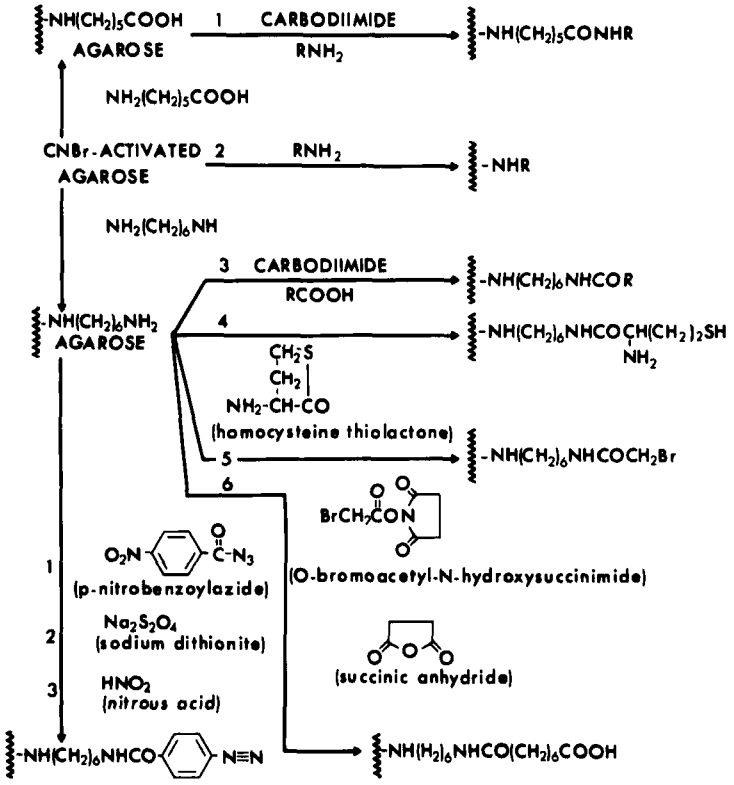


Figure 4

using more drastic eluting conditions (e.g. increased salt concentration, change in pH) provided the specific biocomplex remains intact. The effect of the geometry of the column, the concentration of the affinity ligand and the total of ligand in the column are the three basic parameters that determine both the capacity and extent of interaction. In high affinity systems and when using high concentrations of the bound ligand, column length is of little importance. In practical terms this means that columns that contain a high concentration of ligand can be used for the concentration of very dilute solutions of the macromole of interest. However, for interactions with low affinity, column geometry is an important factor for the overall success of the system.

7 ELUTION

The elution of adsorbed materials from affinity matrices can be the most critical step in the chromatographic process. The success of the purification may depend on the choice of method used to recover the macromolecules. There are several general methods of elution that are commonly and successfully used for a wide variety of affinity matrices (see Table 7).

7.1 Elution by Temperature Changes

The adsorption of a dissolved substance from a liquid phase onto a inert, stationary matrix is generally exothermic, cf. Eq. [1], and thus according to Le Chatelier's principle, elevated temperatures will move the equilibrium in the direction of heat absorption. Under affinity chromatography conditions, an increase in temperature thus will shift the equilibrium to a higher relative concentration in the mobile phase, i.e. dissociation of the complex is favored. In general, the more exothermic the adsorption process, the more susceptible it will be to elution by a temperature gradient[49]. When macromolecules with different enthalpies of adsorption ($\Delta H°$) are adsorbed to the same matrix, the dependence on temperature can be utilized for separation. Linear temperature elution gradients have been particularly useful in the separation of groups of kinase or dehydrogenase enzymes

adsorbed to a general ligand such as AMP-Sepharose. The advantage of the thermal elution system is that the recovered enzymes are not mixed with elution reagents such as salts, nucleotides or chaotropic agents.

7.2 Elution by Dissociation of Coulombic and van der Waals Bonds

Electrostatic (or Coulombic) bonds originate in the attraction between positively and negatively charged groups on the interacting molecules. A close geometric complementarity between the positive and negative alternating series of a certain pattern on the ligand and an oppositely charged series on the counter-ligand will obviously permit an enhanced attraction between molecules. This attraction can be reduced to close to zero by ion shielding (by increasing the ionic strength) or even reversed into a repulsion (by altering the pH of the liquid medium), thereby favoring desorption. In addition, in those situations where plurivalent cations are involved in mediating the

TABLE VII

Elution Techniques in Affinity Chromatography

a. Non-specific	
	Temperature elution
	Ionic strength alterations
	Buffer and/or pH changes
	Solvent changes
	Chaotropic reagents
	Electrophoretic desorption
b. Specific	
	Hapten competition
	Inhibitor competition
	Co-substrate elution

sorption process, chelating agents may be incorporated into the elution buffer in order to effect desorption.

