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AFFINITY CHROMATOGRAPHY

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INTRODUCTION

Within recent years a great deal of attention has been placed on the preparation and uses of immobilized biologically active materials, including enzymes,[1,2,3] antigens, antibodies,[4-9] and hormones.[10-12] Similarly, considerable interest has been directed to the preparation and use of immobilized derivatives for the isolation and purification of biologically active substances, particularly enzymes. This approach to the isolation and purification of biologically active molecules has been termed "affinity chromatography".[13-15] The technique is based on a unique biological property of many proteins, viz. the ability of a biological molecule to bind a ligand specifically and reversibly. Therefore, unlike standard purification methods, which depend on physicochemical differences between proteins, this specific adsorption method exploits the unique property of selective binding.

Purification of proteins based on "biospecific adsorption" is not new. It was first applied to the isolation and purification of antibodies with solid-phase immuno-adsorbents first described in detail by Campbell, et al.[16] For a review of the subject see Silman and Katchalski,[1] Weliky and Weetall,[4] and Weetall.[17]

A biospecific adsorbent is prepared by covalently coupling to a water-insoluble carrier, a ligand specific to the macromolecule of interest. The choice of carrier, coupling method, and purification procedures are of extreme importance and will be discussed in some detail.

Purification of a molecule such as an enzyme is generally accomplished by passing a crude extract or solution containing the protein of interest through a column containing the carrier to which the specific ligand has been covalently coupled. Those molecules not exhibiting any specificity for the bound ligand will pass on through the column, while those molecules having a binding affinity for the ligand will be retarded. The degree of retardation will depend on the binding constant of the macromolecule for the ligand. A molecule with a weak binding affinity might be retarded enough to prevent its emergence with the first void volume as the solution is passed through the column. A molecule with a slightly greater binding affinity may require several void volumes before emerging from the column, while a molecule with a very high binding affinity might for all practical purposes never elute from the column without changing the elution conditions. Thus, in the case of molecules tightly bound to the carrier, it is necessary to change the solvent conditions in order to elute the bound species. This is usually accomplished by changing one or a combination of parameters, such as ionic strength, pH, adding competitive inhibitor or substrate, by adding protein denaturants such as urea, quanidine, or detergents and in some cases even changing temperature.

It is apparent that for the successful application of affinity chromatography the proper choice of

ligand must be made. The binding affinity between the macromolecule to be isolated and purified must be great enough to prevent its elution with the nonspecific material, yet it must be weak enough to permit elution without irreversible denaturation. The bonding affinity while strongly influenced by the choice of ligand is also dependent upon, as previously mentioned, carrier and coupling method, as well as distance of ligand from carrier surface.

Application of affinity chromatography or methods similar in nature include enzyme purification, antigen and antibody purification, separation of hormones or hormone receptors, repressor proteins, vitamin binding proteins, transport proteins, sulfhydryl containing proteins and even cell populations.

The major advantages of this approach to the purification of materials is extreme specificity for biologically active materials, rapidity, and ease of handling. Thus, one can separate a biologically active enzyme from a nonactive form of the same enzyme quite simply and efficiently by affinity chromatography.

CARRIERS FOR AFFINITY CHROMATOGRAPHY

Carriers for affinity chromatography should have the following general characteristics:

a) The carrier should interact weakly with proteins. This means that nonspecific adsorption of proteins in a solution under operational conditions should be minimal. Thus, the ligand groups will not be sterically hindered by adsorbed protein reducing capacity. When the specific protein is eluted, the nonspecifically adsorbed material will not contaminate the final product.

b) The carrier should have good flow properties. Flow rates should be reasonably fast to permit large volumes of solution to pass through the column in some reasonable period of time.

c) The carrier should possess a reasonable number of functional groups which can be activated or modified under conditions that will not affect the matrix structure and will allow chemical attachment of the ligand under reasonably mild conditions.

d) The final derivative must be physically and chemically stable under conditions of adsorption and desorption of the desired species. This means that the matrix must retain its integrity under various conditions of pH, ionic strength, temperature and in the presence of denaturants including detergents, urea, and/or guanidine.

e) The particles should be relatively homogeneous in shape; if possible, spherical is best because of the even packing achievable with spheres. A high degree of porosity is desirable since this gives greater surface area per unit weight and proportionally high specific loadings. Also, high concentration of ligand available to the protein will permit greater interaction between ligand and protein as the protein passes through the column, thus increasing the rate of retardation. Straight chain polymers or carriers of a relatively nonporous nature do not appear to work as well as porous materials for affinity chromatography.

Table I lists several materials which have been used as carriers for affinity chromatography and the materials purified on them.

