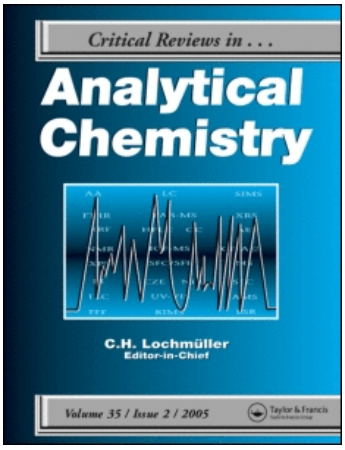

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AFFINITY CHROMATOGRAPHY AND METAL CHELATE AFFINITY CHROMATOGRAPHY

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I. INTRODUCTION

The extremely rapid growth in the field of protein chemistry during the past 50 years has been due in large part to the development of very effective techniques of protein separation and analysis. A number of techniques such as electrophoresis or isoelectric focusing, based on the overall charge and mass of a biomolecule, have been widely applied. However, preparative isolation of proteins often involves some form of chromatography. The two modes of chromatography that have been most widely accepted and used for separation of biomolecules are partition chromatography and adsorption chromatography which includes affinity chromatography. The technique of affinity chromatography exploits the unique biological specificity inherent in a ligand-macromolecule interaction. Thus, among separation procedures, a group of methods based on the molecular affinities found in biological systems have become increasingly important.

A recent extension of affinity chromatography, metal chelate chromatography, is a result of a search for new techniques for the separation of complex biological mixtures, including enzymes and proteins. The development of metal chelate affinity chromatog-

raphy follows a series of milestones such as ion- or ligand exchange chromatography and use of functionalized resins or insoluble polymers as matrices for ligand immobilization. A historical survey shows that the development of metal chelate affinity chromatography as a biospecific adsorption chromatographic technique has been a continuous, evolutionary process. Over 200 enzymes have successfully been purified by use of metal chelate techniques. Additionally, this technique has proved to be valuable for the purification of other proteins such as interferon.

This review comprises a historical survey of the various scientific advances during the past 50 years which have made the development of metal chelate chromatography possible. The mechanisms of metal-protein interactions, the structures of metal-protein coordination complexes, and numerous other factors had to be understood in detail before the application of metal chelate chromatography to the separation of complex biochemical systems could be made. Thus, the first applications of biospecific adsorptions using metals for protein separation drew upon knowledge derived from a variety of experiments, as summarized here. The text further explores possible directions of future developments of this new technique for the determination of biomolecules. The literature survey covers a broad time period, beginning at about 1930, with emphasis on references from 1970 through 1986.

II. EARLY STUDIES OF METAL-PROTEIN INTERACTION

The study of the formation of complexes between metallic cations and amino acids, peptides, and proteins has a long-standing and rich history. Throughout biological systems, proteins are found intimately associated with metallic cations, and among the great variety of natural, metal-protein complexes, some specific metal atoms play a critical role in the structure of the protein.

In their early, monumental review,[1] Gurd and Wilcox considered several important factors regarding the interaction of metal ions with proteins that are still valid today. These factors are (1) in proteins, the side-chain groups, such as carboxyl, imidazole, or sulfhydryl, are much more important in binding the metal ions than are terminal amino or carboxyl groups, (2) in proteins, there are several different ligand groups that have different affinities for binding with a metal ion, (3) each metal ion may combine with only one coordinating group of the protein, (4) the protein molecule bears a considerable number of positive and negative charges whose net potential field favors or hinders the approach of the metal ion, and (5) in certain proteins, a few ligand groups will be found in arrays which are sterically favorable for the chelation of particular metal ions. These factors are important in the equilibrium study of metal-protein interactions (to give the affinity constant).

It has been known for a long time that transition-metal ions form stable complexes with amino acids;[1,2] histidine and cysteine, for example, are known to form rather stable complexes with Cu(II), Zn(II), or Co(II) ions in nearly neutral aqueous solutions.[3,4] Also, it has been reported[5,6] that bovine serum albumin binds Cu(II) ions and other metals to the amino-terminal region, and the nitrogen atom of the peptide linkage and the N terminal amino group may coordinate to various transition-metal ions.[7] It is also known[8,9a-c] that transition metal ions: (1) account for the binding of nucleic acids to proteins, (2) are essential to the functioning and structure of nucleoproteins, and (3) influence protein synthesis and heredity information. They also provide control for the denaturation process and affect stabilization of nucleic acid structure via metal-to-base bonding.[9a,b]

III. EARLY USE OF METAL SALTS IN SEPARATING MIXTURES OF PROTEINS

Early attempts at fractionation of proteins are based on the fact that various proteins show characteristic pH ranges over which their solubility is affected by the presence of different metal ions.[1,10-12] Thus, proteins may vary widely in the solubility of their various metal complexes, and this permits some fractionation of mixtures. The procedure is not fully understood[1,2] and is apparently of limited scope;[11b] however, under certain conditions, the addition of metal ions to proteins appears to be useful. For example, the interaction of β-lipoprotein with γ-globulin is increased by the presence of Zn(II) ions, and glycine appears to combine with Zn(II) that is itself bound to serum albumin,[11a] even though glycine does not combine with the protein in the absence of Zn(II) ions.[11a,13] The mechanism of fractionation of proteins by addition of certain transition metal salts, however, is different from that operating in salting out of proteins.[11b,c]

IV. RECENT STUDIES OF METAL-PROTEIN INTERACTION

A. Copper Complexes in Biochemistry and Pharmacology

Copper is an element essential for life. Copper-protein complexes, for example, are required by all cells in man. Copper complexes, in particular, are remarkably active pharmacological agents;[14,15] this activity is not shared by copper ions or the parent ligands on their own. Copper-protein complexes also serve as enzymes needed for normal metabolic processes. Some of the best understood copper-dependent enzymes and their chemical function are (1) cytochrome c oxidase (reduction of oxygen), (2) superoxide dismutase (dismutation of superoxide), (3) tyrosinase (hydroxylation of tyrosine), (4) dopamine-β-hydroxylase (hydroxylation of dopamine), (5) lysyl oxidase (oxidation of terminal amino group of lysyl amino acids in specific peptides to an aldehydo group), and (6) amino oxidases (oxidation of primary amines to aldehydes).[14,16-22]

Oxygen transport in several species of marine organisms utilizes the binuclear copper protein hemocyanin.[23] The implication for the reversible binding of oxygen by hemocyanin has recently been demonstrated in a study of the reaction of a model phenoxy-bridged Cu(II) dimer with dioxygen, e.g., O_2^{2-}, to form a Cu(II) peroxide adduct (e.g., Cu-O_2-Cu).[24,25]

The recent development of interest in binuclear metal complexes is due largely to their potential as models for that group of metalloproteins for which the biological function is known, or believed, to be associated with the occurrence of the metal centers in pairs. Prominent among these are several of the copper-containing proteins[9a,21,23,26] and several dioxygen transport proteins.[23-26]

B. Copper-Containing Proteins

The interaction of heavy-metal ions with biological species has been a topic of numerous studies. Much recent interest has centered around the elucidation of loci of the active site of copper-containing enzymes.[26-29] Like most metals that perform a key role in living cells, copper carries out its various physiological functions in association with specific proteins. For example, the metabolic roles of copper metalloenzymes (e.g., copper-containing proteins) involve:

1. Transferring electrons from a substrate molecule to molecular oxygen
2. Participation in electron-transfer reaction
3. Transport and storage of the metal itself[9,30-34]

The mononuclear Cu(II) site of the blue copper proteins has unusual optical and magnetic properties.[30] The electronic spectrum is characterized by an intense absorption near 600 nm ($\varepsilon - 10^3 - 10^4$), in contrast to the weak absorption ($\varepsilon \leqslant 10^2$) exhibited by mononuclear copper complexes of low molecular weight. It has also been suggested, in the past from model-compound studies,[35,36] that the intense blue color of copper proteins is due to RS → Cu charge-transfer excitation[30,37] and that the unpaired electron is substantially delocalized onto the sulfur ligand.[38]

For two blue copper proteins, poplar leaf plastocyanin[39] and *Pseudomonas aeruginosa* azurin,[40] the spectroscopically derived structural model has been substantiated by crystal-structure analyses. In each case, the copper is coordinated by two histidine nitrogen atoms and cysteinyl sulfur, with another sulfur, from methionine, nearby. A comprehensive study of the magnetic properties of six Cu(II) blue copper proteins by electron[41] nuclear double resonance (ENDOR) has recently been reported. By application of a spin restricted self-consistent field-X_α-scattered wave calculations, the electronic structure and bonding of the blue copper site in protein plastocyanin has recently been determined.[41a]

C. Additional Recent Studies on Metal-Protein Interactions

The interaction of metal ions with enzymes, including the structure of the metal-enzyme complex, has been unequivocally demonstrated in recent years. For example, strong binding of polypeptide hormone glucagon to the immobilized Cu(II) and Co(II) ions has been ascribed as due to chelation by the N terminal histidine residue and the aspartate residue,[42] metal binding being enhanced by hydrogen bonding of the histidine with the acidic residue.[43] It has also been observed[44] that, in the zinc-insulin complexes, the imidazole residues are the most important binding sites.

Carbonic anhydrase is an enzyme widely distributed in plants, bacteria, and mammals, catalyzing the interconversion of CO_2 and HCO_3^-. This enzyme is well recognized as one of the typical Zn(II)-containing enzymes.[45,46] Zn(II) is known to be bound in the enzyme to three imidazoles arranged in a nonplanar orientation.[45-48] A large number of Zn(II)-containing metalloenzymes are known whose physiological role stems from the ability of the active site metal to promote hydrolysis or a hydration reaction.[49] For example, carboxypeptidase A is a Zn(II) metalloenzyme that catalyzes the hydrolysis of peptides and O-acyl derivatives of α-hydroxy carboxylic acids; the metal ion presumably complexes the carbonyl oxygen atom of peptide substrates.[50a-e] The Cu(II), Zn(II)-superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of two superoxide radical-anions to oxygen and hydrogen peroxide; the mechanism of this action has recently been reported.[51]

Copper-sulfur interactions have attracted attention for some time, originally because of the intense colors associated with such systems[52,53] and, more recently, because of the chain of logic[54-57] leading to the conformation of cysteine thiolate and methionine thioether sulfur as active-site ligands to pseudotetrahedral copper in three copper proteins.[58-61]

Amino acid residues in proteins that possess an ionizable proton can display ligand properties. Nitrogen donors include the imidazole group in histidine, the ε-amino group in lysine, and the guanadinium group of arginine. The carboxyl groups of aspartate and glutamate, and the hydroxyl group of tyrosine, can function as oxygen donors, and the thiol group of cysteine can act as a sulfur donor.[42]

Hydrophobic interaction (often called hydrophobic bonding) is of great importance in determining the shape that proteins assume in an aqueous environment.[62] Thus, by increasing the ionic strength of the solution (via blocking of the charge interaction of the protein), the hydrophobic function of the protein can be enhanced. For example,

hydrophobic interaction causes the hydrocarbon parts of large molecules to cluster together in aqueous solution.

D. Recent X-Ray Studies of Biological Model Compounds

The coordination chemistry of copper(I) salts with polydentate ligands has recently become an important research area with respect to the search for model compounds that can either mimic, or even (ideally) duplicate, one or more of the important specific physical and chemical properties of certain copper proteins, in which the metal exists in the reduced Cu(I) state.[9,63-66] A recent X-ray crystal structure determination of copper(I) and copper(II) complexes involving a model tetradentate chelating imidazole-thioether ligand, i.e., 1,6-bis (*N*-benzylimidazole-2-yl) 2,5-dithiahexane, showed[64] that the Cu(II) atom is in trigonal-bipyramidal coordination geometry, with two axial imidazole N donor atoms (Cu-N, 193 and 194 pm) and two thioether S atoms (Cu-S, 256.5 and 247.0 pm).

In another recent study,[65] the spectral analysis and X-ray crystallography of several complexes of copper(II) and zinc(II), with use of multidentate ligands entailing thiolate, phenolate, imine, and thioether donor atoms, have been presented and the relationship of the structural and redox chemistry of these model compounds to that of certain copper proteins was discussed and compared. Recent pertinent studies include physical properties of the mixed ligands in the Cu(II)-peptide complexes,[66] X-ray structures of Cu(II)-bovine plasma amine oxidase complexes,[66a] Cu(II)-hemocyamin complexes,[66b] and tris(imidazole)-containing phosphine Zn(II) complexes as a biomimetic catalyst,[67] and model tetranuclear Cu(II) complexes using N,N-diethylnicotinamide as a ligand;[68] also, calcium-binding proteins: calcium(II)-lanthanide(III) exchange in carp parvalbumin,[69] and new aspects of the chemistry of metalloproteins.[70] The active site structure in the biologically important cytochrome c oxidase may be binuclear in copper and heme; in support of this, the model bimetallic bridged Cu(II) — Fe(III) complex has been proposed as a possible structure.[70a]

Another recent study[83] reported on the static properties of imidazole-bridged heteronuclear Cu(II)-Zn(II) complexes as new model compounds for the active center of the enzyme superoxide dismutase. For example, the imidazolate-bridged homo-binuclear Cu(II)-diethylenetriamine complex showed a visible absorption maximum at 586 nm; the maximum shifted to a shorter wavelength when the structure of the complex was changed from mononuclear to binuclear and pentanuclear forms. As the absorption in this region arises from a d → d* transition of the Cu(II), the spectral shift suggests changes in the d-orbital of the Cu(II) atom. The visible spectroscopy and electron spin resonance (ESR) studies of model mononuclear Cu(II) and binuclear Cu(II)-Zn(II) complexes appeared to indicate that Zn(II) has an effect on the ligand field of the Cu(II). In the case of bovine superoxide dismutase, where Cu(II) and Zn(II) are bridged by an imidazolate bridge, Zn(II) affects the electronic properties of Cu(II) (and may also control the two-step, inverse electron-transfer between superoxide and the active center).[83] Recently, some imidazolate-bridged dicopper(II) complexes have been examined as models for the active site of copper superoxide dismutase.[84,85]

E. X-Ray Studies of Biological Compounds Containing Copper and Zinc

The geometry of the copper-binding site in the so-called "blue" or type I copper proteins has been known since 1980, when the first X-ray structure of the blue protein plastocyanin was solved.[58] In this protein, the Cu(II) is coordinated by two histidine imidazole N atoms, a cysteine thiolate S atom, and a methionine S atom in a distorted-tetrahedral geometry. The methionine S atom has only a weak interaction with Cu(II) (Cu-S, 290 pm at pH ~ 6). The Cu(II) in the reduced form of the protein is coordinated

by the same set of ligand atoms in a geometry that is strongly pH dependent.[9a,59] A crystallographic study of another blue copper-protein, namely azurin, indicated a similar coordination geometry for Cu(II).[40] On the basis of spectral and redox properties, it has been suggested, that, in the blue copper-protein stellacyanin and rusticyanin, the two sulfur ligands in each protein are two cysteines or two methionines, respectively.[63] The rigid conformation of the protein having a cysteine residue near the copper-binding site obviously prevents the formation of disulfide and Cu(I). Nevertheless, it is rather easy to transfer an electron from Cys(S^-) to Cu(II) as is evidenced by the low energy of the Cys(S^-) → Cu(II) charge-transfer band (near 600 nm), which is responsible for the intense blue color of these proteins.[64]

The active center of bovine erythrocyte superoxide dismutase, which catalyzes the dismutation of toxic superoxide to dioxygen and hydrogen peroxide,[71,72] has been shown by X-ray crystallographic studies[73,74] to consist of a copper and a zinc ion bridged by a histidyl imidazole residue. The X-ray structure of SOD has definitely shown that zinc(II) is bound to two histidines (His 69 and His 78) through their ND_1 nitrogens, to an aspartate residue (Asp 81) and to a histidine (His 61) which is also bound to copper(II). The Zn(II) ion is bound to three more histidines (His 44, His 46, and His 118) in a flattened tetrahedral structure and to an apical water molecule.[74a] Bovine erythrocyte superoxide dismutase in which the native Zn(II) is substituted by cobalt(II) has recently been investigated through ^1H NMR spectroscopy.[74b] The Cu(II) was shown to play a direct role in the catalytic electron-transfer mechanism.[75] The role of Zn(II), however, has not yet been clearly explained, but it may organize the peptide chain of the active center[76] or attenuate the pKa value of the imidazole hydrogen atom.[77] It is of interest to know whether or how the Zn(II) affects the properties of the Cu(II). Model complexes have been synthesized and their magnetic exchange couplings,[78,79] solution properties,[80] and reduction features[81] or redox properties[82] have been reported. All of these complexes have homo-binuclear Cu(II) centers.