Generally in addition to these electrostatic (ionic) forces, van der Waals (or hydrophobic) forces also play an important role in mediating the specific interaction between the bound ligand and the counter-ligand. However, it is not generally realized that Van der Waals interactions between the different components, immersed in a liquid, also easily can be made repulsive, by modifying the van der Waals constant (or Hamaker coefficient) of the liquid. This is done by modulating the surface tension of the liquid medium[50]. Thus in most cases, for elution both the Coulombic and the van der Waals interactions generally need to be reversed, which in each case requires careful study of the appropriate changes to be made in the physicochemical properties of the liquid medium. In the case of Coulombic interactions this is easily achieved, as discussed above, by appropriate pH or ionic strength changes. The net van der Waals interaction between two different materials immersed in a liquid is repulsive, when the surface tension of the liquid medium has a value intermediate between the values of the surface tensions of the two different interacting materials. In practical terms, effecting a van der Waals repulsion involves lowering the surface tension of the aqueous medium through the addition of varying amounts of water miscible organic solvents such as ethylene glycol, dimethyl sulfoxide, dimethyl acetamide, propanol or ethanol. Experimentally the possibility of repulsive van der Waals forces between two interacting macromolecules has been employed to achieve the complete dissociation of antigen-antibody complexes[51,52], to explain the mechanism of elution in hydrophobic chromatography[53,54] and in affinity chromatography[55].

7.3 Elution by Substitution with Low Molecular Weight Compounds

Dissociation of the ligand-counter ligand complex may also be achieved through the addition of an excess of a low molecular

weight substance which corresponds to the binding site of the ligand. This will shift the equilibrium to the right (cf. Eq. [1]) thereby favoring dissociation of the complex. This principle has been most successfully used in the field of immunochemistry where haptens have been employed to dissociate antigen-antibody complexes, e.g. low molecular weight sugars, e.g. D-galactose, corresponding to antigenic determinants in certain blood group antigens, have been used to dissociate specific blood group antigen-antibody complexes. This technique has also been used extensively in enzymology where cofactors, coenzymes or nucleotides are employed to achieve separation (elution) of the ligand bound complex.

7.4 Elution by (Reversible) Denaturation

The specific biocomplex of the bound substance can be disrupted after their (reversible) steric modification, for example with urea, guanidine salts or chaotropic ions. However, when these reagents are employed the possibility exists that the components of the complex might be irreversibly denaturated during the dissociation process. The use of solutions of salts for elution from affinity columns has been investigated by several workers[56-58]. The elution strength of anions corresponded directly to the order of the Hofmeister or "lyotropic" series as listed below in order of increasing elution effectiveness (chaotropicity).

$$PO_4^{3-} < SO_4^{2-} < CH_3COO^- < Cl^- < Br^- < NO_3^- < ClO_4^- < I^- = SCN^-$$

The Hofmeister series, in order of increasing elution effectiveness (chaotropicity), for cations is listed below:

$$(CH_3)_4N^+ < NH_4^+ < Rb^+, K^+, Na^+, Cs^+ < Li^+ < Mg^{2+} < Ca^{2+} < Ba^{2+}$$

It has also been shown that ions of electrolytes such as LiCl or $MgCl_2$, having a high charge density, are less effective than

monovalent ions such as Cs^+, K^+ or Br^-, in promoting the dissociation (or elution) of specific complexes. It should be mentioned however that this order of chaotropicity is not always the same for all proteins. Such procedures cause changes in the tertiary configuration of the macromolecules which decreases the "geometric fit" between the interacting species. Dissociation may also be achieved by incorporating urea (6-8 M) or detergents into the eluting buffer.

7.5 Elution by Electrophoresis

In 1975 Dean and Harvey[59] suggested that an electrophoretic procedure could be used as a direct elution technique in affinity chromatography. Subsequently several reports have appeared in the literature of the application of electrophoretic desorption to various systems. The technique however has been applied most often to immobilized antigen-antibody systems. The theoretical aspects of the technique have been described by Morgan et al.[60]. When an electrical current is passed across a loaded affinity matrix at a pH such that the adsorbed material is charged, then the specifically adsorbed macromolecules will migrate towards the appropriate electrode. Dissociation of the ligand-counter ligand will be induced by the potential difference across the matrix and the charge on the macromolecules. As the ligand is covalently bound it will not be able to migrate and thus dissociation will occur. Reassociation may occur en route but the electrophoretic migration will continue after a further dissociation event. The consequences of this mechanism are that (a) electrophoretic desorption is applicable in all situations where the bound macromolecule to be eluted has an overall net charge, (b) the yields can be expected to be very high provided adequate time for desorption is available. In view of the relatively low current necessary to achieve desorption in typical affinity situations the procedure is very mild and does not involve exposing the macromolecules to any harsh conditions. Not all matrix materials are suitable when using this technique for desorption. Nylon mesh, for example, gives rise to considerable

electroendosmosis. Table 8 lists some of the systems in which this technique has been applied. The technique, in a modified form known as "affinity electrophoresis", has in addition, been used to determine the dissociation constants of protein-ligand complexes (8.1.c.).