TABLE I

Some Macromolecules Which Have Been Purified by Affinity Chromatography

Macromolecule	Carrier	Ligand	Reference
acetylcholinesterase	agarose	[N-(ε-aminocaproyl)-p-aminophenyl] trimethylammonium bromide	24
acetylcholinesterase	agarose	substrate analogs	68
anti-A phytohemagglutinin	agarose	blood group A substance	69
avidin	cellulose	biotinyl chloride	64
avidin	agarose	biocytin	59
carboxypeptidase B (porcine)	agarose	D-Ala-L-Arg.	61
carboxypeptidase A	agarose	L-tyrosine-D-tryptophan	72
chymotrypsin	agarose	ε-aminocaproyl-D-tryptophan methyl ester	70
chymotrypsin	agarose	4-phenylbutylamine	71
3-deoxy-D-arabinohep-tulosonate-7-phosphate synthetase	agarose	L-tyrosine	74
asparaginase	agarose	cross-linked dextran	75
flavokinase	cellulose	flavins	57,76
β-galactosidase	cross-linked bovine gamma globulin	p-aminophenylthiogalactoside	77

Macromolecule	Carrier	Ligand	Reference
β-galactosidase	agarose, polyacrylamide	p-aminophenylthiogalactoside	23
glyceraldehyde-3-phosphate	agarose	adenosine monophosphate nicotinamide adenine dinucleotide	55
glyceraldehyde-3-phosphate	agarose	adenosine monophosphate nicotinamide adenine dinucleotide	56
glycerol-3-phosphate dehydrogenase	agarose	halogenated guanisine monophosphate	24
hemoglobin	agarose	p-chloromercuribenzoate	53
lactate dehydrogenase	agarose	oxamate	63
lactate dehydrogenase	agarose	adenosine monophosphate nicotinamide adenine dinucleotide	55
lactate dehydrogenase	agarose	adenosine monophosphate nicotinamide adenine dinucleotide	56
alcohol dehydrogenase	porous glass	nicotinamide adenine dinucleotide	78
mercaptopapain	agarose	p-aminophenylmercuric acetate	79
amyliod protein (human)	agarose	congo red dye	15
nuclease (staphyloccal)	agarose polyacrylamide	p-aminophenyl-pdt	53,59,80, 81

Macromolecule	Carrier	Ligand	Reference
nuclease (staphyloccal) synthetic peptide of P_2	agarose	P_3-peptide	82
papain	agarose	Gly-Gly-(O-benzyl)-L-Tyr-L-Arg	83
plasminogen	agarose	L-lysine	84
proteins (sulfhydryl)	cross-linked dextran	3,6-Bis(acetatomercurimethyl)-dioxane	85
pyruvate kinase	agarose	adenosine monophosphate	56
ribonuclease A (pancreatic)	agarose	p-aminophenyl p-U-cp	86
ribonuclease-S-peptide (synthetic)	agarose	S-protein	87
ribonuclease inhibitor (liver)	carboxymethyl cellulose	ribonuclease	88
t-ribonucleic acid (isoleucyl)	agarose	specific t-RNA synthetase	89
thrombin	agarose	p-chlorobenzylamide-ε-aminocaproic acid	90
tetrahydrofolate dehydrogenase	agarose	methotrexate	91
tyrosinase	cellulose	aminophenol	47
thyroxine binding protein	agarose	L-thyroxine	65
trypsin	agarose	ovomucoid	62

Macromolecule	Carrier	Ligand	Reference
tyrosine aminotransferase	agarose	pyridoxamine	54
trypsin	agarose	ovomucoid	92

Immunoadsorbents for the isolation and purification of antigens and antibodies have not been included.

Cuatrecasas[15] asserts that derivatives of cellulose are generally less useful than agarose derivatives for enzyme purification because of their fibrous nature and their nonspecific character. The nonuniformity "impedes proper penetration of large protein molecules." Very likely the major difference is in available surface area and the apparent pore diameters of cellulose particles vs. agarose particles, since pore diffusion rates for similar size pores should be the same if there are no additional interactions. Therefore, probably less of the ligand on cellulose is available to the protein being purified because the large molecule cannot reach the ligand.

Highly hydrophobic carriers do not wet very well; wetting, of course, is a necessary condition for interaction between the surface of the carrier and the solution. However, some hydrophobic carriers such as polystyrene are notorious for their nonspecific adsorption of protein. A macromolecule is three dimensional and, therefore, has an "inside." The inside of the molecule is not in contact with the aqueous environment and is hydrophobic in nature. It is possible that these hydrophobic regions of a macromolecule interact with the hydrophobic surface of carriers such as polystyrene, literally turning a portion of the molecule inside out.