The literature on metal — protein interactions and the structure of metal — protein complexes is enormous and will not be discussed here in detail. A few related topics recently surveyed were concerned with long-range, electron transfer in proteins;[86a-k] metal chelates as probes of biological systems;[23,87-90] metals in biological systems;[23,91-93] the binding of heavy metals to proteins;[94] the structure and function of copper and iron proteins;[94-96] the spectroscopic investigations of the binuclear copper-protein hemocyanin;[23,97-99] the reversible binding of dioxygen by hemocyanin;[100] and zinc and copper in clinical medicine.[101] Also surveyed were the reactions of copper oxidases and dioxygen-binding proteins (the reversible dioxygen-binding);[102] other copper complexes that bind dioxygen reversibly[25] or stable copper(II) — superoxide complexes;[103] differential geometry and protein folding;[104] in addition to chemical reagents for protein modification,[105] structure-activity relationships of dihydrofolate reductase inhibitors,[106] and recent reviews on the chemistry and biochemistry of peptides and proteins.[107,108]

V. LIGAND-EXCHANGE CHROMATOGRAPHY

Ligand-exchange chromatography (LEC) was introduced and first interpreted by Helfferich[109,110] over 20 years ago. It is a separation method based on selective adsorption and displacement from matrix-bound heavy metals of amines, hydrazines, hydroxy and keto compounds, nucleic acids, amino acids, peptides, and even proteins. LEC has been defined as a process in which interaction of the stationary phase and the molecules to be separated occurs during the formation of coordination bonds inside the coordination sphere of the complex-forming ion located on the matrix. The topic has been thoroughly reviewed by Davankov and co-workers.[111,112]

LEC differs from ion-exchange adsorption[113] or other types of adsorption chromatography in its basic process of interaction between the adsorbate and the stationary phase.[11b] In this instance, the interaction does not take place directly, but is accomplished via the coordination sphere of the complex-forming metal ion. It was the exchange of ligands bound to the central ion of the metal that suggested the term "ligand-exchange chromatography". Thus, in LEC, the ligands are resolved according to their ability to enter into the coordination sphere of the metal ion. The chromatographic separation of substances proceeds via interactions with the stationary phase through bond formation in the coordination sphere of the transition metal present on the matrix.

LEC is possible only in systems in which the interaction of the mobile ligand with the stationary phase is a reversible process. The coordination bonds between the ligands and the metal ions must break and form readily, i.e., the complexes must be kinetically labile. This mechanism,[109] in addition to the ligand exchange, is certainly true in metal-chelate affinity chromatography[114] discussed later. The authors of two review articles[111,112] regard metal-chelate affinity chromatography as a special type of ligand-exchange chromatography.

LEC in general has been applied mainly to isolation or fractionation of low molecular weight compounds; in particular, amines,[115,116] amino-lactone adducts,[117] amino acids,[118,119] alkaloids,[120] and nucleosides[121] have been successfully fractionated by this method.

LEC has also proved to be a powerful tool for resolving enantiomers of DL-amino acids,[122-125] DL-hydroxy carboxylic acids,[125] or DL-amino alcohols.[126] A new method described[126] for enantiomeric resolution of DL-amino alcohols by liquid chromatography involves derivatization with salicylaldehyde to form the Schiff base followed by LEC of the Schiff base with L-proline-copper(II) as the chiral, stationary phase. The copper(II) ion serves to stabilize the Schiff base, whereas the derivative permits complexation in a manner favorable for resolution and enhanced detection. A possible structure for the mixed complex responsible for separation was suggested. From this structure, a correlation between elution order and configuration was proposed.[126] LEC can also be a valuable technique which complements the methods most commonly used for separating proteins.[127]

VI. USE OF INSOLUBLE, POLYMER-BOUND REAGENTS IN SYNTHESIS AND SEPARATION

A. The Merrifield Solid-Phase Peptide Synthesis

The successful synthesis of peptides on cross-linked polystyrene beads, introduced by Merrifield over 20 years ago,[128-131] focused attention on the advantages provided by insoluble, polymer-bound reagents. The essential feature of solid-phase synthesis of peptides[128] is the covalent attachment of the growing peptide chain to an insoluble, solid support during the course of the chain assembly. This has obvious advantages which include rapid purification of the product peptide at each step by simple filtration and washing and low handling losses. However, it has been realized for some time that there may be a negative influence of the insoluble, resin support on reaction kinetics that could lead to incomplete reaction at each step. To obviate this possibility, a new approach to quantify the synthetic efficiency in solid-phase peptide synthesis as a function of chain length has recently been designed by Merrifield and co-workers.[132]

Before the Merrifield discovery, the most frequent contact of the organic chemist with insoluble, polymer-bound reagents was in the use of ion-exchange resins;[113] since then, these systems have been applied as vehicles for synthetic, catalytic, and

mechanistic studies. Parallel development of other supports and matrices, such as agarose, dextrans (cross-linked and noncross-linked), poly(acrylamide), agarose-poly(acrylamide) copolymers, cellulose, and glass or silica gel with hydrophobic covering help in reporting.[133,134] It is only recently that insoluble polymers have been used in general organic synthesis to solve specific synthetic and separation problems.[135-137] Particularly important was the development of a series of polymeric reagents, e.g., functionalized polymers for use in organic synthesis.[135-149] Synthetic, functional polymers thus have found wide application in organic and bioorganic chemistry.[141,142] The term polymeric reagent is one in which the functional polymer itself reacts in a one-step process to transform a low (or high) molecular weight substrate into product.[142] In many cases, polymer-supported reagents in organic synthesis allow separation of the insoluble polymeric byproduct from the reaction mixture by simple filtration.

Owing to the current increased use of polymers in chemistry[150,151] and biology,[152] a considerable interest in immobilization of enzymes, proteins, and small ligands has developed.[153] These preparations are used for practical applications such as purification of compounds by affinity chromatography[154] and for fundamental studies such as determining mechanisms of chemical[155] or enzymic reactions.[156]

VII. AFFINITY CHROMATOGRAPHY

A. General

Since its introduction by Cuatrecasas et al.,[157-159] affinity chromatography has become a widely used technique for the isolation and purification of numerous, water-soluble, biologically active molecules, including enzymes and proteins. The topic has been thoroughly discussed in reviews by Porath and Kristiansen,[160] Lowe and Dean,[161] Gribnau et al.,[162] Lowe,[163] Scopes,[11b] Parikh and Cuatrecasas,[163a] Walters,[163b] and in monographs.[164a,b]

Selective isolation and purification of enzymes and other biologically important macromolecules by affinity chromatography exploits the unique, biological properties of these proteins to bind ligands specifically and reversibly. Within the past 20 years, however, considerable interest has been aroused in the development and application of chromatographic adsorbents based on biological specificity.[157,160-163] The technique of affinity chromatography exploits the biological specificity of those substances which can form stable, reversibly complexes with matrix-bound ligands. The concept is realized by covalently attaching the ligand to an insoluble matrix and packing the support material into a chromatographic column. In principle, only those macromolecules that display considerable affinity for the immobilized ligand will be retained on such an adsorbent, whereas those having little or no affinity will pass through unretarded. The biospecifically adsorbed species can then be subsequently desorbed by suitable adjustments of the eluent.

The most effective methods of enzyme purification are those that make use of interaction between enzymes and their specific ligands. In affinity chromatography, for example, the ligand is bound to an insoluble support and, depending on the specificity of the ligand-enzyme interaction, a single enzyme, or group of enzymes, can be bound to such an affinity polymer.[161] Disadvantages of such polymers are frequently their instability and high cost.

An alternative approach to enzyme purification is the elution of a bound enzyme with a specific ligand.[154] In this case, proteins are bound nonspecifically to a polymer, and the binding is overcome by complex-formation of the enzyme with a ligand. In general, cation-exchange resins have been used as affinity polymers, since these contain common ligands at which binding occurs, sometimes through groups situated at, or

near, the enzyme-binding site. Upon elution with a specific substrate, these binding sites are shielded against interaction with the ion-exchanger as a result of complex formation between the enzyme and its substrate. Thus, affinity chromatography, which is based on specific interactions between matrix-bound ligands and soluble biochemicals, has become a widely used and powerful technique for the isolation and purification of macromolecules, e.g., enzymes and proteins. Affinity chromatography has been thoroughly discussed in many reviews and books.[154,159-168a-f]

B. Elements of Affinity Chromatography

The critical parameters of affinity chromatography which have been outlined elsewhere[162] are the matrix, the spacer arm, and the ligand. The matrix for affinity chromatography should consist of uniform, spherical beads that are hydrophilic, chemically and biologically stable, and have sufficient modifiable groups to permit a high degree of substitution with the ligand to be immobilized.[160,161] Ideally, the matrix should be rigid and have a very porous network which permits uniform and unimpaired entry and exit of large molecules.[169] Because of their very loose network, cross-linked dextrans (Sephadex®)*, beaded agarose derivatives (Sepharose®), or cross-linked poly(acrylamide) beads are found to be the most suitable matrices for affinity chromatography.[159,170] Cross-linked cellulose, porous glass beads, silica, and glass beads coated with dextran or antigens are also in use. Examination of the thermodynamic and kinetic characteristics of microparticulate affinity chromatography supports has recently been reviewed and discussed.[170a]

The most widely used method for the activation of polysaccharide supports (e.g., agarose) involves treatment of the matrix with aqueous cyanogen bromide[171,172] at alkaline pH. Although cyanogen bromide activation of agarose gel beads[171] is excellent for the preparation of affinity adsorbents, there are a few drawbacks, e.g., the linkage is not stable at alkaline pH and undesired positive charges are introduced.[172a] This, however, can be overcome by O-alkylation of cross-linked agarose gel beads with alkyl chloride in dimethyl sulfoxide using methylsulfinyl carbanion as a catalyst.[172b] Recently, Pierce Chemical Co., (Rockford, Illinois) introduced carbonyldiimidazole-(CDI) activated agarose for affinity columns; these columns are more stable towards ligands and proteins as compared to those activated by cynogen bromide.

Ligands or suitable spacer arms, containing free amino groups, are subsequently coupled to the imidocarbonate-activated matrix. Under these coupling conditions, the matrix-ligand or matrix-spacer arm derivatives which are formed involve positively charged, N-substituted iso-ureas and N-substituted imidocarbonates. These derivatives can behave as "ion-exchange" groups, resulting in enhanced, nonspecific adsorption to the affinity columns.[173,174]

In an alternative, new procedure that is devoid of charged groups, the use of 1,1′-carbonyldiimidazole reagent for the activation of cross-linked agarose, for use in affinity chromatography, has been advanced.[175] The intermediate, activated matrix (an imidazolyl carbonate) formed under these conditions couples readily with N-nucleophiles such as free amino groups in ligands. This method thus provides for affinity chromatography supports that are devoid of additional charged groups by the activation linkage. The efficient purification of bovine trypsin, human thyroglobulin, and sheep thyroid membrane glycoproteins on a 1,1′-carbonyldiimidazole-activated agarose column[176] has demonstrated the suitability of this support for biospecific, affinity chromatography.

* Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for this purpose.

It is now established that, for successful purification by affinity chromatography, the ligand must be placed at a distance from the matrix backbone sufficient to minimize steric interferences. The problem was solved by placing the ligand at the terminus of a long hydrocarbon chain or "spacer arm".[157,159] Systematic studies[177,178] have shown that a hydrocarbon spacer arm comprising six or seven methylene groups, as in immobilized N^6-ω-aminoalkylAMP, e.g., agarose-$NH(CH_2)_n$ R where $n = 2—8$,[178,179] is optimal for maximal interaction of the macromolecule with the immobilized ligand.

A number of considerations govern the selection of the ligand for affinity chromatography.[177,180,181] The ligand must possess an adequate affinity for the macromolecule that is to be separated. The ligand to be immobilized must possess functional groups that can be modified for coupling to the solid support or terminus of a spacer arm without impairing or abolishing its recognition by the complementary macromolecule. In selection of spacer arms for affinity chromatography,[182] the use of hydrophilic arms to control or eliminate nonbiospecific adsorption effects has to be considered.[183]

A few additional important factors in established procedures for the separation of proteins by adsorption chromatography should be mentioned. These involve either hydrophobic adsorption chromatography via an interaction between the hydrophobic group of the protein and a stationary phase (e.g., agarose)[185] or protein separation via a nonionic interaction (e.g., other than hydrophobic), possibly through aromatic π—π interaction or hydrogen binding.[160,186]

In 1972, Pharmacia Fine Chemicals developed cyanogen bromide-activated Sepharose® 4B,[164] a stable, ready-to-use support for immobilizing ligands, thus making simple and rapid affinity chromatography readily available to any laboratory. Today, affinity chromatography occupies a unique place in separation technology because it is the only technique that enables purification of almost any biomolecule on the basis of its biological function. In regard to limitations of affinity chromatography, occasional solvolytic detachments of ligands from polymeric supports have been observed.[184]

Affinity chromatography has been utilized most fruitfully in preparative applications. The technique has achieved some spectacular, single-step purifications of numerous enzymes and proteins.[163,164,166,168,187-199] For example, affinity chromatography on vitamin B_{12}— agarose resulted in a 24,000-fold purification of transcobalamine II from human plasma.[194]

VIII. DYE-LIGAND AFFINITY CHROMATOGRAPHY

A. Triazine Dyes as Ligands

The development and extensive use of biospecific affinity adsorbents of the "group specific" type have proven to be a most valuable addition to the range of techniques available for enzyme purification.[163,180,188] The term "dye-ligand chromatography" refers to a variant of affinity chromatography in which certain synthetic textile dyes having a triazine structure are used instead of the natural substrates, cofactors, or effectors commonly employed as immobilized ligands.

Since the 1968 discovery that the dye chromophore rather than the matrix is responsible for the interaction with enzymes,[200,201] triazine-dye "affinity" chromatography adsorbents have been shown to bind a wide variety of different proteins. In recent years, triazine dyes immobilized on agarose or dextran have been applied to serve as "group-specific and selective" affinity chromatography ligands in the purification of a broad range of enzymes and proteins; the topic has been reviewed and discussed in detail by Dean and Watson,[202] Fulton,[203] Scopes,[11b] and most recently by Subramanian[204] and others.[161,163,191,205-213] The application of dye-ligand chromatography has been greatly extended to include the purification of nearly 200 enzymes and proteins, as well as a number of new dye-ligands.

B. Structure of Triazine Dyes

The structures of some triazine dyes, best known by their commercial names, such as Cibacron Blue F3G-A (Procion Blue H-B), Procion Red HE-3B, Procion Yellow H-A, and Procion Yellow MX-B, are shown in Figure 1.

The dyes depicted in Figure 1 are derivatives of mono- and dichlorotriazine (cyanuric acid analogs); four structural components, for example, of Cibacron Blue dye-ligand comprise: (1) sulfonated anthraquinone (chromophore), (2) sulfonated benzene (bridge), (3) triazine ring (active chlorine group), and (4) sulfonated benzene (terminal, a mixture of *meta* and *para* isomers). Modification of one of the structural groups may result in a new dye-ligand. Due to the structural features, a wide variety of proteins having hydrophobic binding regions are able to accept parts, or all, of the sulfonated aromatic rings of these dyes.[202,214] The presence of metal ions in some dyes, such as Procion Brown MX-5BR [Cr(II)] and Procion Turquoise H7-G [Cu(II)], or aromatic amino groups, as in many of the anthraquinone-containing blue dyes, allows a considerable variety of dye structures to be presented as potential ligands.[208] Recently, Amicon Corporation introduced three new dye-ligands (also triazine derivatives) coupled to cross-linked 5% agarose beads as Matrex Gel Orange A, Green A, and Blue B; all of them are intense, brilliant dyes.[203,204]

As with conventional, affinity chromatography, the success of an interaction between an immobilized dye and a biological macromolecule will depend largely on the experimental conditions chosen. Careful consideration must therefore be given to the nature of the matrix (preferably hydrophilic and chemically and biologically stable), the structure of a dye-ligand, gel-bound ligand concentration, and a host of such operational parameters as pH, ionic strength, temperature, flow rate, column capacity, and column geometry.[161,163]

C. Choice of a Matrix in Immobilization of a Dye

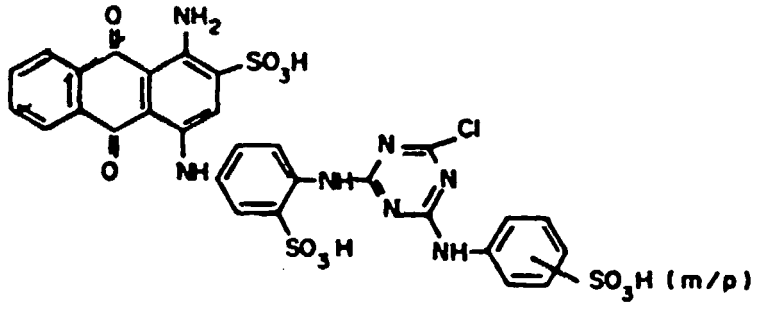
As with many affinity chromatographic ligands, triazine dyes have been immobilized on a wide variety of support matrices in the search for an ideal system.[159,202,204] Cross-linked dextran (Sephadex®), beaded agarose derivatives (Sepharose®), or poly(acrylamide) are suited for immobilization of the dye on the basis of flow properties, mechanical and chemical stabilities of the gel after the coupling, and the ability of the gel to form a loose, porous network; however, it has been shown that agarose is superior to cellulose[215] or poly(acrylamide).[216]

