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Edited by
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Preface

This volume contains most of the papers presented at the Symposium on Affinity Chromatography and Immobilized Biochemicals, which was held on November 7-9, 1973 in Charleston, South Carolina in conjunction with the Southeastern Regional American Chemical Society meeting. The topics of the symposium represent two new biochemical frontiers which have emerged in recent years through the ingenious development and application of solid phase biochemical technologies. Affinity chromatography involves the use of selected ligands, covalently bound to a solid support such as cellulose, glass, Sepharose, or polyacrylamide and exploits the principle of biochemical recognition between the ligand in the solid phase and a selected macromolecule to facilitate the rapid and often quantitative purification of enzymes, antibodies, antigens, hormones, other proteins, etc. The area of immobilized biochemicals includes the use of coenzymes, oligo- and polynucleotides, enzymes, and multistep enzyme systems which are immobilized or entrapped in the solid phase. The goals of the symposium were the review of the status of affinity chromatography and immobilized biochemicals, the presentation of new data and ideas in both areas, and the establishment of a dialogue between research workers in these two evolving disciplines which are so closely interrelated. The papers published in this volume provide the reader with reviews of several topics inherent in the solid phase biochemistry together with a series of timely manuscripts concerning new techniques and applications both in the use of affinity chromatography and in the investigation of immobilized biochemicals.

The symposium was international in flavor and attracted well over two hundred attendees. Support for the symposium was kindly and generously provided by Beckman Instruments, Bio-Rad Laboratories, Miles Laboratories-Research Division, Pharmacia Fine Chemicals, the Worthington Biochemical Corporation, the American Chemical Society, and the National Science Foundation.

I would like to express my thanks to the financial supporters, the presiding officers, and the participants who all contributed to the success of the symposium. Special thanks are due to Professor O. D. Bonner for his assistance in planning the symposium and to Miss Susan Carroll and Mrs. Anne Davis for their secretarial help performed during the planning phases of the symposium as well as in preparing manuscripts for the publisher.

January, 1974

R. Bruce Dunlap

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PART I

AFFINITY CHROMATOGRAPHY

AFFINITY CHROMATOGRAPHY - OLD PROBLEMS AND NEW APPROACHES

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The purification of macromolecules by biospecific adsorption-affinity chromatography (1) is a technique with a long history although its application to a wide variety of problems is comparatively recent. As long ago as 1910 there were reports of the purification of amylase by adsorption to insoluble starch (2). Tyrosinase was purified in 1953 using a cellulose matrix bearing the inhibitor, diazodizine (3). In both of these examples the power of purification lies in the highly selective "insolubilization" of the macromolecule from solution. This reversible phase separation often allows a high degree of concentration of the particular molecular species of interest from dilute solution and the removal, by washing and elution processes, of other molecular species that are cosolutes.

Affinity chromatography, like all chromatographic procedures, represents the application of the law of mass action. Enzyme kinetics can also be expressed, assuming pseudo-equilibrium, in terms of this law (Figure 1). Usually substrates are not used as affinity ligands (1a) although they may prove useful under conditions where catalysis proceeds at a very slow rate while binding is still high, e.g. at pH values distant from the pH optimum, or low temperatures. This hypothetical reaction sequence (1a) also includes a covalent intermediate and such intermediates have also proved useful in purifications (4). More frequently the coupled ligand used is an inhibitor (1b), a hormone (1c), and antibody (1d), or even an enzyme subunit (1e) as in studies on enzyme reconstitution. In these instances the physical parameter of most importance is the association (dissociation) constant. The magnitude of this quantity determines both whether the system will have a strong enough association to be useful and how one will have to treat the adsorbed material to recover it from the column.