The recent use of porous glass as a carrier for the immobilization of enzymes[18-21] and antigens and antibodies[22] has proven quite successful. This carrier has many of the characteristics desired for affinity chromatography. The carrier does not change shape or configuration at various pH values or in different solvents. The particles are relatively uniform in size and porosity can be controlled to within less than

±10%. A wide variety of organic functional groups can be applied to the carrier and it can be activated by the same techniques as any other support. A major drawback of porous glass is that unless treated, it tightly binds protein nonspecifically. Silanization decreases this nonspecific binding appreciably.

Most of the carriers used today can be purchased with organic functional groups already attached. The most frequently used carrier is agarose, a polysaccharide polymer, which can be purchased already activated with cyanogen bromide or can easily be activated in the laboratory after which many functional groups can be attached. Porous glass is not yet available with a wide variety of functional groups, but these can also be prepared in the laboratory. For those not familiar with the activation of agarose with cyanogen bromide or the silanization of porous glass, the procedures for their preparations are given below. These procedures have been used by this author and found to work quite well.

Preparation of Agarose Activated with Cyanogen Bromide[37-43]

To 1.0 g of agarose suspended in distilled water and adjusted to pH 11.0 with a 50% solution of NaOH is added 3.0 g CNBr. The CNBr is first ground to a powder with a mortar and pestle and added slowly maintaining pH with the NaOH solution. Temperature is maintained at 15°C-20°C by addition of small pieces of ice. The reaction is continued for 30 minutes after addition of all the CNBr. The activated agarose is then washed exhaustively with ice-cold water. The product can be stored at 4°C as a moist gel at this point or used for coupling to some additional molecule.

Preparation of Alkylamine Porous Glass[17-22]

Porous glass as purchased must be cleaned rigorously before use. In our laboratory we find that the pores are still filled with silica gel, which must be removed by sonication at 60°C-70°C for 3-4 hr. The porous glass is cleaned by boiling in a 5% solution of nitric acid for one hour. The glass is then filtered and can be dried and stored in a closed container.

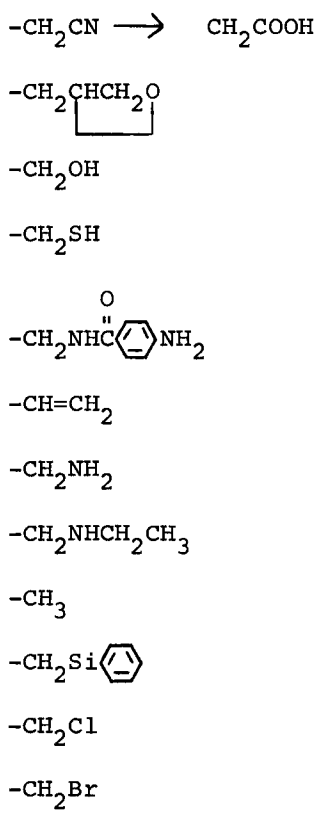
Aqueous silanization appears to give the best results for durability. To 1.0 g of porous glass, add 20 ml of a 10% solution of γ-aminopropyltriethoxysilane (Union Carbide A-1100). The preparation is adjusted to pH 3.5 with 6N HCl and placed in a water bath at 70°C for 2.5 hr. The product is placed on a Buechner funnel and washed with only one volume of distilled water. It is then placed in an oven and dried overnight at 115°C. For silanes not soluble in water the porous glass should be refluxed in a 10% solution of the silane in some organic solvent such as xylene, benzene, or toluene, washed with the same solvent, air dried and then heated in an oven at 115°C overnight. Silanes with many functional groups are commercially or experimentally available (see Table II). Additional functional groups can be added by treating the silanized glass with some organic intermediate.

THE LIGAND

The ligand to be used for the biospecific adsorption of proteins or other large molecules must meet two important criteria. First, it must show affinity for the macromolecule to be isolated. Second, it must have a functional group useful for attachment to the carrier, which when blocked will not destroy its affin-

TABLE II

Functional Groups Available on Silanes



ity for the molecule to be purified. The ligand generally is a substrate, substrate analog, effector, cofactor, or a reversible inhibitor. The use of a substrate for the ligand creates an additional problem in that the conditions of adsorption should be such that catalysis will not occur, i.e., in the absence of an activator, at low temperature, or at non-optimal pH. If an inhibitor is used, one can figure as a rule of thumb that there is at least a three-order-of-magnitude decrease in affinity on bonding to the carrier. Thus

a ligand with a small dissociation constant of 10^{-9} may become a useful ligand with 10^{-6} M dissociation constant, when attached to the carrier. This simply means that a very tightly binding ligand may, when attached to a carrier, become a reasonably dissociable ligand.