The literature describes several general procedures that require activation of agarose with cyanogen bromide (CNBr) to immobilize the dye.[166,204] An alternative method for the preparation of the agarose-bound dye that is devoid of the cyanogen bromide treatment has been described;[211] it can be applied for immobilization of both mono- and dichlorotriazine dyes. Agarose is added to an aqueous solution of the dye, followed by the addition of solid sodium chloride to 2% (w/v) final concentration. Following physical adsorption of the dye onto the matrix, the pH of the solution is raised to 10.5 by the addition of sodium carbonate, wherein the necessary nucleophiles are generated and attack on the dye ensues. No definitive studies have been performed to establish the optimal coupling time, although 24 to 120 hr is commonplace.[206]

D. Mechanism of Protein Binding

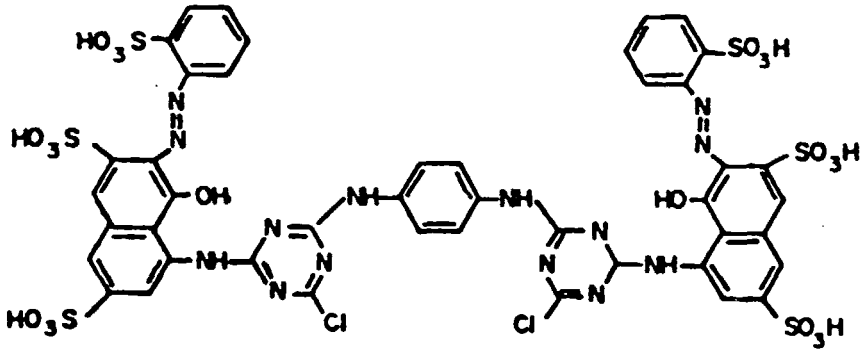
The mechanism of interaction between the dye-ligands and proteins has not yet been fully explained. However, one important factor, among others,[203,204] that can be agreed on is that the interaction of the dye with proteins is strongly ionic in nature (anionic dye with cationic protein sites), but is influenced, or even dominated, in many cases, by apolar (hydrophobic) effects. The binding of proteins on immobilized dye columns

(a)



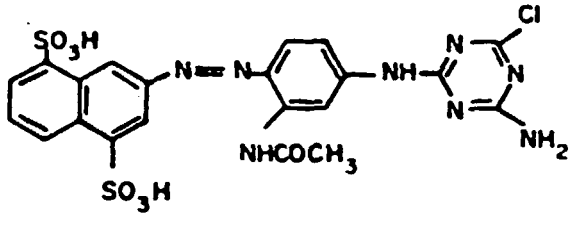
Cibacron blue F3G-A (Procion blue H-B)

(b)



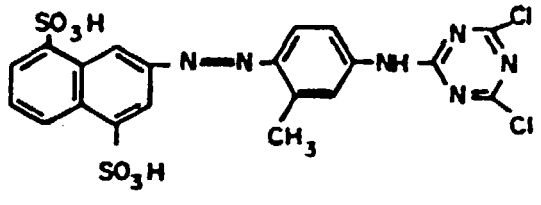
Procion red HE-3B

(c)



Procion yellow H-A.

(d)



Procion yellow MX-R

FIGURE 1. The structures of four typical triazine dyes: (a) Cibacron Blue F3-GA (Procion Blue H-B), (b) Procion Red HE-3B, (c) Procion Yellow H-A, (d) Procion Yellow MX-B.

has been characterized as "nucleotide-specific", "ionic", and "hydrophobic".[204] Depending on the environment, whether hydrophobic or electrostatic, the dye seems to optimize the available interactions. For example, most dehydrogenases and kinases bind to the immobilized dye columns via their nucleotide-binding sites or "biospecific" binding.[217,218] A clear-cut case of hydrophobic vs. electrostatic interaction of a protein with the blue dye chromophore was demonstrated for interferon.[219] Because the triazine dye chromophore contains both amino and sulfonate groups, the so-called "nonspecific" ion-exchange effects would also be expected. It has been shown[213] that a wide variety of proteins, ranging from albumin and aldolase to hemoglobin and cytochrome c, exhibit nonspecific binding to Blue Sepharose® at low ionic strength. The key to nonspecific binding is low ionic strength. Nonspecific binding can be either hydrophobic or ionic or both. Usually, ionic binding is disrupted by high concentrations of chaotropic salt (e.g., LiBr, NH_4SCN, KSCN). However, hydrophobic interactions can be enhanced in the presence of high concentrations of either ammonium or sodium sulfate or potassium hydrogen-phosphate; such salts also tend to inhibit protein denaturation.

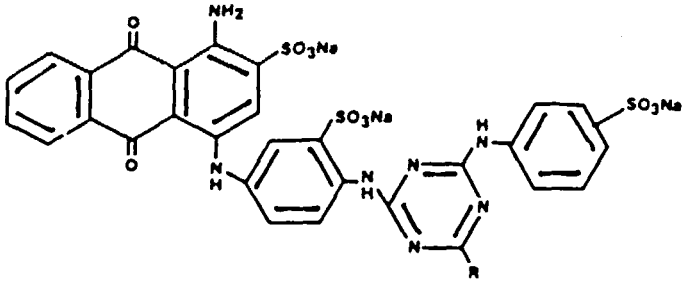
E. Application of Immobilized Dyes

The literature contains numerous accounts of immobilized dyes used to purify individual proteins.[202-204,207] For example, immobilized Procion Brown MX-5BR and Green HE-4BD, respectively, may be utilized to purify tryptophanyl-tRNA synthetase 137-fold and methionyl-tRNA synthetase.[208] Immobilized Cibacron Blue F3-GA has been established as an effective adsorbent for the purification of both NAD^+ and $NADP^+$-dependent dehydrogenases, kinases, glycolytic enzymes, blood proteins,[202,205] and crude interferon.[219,220] By the use of the Matrex Gels Orange A, Green A, and Blue B as dye-ligands,[207] it has been possible to separate a rabbit muscle pyruvate kinase from lactate dehydrogenase and achieve simultaneous purification of both enzymes. The same group[207] also found that chondronectin binds only to the Orange A, from which it can be recovered in high yield.

Other triazine dyes, such as Procion Red HE-3B, Procion Yellow MX-8G, Procion Scarlet MX-G, Procion Green H-4D, and Procion Brown MX-5BR have also been found to be suitable ligands for the selective purification of individual pyridine nucleotide-dependent dehydrogenases, kinases, plasminogen, carboxypeptidase G2, alkaline phosphatase, and L-amino acid-tRNA synthetase.[205,208,209,211,221-224] It has been suggested that dye binding occurs predominantly at sites overlapping the substrate, coenzyme, or effector binding sites,[225] and the ability to elute proteins biospecifically from triazine dye adsorbents with substrates[211] and coenzymes[224,226,227] supports this view.

F. Effect of Metal Ions on Binding Proteins to Immobilized Triazine Dye Ligands

The development and extensive use of biospecific affinity adsorbents of the "group-specific" type (including dyes) have proved a most valuable addition to the range of techniques available for enzyme purification.[161,163,180,188] The ability of metal ions to promote the binding of proteins to immobilized, triazine dye adsorbents is a useful chromatographic procedure developed only recently. It has been found[228] that low concentrations of such metal ions as Zn(II), Co(II), Mn(II), Ni(II), and Cu(II) promote the binding of proteins to a number of immobilized, triazine dye affinity adsorbents. For example, Zn(II) ions promote binding of carboxypeptidase G2, alkaline phosphatase, and yeast hexokinase to immobilized triazine dyes, such as Procion Red H8-B4, Procion Yellow H-A, and Cibacron Blue F3-GA, respectively. Also, the binding of ovalbumin to immobilized Cibacron Blue F3-GA and Procion Orange MX-G is selectively enhanced in the presence of Al(III) ions.[228] Also, it is known from circular di-



R = -Cl = Cibacron Blue
R = -O-Dextran = Blue Dextran
R = -O-Sepharose = Blue Sepharose

FIGURE 2. The structure of Cibacron F3-GA. The dye is a mixture of meta and para isomers with respect to the sulfonate group on the terminal phenyl ring. In Blue Dextran Sepharose®, Blue Dextran is linked via the 1-amino group of the anthraquinone ring to the Sepharose®.

chroism studies[228] that these dyes are conformationally mobile and do not assume a single, unique conformation on binding to different proteins. Because Zn(II) contains a full (d^{10}) electronic configuration, no crystal field effects occur, and the stereochemistry of the complex is decided entirely by electrostatic and covalent binding forces and by the ligand size.[180] It is conceivable therefore that the interaction of Zn(II) with functional groups appended to the dye chromophore should stabilize a particular conformation of the dye that is especially acceptable to the protein. Therefore, it seems most probably that the metal ion forms an essential bridge between the dye and the protein, to generate a highly selective, protein-substrate-like ternary complex which displays considerable specificity for the dye chromophore, the metal ion, and the protein. Although the mechanism by which the separation proceeds is still to be defined, the data available suggest that the metal ion-promoted effects involve the formation of specific, dye-metal-ion-protein coordination-complexes.[228]

G. The Interaction of Cibacron Blue F3-GA with Proteins and Enzymes

In a series of triazine dyes studied, Cibacron Blue F3-GA (Figure 2) has acquired the distinction of being a "universal pseudoaffinity ligand".[202,204,210] In addition, the dye has been described as possessing a discriminating ability to bind to selected proteins containing a specific structure called the "dinucleotide fold".[191,217] The versatility of Cibacron Blue arises from the proper mix of aromatic (nonpolar, hydrophobic) and sulfonate (ionic) groups; it thus constitutes a specialized case of a molecule that combines structural and stereochemical features together, so as to be able to offer a pseudospecificity to a large number of proteins. By appropriate modification, the dye can be, and has been, "fine tuned" for more specific needs. It is probably not inappropriate to suggest that the dye has only now begun a long and pioneering journey in the exploration of newer techniques of protein isolation and purification.

In the presence of binding enzymes, the absorbance spectrum of Cibacron Blue dye exhibits a significant bathochromic shift, going from λ_{max} (H_2O) at 610 to 620 nm in free solution to a λ_{max} at 660 to 690 nm in the presence of an enzyme. This shift is presumed to be due to the insertion of the dye into a hydrophobic site in the protein, which alters the absorbance of the aromatic ring.[215] The dye showed the ability to bind strongly to many enzymes; for example, the dye bound to NAD kinase was not removed by dialysis, gel filtration, or charcoal adsorption and was probably bound ir-

reversibly.[229] Immobilized Cibacron Blue F3-GA has the remarkable ability to separate 27 different plasma proteins.[229a]

Cibacron Blue has been applied extensively for purification of numerous, diverse proteins and enzymes that include[204] dehydrogenases and kinases, phosphodiesterase, reductases, enzymes of polynucleotide metabolism (e.g., RNA polymerases), interferons, flavoproteins, endonucleases, β-lactamases, arylsulfatases A and B, muscle proteins, serum proteins, basic proteins (histones), alkaline phosphatase, and many other substrates.

Dye-ligand chromatography often is superior to conventional, natural ligand affinity chromatography in terms of column-binding capacity, biological and chemical resistance of the media, versatility of operating conditions, reusability, and low cost. On the laboratory scale, dye-ligand chromatography is a cost effective and versatile improvement over conventional, physical separation (gel filtration, ion exchange, etc.) and can augment, or often replace, normal affinity techniques.

H. Affinity Electrophoresis on Cibacron Blue F3-DA-Agarose-Poly(acrylamide) Gels

Affinity electrophoresis in poly(acrylamide) gels was first used for the study of the interaction between phosphorylase and glycogen[230] and the interaction of several proteins with Blue Dextran.[231] The theoretical aspects concerning this technique have been described elsewhere.[232,233] Agarose-poly(acrylamide) gels have been shown to possess ideal mechanical strength and porosity for the manipulations involved in electrophoresis (e.g., staining and destaining of ribonucleic acids).[234] Affinity gel electrophoresis using Cibacron Blue agarose-poly(acrylamide) gels and using bovine serum albumin as a model protein have been found useful in determining dissociation constants for protein-ligand interaction.[235]

In a related technique,[236] immunoaffinity electrophoresis and affinity electrophoresis in slabs of 1% agarose have been used to predict the success of ligands in preparative, affinity chromatography. Similarly, immunoaffinity chromatography has been found useful for purification of many polypeptides.[237] Alternatively, an application of 1,1-carbonyldiimidazole-activated agarose for biospecific, affinity chromatography has been described; the efficient purification of bovine trypsin, human thyroglobulin, and sheep thyroid membrane glycoproteins has been demonstrated, using the aforementioned activated matrix.[238]

To the list of various procedures for affinity chromatography[11b] may be added antibody affinity chromatography,[239-240] special ligand affinity chromatography,[241,242] hydrophobic affinity chromatography,[243] and gel chromatography.[243] The relevant work also may include adsorption of bacteria onto ion-exchange resins,[244,245] immobilization of microorganisms on hydroxides of transition metals,[246] or immobilization of enzymes onto titanium-cellulose matrix[247,248] or onto Fe(III)-pectate iminodiacetate matrix.[249] Although affinity chromatography and related methods have afforded considerable improvements to protein purification during the past decade, the search for purification techniques based on new principles still remains a very challenging motivation.

IX. CROCONATE DYES: POTENTIAL REAGENTS FOR BIOLOGICAL SYSTEMS

A new class of anionic dyes, condensation derivatives of oxocarbons, such as Croconate Orange, Croconate Violet, and Croconate Blue (Figure 3) has been found to have excellent semiconductor properties. These salts are all highly intense dyes; for example, Croconate Violet absorbs in the visible spectrum at $\lambda^{H_2O}_{max}$ 533 nm, ($\varepsilon \sim 100{,}000$). The conjugates of these salts are strong acids; indeed, the conjugate acid

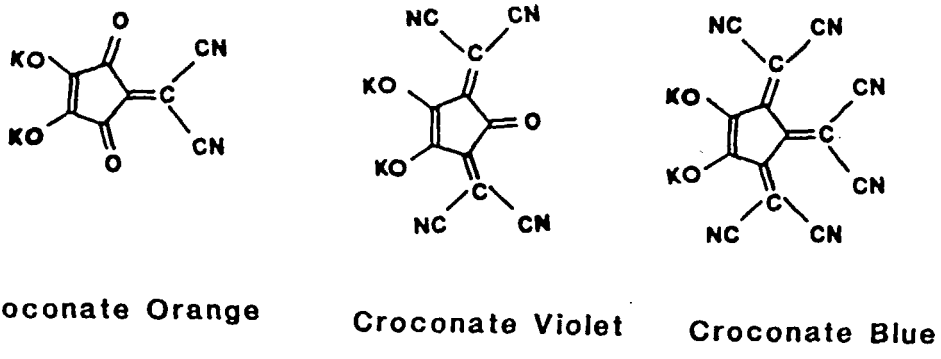


FIGURE 3. Structure of Croconate anionic dyes: Croconate Orange, Croconate Violet, and Croconate Blue.

of Croconate Violet has immeasureably large pK_1 and pK_2 ~0.07. These salts are quite soluble in water, yielding stable solutions.[250-252] In the presence of a binding protein (e.g., bovine serum albumin), these dyes exhibit some bathochromic shifts, indicating a protein-dye interaction. For example, $\lambda^{H_2O}{}_{max}$ for Croconate Orange goes from 444 to 450 nm, Croconate Violet goes from 533 to 544 nm, and Croconate Blue goes from 599 to 618 nm.

Many functionalized polymers, such as cross-linked poly(4-vinylpyridine), cross-linked poly(vinylpyrrolidone), polyamide-CC6 [poly(caprolactam)], or poly(vinyl-styrene)diiminoacetic acid, are good adsorbents for immobilization of croconate dyes; however, hydrophilic matrices such as agarose or dextran show weak coupling with these dyes. The adsorption of croconate anionic dyes, for example, on cross-linked poly(4-vinylpyridine) may be ascribed to hydrophobic interactions and in part, to a donor-acceptor adsorption involving an electron transfer to give an ion pair.[253] Thus, immobilized croconate dyes can be used either for a donor-acceptor adsorption (e.g., charge-transfer chromatography)[254,255] or for affinity chromatography,[253] depending on the reaction conditions applied.

A preliminary study also indicated[256] that these dyes are promising reagents for specific biological staining. For example, Croconate Violet shows a strong affinity for globulins. The dye can discriminate between α-, β-, and γ-globulins on thin-layer, isoelectric-focusing gels. These and other observations warrant further exploration of these unusual, anionic dyes.

X. PYRIDINE-CONTAINING POLYMERS: NEW MATRICES FOR PROTEIN IMMOBILIZATION

Owing to the increased use of polymers in chemistry and biology, considerable interest has developed in the immobilization on the polymers of such entities as enzymes, cells, and small ligands. The advantages of using immobilized species are numerous and include possibility for reuse, ease of separation from the reaction mixture, and increased stability against heat, autolysis, and chemical effects.