a) $E + S \rightleftarrows E \cdot S \rightarrow E\text{-}S \rightarrow E + P$

b) $I + E \rightleftarrows I \cdot E$

c) $H + R \rightleftarrows H \cdot R$

d) $H + A \rightleftarrows H\text{-}A$

e) $_nE \rightleftarrows E_n$

E enzyme
S substrate
I inhibitor
H hormone
A antibody

· indicates reversible complex
- indicates covalent intermediate

Figure 1

The transition, historically, from early isolated affinity purifications to the widespread usage we see now was made possible by three events. First was the publication, by Axen et al. (5), of a simple chemical procedure (CNBr) for activating polysaccharide matrices. Second was the commercial availability of a near ideal polysaccharide matrix, agarose, which possessed the necessary features of chemical and biological inertness, good chromatographic properties and ease of activation by cyanogen bromide (CNBr). Third, was the publication of several papers demonstrating that systematic approaches could be applied successfully to practical problems (6,7,8). After demonstration of the ease with which purifications could be accomplished using affinity systems, especially as compared to classical methods, and after a rational foundation was established for the design of absorbents, there was a very rapid increase in the use of affinity chromatography as a biochemical tool.

Enzyme purification continues to be the primary application of affinity chromatography. In this chapter, however, we would like to survey some of the more novel applications and to suggest some potential uses that have not yet appeared. During the purification of E. coli β-galactosidase it was noted that the catalytically inactive monomeric subunit was purified, by affinity chromatography, along with the active tetramer (6). This purification by binding, even when catalytic activity is lost through mutation or chemical modification, is an aid to studying catalytic mechanisms and structure-function relationships.

Particulate systems can also be analyzed by affinity chromatography. Hormones bound to agarose beads interact with fat cells and can stimulate biological activities similar to those observed following administration of the hormone in solution. Micrographs of fat cells sticking to agarose-insulin beads show that the

interaction between the cells and beads includes changes in the cell shape resulting in maximization of the cell surface in contact with the bead (9). This active cell participation in the cell-bead interaction can result in such tight binding that the buoyant density of the cell-bead complex changes to the point that cells that would normally float to the top of the solution when centrifuged now sediment with the agarose (10).

Studies on lymphocytes predate the term affinity chromatography (11,12). For instance, glass beads coated with antigen can remove from a mixed lymphocyte sample those cells bearing the specific antibody directed against the coated antigen. Sensitized cells can be adsorbed to the beads and removed (13). Lymphocytes bearing anti-DNP, guinea pig albumin receptors were shown to be different from those cells bearing anti-DNP keyhole-limpet hemocyanin receptors indicating that different cell clones exist (14). More recently, lymphocytes sensitized to DNP antigens were removed from mixed cultures by binding to nylon fibers which had the DNP-antigen coupled to them. The fiber can be transferred to fresh medium and plucked to remove the bound cells (15).

Organelles can also be purified on affinity columns. Utilizing the ability of a newly synthesized enzyme to bind to its substrates even before the enzyme is released from the ribosome, polyribosomes capable of synthesizing tyrosine aminotransferase (TAT) were bound to a column containing coupled pyridoxal phosphate, a cosubstrate of the transfer reaction. A 100-fold purification of TAT synthesizing ribosomes was possible using this technique (16).

Viruses can also be purified (17). Using a neuraminidase inhibitor column a supposedly homogeneous preparation of influenza virus particles was purified two-fold through the interaction of a viral neuraminidase coat protein with the coupled ligand (18).

An exciting new area is the use of plant lectins as coupled ligands. Plant lectins are useful for several reasons. They allow purification on the basis of often complex carbohydrate structure and frequently simple sugars are effective competitors for binding, thus allowing easy elution of the bound material. Lectins have been used to purify blood group substances and glycoproteins like FSH and LH. Work has started on cell typing with coupled lectins and they are being used in studies on the differences between normal and neoplastic cells.