Sterrs, et al.[23] elegantly showed that the length of the chain holding the ligand to the carrier plays a significant role in the ability of the ligand to bind the macromolecule to be isolated. This was clearly

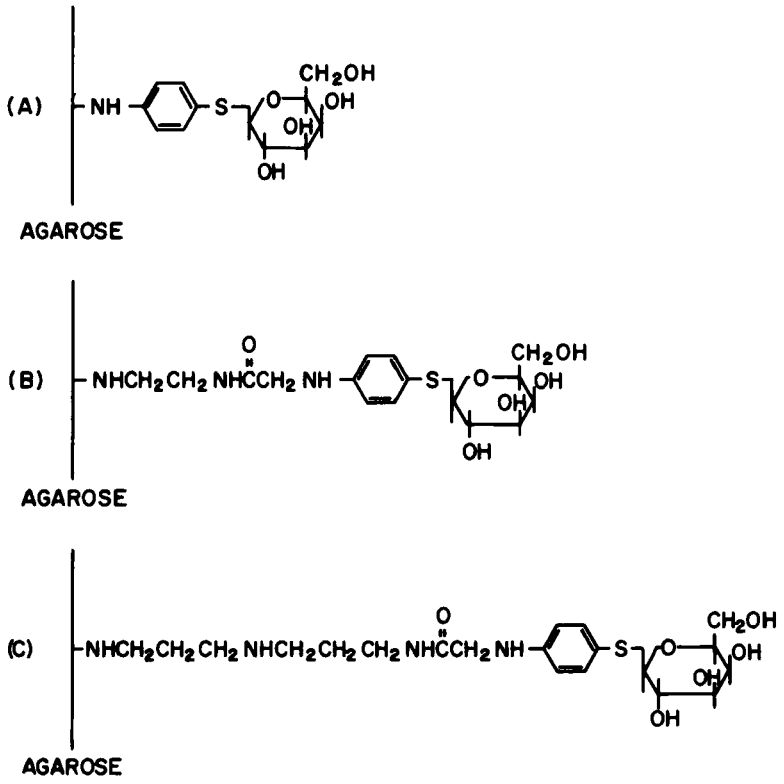


FIGURE 1

Derivatives of p-amino-β-thiogalactopyranoside used for the purification of β-galactosidase

shown with the isolation and purification of E. coli β-galactosidase with an agarose derivative of p-aminophenyl-β-thiogalactopyranoside. Figure 1 shows three derivatives tested for their bonding affinity to the enzyme. No specific binding was observed when a crude mixture containing the enzyme was passed over derivative 1A, slight affinity was observed with derivative 1B, and excellent affinity with derivative 1C. Similar results have been observed with other affinity chromatography systems.[24,25] Chain length becomes less important as the size of the ligand is increased.

There are several important parameters one must remember on choosing the proper ligand. Cuatrecasas[13] has shown that if one starts with an inhibitor at a concentration of 10^{-3} M on the matrix of a carrier, and one requires 99% retention of enzyme on that carrier from a sample of crude enzyme containing 10^{-5} M enzyme in three times the volume of the matrix, the K_i (inhibition dissociation constant) for the bound ligand cannot exceed 10^{-4} M.

If one looks at a relative adsorption isotherm for affinity chromatography[26] (Figure 2), the actual isotherm is somewhere between the ideal and the nonspecific. In the ideal case ΔF° (free energy of reaction) is relatively large and of negative sign. Adsorption stops when the ligand sites are no longer accessible. The ΔF° for affinity chromatography is the sum of the ΔF°'s for nonspecific binding and specific binding.

Thus, generally speaking, $\Delta F^\circ_{ads} = \Delta F^\circ_{ac} + \Delta F^\circ_{ns}$ where ΔF°_{ads} represents free energy of actual affinity chromatography system. Also, ΔF°_{ac} represent free

energy of specific absorption to the ligand; ΔF_{ns} represents nonspecific adsorption to the carrier. For a soluble system

$$E + \overline{I} \rightarrow [E\overline{I}] : \Delta F^{\circ}_{ac}$$

E represents enzyme

$\overline{I}$ represents immobilized inhibitor

and $\Delta F^{\circ}_{ac} = RT\ln K_i$.

By substituting 10^{-5} M for K_i one obtains ΔF°_{ac} of -6.82 Kcal/mole at 25°C for the free energy of specific adsorption to the ligand.