Pyridine-containing polymers have been shown to be good matrices for protein immobilization.[153] The basis of the new method involves the reaction of cyanogen bromide with pyridine to give a cyanopyridinium bromide which is readily hydrolyzed in water to a dialdehyde. When bound to a resin matrix, the dialdehyde readily reacts with the NH_2 group of proteins. Poly(4-vinylpyridine) resins or cross-linked copolymers of styrene and vinylpyridine can be used. The amount of aldehyde functionality formed is readily controlled by the amount of cyanogen bromide added. Also, excess

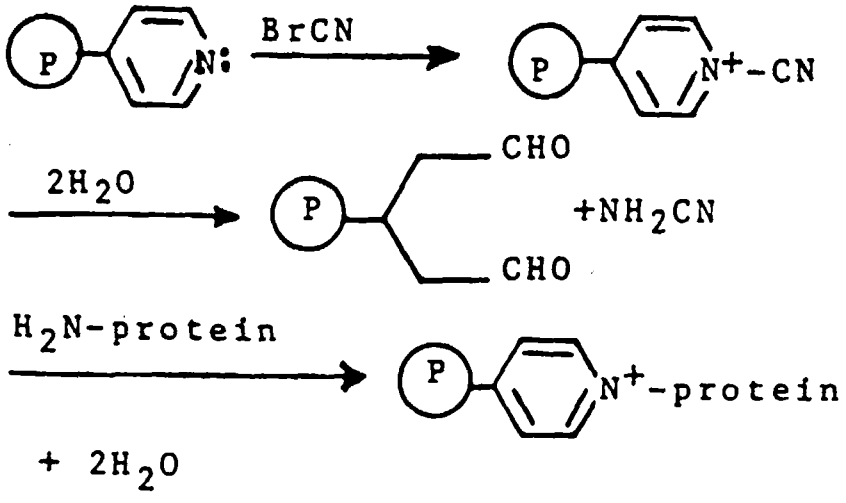


FIGURE 4. Schematic overall reaction for the protein immobilization on pyridine-containing polymers.

aldehyde groups are conveniently reduced by using $NaBH_4$, or they may be reconverted into pyridine moieties by treatment with ammonia. The overall reaction is summarized in Figure 4 and is exemplified by immobilization of such enzymes as trypsin and α-chymotrypsin. Because the pyridine moiety is found in many synthetic polymers, copolymers, membranes, and ion-exchange resins, this method opens up a new avenue for immobilization and separation of many proteins. Thus, proteins as well as such smaller molecules as amines, amino acids, or peptides can be bound to any of the aforementioned polymers.

XI. METAL CHELATE AFFINITY CHROMATOGRAPHY

A. The Porath Method

Affinity chromatography represents the ultimate extension of adsorption chromatography since it embodies the complex set of van der Waals', hydrophobic, steric, and electrostatic forces involved in the specific binding of substrates and other ligands to proteins. Biospecific affinity chromatography is based on the affinity between the ligand and the adsorbate molecule, and this relationship may be used to separate a complex biological mixture, by attaching one of the components to an insoluble matrix, e.g., a gel.[115,160]

Porath et al. demonstrated[257] in 1975 that not only organic molecules (e.g., immobilized organic ligands) but also immobilized metal ions, such as the divalent cations Zn(II) and Cu(II), can be utilized to separate proteins that are able to bind these metals. These authors reported, for the first time, a model system using a Zn(II) and Cu(II) column in tandem for the fractionation of serum proteins. They attached these metal ions to a chelate (iminodiacetic acid [Na-salt]), which was covalently bound (immobilized) to the insoluble matrix (oxirane-activated agarose) (Figure 5).[160,258,259] After formation of the metal chelates, the gels were equilibrated at weakly alkaline pH for sample application. After washing away unbound material, bound proteins could be recovered by changing conditions to favor desorption. A gradient or step-wise diminution of pH to 3 to 4 is often suitable. An alternative way[114] to achieve selective desorption of protein, apart from changing the pH, is to incorporate certain solutes in the eluant that have a higher affinity for the adsorption sites (coordination sites) on

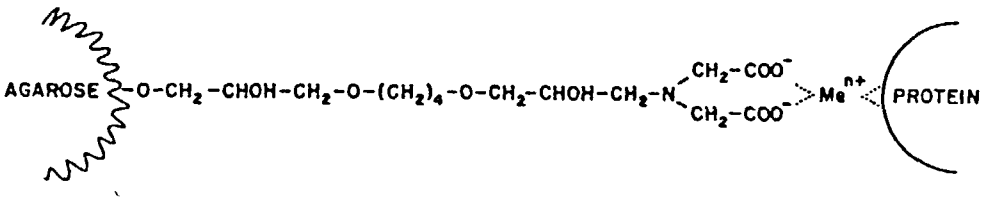


FIGURE 5. Schematic structure of binding of a protein to a metal (Me^{n+}) (with a coordination number of 4) chelated to iminodiacetate acid coupled to oxirane-activated agarose.

the proteins than does the immobilized metal ion or, alternatively, to use solutes that effectively compete with the proteins binding to the metal. In the former case, other kinds of metal ions can be used, whereas, in the latter, specific displacers that contain such groups as -NH_2, -COOH, -SH, ammonium chloride, and glycine (with increasing concentration), or imidazole, histidine, and histamine (selective-gradient substances), may be effective. A third method of elution consists of adding a chelating agent such as EDTA to the mobile phase. It is good practice to maintain a high ionic strength throughout (e.g., with 0.1 to 1.0 mol/*l* of NaCl) to avoid unwanted, ion-exchange effects. Using this method, Porath and associates were able to separate several metal-binding proteins, including ceruloplasmin, α_2-macroglobulin, serum albumin, and transferrin. Chelating Sepharose® 6B, a ready-to-use agarose gel for metal-affinity chromatography, is commercially available (Pharmacia Fine Chemicals).

This new approach for protein separation and isolation was named "metal chelate affinity chromatography" by Porath and co-workers;[257] however, as pointed out by Davankov and Semechkin,[111] this type of chromatography is, in fact, a form of ligand-exchange chromatography, a term first used by Helfferich.[109] Subsequently, Porath and Olin[114] and Porath and Belew[260] introduced the term "immobilized metal affinity adsorption" (IMA adsorption) and "immobilized metal ion affinity chromatography" (IMIA chromatography) to cover all of the types of interactions between solutes and immobilized metals of whatever form; in this way, LEC and metal chelate chromatography can be treated as subdivisions of IMA chromatography or immobilized metal affinity chromatography (IMAC). To obtain some insight into the mechanisms underlying IMAC, the Porath group has recently undertaken a systematic study of the behavior of model compounds, e.g., amino acids and oligopeptides on immobilized nickel iminodiacetate (IDA-Ni^{2+}).[260a,b] As shown by Hemdan and Porath,[260a] the interaction of different amino acids with IDA-Ni^{2+} is complex and is largely dependent on the particular nature of the amino acid under study. For example, the amino acids such as L-cystine, L-histidine, and L-tryptophan were retained very strongly, but L-glutamic and L-aspartic acids were not retained at all. The chromatographic behavior (e.g., retention) of oligopeptides on IDA-Ni^{2+} as found is the sum of the individual contributions of the constituent amino acids.[260b]

Two iminodiacetate (IDA) gels coupled to a Sephadex® G-25 were recently prepared by different synthetic routes and their retention of Cu^{2+} ions was measured.[260c] The preparation of the metal chelate affinity support, e.g., iminodiacetic acid-agarose, has also been described elsewhere.[259a] Metal chelate-interaction chromatography (MCIC) of proteins with iminodiacetic acid-bonded stationary phases on silica support has recently been described.[260d] A new support for immobilized metal chelate affinity chromatography (IMAC) has recently been described;[260e] it is prepared by introducing iminodiacetic acid into TSK gel G5000 PW, which is a hydrophilic resin-based material of larger pore size (particle diameter 10 μm) employed for high-performance gel filtration. Recently, Sulkowski[260f] discussed at length the mechanism of the interaction between metal chelate and protein and cited some examples of what kind of information can be

extracted from the Porath's IMA chromatography (IMAC). The review focused on the discussion of structural requirements underlying the metal chelate-protein recognition.

B. Application of Metal Chelate Affinity Chromatography

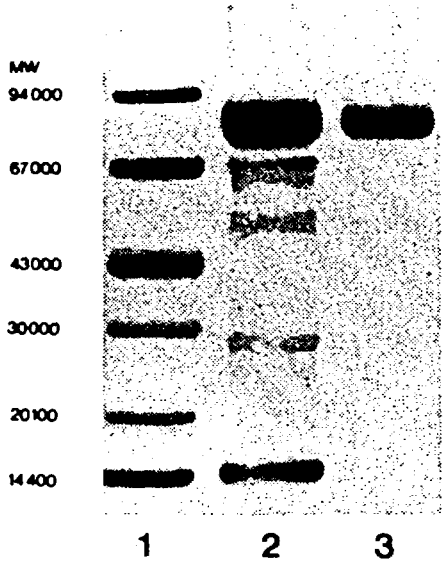
Metal-chelate affinity chromatography has found wide application for enzyme and protein fractionation and purification. Porath and co-workers,[261] using metal-chelate chromatography, reported successful purification of an iron-binding protein, lactoferrin, from human milk by utilizing the Cu(II)-binding property of this protein (Figure 6). Recovery of lactoferrin from the gel was found to be ~100%. Furthermore, the biological property of lactoferrin to inhibit bacterial growth was intact after the purification procedure, indicating that the purification method does not detectably damage the protein.

The plasma proteins α_2-macroglobulin[262-265] and α_1-proteinase inhibitor,[262,266] for example, have been purified to homogeneity on zinc chelate columns. Zn(II) chelate chromatography has also been used to provide immunologically and physiochemically pure α_2-HS glycoprotein from plasma for studies of its structure and function in pathological conditions.[267,268] Plasminogen activators from both normal tissue (human uterus) and human melanoma cells have been isolated by Zn(II) chelate chromatography and found to be very similar molecules that differ from urokinase in a number of properties.[269,270] Recently, Andersson,[270a] using Zn(II) and Cd(II) iminodiacetate and tri(carboxymethyl)ethylenediamine chelates, successfully fractionated human serum proteins; in the case of Zn(II) iminodiacetate-Sepharose® 6B, a "train" tandem of seven columns has been employed.

An example of a difficult purification successfully achieved by zinc-chelate affinity chromatography is the isolation of collagenase from tissue culture medium.[271] This source has only a low concentration of enzyme and large losses occur during conventional extraction procedures. A critical point was the inclusion of detergent throughout the purification scheme. Other proteins and polypeptides separated by using metal-chelate affinity chromatography include rat liver nucleoside diphosphatase [on Zn(II) chelate],[272] granulocyte granule proteins [on Cu(II) chelate],[273] *Dolichos biflorus* seed lectin [on Cu(II) and Zn(II) chelate],[274] GI polypeptides [on Zn(II) chelate];[275] also, serum albumin [on Zn(II) chelate],[276] inter-α-trypsin inhibitor [on Zn(II) chelate],[277] isoinhibitor of trypsin from swine colostrum [on Zn(II) chelate],[278] aminoacylated tRNA [on Cu(II) chelate];[279] also, thiol proteinase inhibitor [on Cu(II) chelate],[280] nonhistone proteins [on Cu(II) chelate],[281] histone-rich RNAase [on Zn(II), Cu(II), and Ni(II) chelates],[282] a protein from *Escherichia coli* cells [on Cd(II) chelate],[283] and NADH nitrate reductase [on Zn(II) chelate].[284]

Various techniques for partial purification of human fibroblast interferon have been described;[285] however, use of metal-chelate affinity chromatography for purification of interferon proteins is of potential future interest. This technique deserves special mention, because metal-chelate affinity chromatography using zinc, copper, cobalt, or nickel has been applied with great success for the isolation and characterization of interferons from many sources. Interestingly, human-fibroblast interferon displays an affinity for Zn(II), whereas human leucocyte interferon does not;[286] the Syrian hamster interferon also fails to bind the Zn(II) chelate, but is retained on Cu(II) chelate columns.[287] Human fibroblast and human leucocyte interferons display a strong affinity for the copper chelate of bis(carboxylmethyl) amine-agarose, binding tenaciously over a wide range of pH (7.4 to 4.0).[288]

The affinity of interferons for metal chelates — aside from the practical use for purification — may also give clues about their structural similarities, as reflected in the surface topography of certain amino acid residues.[289] Metal-chelate affinity chroma-



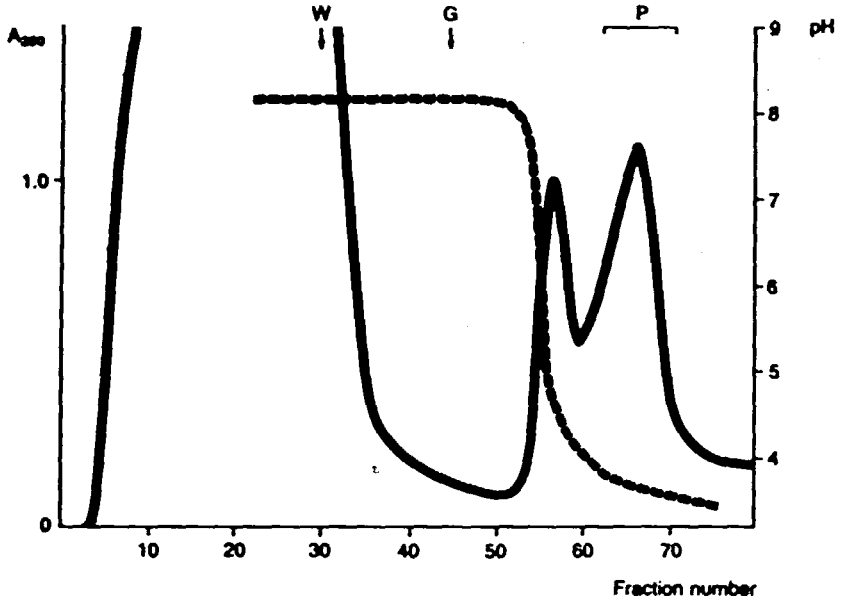


FIGURE 6. Insolation of lactoferrin from human milk. Sample: 70 m*l* defatted, casein-free human milk equilibrated with the starting buffer. Column: C 10/20. Bed height: 16.3 cm, charged with Cu(II) for 10.3 cm. Eluants: W = wash with starting buffer 0.05 mol/*l* tris-acetate pH 8.2, 0.5 mol/*l* NaCl, G = development to final buffer, 0.05 mol/*l* Tri-acetate pH 2.8, 0.5 mol/*l* NaCl. Flow rate: 25 m*l*/h. Fraction size: 2.5 m*l* Insert shows gradient gel electrophoresis in SDS. Gel: PAA4/30. Lane 1: LMW calibration kit. Lane 2: Defatted, casein-free human milk. Lane 3: Pooled material eluted from column (P). (Work from Pharmacia Fine Chemicals).

tography has also been used for purification of primary, human-amnion interferon [on Zn(II) chelate],[290] human-lymphoblastoid interferon (achieving purity of protein to a specific activity of 7.6×10^8 IU/mg) [on Cu(II) chelate];[291] for purification of human-fibroblast interferon to a specific activity of 2×10^8 to 8×10^8 IU/mg of protein [on Zn(II) chelate];[292] for purification of human leucocyte interferon [on Cu(II) chelate];[293,294] or for purification of human cornea-cell interferon [on Zn(II) chelate];[295] and to study the binding of fibrinogen to such metal ions as Ca(II) or Zn(II) [on Zn(II) chelate].[296] A very interesting combination technique for purification of the N^a-acetylencephalin carboxypeptidase (from monkey kidney) has recently been reported; the method involves, first, Cibracron Blue F3-GA-Sepharose® chromatography followed by Ni(II) — and Zn(II) — chelate affinity chromatography; almost 90% of the activity in the homogenate was recovered.[297] Finally, Porath's group demonstrated the potential usefulness of metal-chelate affinity chromatography for nucleotide separations[298,299] and for immobilization of enzymes,[300] thus extending the field of applications still further. A recent discussion of the Porath method was in a symposium that included presentations of kinetic and diffusional effects in affinity chromatography[301] and metal-chelate chromatography, e.g., separation of ribonuclosides and carboxypeptidase G on a Zn(II) gel.[302] A very interesting and promising report in the same symposium was on liquid chromatography with immobilized metal adsorbents that included rare earths and transition metals. For example, lactoferrin was separated from milk on an immobilized Cu(II) column or on a Sm(III) column; similarly, trypsin, snake venom phospholipase, and human serum albumin were separated on immobilized Tb(III), and plasma proteins were separated on tandem columns of immobilized Sm(III) and La(III) (with glycine buffer, pH 9).[303] Immobilization of nuclease P_1 has been effected on a titanium-pumice complex.[304] Immobilization of enzymes on inorganic supports has recently been reviewed.[304a]

1. Immobilized Fe^{3+}-Gels as Supports for Separation of Proteins

The Porath group expanded the use of the principle of IMA adsorption along two lines: (1) exploration of new ligands for metal immobilization and (2) concurrent introduction of a new type of metal. The synthesis of the tris(carboxymethyl)ethylenediamine ligand (TED) allowed the stable immobilization of several additional metals: Fe^{3+}, Al^{3+}, Ga^{3+}, In^{3+}, and Tl^{3+}, and their evaluation as sorbents for protein resolution to utilizing serum as a model mixture.[114,304b]

Several novel metal chelating ligands of mono-, bis-, and trishydroxamic acid series with single hydroxyl functions have recently been synthesized[304c-f] and coupled to the epoxy-activated Sepharose 6B support. All of them have been found strongly to chelate several metals, particularly Fe^{3+} ions. Thus, monohydroxamic acid-Sepharose® 6B sorbent charged with Fe^{3+} was found to bind strongly several proteins and was used for chromatography of human serum[304c] or serum proteins.[304d] Similarly, the adsorbent glycinehydroxymate-Sepharose® 6B, charged with Fe^{3+}, was successfully applied for chromatography of the following proteins: lysozyme, cytochrome c, avidin, bovine pancreatic RNAase, myoglobin, ovalbumin, and human serum albumin.[304e] Recently, Andersson and Porath[304f] found that phosphoproteins and phosphoamino acids bind to ferric ions immobilized on iminodiacetate-agarose gel (IDA-Fe^{3+} gel) and can be eluted by increasing pH or introducing phosphate ions to the eluant. This procedure was utilized for fractionation of ovalbumin into three components of varying phosphate content.