Affinity techniques have been applied to the purification of membrane constituents, virtually always in the presence of detergents. A solubilized fat cell membrane fraction was enriched for the insulin receptor using wheat germ agglutinin and Concanavalin A. Rhodopsin from bovine retinal cell outer rod membrane, can be purified on Concanavalin A columns (20). Glycopeptide

purification and sequencing make use of a wide variety of lectins. Lectins may also prove useful in the separation of cytoplasmic membranes from intracellular membranes if their carbohydrate composition proves to be different.

Membrane receptors can also be purified using more functional ligands. The insulin receptor has been purified on insulin columns (21). Recently the cytoplasmic estradiol receptor has been purified on 17-substituted estradiol columns. These adsorbents were extremely effective in extracting the estradiol receptor only when very long spacer arms, e.g., albumin and poly-L-lysine-alanine copolymers were interposed between the sterol and the agarose matrix. Use of macromolecular spacer arms resulted in purification approaching 100,000-fold in one step with 30-50% recovery (22).

Returning again to soluble systems a vitamin B-12 binding protein has been isolated from human tissues on columns coupled to the partially hydrolyzed vitamin through amide bonds (23). The use of affinity techniques in radioimmunoassay is undergoing very rapid growth.

Another technique is the use of coupled ligands as probes of cell surfaces. When isoproterenol bearing beads are placed in contact with muscle cells there is a prompt inotropic effect which does not diminish with time as it would if the response were caused by the drug in solution (24). Control studies eliminated the possibility that ligand released from the beads was the cause of the inotropic effect. Removal of the beads resulted in prompt relaxation of the muscle.

Affinity chromatography is unique as a separation tool in that its application requires a good deal of knowledge of the biochemical system in which it is to be used before the affinity absorbent can be designed. The problem of selecting the matrix and ligand reoccurs with every new system approached. A useful ligand must have a high affinity (K_D typically 10^{-5} or greater) for the molecule that is to be purified. Having such a ligand, a point of chemical modification of the ligand must be available. The aim is to retain the biological binding activity of the ligand after it is coupled. Frequently one can study the effect of chemical modification of the potential ligand with soluble model systems. One can synthesize a soluble "armed" derivative, for example, and test its potency. Tests of this type may show that the modified ligand has an enhanced affinity supposedly due to hydrophobic contributions to binding resulting from the presence of the spacer. Enhancement of binding is not a guaranteed result of such modifications, however.

Coupling of the ligand to the matrix may involve reactions that are not completely defined. The use of gluteraldehyde as a

coupling agent is an example of this. Even when the coupling chemistry is reasonably well understood, as in the reactions of CNBr activated agarose, the actual product may not be known. Protein ligands are assumed to couple the CNBr activated agarose through ϵ-amino groups. However, other polar groups may also react. Model studies done in our laboratory demonstrate that N-acetylated cysteine can compete with alanine in the coupling reaction. The blocked cysteine inhibits alanine binding by 50%, when present in 4-10 fold molar excess. The importance of sulfhydryl groups in coupling reactions involving proteins is unknown.

No coupling procedure is universally applicable. Some coupling methods are incompatible with useful matrices. Some coupling agents inactivate a large fraction of the ligand during coupling. One alternative that may not be immediately obvious is the possibility of activating the ligand to react with the matrix. The advantages of using an active ligand is that one can easily control the degree of activation, and the unreacted material is soluble and can be removed by washing. This may reduce the number of by-products coupled to the gel and may produce a more ideal matrix.