The adsorption energy of the nonspecific binding must be as low as possible or the carrier will pick up more nonspecific protein than specific protein. Therefore it is always important to remember that the K_i or K_m (Michaelis constant) of the ligand must be less than

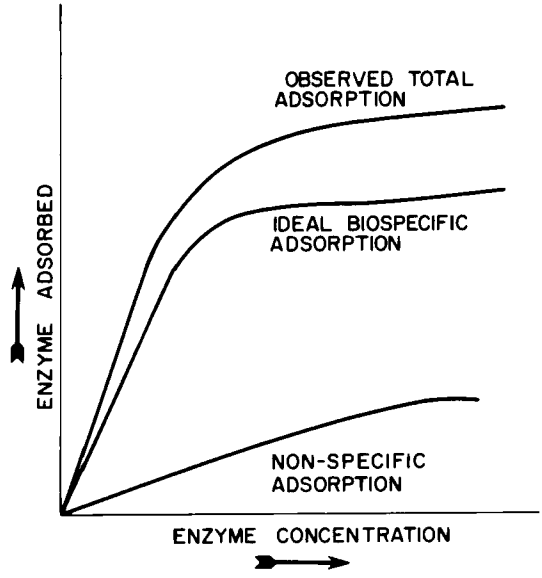


FIGURE 2

The relative adsorption isotherms for affinity chromatography

10^{-4} M whenever possible or little specific adsorption will occur, since the ΔF_{ads} will come almost entirely from the ΔF_{ns}.

One of the causes of nonspecific adsorption to a carrier is carrier charge. Charged groups on the carrier surface are in fact ion-exchange groups. Undesired ion-exchange properties can be minimized by proper choice of matrix, proper choice of adsorption pH and matrix,proper choice of adsorption pH and temperature, and control of ionic strength. It has been shown[27] that interaction between a hydrophobic surface and a protein in solution containing hydrophobic groups is weaker than the interaction between a protein and an ion-exchange resin, but stronger than that between a protein and a hydrophilic surface. Thus, control of ionic strength and pH becomes very important when using a hydrophilic matrix. One would rather have nonspecific adsorption on a surface such as polyhydroxylated dextran than on functional ion-exchange groups.

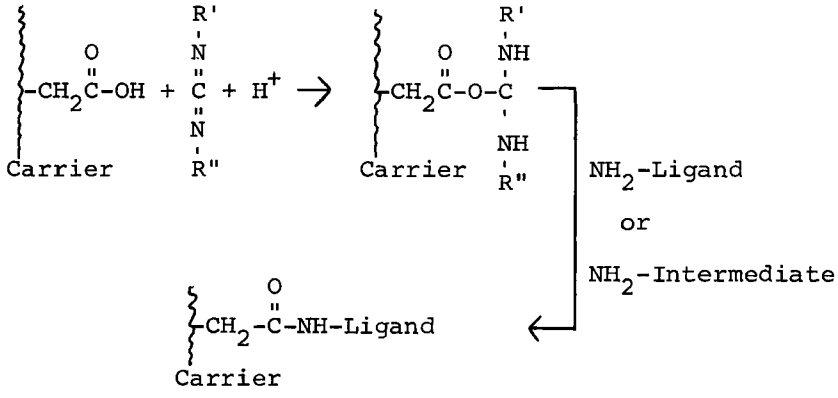
METHODS OF COVALENTLY LINKING LIGANDS OR OTHER INTERMEDIATES TO WATER-INSOLUBLE CARRIERS

The methods used for the covalent attachment of ligands to water-insoluble carriers are identical to those employed for the covalent attachment of enzymes, antigens, antibodies, and other organic moieties to carriers.[2,3,8,15,17] It is not within the scope of this review to describe these methods in great detail. However, the covalent attachment of the ligand is one of the major steps in the sequence of events required for the isolation and purification of a macromolecule by affinity chromatography. Therefore, a general familiarity with the major coupling methods and their applications are of value.

The carriers utilized for these techniques can be purchased commercially and include: celluloses, polystyrenes, polyamines, acrylics, cross-linked dextrans, agarose and inorganic carriers having functional groups prepared as previously described.