7.6 Elution by Destruction of the Spacer Arm or Matrix

An interesting elution technique is that based on the enzymatic degradation of either the matrix or of the spacer arm. One example of this method is the purification of albumin on a gelatin matrix which was subsequently digested by trypsin[61] thereby releasing the albumin. The same principle was employed in the separation of mouse chymotrypsin on Sepharose 4B. In this report the ligands were various preparations of soybean trypsin inhibitor which varied in their reactivity with chymotrypsin[62].

8 THEORETICAL AND QUANTITATIVE ASPECTS OF AFFINITY CHROMATOGRAPHY

8.1 Equilibrium and Dissociation Constants

Theoretical models of the process of affinity chromatography have been developed by Dunn and Chaiken[63-65], Nishikawa et al. , Porath and Kristiansen[4], and Graves and Wu[67]. Of these models perhaps the most instructive is that of Graves and Wu which is based on the theory of gel chromatography combined with simple kinetics. As a first approximation only equilibrium relationships were assumed. The main prerequisite for affinity chromatography is the formation of a specific complex (CL) between the isolated counter-ligand (C) and the immobilized ligand (L). For this process the following equations apply:

$$C + L \underset{K_{diss}}{\overset{K_{ass}}{\rightleftharpoons}} CL \qquad [2]$$

and

$$K = \frac{[C]\,[L]}{[CL]} \qquad [3]$$

where K is the equilibrium constant for the ligand-counter ligand (association) interaction during the adsorption step. Schematically this may be represented as follows:

$$C + \underline{LM} \rightleftharpoons \quad [4]$$

$$C \rightleftharpoons \underline{C} + \underline{LM} \rightleftharpoons \underline{C - LM} \quad [5]$$

C: free counter ligand

LM: ligand coupled to the matrix

C: free counter ligand present in the matrix

C-LM: reversibly bound counter ligand

When the matrix material used has a high exclusion limit (10^6-10^7 daltons) and the interacting species a molecular mass considerably lower than this value, then the amount of counter ligand which is attached to the outer surface of the matrix will be negligible in comparison to the concentration of counter ligand bound to the interior of the gel. Thus equilibrium conditions for Eq.[5] only need to be considered. (In addition steric hindrance of the incoming, free in solution, counter ligand molecules by the previously bound counter ligand molecules is not considered.) The dissociation constant (K_{diss}) for the complex is given by:

TABLE VIII

A selection of the materials that have been successfully electrophoretically desorbed from a variety of different matrices

SPECIES DESORBED	ADSORBENT
Ferritin	Antibody
Steroid (E_3-Gluc)	Antibody
Steroid antibody	E_3-Gluc-BSA
IgG	Protein A
IgG	Con A
Anti IgG	IgG
HSA	Cibacron Blue-Sepharose
LDH	Cibacron Blue-Sepharose
LDH	5-AMP-Sepharose
LDH	Hexyl-Sepharose
LDH	SEAE-Sepharose
Cytochromes	Cytochrome-C
E_3-Gluc	Amberlite XAD-2
Amino Acids	Amberlite IR 120
Amino Acids	Charcoal
ATP	Charcoal

$$K_{diss} = \frac{[\underline{C}]\ [\underline{LM}]}{[\underline{C - LM}]} \qquad [7]$$

In 1968 Determan[68] considering normal gel permeation chromatography indicated that:

$$V_e = V_o + K_d \cdot V_i \qquad [8]$$

where V_e: elution volume of the enzyme

V_o: void volume of the column

V_i: internal (bed) volume of the column

K_d: distribution coefficient ($K_d = [\underline{C}]/[C]$)

In affinity chromatography however there is an additional interaction between the counter ligand and the gel beads, due to the covalently attached ligands which specifically bind the counter ligand. Thus

$$V_e' = V_o + K_d' \cdot V_i \qquad [9]$$

and

$$K_d' = \frac{[\underline{C - LM}] + [\underline{C}]}{[C]} \qquad [10]$$

Rearranging and substituting for [C]

$$K_d' = K_d \frac{[\underline{C - LM}]}{[\underline{C}]} + K_d \qquad [11]$$

Substituting for $[\underline{C - LM}]$ using [6]

$$K_d' = K_d \cdot \left(\frac{\underline{LM}}{K_{diss}}\right) + K_d \qquad [12]$$