It has been found[304e] that proteins can be adsorbed on immobilized Fe^{3+} over a wide range of pH. The adsorption affinities of proteins towards the IMA-Fe^{3+} sorbent are highly dependent on the nature of the buffer and the capacity of the adsorbent; for

example, α-aminohydroxamate adsorbent charged with Fe^{3+} glycine shows selective ion-exchange properties. The adsorption, however, is different from the ordinary ion-exchange processes and is interpreted on the basis of interactions that depend on the coordination characteristics of proteins with metal ions at various pHs.

Metal-chelate affinity-chromatography has been reviewed by Davankov and Semechkin,[111] Lonnerdal and Keen,[305] Sulkowski and co-workers,[289] Sulkowski,[260f] and Porath and Belew;[260] the topic has also been briefly discussed in a technical bulletin.[306] The influence of various parameters important to metal-chelate affinity chromatography, such as pH dependence, ionic strength, temperature, and the contribution of hydrophobic interactions in the substrate-retention process, have been discussed by Hubert and Porath[298] and Sulkowski;[260f] the fundamental principles of the related, biospecific affinity chromatography have been presented in a comprehensive review by Porath and Kristiansen.[160]

C. Protein Affinity for Metal Chelates

As shown earlier,[257] copper and zinc have been the metals most commonly used for metal-chelate affinity chromatography. For a biological fluid, such as serum, Cu(II) chelate gels have been shown to be more efficient than those with Zn(II) with regard to capacity to adsorb serum proteins. Binding of most serum proteins to the Zn(II) gel is weaker than to the Cu(II) gel, as evidenced by the fact that a pH of 6 is sufficient to elute bound proteins from a Zn(II) gel, whereas a pH of 4 is necessary for elution of bound proteins from a Cu(II) gel. Certain interferon proteins, depending on their structure, display affinity for Zn(II) rather than Cu(II) gels;[286] in another case, interferon binds to the Cu(II) gel and not to the Zn(II) gel.[287,288] Other cations, such as Ca(II),[274,307] Cd(II),[282,283] Co(II), Ni(II), Mn(II),[260,282,289] and Fe(III),[249] have been tested for their affinity, and many of them have been used. Also, gels charged with such trivalent metal ions as Al(III), Ga(III), In(III), and Tl(III) at various pH values[308] have been used to separate serum proteins. It is important to note that, in regard to the metal-protein interactions, different kinds of mechanisms are responsible for the adsorption, with ionic interaction prevailing at low pH, and coordinate covalent bonding playing an increasingly important role at neutral and alkaline pH values.[308]

D. Nature of Metal Ions as Binding Ligands

Metals such as Zn(II) and Cu(II) and, to a lesser extent, Mg(II), Ca(II), Hg(II), Co(II), and Ni(II), are known to form stable complexes under appropriate conditions with cysteinyl, α-amino, ε-amino, carboxyl, histidinyl, tyrosinyl, and guanidinium functional groups of proteins.[7,257] The empirically determined series of metal-complex stabilities[309] is applicable to ligands where oxygen and nitrogen serve as the donor atoms; the order of metal ligation generally follows the Irving-Williams series of stability:[310] Zn(II) < Cu(II) > Ni(II) > Co(II) > Fe(II) > Mn(II) > Mg(II). However, when the large and highly polarizable sulfur atom is substituted for oxygen or nitrogen as one of the donors, the binding-stability constant is increased; particularly Zn(II) complexes are greatly stabilized relative to Mn(II) or even to Ni(II).[309,311] Reasoning for the complex stability can be seen in the electronic configuration of the metal ion; for example, Zn(II) contains a full (d^{10}) configuration; no crystal field effects occur.[312] Zn(II) also promotes binding with such enzymes as carboxypeptidase G2, alkaline phosphotase, and the Mg(II)-dependent enzyme hexokinase;[313,314] and the stability constants of a number of transition metallo-carboxypeptides follows the Irving-Williams series.[310]

All complexes of metal ions having electrons in e_g orbitals are labile. Tripositive iron, with its spherically symmetrical, d^5 configuration and five spin-unpaired electrons, forms labile, outer-orbital complexes. In contrast, metal ions having electrons in the

d^8 configuration offer larger field stabilization when subjected to such strongly splitting ligands as those occurring in some proteins. Dipositive nickel (d^8) has two unpaired electrons and, with eight d electrons, Ni(II) is likely to give complexes that are more inert than those of Fe(II), with no ligand-field stabilization.[114,315]

Iron and nickel ions can be compared from another aspect. According to the "hard-soft", metal-ion concept based on the polarizability of interacting ions,[316-318] Fe^{3+} can be considered to be a "hard" Lewis acid or electron acceptor. Cd^{2+} and Hg^{2+} are examples of Lewis "soft" acids, and Ni^{2+} (also Co^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}) are on the borderline for "soft" acids. Accordingly, Fe^{3+} may associate more strongly with oxygen as the ligand atom, whereas Ni^{2+} has a preference for nitrogen and sulfur.[114]

E. Anticipated Structure of Metal-Protein Coordination Complexes

The coordination of tyrosine to metal centers is a structural feature recently found for many of the metalloproteins.[9b,c] In the formation of most stable coordination centers, quite a number of polyligands participate via two functional groups per divalent metal ion, ML_2. Hydrated ions may also give rise to centers of composition, $ML_2(H_2O)n$.[319] Solvents used in the complexing processes may enter the coordination sphere to produce centers of the type $ML_2(H_2O)n\ S_m$.[319] This is not necessary in the case of Cu^{2+} ions (d^9 configuration), which are most likely to give coordination centers having a planar structure. With other transition metal ions such as $Ni^{2+}(d^8)$, $Co^{2+}(d^7)$, $Co^{3+}(d^6)$, $Mn^{2+}(d^5)$, and $Mn^{3+}(d^4)$, of which the octahedral or tetragonal environment is characteristic, the structure of the coordination center undergoes distortion. In practice, when bound to a polycomplex, the coordination sphere of such ions may involve any anion that occurs in the reaction mixture. Even a uniform, polymeric iminodiacetic acid may act as a monobasic or dibasic acid, to give rise to purely ionic, or more covalent, bonding in the same macromolecule.[7,319,320]

F. Mechanism of Binding in Metal-Chelate Affinity Chromatography

A number of studies (and corresponding techniques) on protein-substrate complexation in solution[321-329] have been described recently, however, reports on the binding of proteins with matrix-immobilized, organic or metal ligands are scarce. Considering the mechanism of the sorbate-sorbent interaction, Porath and associates[257] assumed that the chromatographic behavior of a protein is governed largely by its number, or density, of exposed surface imidazole and thiol groups that are able to form metal complexes. The authors[257,260,300] also postulated that metal ion-protein interactions, including electrostatic, hydrophobic, and charge-transfer effects, may involve the formation of the specific, metal ion-protein, coordination complex, the reversibility of which is pH dependent.

The binding of protein, peptides, and nucleotides is believed to be the result of the ability of electron-rich ligands, such as histidine, cysteine, and tryptophan, to substitute for weakly bonded ligands (such as water) in the metal complexes.[300] The metal may donate to or accept electrons from the ligand atoms. Thus, when a protein with surface-exposed amino acids having electron-accepting or donating capacity is exposed to a metal chelated to a negatively charged gel, with a capacity for forming additional coordination bonds, the protein can bind strongly by multipoint attachment. It is evident that this binding is strong because it is stable even in mol/ℓ NaCl, precluding the possibility that ionic interaction is the principal force in the binding, in contrast to ion-exchange chromatography. It is important to note that several of the proteins isolated to date have been recovered in their initial, metal-saturated form. Thus, an iron-containing protein (Fe-lactoferrin) can be isolated on a Cu(II) chelate gel, and subsequently eluted, and recovered in its initial iron-saturated state.[305] This observation ne-

gates the possibility that the mechanism of attachment is through ligand exchange involving the metal on the protein and the metal on the gel, but does not preclude the possibility that there are weaker, secondary binding sites on the surface of the protein, which could form multipoint attachment to the metal chelate gel. However, these groups do not bind "free" metal ions in solution under normal conditions. For example, an excessive binding of human interferon to Cu(II) chelate-agarose in comparison to that of Zn(II) chelate-agarose has been explained[288] as due to the formation of additional linkages. Another explanation has also been considered, namely that, at a certain metal density of the sorbent, some possible bonds are not formed between protein molecules and the sorbent. An increase in metal density would result in the formation of multiple coordination bonds.

Another potential mechanism is based on the possibility that some type of coordination bond is formed between unoccupied coordination sites at the primary, metal-binding site(s) in the protein and the complexed metal at the chelate on the gel. This would be in accord with the definition that describes exchange LEC[111] as "a process in which interaction between the stationary phase and the molecules to be separated occurs during the formation of coordination bonds inside the coordination sphere of the complex-forming ion". The latter mechanism also seems likely from the point of view that many proteins isolated by means of metal-chelate affinity chromatography are metalloproteins. However, it is possible that there is a combination of the previously discussed mechanisms responsible for the binding. Also it is possible that different types of binding occur, depending on the nature of the protein studied. Further experiments are needed to clarify the mechanism of binding in this type of chromatography.

XII. NEW APPLICATIONS OF METAL POLYMERS

A. New Application of Styrene-Divinylbenzene Metal Chelates for the Separation of Proteins

A copper polymer prepared by treating a styrene-divinylbenzene copolymer containing iminodiacetate ions with copper sulfate solution[330] was used by Fréchet and coworkers[331] for separation of amine-lactone adducts from complex mixtures; Cu(II) ions are known for their affinity for amino derivatives.[316] As described earlier, the Porath method[257] requires IDA as a metal-chelating ligand; however, in the procedure reported,[331] a hydrophobic, cross-linked polystyrene matrix was used instead of the agarose carrier. Both resins have recently been applied in the author's laboratory[332] for the study of the mechanism of separation of serum proteins by metal-chelate affinity chromatography, as shown in Figure 7 for a Cu(II) chelate column.

The results of this study[332] may be summarized as follows. Immobilized metal-ion affinity columns have been prepared by chelating transition-metal ions, Cu(II), Ni(II), Zn(II), Co(II), and Mn(II), to IDA derivatives of styrene-divinylbenzene and Sepharose® 6B beads. The ability of these materials to resolve proteins was tested at various pH values by adsorbing human serum to column packings to which a single metal had been chelated. The proteins were eluted by increasing the buffer concentration (0.05 to 0.5 mol/*l*) and by increasing the metal-ion concentration (0.01 to 5%). The elution profile for each styrene-divinylbenzene metal chelate varied, but examination of protein fractions by isoelectric focusing indicated that the presence of the metal ion influenced the ionic strength at which the proteins were eluted. Albumin and acidic proteins were eluted at 0.05 mol/*l* buffer; very basic and some neutral species were eluted at 0.15 mol/*l* buffer; and a few slightly basic proteins were eluted at 0.5 mol/*l* buffer. Similar results were obtained with the Sepharose® 6B metal-ion chelate. As the pH was increased, the protein fractions were more strongly bound and increasing concen-

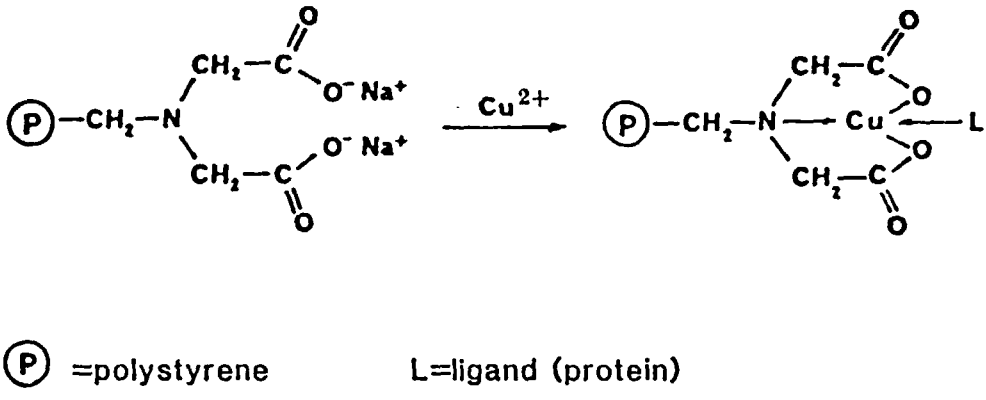


FIGURE 7. Schematic reaction of the preparation of a styrene-divinylbenzene-Cu iminodiacetate column; L (ligand) indicates a possible attachment of a protein to the metal-polystyrene matrix.

trations of metal ion were required for protein elution. High concentration (1 mol/ℓ) of salt did not increase the binding of protein to the columns. These results suggest that those polymers that bind metal ions by charge-transfer complexing behave as ion-exchange resins. Each metal modifies the properties of the polymer-metal complex to some extent.

A typical fractionation of human serum in styrene-divinylbenzene-Cu IDA column is shown in Figure 8; it may be noted that fraction B is enriched with globulins not eluted from the column with 0.05 mol/ℓ NaOAC buffer. Further studies on the mechanism of protein separation on immobilized, metal-ion columns are in progress.

B. Analytical Application of Poly(Vinylpyridines)

Polymer beads have found a wide range of uses in organic chemistry, cross-linked polystyrene resins usually being employed, whereas cross-linked poly(vinylpyridine) or cross-linked copolymers with styrene and vinylpyridine have received much less attention.[135,142,146,147,150,333] Pyridine has broad applications in organic synthesis either by itself or in conjunction with other reagents. Poly(vinylpyridine) was found to be useful as an HCl acceptor.[334] Functionalized PVPs have found a variety of useful applications: poly(vinylpyridinium hydrobromide perbromide) resins are employed as brominating agents[335] or stereoselective brominating agents;[336] poly(vinylpyridinium chlorochromate) as an oxidizing agent;[337] poly(vinylpyridine borane) as a reducing agent[338] or poly(4-vinylpyridine) as an electrode oxocarbon-impregnated, polymer-film coating for improving photoelectrochemical sensitivity;[339,340] poly(vinylpyridine) as a matrix for protein immobilization,[153] selective adsorption of metal ions,[341] or complexation of Cu(II);[342] poly(vinylpyridine)-copper as a complex catalyst for polymerization[343] or as a catalyst for oxidation of sulfur compounds,[344] the oxidative coupling of phenols, or the oxidative polymerization of phenols;[345] and PVP-*bis*(2,2′-bipyridyl)ruthenium complexes as catalysts or sensitizers for photochemical reactions.[346]

The stability of cross-linked poly(4-vinylpyridine) (PVP) beads has recently been examined[347] by laser ionization mass spectrometry; the possible positive ion fragments *m/z* 77 through *m/z* 209 are shown in Figure 9.