The most convenient method of matrix activation is no activation at all. Adsorption, like that of protein on glass, polymerization trapping, e.g. enzymes trapped in acrylamide gels, or binding to hydrophobic columns (25), requires no chemical manipulation of the ligand and, in some cases, produces matrices of useful short-term stability. Usually, however, some form of chemical activation is required to covalently couple molecules to the matrix. The most frequently used coupling reaction is the activation of agarose with CNBr. The intermediate cyanate produced is very unstable and rapidly reacts with hydroxyl radicals to form the more stable imidocarbonate. The imidocarbonate can then react with amino groups to form a variety of products, the major one probably being an isourea (26,27). Isoureas are protonated and positively charged at physiological pH. From this basic reaction a whole series of alternate pathways radiate. Amino compounds of all types, including compounds with reactive radicals can be coupled to modify the matrix for additional coupling reactions (28). Another frequently used matrix is polyacrylamide, which is also available in beaded form (29). Acrylamide is activated by treatment with hydrazine followed by nitrous acid to form reactive acyl azides. Potentially, polyacrylamine has a higher coupling capacity than agarose. The low porosity of the useful acrylamide beads has limited the utility of this matrix. The restricted porosity can be useful, however, in the preparation of particulate adsorbents. The importance of porosity is emphasized elsewhere in this volume in discussions on the use of glass as a matrix.

Activation of the matrix may produce some undesirable changes in the matrix properties. Frequently the substituted matrix is more hydrophobic. Many ionic residues may be introduced into a normally non-ionic polymer. Neither of these effects, however, has seriously impaired the use of such matrices. It should be mentioned that increases in hydrophobicity may increase binding in some cases and virtually all biological environments possess some ion-exchange properties. The degree of activation may be critical when single bond attachment is necessary for retention of activity. Most low molecular weight ligands can be designed to attach only at one point. Polymeric ligands may possess several reactive sites. Chemical modification of some of the sites may destroy the activity of the ligand. This is especially likely if the ligand couples to the matrix at more than one site. To lessen the likelihood of such complications lesser degrees of activation of the matrix may be necessary. Alternatively, the pH or temperature at which the coupling reaction is done can be adjusted to favor coupling at a particular site.

Leakage, release of the coupled ligand into the solvent, may be a problem. Leakage is important in at least two kinds of systems. Industrial usage often requires the use of high flow rates and high temperatures which accentuate otherwise minor leakage effects. The chapters in this volume on glass-enzyme reactors illustrate this effect. Secondly, systems of very high affinity and low concentrations of the desired macromolecule can be severely affected by the presence of relatively small amounts of released ligand. Hormone-receptor systems are an example of this group. If one obtains two micrograms of estrogen receptor protein in a homogenized calf uterus one can estimate that the molar concentration of receptor will be very low. The leakage of a few nanomoles of the high affinity ligand from the column could functionally inactivate all of the receptor in solution and prevent its binding to the column. Prevention of this effect often requires the dilution, with unsubstituted matrix, of the affinity matrix until there remains only enough ligand in the column to remove the species of interest from solution.

A model study performed on ligands coupled to agarose with CNBr and stored at 4° for 30 days suggested that about 0.1% of the alanine coupled was released per day. Albumin, coupled and stored under the same conditions eluted at a rate approximately 1/5 as fast (30). These columns were washed with 0.1 M carbonate buffer, pH 10, 2 M urea and 0.1 M acetate buffer, pH 4, all prepared in 0.5 M NaCl, before storage to reduce the amount of adsorbed ligand. Treatment of the coupled ligand with sodium borohydride to reduce the isourea did not reduce the rate of leakage. Methods of reducing leakage are discussed below.

The presence of a polyvalent matrix, such as is formed for affinity chromatography, may have pronounced effect on biological systems, even in the absence of any chemical effects. Incubation of insulin coupled to agarose beads with murine mammary cells rapidly causes increased transport of a non-metabolizable amino acid into the cells. Incubation of the same type of cells with insulin free in solution does not cause increased transport unless the cells have been preincubated for 24 hours with prolactin (31). This polyvalent affect may be related to the "patching" phenomenon observed with lymphocytes treated with fluorescent antibody. There may be a redistribution of surface receptors under the influence of the polymeric-coupled ligands. This effect may complicate the interpretation of experiments using affinity columns especially those involving particulate materials.