Substituting for K_d using [7]

$$K_d' = \left(\frac{V_e - V_o}{V_i}\right)\left(\frac{\underline{LM}}{K_{diss}}\right) + \frac{V_e - V_o}{V_i} \qquad [13]$$

Substituting for K_d' using [8]

$$\frac{V_e' - V_o}{V_i} = \left(\frac{V_e - V_o}{V_i}\right)\left(\frac{\underline{LM}}{K_{diss}}\right) + \frac{V_e - V_o}{V_i} \qquad [14]$$

Rearranging and multiplying by V_i

$$V_e' = V_e + \frac{(V_e - V_o)\ [\underline{LM}]}{K_{diss}} \qquad [15]$$

Thus it can be seen that the increase in elution volume (V_e') in affinity chromatography is proportional to the degree of substitution of the ligand on the matrix ([LM]), if the concentration of the soluble ligand is constant and is inversely proportional to the dissociation constant (K_{diss}) of the interaction between the bound ligand and the counter ligand. Equation [14] also provides a quantitative basis for a statement made earlier in this text (Section 4.2) that when the affinity of the ligand for its substrate is low then the amount of ligand coupled to the matrix should be increased, in order to achieve successful separation.

8.1.1 Elution Analysis

The most commonly employed method in quantitative affinity chromatography is based on the elution of a macromolecule from the matrix-ligand complex with varying concentrations of the ligand in free (unbound) solution. Schematically this may be represented as follows (cf. 63, 64).

$$\begin{array}{ccccc} C & \rightleftharpoons & \underline{C} & + & \underline{LM} \rightleftharpoons \underline{C - LM} \\ + & & + & & \\ S & & \underline{S} & & \\ \Updownarrow & & \Updownarrow & & \\ CS & & \underline{CS} & & \end{array}$$

where S, $\underline{S}$, CS and $\underline{CS}$ represent the soluble ligand (or competitive inhibitor) and the ligand complex in and outside the matrix as described for Eq. [5]. As mentioned previoulsy (Eq. [15]) the elution volume, V_e, is directly proportional to the concentration of the bound ligand [$\underline{LM}$] and is indirectly proportional to the concentration of the soluble ligand if the concentration of the bound ligand is constant. An analogous expression to Eq. [14] can be derived to represent this procedure as follows:

$$V_e' = V_e + \frac{(V_e - V_o)\,[\underline{LM}]}{\dfrac{K_{diss}}{\left(1 + \dfrac{S}{K_s}\right)}} \qquad [16]$$

where K_s is the dissociation constant for the ligand-counter ligand ($S + C \rightleftharpoons S - C$) binary complex in solution. This value (K_s) is usually determined by direct analysis using techniques such as equilibrium dialysis, ultrafiltration, spectroscopic methods or steady state kinetics. The validity of this equation was experimentally verified by Dunn and Chaiken[65] using staphylococcal nuclease chromatographed on a thymidine 3'-(p-Sepharose-amino-phenylphosphate)5'- phosphate matrix with elution being achieved with various concentrations of nuclease. The advantage of this rather simple model is that the derived theoretical expressions can readily be tested experimentally in contrast to the more rigorous and complex models developed by other workers[67,69,70] and which take into account factors such as flow rate, steric hindrance, reaction rate and diffusion rate.

On the basis of Eq. [16], if an experimental plot of the elution volume, V_e, versus the concentration of the bound ligand ($[\underline{LM}]$) is drawn, then the resulting slope can be described by the following expression:

$$\text{Slope} = \frac{(V_e - V_o)/K_{diss}}{\left(1 + \frac{S}{K_s}\right)} \qquad [17]$$

Hence when one of the dissociation constants and the slope are known, then the other dissociation constant may be calculated.