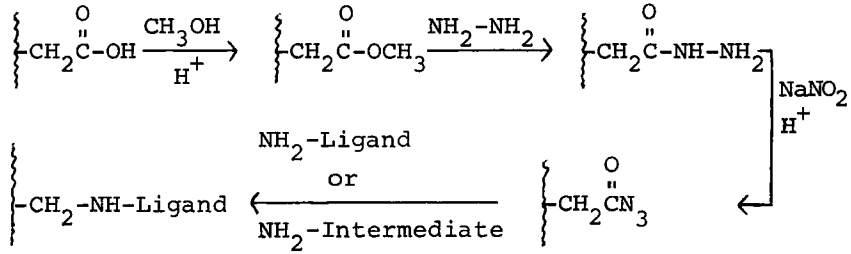
1. Carbodiimides

Basic amines can be coupled to carboxyl groups directly in the presence of a carbodiimide.[28,29,30] The carbodiimides can be used in either aqueous environments or in organic solvents making them very useful coupling agents. The reaction mechanism is as follows:



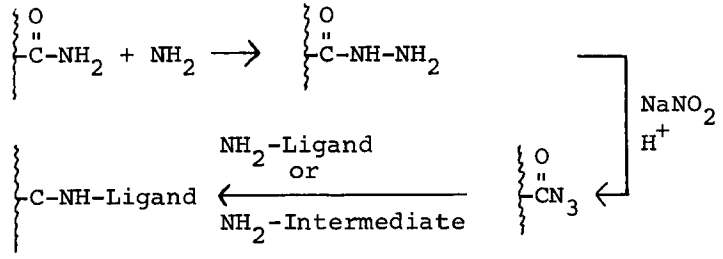
2. Azides

This method[31-35] works particularly well when the ligand or intermediate is a primary aliphatic amine.



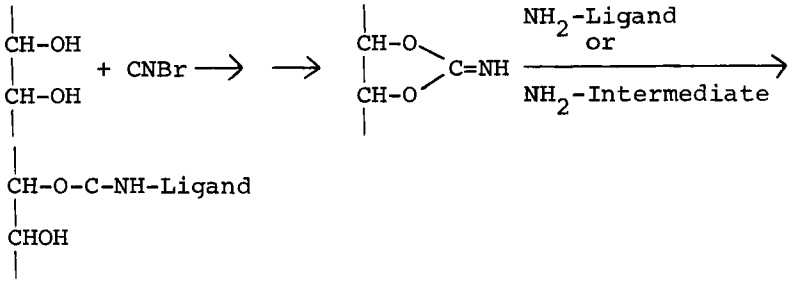
3. Hydrazides

The reaction[36] is similar to that above except the carrier is a primary amine rather than a carboxyl group.



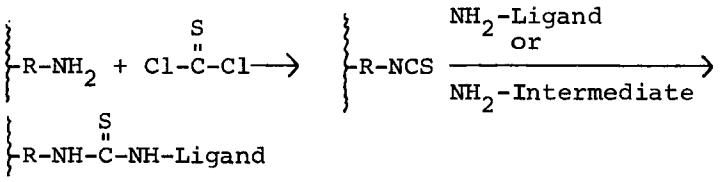
4. Cyanogen Bromides

This method[37-41] is one of the most versatile and is commonly used with agarose and cross-linked dextrans as the coupling method of choice. Although the reaction is shown as a hydroxyl activator it may be used to treat primary amines.



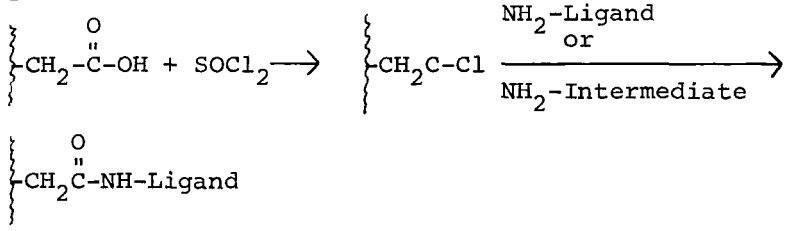
5. Isothiocyanates

This method[42-43] works well with amine derivatives and can be used with both alkyl- and aryl- amine carriers.



6. Acid Chlorides

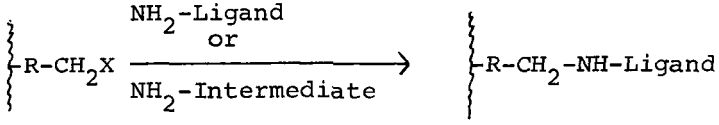
The acid chloride[44,45] of some derivatives can be prepared and used for the covalent attachment of the ligand or ligand intermediate, particularly if the entire reaction can be carried out in organic solvents. If the ligand or the intermediate is only soluble in water, this approach may not be as rewarding as many others, since the acid chloride is quickly hydrolyzed by water.