C. Poly(4-Vinylpyridine)-Cu Chelate as a Possible Column Matrix

Cross-linked PVP or PVP-metal complexes are being examined at NBS as possible column-packing materials for the affinity chromatography of proteins. The stability of PVP beads and PVP-metal complexes comprising a pendant (monodentate) pyridine

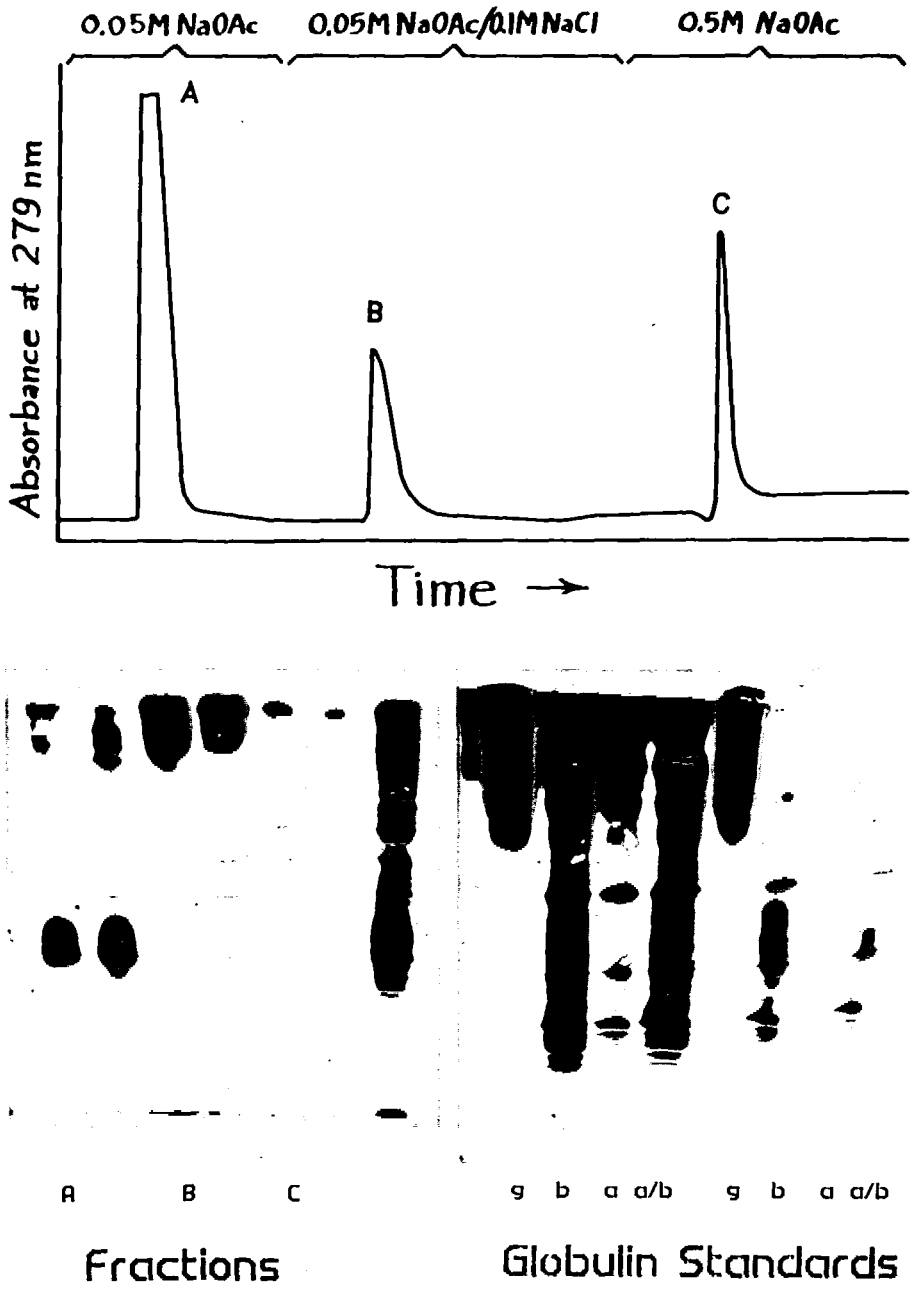


FIGURE 8. Separation of human serum on a styrene-divinylbenzene-Cu iminodiacetate column. Three major protein peaks are eluted consecutively using a three-step sodium acetate pH 5.5 gradient with increasing ionic strength. The first peak (A) contained albumin and many other proteins. There is a large proportion of protein in the second peak (B) that has a basic charge and migrates to an apparent isoelectric point (pI) of about 9 and is likely due to globulins; the peak (B) appears to be devoid of albumin. The third peak (C) has a more neutral distribution of proteins, with the majority of protein having a pI of about 7; albumin is also absent from this peak.

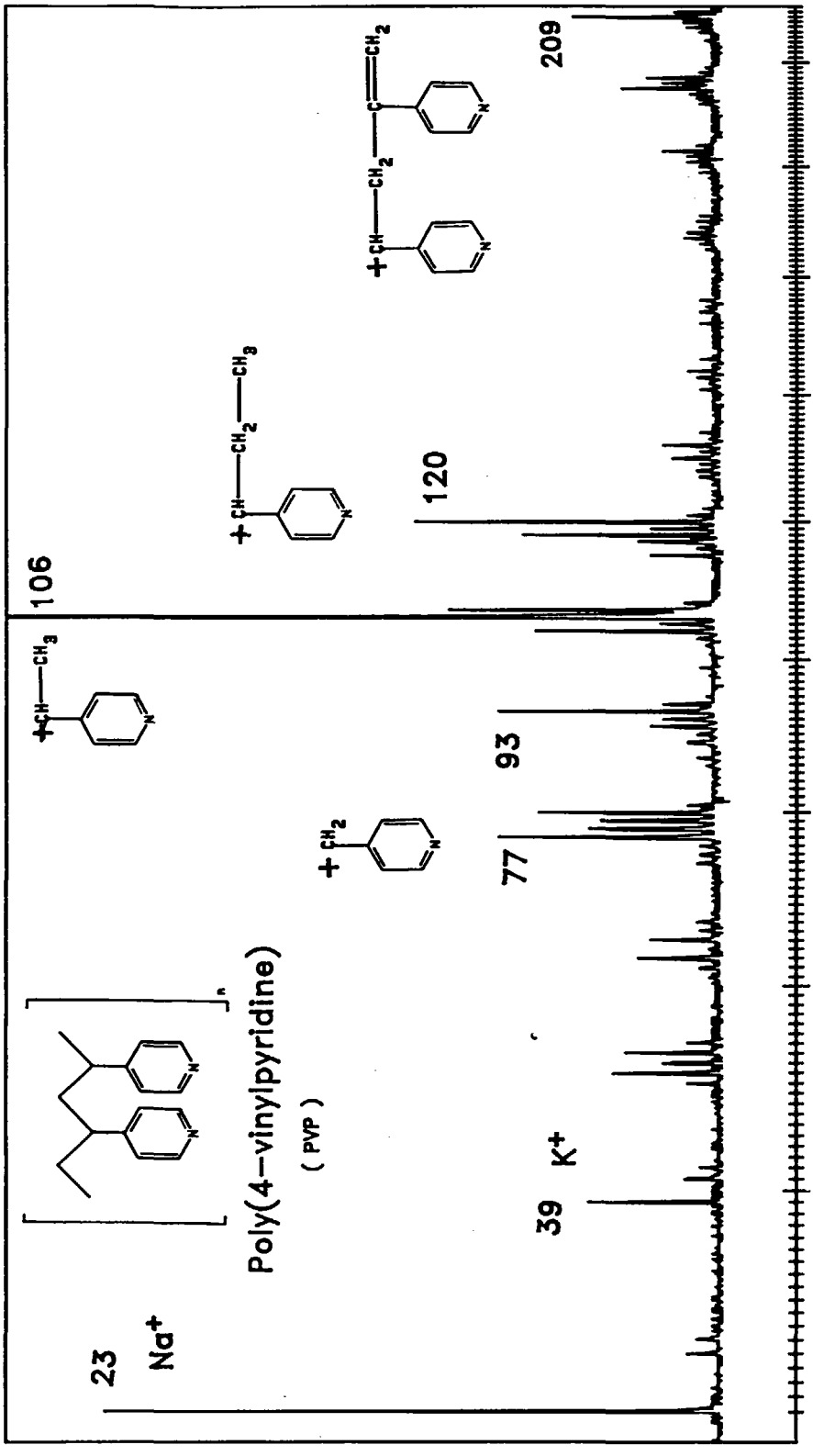


FIGURE 9. Laser ionization mass spectrometry of poly(4-vinylpyridine) (PVP); positive ion spectrum and positive ion fragments of commercial PVP.

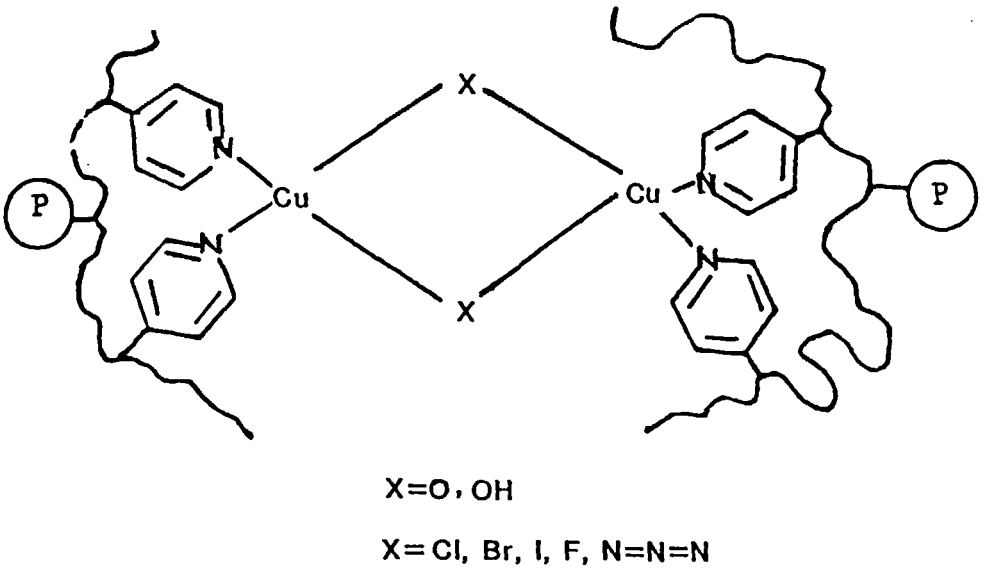


FIGURE 10. The proposed structure for the binuclear PVP-Cu complex with hydroxide bridging-ligan (X = O^- or OH^-) and their replacement with halide (or azide) bridging-ligands (X = Cl^-, Br^-, I^-, F^-, N_3^-).

ligand is considerably less than that of styrene-divinybenzene copolymer comprising a multidentate IDA acid ligand; however, the stability of PVP beads can be increased either by suitable cross-linking[150] or chemical modification.

As found here,[253] PVP can act as a ligand in the presence of Cu(II) ions and the blue PVP-Cu beads thus obtained indeed show affinity for proteins; however, the resin was not stable during prolonged treatment with elutant buffers. It has been observed further in this laboratory[253] that, when PVP-Cu chelate is stirred with a solution of a halide, ready transformation of the resin takes place and the color of the beads turns from the original blue to green, brown, black, and deep blue following successive treatment with aqueous sodium chloride, bromide, iodide, or fluoride. Sodium azide treatment produces dark brown-green beads. The above reactions can be explained as due to replacement of the bridging hydroxide groups apparently present in the original binuclear PVP-Cu complex with such stronger bridging groups (e.g., ligands) as Cl^-, Br^-, I^-, F^-, or N_3^-. Existence of binuclear O^-- or OH^--bridged Cu(II) complexes is known,[348-350] and replacement of bridging hydroxide ligands with halogen bridging ligands has also been reported for certain binuclear copper complexes,[89,333,351,352] but not for the cross-linked PVP-Cu beads. The structure proposed for the binuclear PVP-Copper complex with hydroxide bridging-ligands (X = O^- or OH^-) and their replacement with halide (or azide) bridging-ligands (X = Cl^-, Br^-, I^-, F^-, N_3^-) is shown in Figure 10. The laser Raman spectra of the PVP-Cu complex is shown in Figure 11; it may be noted that the Raman frequency shifts in PVP following complexation (to yield the PVP-Cu complex).[353] Further structural and application work on these metal-ion resins is in progress.

Another group of metal-ion resins should receive consideration as possible matrices for separation techniques; this group includes binuclear copper-Schiff base complexes.[354] The thermal stabilities of some of the transition metal-Schiff base coordination polymers have been reported.[355]

The feasibility of photoacoustic infrared spectroscopy for structural studies of biological metal salts and beads is being investigated in this laboratory. Although still in a research stage, this technique appears to be a useful complement to laser Raman

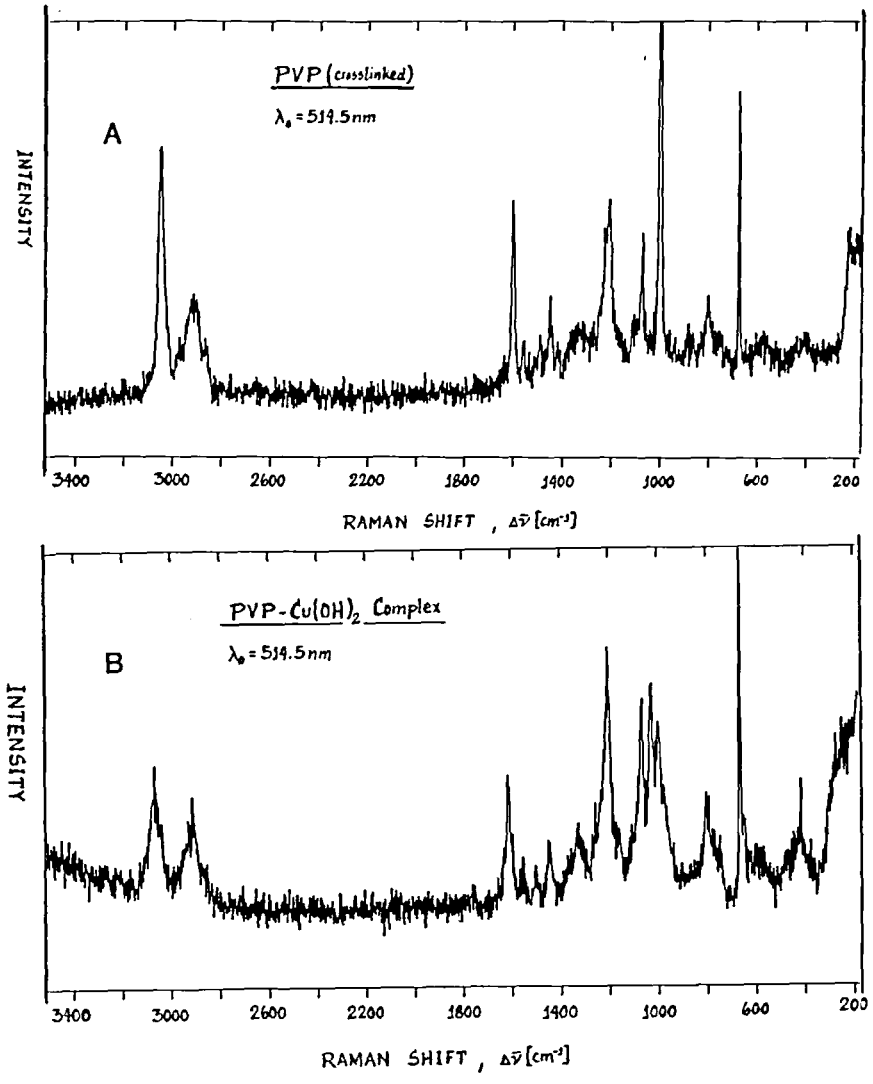


FIGURE 11. The laser Raman spectra: commercial PVP (spectrum A), PVP-Cu complex (spectrum B) and superimposed A and B (spectrum C) showing definite Raman shifts following complexation.

spectroscopy. Preliminary photoacoustic infrared spectra of several amino acid alkali salts, including the DNA sodium salt and their PVP-Cu complexes, are shown in Figure 12.[253]

The systematic exploration of interactions of proteins with a wide variety of supports to understand further the mechanisms of interactions of the metal-binding ligands with individual protein molecules is one of our goals. By studying these specific interactions, we expect to gain a greater knowledge of protein structure and the mechanisms of separation. Use of metal supports may play an important analytical, as well as industrial role, in biomolecule separations. In regard to biotechnology,[356-358] a recent report[359] emphasized the importance of affinity chromatography in the purification of the products of genetic engineering.

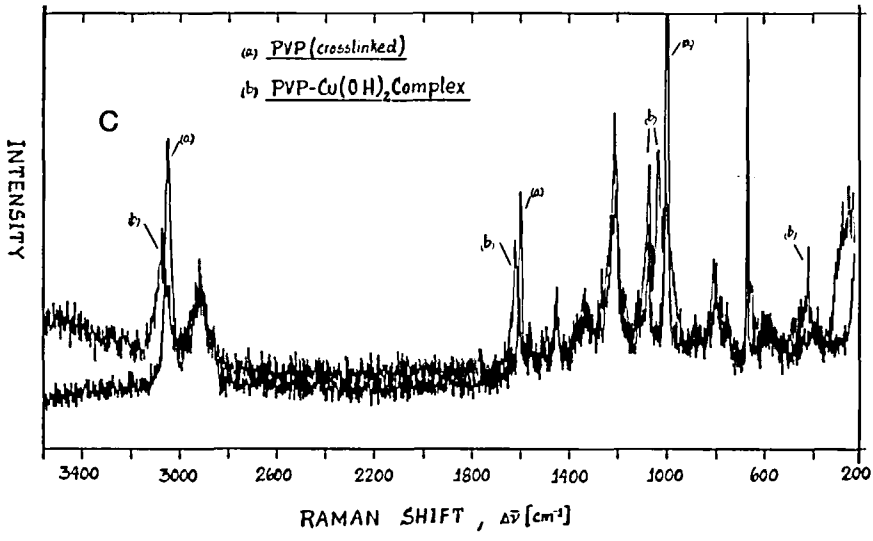
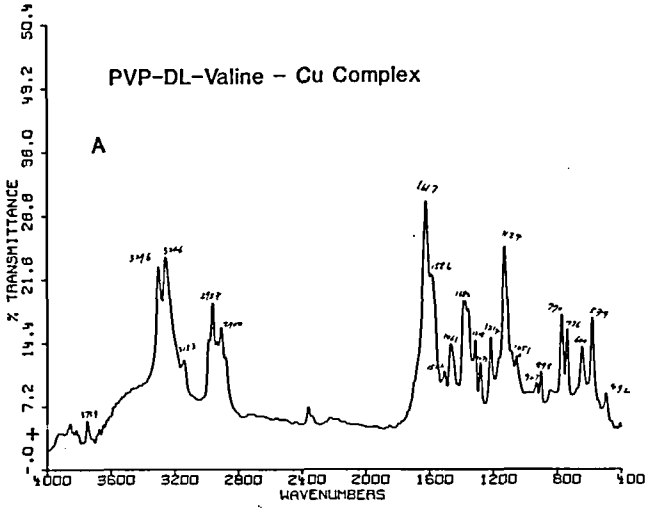


FIGURE 11C.