In addition Eq. [16] may be transformed into the following form:

$$\frac{1}{V_e' - V_e} = \frac{1}{\frac{(V_e - V_o)\ [\underline{LM}]}{K_{diss}}} + \frac{[S]}{\frac{K_s(V_e - V_o)[\underline{LM}]}{K_{diss}}}$$

$$= \frac{K_{diss}}{(V_e - V_o)[\underline{LM}]} + \frac{K_{diss}}{K_s(V_e - V_o)[\underline{LM}]} \cdot [S] \qquad [18]$$

Thus a graph of $1/(V_e' - V_e)$ versus [S] should provide a linear plot of the form: $y = mx + c$ with an intercept equal to $K_{diss}/(V_e - V_o)([\underline{LM}])$ and the slope equal to $K_{diss}/K_s(V_e - V_o)[\underline{LM}]$. ([S] may be obtained from elution

volumes). It follows from the above that the ratio of the intercept:slope is:

$$\frac{\text{intercept}}{\text{slope}} = \frac{K_{diss}}{(V_e - V_o)[\underline{LM}]} \cdot \frac{(V_e - V_o)\,[\underline{LM}]\,K_s}{K_{diss}}$$

$$= K_s \qquad [19]$$

Since the expression for the intercept contains the dissociation constant for the immobilized ligand-substrate complex, K_{diss}, may be calculated from that expression.

8.1.2 Frontal Analysis

Equilibrium and dissociation constants (in affinity chromatography systems) may also be obtained by frontal analysis according to the method of Kasai and Ishii[71]. This technique has several theoretical advantages over the method of Dunn and Chaiken described above. Frontal analysis is extremely sensitive and elution volumes can be accurately determined because their dependence on concentration is negligible.

8.1.3 Affinity Electrophoresis

Affinity electrophoresis is a technique that makes use of the advantages of both affinity chromatography (separation according to biospecific interactions between macromolecules) and polyacrylamide gel electrophoresis (where macromolecules are separated according to their electrophoretic mobility). The method was first described by Takeo and Nakamura who used it to determine the dissociation constants of glucan phosphorylases[72]. The term "affinity electrophoresis" was introduced by Horejsi and Kocourek who used the technique for the study of phytohemagglutins[73]. The principle of the method consists of the electrophoresis of macromolecule on a matrix (usually polyacrylamide) which contains covalently bound immobile ligands for which the macromolecules exhibit a specific affinity. The macromolecules which have combining sites complementary to the ligands will bind to them and do not migrate whereas other proteins (which do not exhibit the required specificity) undergo a normal separation based on their individual electrophoretic

mobilities. Technically the procedure requires the formation of a polyacrylamide gel consisting of three regions:

(1) Top. This is a "stacking gel" of large pore size which is approximately 5 mm in length and is used to concentrate the applied mixture to yield a narrow, well defined starting region or origin.

(2) Middle. This is the "affinity gel" to which the ligand has been covalently bound to the matrix and is also about 5 mm in length. This is a large pore gel.

(3) Bottom. This is the "separating gel" of small pore size and is generally about 5-6 cm in length. It is in this region that separation of the non-binding macromolecules occurs.

In practice when dissociation constants are to be determined by this method disc gels are prepared which contain varying amounts of the covalently attached ligand. After electrophoresis of the sample the whole gel is stained and the position of the macromolecule exhibiting specificity for the bound ligand is determined. The distance migrated is then expressed relative to the distance migrated by the macromolecule in a control gel which does not contain any ligand.

Nakamura and Wakeyama have published a mathematical relationship which describes the electrophoretic mobility of macromolecules on a medium containing a reacting substance[74] which can be used for determining the dissociation constant. It is assumed that an equilibrium is established in the reaction between the ligand (L) and the counter ligand or specific macromolecule (C):

$$CL \rightleftharpoons C + L \qquad [19]$$

The dissociation constant is then defined as:

$$K_{diss} = \frac{[C][L]}{[CL]} \qquad [20]$$

It is also assumed that the concentration of the ligand, [L], is much greater than the concentration of the specific counter ligand [C] and is taken to be equal to the concentration of the ligand bound to the matrix [L]. From (ref. 74) it can be shown that:

$$\frac{x - y}{y} = \frac{[L]K_{diss}}{(K_{diss} + C)^2} \quad [21]$$

where x is the distance migrated by C in the presence of a certain concentration of bound ligand and y is the distance migrated by C in the absence of any bound ligand. The concentration of free counter-ligand [C] is assumed to be very much smaller than the concentration of bound [C] and thus Eq. [21] may be written as:

$$\frac{x - y}{y} = \frac{[L]\ K_{diss}}{K_{diss}} \quad [22]$$

The mobility of the counter-ligand, in the presence of varying concentrations of bound ligand, may be expressed relative to the mobility of a tracking dye in the same gel as follows:

$$R_m = \frac{(C_a - O)}{(D_a - O)} \quad [23]$$

where the subscript "a" indicates the position of the band after electrophoresis; O, the position of the origin; C, the position of the counter ligand; and D, the position of the tracking dye. Relative mobilities are used since the absolute mobilities expressed in Eq. [22] cannot be determined in these experiments. The value of the dissociation constant can be determined from relative mobilities. Eq. [22] may be rewritten as:

$$K_{diss} = \frac{[L]\ y}{x - y} \quad [24]$$

$$= \frac{[L]Rm_x}{(Rm_y - Rm_x)} \quad [25]$$

where Rm_y and Rm_x are the relative mobilities measured in the absence and presence of the ligand, respectively. Equation [25] can be transformed into

$$\frac{1}{Rm_x} = \frac{1 + \frac{[L]}{K_{diss}}}{Rm_y} \quad [26]$$

Thus from a plot of $1/Rm_x$ vs [L] a straight line may be obtained with the

$$\text{slope} = \frac{1}{Rm_x \cdot K_{diss}} \qquad [27]$$

The intercept on the ligand concentration axis ($1/Rm_x = 0$) will yield ($- K_{diss}$) and the intercept on the $1/Rm_x$ axis ([L] = 0) will yield the reciprocal of the theoretical mobility of the counter-ligand ($1/Rm_o$) on the matrix in the absence of any bound ligand. The dissociation constant may also be obtained from the product of

$$\frac{\text{intercept}}{\text{slope}} = \left(1/Rm_x\right)\left(K_{diss} \cdot Rm_x\right) \qquad [28]$$

$$= K_{diss}$$

Experimentally it has been found that the dissociation constants found by this method are usually lower than the K_{diss} values found for the same system when the ligand is not bound and using, e.g. equilibrium dialysis. The technique has the advantage that systems having relatively high values of dissociation constants (weak interactions) can be determined. Also, very small amounts of sample (which may be inhomogenous) can be used.

This technique has been used to determine the dissociation constants of several different systems: polysaccharide-phosphorylase[72], concanavalin A-glycoprotein[75], mannose-phytohemagglutins[73]. The method also permits the detection of inactive/denatured macromolecules.

8.2 Determination of Rate Constants

Denizot and Delaage[69] have proposed that affinity chromatography can be used to determine the association (forward) and dissociation (reverse) rate constants of the binding of macromolecules to immobilized ligands. Evidence reported to date suggests that this contention cannot be experimentally verified. The results obtained appear to reflect more a measure of the mass transfer processes involved, than the primary binding events of

the macromolecule to the ligand. If this technique is to be employed for the determination of rate constants then it probably will be necessary to employ matrix systems with a very low porosity.

8.3 Uses in Functional Characterization

Affinity chromatography appears to offer a relatively simple and widely applicable method for determining and characterizing several features of the macromolecule binding site. The method can be readily used to determine the quantitative recognition by a specific macromolecule, or set of related macromolecules, of a range of compounds. In addition, the technique provides increased versatility for the determination of a wide range of binding strengths and these affinity constants may be gathered from a large range in the size of the macromolecules. The more conventional techniques, such as equilibrium dialysis, are generally restricted by the size of pores of the separating membrane. In addition to the specificity of the interaction, affinity chromatography is a sensitive analytical tool which can be used to determine the precise location of the binding or active site of the macromolecule. This can be done by determining the ligand-macromolecule binding affinity before and after selected chemical modification of the active groups on the macromolecule or ligand.

9 RELATED TECHNIQUES

9.1 Chromatography with Organic Dyes as Ligands

Often the interaction of a specific ligand with its corresponding counter-ligand is so strong that it is difficult to dissociate the complex under conditions which retain the native activity or structural integrity of the macromolecule. In such cases, it is often desirable to prepare a ligand which has a lower but sufficient affinity to serve as an adsorbent. One way of achieving this is to prepare fragments of the native ligand. For example, adenosine 5'-phosphate may be considered a fragment of nicotinamide adenosine diphosphate (NAD) and can in some cases serve as a general ligand for NAD-binding enzymes. Another

approach is to prepare affinity matrices by covalently coupling organic dyes to the insoluble carrier. These dyes generally have a broad specificity and intermediate binding affinity for a group of proteins. The best known of this type of affinity matrix is Cibacron^{R} Blue FG3A which is a polyaromatic, sulphonated blue dye. The dye appears to be able to mimic NAD, NADP or ATP and consequently its potential range is extremely wide. As of 1980, about 50 proteins with affinity for nucleotides had been purified by Cibacron Blue affinity chromatography[76]. The matrix most commonly employed in conjunction with this dye is crosslinked agarose. Cibacron Blue is a triazine-activated compound which will react with the free hydroxyl groups in agarose. It should be remembered that dextrans have more hydroxyl groups than agarose and consequently higher coupling efficiencies are to be expected with dextran. Using the activated dye has the advantage that it does not introduce any further reactive groups into the matrix. This is often a problem when the gel is activated imparting an ionic or hydrophobic nature to the matrix and results in increased non-specific adsorption.