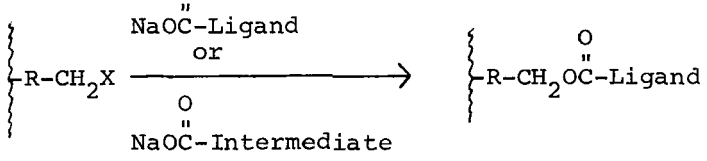
7. Alkyl Halides

Alkyl halide derivatives should form secondary amines with primary amines. They will also react with salts of acids to form esters and will react with alcohols to form ethers.[46,47,48]

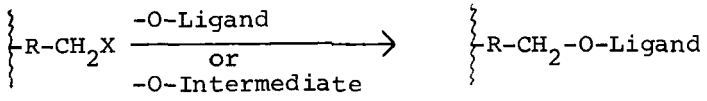
a. Secondary amines



b. Esters

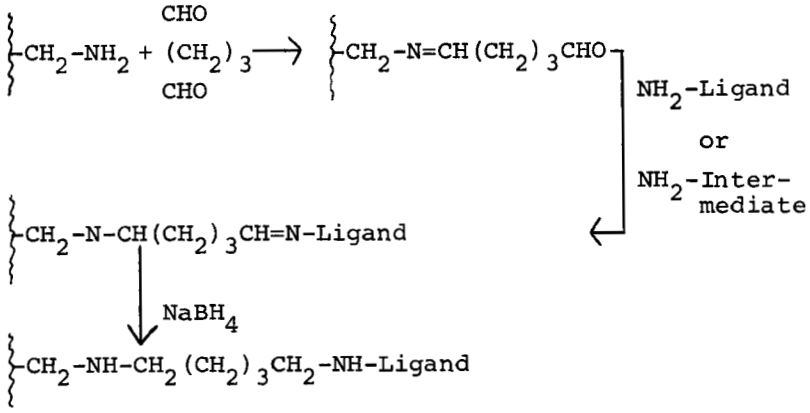


c. Ethers



8. Glutaraldehyde

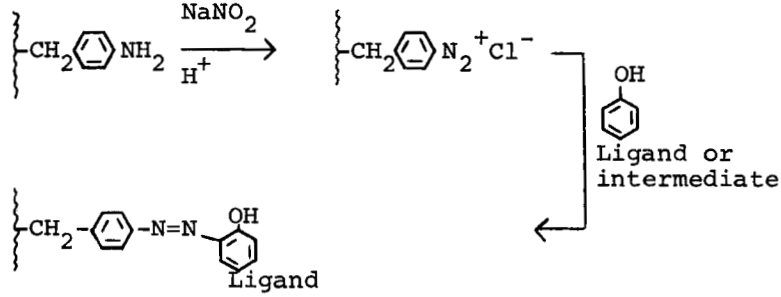
A reagent commonly used for cross-linking to amines[49,50] can also be used for linking two amines without any cross-linking occurring.[21] This technique is quite simple and yields a very stable derivative.



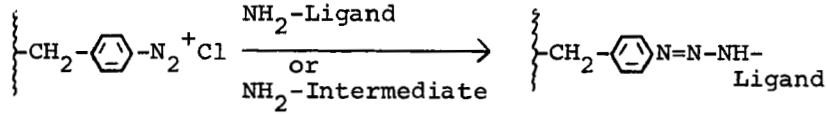
9. Diazonium Salts

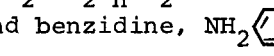
Diazonium salts[2,3,4,9,18,19,51] will react with phenolic compounds and many other heterocyclics such as histidine and arginine. The diazonium salts will also react with amines to form triazines.

a. Azo formation



b. Aryl-triazine formation[52]



Typical intermediate groups used to extend the ligand the desired distance from the carrier are aliphatic diamines such as $NH_2(CH_2)_nNH_2$; dicarboxylic acids, $HOOC(CH_2)_n$-COOH; and benzidine,  .

By using one of the methods described above, it should be relatively simple to prepare an immobilized ligand at the proper distance from the carrier and attached by the desired functional group.

CHROMATOGRAPHY ON BIOSPECIFIC ADSORBENTS

The method of applying the macromolecule to the adsorbent for chromatography is varied and no one method a priori is best. The adsorbent may be used in a slurry and handled entirely by a batch method. The entire procedure could also be carried out in a column or one may even use a combination of the two methods. The importance of the method of application and elution is not carefully defined. Experience with immunoadsorbents has shown us that columns generally work better because there is less dilution, the antibodies are eluted from the column as a front, and there is less nonspecific readsorption of antibody. The nonspecific protein is easily washed out of a column before elution. These same general characteristics apply to affinity chromatography. In addition, if the enzyme is not tightly bound to the ligand, but is only retarded in its passage through a column, then a batch-type operation would be of no use at all. In a column system with the above characteristics, the protein of interest is generally eluted as a broad dilute peak. Preferably, one would like to obtain the product only after some change in buffer, pH, or ionic strength. In this manner the product should elute as a narrow band or peak. If a denaturant is required for elution, the recovered

protein should immediately be neutralized, diluted, if necessary, and the denaturant removed by dialysis or some similar process.