A

FIGURE 12. Photoacoustic infrared spectra of some biological solids: DL-valine Na-salt (spectrum B); PVP-DL-valine-Cu complex (spectrum A); L-methionine Na-salt (spectrum C), PVP-L-methionine-Cu complex (spectrum D); DNA Na-salt (spectrum E), PVP-DNA-Cu complex (spectrum F).

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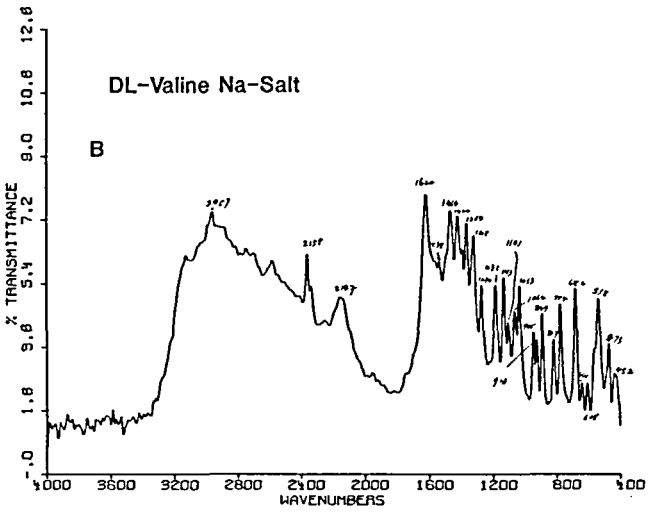

FIGURE 12B.

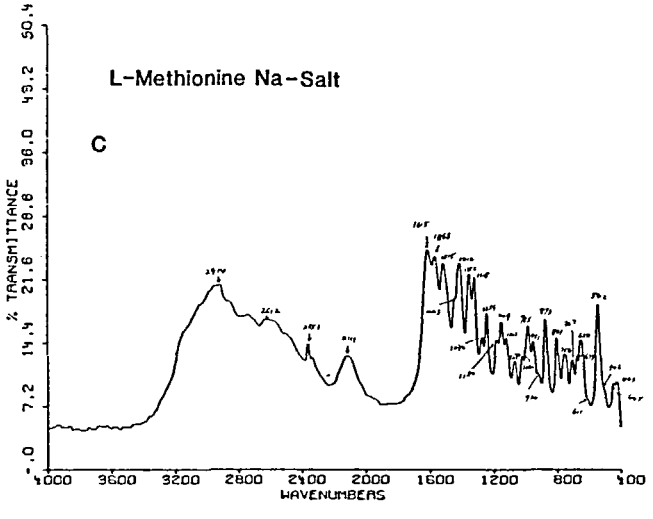

FIGURE 12C.

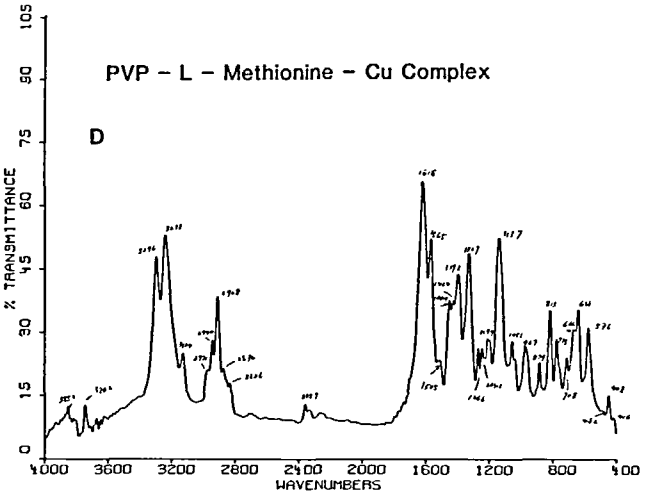

FIGURE 12D.

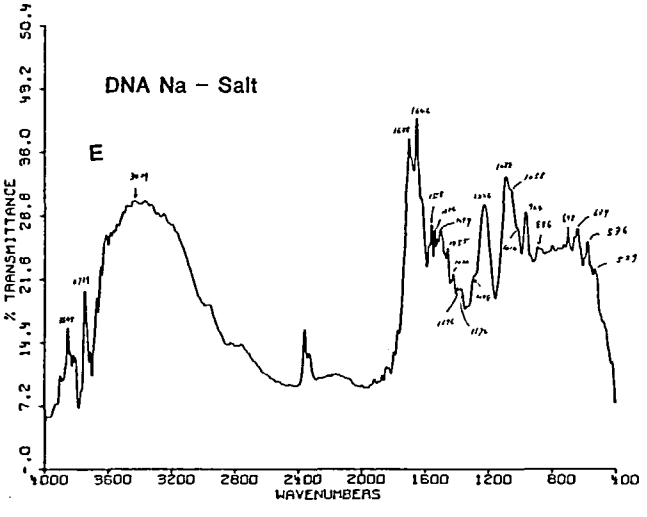

FIGURE 12E.

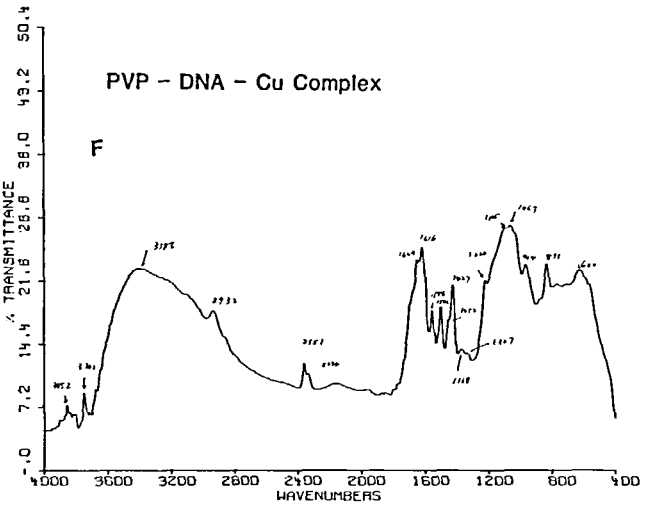

FIGURE 12F.

REFERENCES

1. Gurd, F. R. N. and Wilcox, P. E., *Adv. Protein Chem.*, 11, 311, 1956.
2. Barrett, G., Ed., *Chemistry and Biochemistry of the Amino Acids*, Chapman and Hall, London, England, 1984; Jakubke, H. D. and Jeschkeit, H., *Amino Acids, Peptides, and Proteins*, Halstead Press-Wiley, New York, 1977.
3. Hearon, J. Z., *J. Natl. Cancer Inst.*, 9, 1, 1948.
4. Schubert, M. P., *J. Am. Chem. Soc.*, 53, 3851, 1931.
5. Bradshaw, R. A., Shear, W. T., and Gurd, F. R. N., *J. Biol. Chem.*, 243, 3817, 1968.
6. Taylor, R. P., *Albumin Structure, Function and Uses*, Rosehoer, V. M., Orats, M., and Rothschild, M. A., Eds., Pergamon Press, Oxford, 1977, 183.
7. Vallee, B. J. and Wacker, W. E. C., *The Proteins*, Vol. 5, Neurath, H., Ed., Academic Press, London, 1970, 61.
8. Davydova, S. L. and Plate, N. A., *Coord. Chem. Rev.*, 16, 195, 1975.

9a. Beinart, H., *Coord. Chem. Rev.*, 33, 55, 1980.

9b. Que, L., Jr., *Coord. Chem. Rev.*, 50, 73, 1983.

9c. Pyrz, J. W., Roe, A. L., Stern, L. J., and Que, L., *J. Am. Chem. Soc.*, 107, 614, 1985.

10. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittleman, D., Mouton, R. F., Schmid, K., and Uromou, E., *J. Am. Chem. Soc.*, 72, 465, 1950.

11a. Gurd, F. R. N., *Ion Transport Across Membranes*, Clarke, H. T., Ed., Academic Press, New York, 1954, 246;

11b. Scopes, R. K., *Protein Purification, Principles and Practices*, Springer-Verlag, New York, 1982.

11c. Lascu, I., Abrudan, I., Muresan, L., Presecan, E., Vonica, A., and Proinove, I., *J. Chromatogr.*, 357, 436, 1986.

12. Schmid, K., *J. Am. Chem. Soc.*, 75, 2532, 1953.
13. Hunter, M. J. and Commeford, S. L., *J. Am. Chem. Soc.*, 77, 4857, 1955.
14. Sorenson, J. R., *J. Chem. Br.*, 20, 1110, 1984; Page, M. I., Ed., *New Comprehensive Biochemistry, The Chemistry of Enzyme Action*, Elsevier, Amsterdam, 1984; Abraham, A. K., Ikhom, T. S., and Pryme, I. F., Eds., *Proteins Synthesis: Translational and Posttranslational Events*, Humana Press, Clifton, N.J., 1984.
15. Towsend, S. F., and Sorenson, J. R. J., Trace elements in the pathogenesis and treatment of inflammatory conditions, in *Agents and Actions*, Rainsford, K. D., Brune, K., and Whitehouse, M. W., Eds., Suppl. 8, Birkhauser, Basel, 1984, 389.

16. George, P., *Oxidases and Related Redox Systems,* Vol. 1, King, T. E., Mason, H. S., and Morrison, M., Eds., Wiley-Interscience, New York, 1965, 3.
17. O'Dell, B. L., *Trace Elements in Human Health and Disease, Zinc and Copper,* Vol. 1, Prasad, A. S. and Oberless, D., Eds., Academic Press, New York, 1976, 391.
18. Underwood, E. J., *Trace Elements in Human and Animal Nutrition,* Academic Press, New York, 1977, 57.
19. Sorenson, J. R. J., *Copper in the Environment, Part 2, Health Effects,* Nriagu, J. O., Ed., Wiley-Interscience, New York, 1979, 83.
20. Hamilton, G. A., *Copper Proteins,* Spiro, T. G., Ed., Wiley-Interscience, New York, 1981, 193.
21. Sorenson, J. R., *J. Biol. Trace Element Res.,* 5, 257 (1983); Lontie, R., *Inorganic Biochemistry,* Eichhorn, G. I., Ed., Elsevier, New York, 1973.
22. Sorenson, J. R. J., *Oxygen Radicals in Chemistry and Biology,* Bors, W., Saran, M., Tait, D., Eds., Walter de Gruyter, Berlin, 1984, 821.
23. Solomon, E. I., *Metal Ions in Biology, Copper Proteins,* Vol. 3, Spiro, T. G., Ed., Wiley-Interscience, New York, 1984, 41; Packer, L., Ed., *Oxygen Radicals in Biological Systems. Methods in Enzymology,* Vol. 105, Academic Press, Orlando, Fla., 1984; Spira-Solomon, J., Allendorf, M. D., Solomon, E. I., *J. Am. Chem. Soc.,* 108, 5318, 1986.*
24. Sorrell, T. N. and Borovik, A. S., *J. Chem. Soc. Chem. Commun.,* 1489, 1984.**
25. Karlin, K. D., Cruse, R. W., Gultneh, Y., Hayes, J. C., and Zubieta, J., *J. Am. Chem. Soc.,* 106, 3372, 1984; Parcker, E., Ed., Oxygen radicals in biological systems, in *Methods in Enzymology,* Vol. 105, Academic Press, Orlando, Fla., 1984.
26. Peisach, J., Aisen, P., and Blumberd, W. E., *The Biochemistry of Copper,* Academic Press, New York, 1966.
27. Malkin, R. and Malström, B. G., The state and function of copper in biological systems, in *Advances in Enzymology,* Nort, F., Ed., Wiley-Interscience, New York, 1970, 170.
28. Vanngard, T., *Copper Proteins in Biological Applications of Electron Spin Resonance,* Swartz, H. M., Bolton, J. R., and Bory, D. C., Eds., Wiley-Interscience, New York, 1972, 411.
29. Evan, G. W., *Phys. Rev.,* 54, 535, 1973.
30. Fee, J. A., *Struct. Bonding (Berlin),* 23, 1(1975); Gray, H. B., *Chem. Soc. Rev.,* 15, 17, 1986.
31. Hughes, M. N., *The Inorganic Chemistry of Biological Processes,* Wiley-Interscience, London, 1981, 164.
32. Dorfman, J. R., Bereman, R. D., and Whangbo, M.-H., *Copper Coordination Chemistry: Biochemical and Inorganic Perspectives,* Karlin, K. D. and Zubieta, J., Eds., Adenine Press, Guilderland, 1983, 75.
33. Solomon, E. J., *Copper Coordination Chemistry: Biochemical and Inorganic Perspectives,* Karlin, K. D. and Zubieta, J., Eds., Adenine Press, Guilderland, 1983, 1.
34. Agnus, Y. L., *Copper Coordination Chemistry: Biochemical and Inorganic Perspectives,* Karlin, K. D. and Zubieta, J., Eds., Adenine Press, Guilderland, 1983, 371.
35. Thompson, J. S., Marks, T. J., and Ibers, J. A., *Proc. Natl. Acad. Sci. U.S.A.,* 74, 3114, 1977.
36. Jones, M. H. Levason, W., McAuliffe, C. A., Murray, S. G., and Johns, D. M., *Bioinorg. Chem.,* 8, 267, 1978.
37. McMillan, D. R., *Bioinorg. Chem.,* 8, 179, 1978.
38. Malmström, B. G. and Vänngard, T. J., *J. Mol. Biol.,* 2, 118, 1960.
39. Freeman, H. C., *Coordination Chemistry-21,* Laurent, J. L., Ed., Pergamon Press, Oxford, 1981, 29; Guss, J. M. and Freeman, H. C., *J. Mol. Biol.,* 169, 521, 1983; Sykes, A. G., *Chem. Soc. Rev.,* 14, 283 (1985).
39a. Cookson, D. J., Hayes, M. T., and Wright, P. E., *Nature (London),* 283, 682, 1980; *Biochim. Biophys. Acta,* 591, 162, 1980. Cass, A. E. G. and Hill, H. A. O., *Copper Proteins and Copper Enzymes,* Vol. 1, Lontie, R., Ed., CRC Press, Boca Raton, Fla., 63.
39b. Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Morris, V. A., Ramshaw, J. A. M., and Vekatappa, M. P., *Nature (London),* 272, 319, 1978;*** Maret, W., Shiemke, A. K., Wheeler, W. D., Loehr, T. M. and Sanders-Loehr, J., *J. Am. Chem. Soc.,* 108, 6351, 1986.
40. Adman, E. T., Stenkamp, R. E., Sieker, L. C., and Jensen, L. H., *J. Mol. Biol.,* 123, 35, 1978; Norris, G. E., Andersson, B. F., and Baker, E. N., *J. Am. Chem. Soc.,* 108, 2784, 1986.

* On vibrational circular dichromism of amino acids and peptides (spectroscopic studies), see Oboodi, M. R., Lal, B. B., Young, D. A., Freedman, T. B., and Nafie, L. A., *J. Am. Chem. Soc.,* 107, 1547, 1985.

** On use of the coordination complex $(OP)_2 Cu^+$ in the analysis of DNA structure or study the binding of RNA polymerase see recent overview, Sigman, D. S., *Acc. Chem. Res.,* 19, 180, 1986.

***On structure and electron-transfer reactivity of the blue copper protein plastocyanin, see recent review by Sykes, A. G., *Chem. Soc. Rev. (London),* 14, 283, 1985.