The available experimental evidence seems to indicate that the specificity of the protein-dye interaction is due to a structure, common to many enzymes, known as the dinucleotide fold. This fold consists of about 120 amino acids in a β-sheet configuration of 5-6 parallel strands stabilized by α-helical loops. Thompson *et al.*[77] and Wilson[78] have found that all proteins known to possess the dinucleotide fold, from X-ray crystallographic studies, bind to Cibacron Blue and could be eluted in a specific manner with a low concentration of the appropriate nucleotide. In each case, the most effective nucleotide for elution was the soluble nucleotide which had the highest affinity for the enzyme. Binding of the dye to the dinucleotide fold probably involves both electrostatic and hydrophobic interactions. Albumin is alo known to bind strongly to Cibracron Blue. The mechanism of this attachment has not yet been clarified. Recently a different blue agarose has been introduced: Remazol^{TM} Blue Agarose which does

not bind to albumin and exhibits increased selectivity for those proteins which possess the dinucleotide fold.

Recently two other dye ligands have been introduced viz. Congo Red (for the isolation of proteins possessing the dinucleotide fold) and the procion dyes (which appear selectively to bind many restriction endonucleases).

9.2 Covalent Chromatography

This technique differs from normal "affinity" chromatography in that covalent bonds are formed between a solute and the immobilized ligand and thus separation is not dependent on the non-covalent biospecific interactions between the two interacting macromolecules. This nevertheless is a form of affinity chromatography in the sense that the macromolecules show a disposition to interact specifically with certain inorganic molecules. Covalent chromatography is most frequently applied to the purification of sulfhydryl proteins. In this technique the substance containing the thiol groups is attached covalently to the chromatographic support. Several reaction mechanisms for this purpose are indicated in Fig. 5. The macromolecule (i.e. counter-ligand) is then bound to the activated thiolated matrix through the selective attachment of thiol groups. This involves a thiol-disulphide exchange with the formation of a mixed disulphide. Since this coupling reaction is reversible the thiol-containing macromolecule can be eluted through the reduction of the disulphide bond using dithiothreitol or 2-mercapoethanol.

These thiolated matrices have been successfully employed in the following areas:

* Specific isolation of thiol containing proteins or peptides.

* Separation of thiolated from non-thiolated proteins or peptides.

* Immobilization and isolation of mercurated polynucleotides.

* Isolation of enzymes containing thiol groups.

Thiolated affinity matrices should not be stored for prolonged periods in the free thiol form as the thiol group is susceptible to oxidation. In addition merthiolate and phenylmercuric salts

should not be used as bacteriostatic agents as they will bind with the thiol group.

9.3 Metal Chelate Chromatography

In 1975 Porath et al.[79] introduced the term "chelate chromatography" to describe a separation procedure based on the affinity of proteins for heavy metal ions. The technique is mainly used for the isolation of histidine- and cysteine-containing macromolecules. The imidazole and thiol groups form stable co-ordination complexes with the ions of heavy metals such as zinc, copper, cadmium, mercury, cobalt and nickel. Of the metal chelates tested the adsorption capacity for serum proteins decreased in the order $Cu^{2+} > Zn^{2+} > Ni^{2+} > Mn^{2+}$.

As these metals are transition elements, the binding affinity is pH dependent. At neutral pH (pH 6-8) adsorption will occur

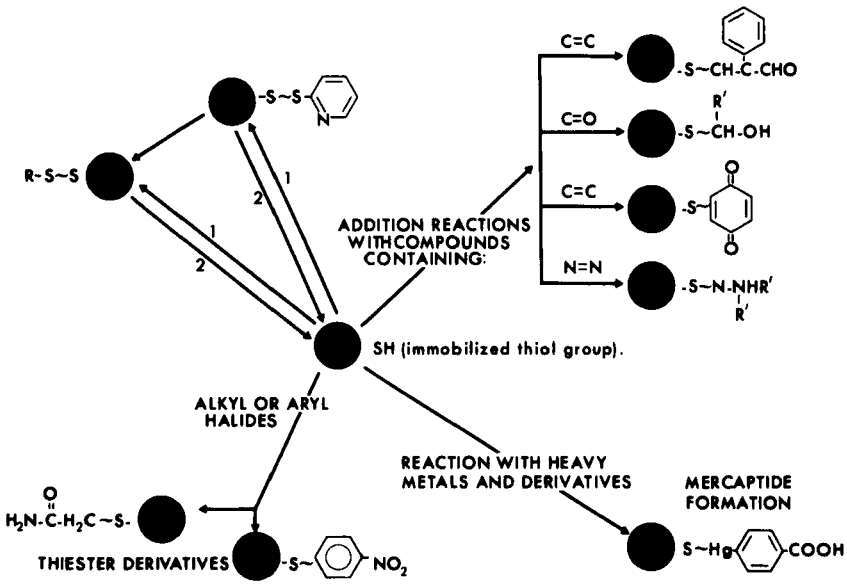


Figure 5

through histidine and cysteine whereas at alkaline pH (pH 8-9) co-ordination with other amino acids may occur. This means that at high pH values adsorption will be more effective but less specific.