Once the ligand is coupled and the desired molecule is absorbed to the column, one has the final problem of elution. Elution by denaturation has several advantages. The reaction is rapid, leading to the prompt release of the protein in a small volume. The macromolecule must have the ability to renature for this method to be useful and this elution method is not specific. It only works well when there is only one or a few proteins retained by the column. Another common method of elution is by ligand competition. This requires the addition of a soluble ligand which competes for binding with the coupled ligand. If the affinity of the ligand-macromolecule complex is very high, dissociation kinetics may become important. For example, for high affinity complexes the dissociation rates are slow. One must allow five half-lives to pass before the "insoluble" complex is 95% dissociated. Elution may require incubation of the bound material with the competitive ligand for hours with zero flow conditions before dissociation is complete. Ligand competition elution provides the possibility for added specificity in that, by appropriate choice of competitive ligand, one of several adsorbed species may be selectively eluted. This method has been used in the resolution of dehydrogenases coupled to an NAD column (32). The bound molecules may also be removed by extruding the matrix from the column and treating the matrix in suspension. This dilutes the gel and pulls the equilibrium towards the dissociated species. Occassionally the macromolecule can be eluted without the addition of soluble ligand but in any case the kinetic considerations outlined above still are important. This technique results in very dilute solutions of the isolated molecule. Occassionally elution may require chemical or enzymic treatment of the bound-ligand macromolecule complex. An extreme example is the use of dextranase to digest Sephadex columns.

A new approach to the attachment of ligands to matrices is the use of polymeric arms or spacers. It is widely recognized that displacement of the ligand from the matrix by interposing a

spacer-arm may markedly improve the effectiveness of the affinity ligand. Symmetric diamines and diacids have been very useful as spacer arms. Several short arms, a diamine, a diacid, and an amino-acid may for example be added sequentially to build up a longer multimeric spacer arm. Such multimeric arms were used in the purification of the estradiol receptor (8). Multimeric arms can be produced that bear an active functional group on the end distal to matrix. This group may react directly with amino-ligands. Active esters like N-hydroxy-succinimide can be synthesized, stored dry and added directly to solutions of the molecule to be coupled (33). This material is now commercially available (34). Another "activated" matrix, CNBr activated Sepharose, is also commercially available (35).

Recently macromolecular spacer arms have come into use. An example is polylysine-alanine. This material possesses a polylysine backbone whose ϵ-amino groups are substituted with 14 to 16 residue polyalanine peptides. This material has a molecular weight of about 260,000 and a Stokes radius of approximately 7.5 nm. This distance is important because, if one calculates the distance between attachment points in a polymeric matrix substituted to the extent of 10 nmoles per ml, the distance between attachment points is, on the average, 6 nm. The possibility exists therefore that polymeric arms like polylysine-alanine, can attach at two or more points to the matrix. All of the unattached amino terminals are available for the coupling of ligands using, for example, carbodiimides. This polymeric spacer-arm may be synthesized from D-amino acids. The D-polymer should be resistant to attack by most proteases.

Albumin, if coupled in the denatured state, can also be used as a macromolecular spacer arm. Albumin can be an "active" arm if the glutamate and aspartate residues are first converted to acyl-hydrazides. Treatment of hydrazido-albumin-agarose with nitrous acid allows coupling of amino-ligands (30). The potential importance of multipoint attachment of polymeric spacer arms is illustrated by considering the effect of such attachment on the rate of leakage. If a ligand leaks at the rate of 0.1% per day when coupled at one point and the cleavage of one attachment bond does not influence the rate of cleavage of a second attachment bond we may expect that a polymeric spacer arm attached at two points would leak at a rate of 0.0001% per day. The realization of this calculated effect can be seen in the utility of polymeric arms in the purification of the estradiol receptor where a multimeric arm allows a purification of 27-fold, use of albumin increases the purification 4,400-fold and a polylysine-alanine arm allows purification in excess of 100,000-fold (8).

Recently a modified method for activating agarose with CNBr