It is always possible that the specifically bound macromolecule cannot be removed by any elution method. In such a situation it may be necessary to remove the ligand from the carrier. For example, a ligand coupled through azo-linkage may be removed by reduction of the azo groups with sodium dithionite to two primary amines. The ligand must then be removed by some other method such as dialysis against large volumes of water. Destruction of the covalent linkage between carrier and ligand was used successfully by Cuatrecases[15,35] to recover serum estradiol binding protein from an azo estradiol derivative.

A brief description of affinity chromatography of a few materials may be of some value to demonstrate the practical aspects of the method.

Recently Miller, et al.[54] showed that tyrosine aminotransferase can be purified in a column of an immobilized cofactor pyridoxamine phosphate. The dissociation constant or "apparent K_m" for the complexing of the enzyme with this compound is 10^{-7} M. The adsorbent was prepared by combining pyradoxamine phosphate with agarose at three different chain lengths from the carrier. The derivatives used were an ethylamino-agarose which was coupled to (a) ethylenediamine, (b) a succinylated form of ethylenediamine-treated agarose followed by ligand, and (c) a carrier to which the compound 3.3' diaminodipropylamine was attached, after which it was succinylated and coupled to the pyridoxamine (see Fig.3). Enzyme was retained on derivatives

(b) and (c) only . The derivative with the longest chain bound almost ten times the enzyme of the others. Elution was accomplished by increasing salt and pH and adding excess cofactor pyridoxal phosphate. A 650-fold purification was achieved. The column bound 19.5% of the enzyme offered, and of the bound enzyme 76% was recovered for an overall efficiency of approximately 15%.

FIGURE 3

Ethylamino-agarose derivatives used for the purification of tyrosine amino transferase

Mosbach and co-workers[55,56] have successfully purified dehydrogenases on columns of immobilized AMP and NAD. The NAD was coupled through the free amino-group on the adenine ring to a carboxyl group on the carrier, while the AMP derivative was prepared by a laborious procedure from inosine. The results of these experiments indicated that either derivative could bind glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. They could be sequentially desorbed by treating the carrier with NAD followed by NADH. Recovery of the enzymes exceeded 70% of that bound.

Adsorption and purification of flavokinases have been accomplished with both immobilized substrate and immobilized inhibitor.[57] However, most workers find immobilized inhibitors more successful for isolating enzymes. Cuatrecasas has used inhibitors successfully to purify nucleases,[53,58] chymotrypsin[59] and β-galactosidase.[23] Other enzymes successfully purified using immobilized inhibitors include:acetylcholinesterase,[60] carboxypeptidase,[61] thrombin, and trypsin.[62]

Substrate analogs are also frequently used as ligands. Hoboken, et al.[24] purified muscle glycerol-3-phosphate dehydrogenase on derivatives prepared for 1-Cl and 1-Br analogs of glycerol-3-phosphate. O'Carra[63] isolated latate dehydrogenase also on an analog, but an analog of an inhibitor. He substituted oxamine for pyruvate, since the oxamine has a free amino group for coupling to a carrier.

Several nonenzymatic proteins have been isolated by affinity chromatography; these include estradiol binding protein, thyroglobulin,[53] avidin,[64] and thyroxine.[65]

It is obvious that almost any macromolecule that specifically complexes with some organic moiety whether large or small, can be purified by an affinity chromatographic process. Recent studies on hormones and hormone receptors by Ventner, et al.[12] indicate that receptor sites for catacholamines are on the surface of cells, thus making possible the isolation of the receptor or even the entire receptor cell by affinity chromatography. Davie and Paul[66] have utilized hapten immunoadsorbents to fractionate immunocompetent lymphoid cells. Antihapten antibody-producing cells have been isolated on haptens coupled to polyacrylamide beads.[67] These studies in conjunction with the studies of Ventner[12] using immobilized catacholamines strongly indicate that affinity chromatography may find application in separating very specific cell populations. Similarly, affinity chromatography can be applied to the isolation and purification of receptor sites for hormones, which, in turn, may be useful in very sensitive bioassay techniques.

At this time there are no published reports of affinity chromatography being used commercially for the isolation of enzymes. Since the technique offers what appears to be a simple, inexpensive and, in many cases, a one-step purification scheme, it is likely that industrial enzyme manufacturers are already looking at this approach to enzyme purification.

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