9.4 Charge Transfer Chromatography

Fairly recent reports in the literature[80] have documented the possibility of specifically isolating macromolecules on the basis of the interaction between the π-electrons of the macromolecule and the hybrid orbitals on the ligand. Pellizzari et al.[81] have described the use of the technique for the isolation of steroids from biological fluids, Porath[82] has described the use of charge-transfer affinity chromatography for the separation of tryptophan and N-acetyl tryptophan on a column carrying 1,3-dinitrophenyl groups as the ligand.

9.5 Affinity Partition Chromatography

This technique is based on the same principle as phase partition chromatography[83]. Two (or more) phases of soluble polymers are formed by mixing aqueous solutions of the polymers. Classically dextran (500,000 daltons) and polyethylene glycol (6,000 daltons) have been used. After mixing the two phases separate: the upper phase is rich in polyethylene glycol and the lower phase is dextran-rich. If the components to be purified differ in their distribution in the two phases, they may be separated by several extractions or alternatively by counter current distribution. In the affinity partitioning method one of the polymers is modified through the covalent attachment of a ligand. On phase separation nearly all the bound ligands will be in one phase. If the ligand exhibits specific binding characteristics for a particular macromolecule, then on separation the macromolecule will be distributed in that phase which contains the modified ligand. This method was first introduced by Flanagan et al.[84] who isolated acetylcholine receptors in this manner by coupling cholinergic ligands to polyethylene glycol. Shanbhag and Johansson[85] isolated human serum albumin by this method through the attachment of palmitoic acid to polyethylene glycol. This

technique has also been used for the successful separation of blood cells[83]. See also Hofstee who fractionated protein mixtures through differential adsorption on a gradient of substituted agaroses of increasing hydrophobicity.

9.6 Hydrophobic Chromatography

Hydrophobic chromatography is a method with a largely empirical basis and which developed as an outgrowth from affinity chromatography. It was first used in 1967 for the separation of tRNA on a benzoylated DEAE-cellulose column[86]. The first systematic investigations of the nature of the interaction between biopolymers and a hydrophobic matrix were reported by Hofstee[87,88] who called the process "Hydrophobic Affinity Chromatography of Proteins". In the same report[87] Hofstee defined the hydrophobic effect as "an interaction of separate molecules with water, and which cannot be accounted for by covalent, electrostatic, hydrogen-bond or charge-transfer forces." At first "salt effects" were thought to be of the utmost importance but it soon became evident that the elution of biopolymers, especially proteins, from the hydrophobic matrix was greatly facilitated through the incorporation of "non-polar" liquids such as ethylene glycol.

The affinity matrix usually consists of polysaccharide chain to which various hydrocarbon ligands of varying chain lengths have been attached. Retention of the biopolymers on the ligands is not biospecific and is a function of physicochemical variables such as pH, ionic strength and the content of organic solvents in the mobile phase[89]. Extensive experimental data are available in the literature and yet the underlying principle of this type of chromatography has only recently been elucidated[53]. Van Oss et al.[53] approaching the problem from a surface thermodynamic standpoint have suggested that hydrophobic chromatography is a category of liquid chromatography in which coupling between solutes (and/or particles) and the ligand occurs by van der Waals attraction under conditions where the interfacial free energy of the liquid medium is higher than the interfacial free energies of

both solutes (and/or particles) and the ligand. According to this theory elution is effected by changing the van der Waals attraction into a repulsion by lowering the interfacial free energy of the liquid to a value intermediate between that of the solute(s) (and/or particles) and the ligand surface. In polar liquids such as water, electrokinetic interactions (which are influenced by pH and salt effects) will also play a role. The thermodynamic intrepretation by van Oss et al. of the mechanism of hydrophobic chromatography[53] is supported by the recent extensive review article by Srinivasan and Ruckenstein[90] which examines in detail the role of physical forces in this process.

It is far beyond the intent of this article to discuss the specifics of hydrophobic chromatography. Suffice it to say that the technique is now well established as an analytical and preparative tool for macromolecular characterization or purification. Several hydrophobic matrix materials are now available from different commercial suppliers. Several excellent review articles have been published which describe the experimental research and current status of this technique in detail[89,91-94]

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