41. Roberts, J. E., Cline, J. F., Lum, V., Freeman, H., Gray, H. B., Peisach, J., Reinhammer, B., and Hoffman, B. M., *J. Am. Chem. Soc.*, 106, 5324, 1984.
41a. Penfield, K. W., Gerwith, A. A., and Solomon, E. I., *J. Am. Chem. Soc.*, 107, 4519, 1985.
42. Conlon, M. and Murphy, R. F., *Biochem. Soc. Trans.*, 4, 860, 1976.
43. Liljas, A. and Rossmann, M. G., *Ann. Rev. Biochem.*, 43, 489, 1974.
44. Brill, A. S. and Venable, J. H., Jr., *J. Am. Chem. Soc.*, 89, 3622, 1967.
45. Lindskog, S., Ibrahim, S. A., Jonsson, B. H., and Simmonson, I., *The Coordination Chemistry of Metalloenzymes*, Bertini, I., Drago, R. S., and Lucinat, C., Eds., Reidel, Boston, Mass., 1983, 49.
46. Silverman, D. N. and Vincent, S. H., *CRC Crit. Rev. Biochem.*, 14, 207, 1983.
47. Pocker, Y. and Sarkanen, S., *Adv. Enzym.*, 47, 149, 1978.
48. Tabushi, I. and Kuroda, Y., *J. Am. Chem. Soc.*, 106, 4580, 1984.
49. Galdes, A. and Vallee, B. L., *Met. Ions Biol. Syst.*, 15, 1, 1983.
50a. Prince, R. H., *Adv. Inorg. Chem. Radiochem.*, 22, 349, 1979.
50b. Hartsuck, J. A. and Lipscomb, W. N., *The Enzymes*, Vol. 3, 3rd, ed., Boyer, P. D., Ed., Academic Press, New York, 1971.
50c. Lipscomb, W. N., *Acc. Chem. Res.*, 3, 81, 1970.
50d. Ludwig, M. A. and Lipscomb, W. N., *Inorganic Chemistry*, Eichhorn, G. L., Ed., American Elsevier, New York, 1973, 438.
50e. Miller, S. K., van Derveer, D. G., and Marzilli, L. G., *J. Am. Chem. Soc.*, 107, 1048, 1985.*
51. Osman, R. and Bash, H., *J. Am. Chem. Soc.*, 106, 5710, 1984.
52. Klotz, I. M., Urguhart, J. M., Klotz, T. A., and Ayers, J., *J. Am. Chem. Soc.*, 77, 1919, 1955.
53. Klotz, I. M., Czerlinski, G. H., and Fiess, H. A., *J. Am. Chem. Soc.*, 80, 2920, 1958.
54. Brill, A. S., Martin, R. B., and Williams, R. J. P., *Electronic Aspects of Biochemistry*, Pullman, B., Ed., Academic Press, New York, 1964, 554.
55. Jorgensen, C. K., *The Biochemistry of Copper*, Peisach, J., Aisen, P., and Blumberg, W. E., Ed., Academic Press, New York, 1966, 14.
56. Katho, S. and Takiyame, A., *J. Biochem. (Tokyo)*, 55, 378, 1964.
57. McMillin, D. R., Rosenberg, R. C., and Gray, H. B., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 4760, 1974.
58. Colman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., and Venkatappa, M. D., *Nature (London)*, 272, 319, 1978.
59. Ulrich, E. L. and Markley, J. L., *Coord. Chem. Rev.*, 27, 109, 1978.
60. Adman, E. T. and Jensen, L. H., *Isr. J. Chem.*, 21, 8, 1981.
61. Norris, G. E., Anderson, B. F., and Baker, J., *J. Mol. Biol.*, 165, 501, 1983.
62. Franks, F., Ed., *Water, a Comprehensive Treatise*, Vol. 4, Plenum Press, New York, 1975, 1; Ben-Naim, A., *Hydrophobic Interactions*, Plenum Press, New York, 1980; Käiväräinen, A. I., *Solvent-Dependent Flexibility of Proteins and Principles of their Function. Advances in Inclusion Science Series*, D. Reidel, Dordrecht, The Netherlands, 1985.
63. Cass, A. E. G. and Hill, H. A., *CIBA Found. Symp.*, 79, 71 (1980); Griffin, J. F. and Duax, W. L., *Molecular Structure and Biological Activity*, Elsevier, New York, 1982.
64. Birker, P. J. M. L., Godefroi, E. F., Helder, J., and Reedijk, J., *J. Am. Chem. Soc.*, 104, 7556, 1982.
65. Addison, A. W., Rao, T. N., and Sinn, E., *Inorg. Chem.*, 23, 1957, 1984.
66. Nakon, R. and Krishnamoorty, C. R., *J. Am. Chem. Soc.*, 106, 5193, 1984.
66a. Scott, R. A. and Dooley, D. M., *J. Am. Chem. Soc.*, 107, 4348, 1985.
66b. Sorrell, T. N., O'Connor, C. J., Anderson, O. P., and Reibenspies, J. H., *J. Am. Chem. Soc.*, 107, 4199, 1985.
67. Brown, R. S., Zacmkanei, M., and Cocho, J. L., *J. Am. Chem. Soc.*, 106, 5222(1984); Bernarducci, E., Bharadwaj, P. K., Krogh-Jespersen, K., Potenza, J. A., and Schugar, H. J., *J. Am. Chem. Soc.*, 105, 3860, 1983.
68. El-Toukhy, A., Cai, G-Z., Davies, G., Gilbert, T. R., Onan, K. D., and Veidis, M., *J. Am. Chem. Soc.*, 106, 4596, 1984.
69. Williams, T. C., Corson, D. C., and Sykes, B. D., *J. Am. Chem. Soc.*, 106, 5698, 1984.
70a. Ibers, J. A. and Holm, B. H., *Science*, 209, 223, 1980.
70b. Saxton, R. J. and Wilson, L. J., *J. Chem. Soc. Chem. Commun.*, 359, 1984.
71. McCord, J. M. and Fridovich, J., *J. Biol. Chem.*, 244, 6049, 1969.
72. Steinmann, H. M., *Superoxide Dismutase*, Vol. 1, Oberley, L. W., Ed., CRC Press, Boca Raton, Fla., 1982, 11.

* For a ternary enzyme Zn^{2+}-Chelator complex (from bovine liver dihydropyrimidine amidohydrolase), see recent paper by Lee, M. H., Cowling, R. A., Sander, E. G., and Pettigrew, D. W., *Arch. Biochem. Biophys.*, 248, 368, 1986.

73. Richardson, J. S., Thomas, K. A., Rubin, B. H., and Richardson, *Proc. Natl. Acad. Sci.*, U.S.A., 72, 1349, 1975.
74. Tainer, J. A., Getzoff, E. D., Richardson, J. S., and Richardson, D. C., *Nature (London)*, 306, 284, 1983.
74a. Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. J., and Richardson, D. C., *J. Mol. Biol.*, 160, 181, 1982; Valentine, J. S. and Pantolino, M. W., *Copper Proteins*, Vol. 3, Spiro, T. G., Ed., John Wiley & Sons, New York, 1981, chap. 8.
74b. Bertini, I., Lanini, G., Lichiant, C., Messori, L., Monnanni, R., and Scozzafava, G., *J. Am. Chem. Soc.*, 107, 4391, 1985.
75. Fielden, E. M., Roberts, P. B., Bray, R. C., Lowe, D. J., Mauntner, G. N., Rotilo, G., and Calabrese, L., *Biochem. J.*, 139, 49, 1974.
76. Lippard, S. J., Burger, A. R., Ugurbil, K., Pantoliano, M. W., and Valentine, J. S., *Biochemistry*, 16, 1136, 1977.
77. Martin, R. B., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 4346, 1974.
78. Kolks, G., Lippard, S. J., Waszczak, J. V., and Lilienthal, H. R., *J. Am. Chem. Soc.*, 104, 717, 1982.
79. Yokoi, H. and Chikizia, M., *J. Chem. Soc. Chem. Commun.*, 1125, 1982.
80. Kolks, G., Frihart, C. R., Coughlin, P. K., and Lippard, S. J., *Inorg. Chem.*, 20, 2933, 1981.
81. Sato, M., Kodama, K., Uehara, M., and Nakaya, J., *J. Chem. Soc. Chem. Commun.*, 51, 1984.
82. Coughlin, P. K. and Lippard, S. J., *Inorg. Chem.*, 23, 1446, 1984.
83. Sato, M., Nagae, S., Uehara, M., Nakaya, J., *J. Chem. Soc. Chem. Commun.*, 1661, 1984.
84. Martin, A. E. and Lippard, S. J., *J. Am. Chem. Soc.*, 106, 2579, 1984.
85. Strothkamp, K. G. and Lippard, S. J., *Acc. Chem. Res.*, 10, 318, 1982.
86a. Marcus, R. A., *Annu. Rev. Phys. Chem.*, 15, 155, 1964.
86b. Cannon, R. D., *Electron Transfer Reactions*, Butterworths, London, 1980.
86c. Sutin, N., *Prog. Inorg. Chem.*, 30, 441, 1983.
86d. Baum, R. M., *Chem. Eng. News*, 62, 24, 1984.
86e. Raymond, K. N., *Adv. Chem.*, 162, Chaps. 1—7, 1977.
86f. Lever, A. P. B. and Gray, H. B., Eds., *Iron Porphyrins*, Addison-Wesley, Reading, Mass., 1983.
86g. Dolphin, D., Ed., *The Porphyrins*, Academic Press, New York, 1979.
86h. Coughlin, M., Ed., *Molybdenum and Molybdenum-Containing Enzymes*, Pergamon Press, New York, 1980.
86i. Antonini, E. and Brunori, M., *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam, 1979.
86j. Berg, J. M. and Holm, R. H., *J. Am. Chem. Soc.*, 107, 917, 1985.
86k. Creighton, A. M. and Turner, S., Eds., *The Chemical Regulation of Biological Mechanisms*, The Royal Society of Chemistry, London, 1981.
86l. Walsh, C., *Enzymatic Reaction Mechanism*, Freeman, San Francisco, 1979; Guengerich, F. P. and Macdonald, T. L., *Acc. Chem. Res.*, 17, 9, 1984; White, R. E. and Coon, M., *Annu. Rev. Biochem.*, 49, 3154, 1980; Ullrich, V., *Top. Curr. Chem.*, 83, 67, 1979; Burke, T. L., Guengerich, F. P., Willard, R. J., and Macdonald, T. L., *J. Am. Chem. Soc.*, 107, 2549, 1985.
87. Karlin, K. D., Gultneh, Y., Hutchinson, J. P., and Zubieta, J., *J. Am. Chem. Soc.*, 104, 5240, 1982.
88. Karlin, K. D., Dahlstrom, P. L., Cozzette, S. N., Scensny, P. M., and Zubieta, J., *J. Chem. Soc. Chem. Commun.*, 881, 1981.
89. Karlin, K. D., Heyes, J. C., Hutchinson, J. P., and Zubieta, J., *J. Chem. Soc. Chem. Commun.*, 376, 1983; Karlin, K. D., Heyes, J. C., Gultneh, Y., Cruse, R. W., McKown, J. W., Hutchinson, J. P., Zubieta, J., *J. Am. Chem. Soc.*, 106, 2121, 1984.
90. Mears, C. F. and Wensel, T. G., *Acc. Chem. Res.*, 17, 202, 1984.
91. Siegel, H., Ed., *Metal Ions in Biological Systems*, Vol. 7, Marcel Dekker, New York, 1977; Rifkind, J. M., *Met. Ions Biol. Syst.*, 15, 275, 1983.
92. Siegel, H., Ed., *Metal Ions in Biological Systems, Zinc and its Role in Biology and Nutrition*, Vol. 15, Marcel Dekker, New York, 1983.
93. Lontine, R., Ed., *Copper Proteins and Copper Enzymes*, Vols. 1, 2, and 3, CRC Press, Boca Raton, Fla., 1984.
94. Blundell, T. L. and Jenkins, J., *Chem. Soc. Rev.*, 6, 139, 1977.
95. Frieden, E., *Trends Biochem. Sci.*, 1, 273, 1976.
96. Yasunobu, K. T., Mower, H. F., and Hayashi, O., Eds., *Iron and Copper Proteins*, Plenum Press, New York, 1976.
97. Brown, J. M., Powers, J., Kincaid, B., Larrabee, J. A., and Spizo, T. H., *J. Am. Chem. Soc.*, 102, 4210, 1980.
98. Co, M. S., Hodgson, K. O., Eccles, T. K., and Lontie, R., *J. Am. Chem. Soc.*, 103, 984, 1984.
99. Co. M. S. and Hadgson, K. K., *J. Am. Chem. Soc.*, 103, 3201, 1981.

100. Sorrell, T. N. and Borovik, A. S., *J. Chem. Soc. Chem. Commun.*, 1489, 1984.
101. Hambridge, K. M. and Nichols, B. L., Jr., *Zinc and Copper in Clinical Medicine*, S. P., Medical and Scientific Books, London, 1977.
102. Woolery, G. L., Powers, L., Winkler, M., Solomon, E. I., and Spiro, T. G., *J. Am. Chem. Soc.*, 106, 86, 1984.
103. Thompson, J. S., *J. Am. Chem. Soc.*, 106, 4057, 1984; J. Am. Chem. Soc., 106, 8308, 1984.
104. Rackovsky, S. and Scheraga, H. A., *Acc. Chem. Res.*, 17, 209, 1984.
105. Lundblad, R. L. and Noyes, C., Eds., *Chemical Reagents for Protein Modification*, Vols. 1 and 2, CRC Press, Boca Raton, Fla., 1984.
106. Blaney, J. M., Hansch, C., Silipo, C., and Vittoria, A., *Chem. Rev.*, 84, 333, 1984; Saenger, W., *Principles of Nucleic Acid Structure*, Springer-Verlag, Heidelberg, Germany, 1984.
107. Hearn, M. T. W., Ed., *Peptide and Protein Reviews*, Vol. 1, Marcel Dekker, New York, 1983, 1; Weinstein, B., Ed., *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*, Vol. 7, Marcel Dekker, New York, 1983.
108. Hearn, M. T. W., Ed., *Peptide and Protein Reviews*, Vol. 2, Marcel Dekker, New York, 1984, 1; Hearn, M. T. W., Ed., *Peptide and Protein Reviews*, Vol. 3, Marcel Dekker, New York, 1984; Hruby, V. J. and Rich, D. H., Eds., *Peptides: Structure and Function*, Pierce Chemical Co., Rockford, Ill., 1983.
109. Helfferich, F., *Nature (London)*, 189, 1001, 1961.
110. Helfferich, F., *Adv. Chromatogr.*, 1, 39, 1965.
111. Davankov, V. A. and Semechkin, A. V., *J. Chromatogr. Chromatogr. Rev.*, 14, 313, 1977.
112. Davankov, V. A., Kurganov, A. S., and Buchkov, A. S., *Adv. Chromatogr.*, 22, 71, 1983.
113. Helfferich, F., *Ion Exchange*, McGraw-Hill, New York, 1962.
114. Porath, J. and Olin, B., *Biochemistry*, 22, 1621, 1983.
115. Shimomura, K., Dickson, L., and Walton, H. F., *Anal. Chim. Acta*, 37, 102, 1967.
116. Latterell, J. J. and Walton, H. F., *Anal. Chim. Acta*, 32, 101, 1965.
117. Cheminat, A., Benezra, C., and Frechet, J. M. J., *J. Chem. Res. (S)*, 148, 1982; *J. Chem. Res. (M)*, 1618, 1982.
118. Hemmasi, B., *J. Chromatogr.*, 104, 367, 1975.
119. Bellinger, J. F. and Buist, N. R. M., *J. Chromatogr.*, 87, 513, 1973.
120. Murgia, E. and Walton, H. F., *J. Chromatogr.*, 104, 417, 1975.
121. Goldstein, G., *Anal. Biochem.*, 20, 477, 1967.
122. Hare, P. E. and Gile-Av, E., *Science*, 204, 1226, 1979.
123. Davankov, V. A., Bochkov, A. S., Kurganov, A. A., Roumeliotis, P., and Unger, K. K., *Chromatographia*, 13, 677, 1980.
124. Gubitz, G., Jellenz, W., and Santi, W., *J. Chromatogr.*, 203, 377, 1981.
125. Klemisch, W., von Hodenberg, A., and Volimer, K.-O., *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4, 535, 1981.
126. Gelber, L. R., Karger, B. L., Neumeyer, J. L., and Feibush, B., *J. Am. Chem. Soc.*, 106, 7729, 1984.
127. Goździcka-Jozefiak, A. and Augustyniak, J., *J. Chromatogr.*, 131, 91, 1977.
128. Merrifield, R. B., *J. Am. Chem. Soc.*, 85, 2149, 1963.
129. Merrifield, R. B., *Science*, 150, 178, 1965.
130. Barany, G. and Merrifield, R. B., *The Peptides*, Vol. 2, Academic Press, New York, 1979, 1.
131a. Spatola, A. F., *Am. Biotechnol. Lab.*, 14, 1984; Birr, C., *Aspects of the Merrifield Peptide Synthesis*, Springer-Verlag, New York, 1978; Bodanszky, M., *Principles of Peptide Synthesis*, Springer-Verlag, New York, 1984; Bodanszky, M. and Bodanszky, A., *The Practice of Peptide Synthesis*, Springer-Verlag, New York, 1984.
131b. Frey, M. H., Opella, S. J., Rockwell, A. L., and Gierasch, L. M., *J. Am. Chem. Soc.*, 107, 1946, 1985.
131c. Boussard, G. and Marrand, M., *J. Am. Chem. Soc.*, 107, 1825, 1985.
132. Sarin, V. K., Kent, S. B. H., Mitchel, A. R., and Merrifield, R. B., *J. Am. Chem. Soc.*, 106, 7845, 1984.
133. Majors, R. E., *Chromatogr. Sci.*, 15, 334, 1977.
134. Majors, R. E., *Chromatogr. Sci.*, 18, 488, 1980.
135. Regen, S. L., *Angew. Chem. Int. Ed. Engl.*, 18, 421, 1979.
136. Hodge, P. and Sherrington, D. C., Eds., *Polymer-Supported Reactions in Organic Synthesis*, John Wiley & Sons, London, 1980.
137. Mathur, N. K., Narang, C. K., and Williams, R. E., Eds., *Polymer as Aids in Organic Chemistry*, Academic Press, New York, 1980; Waller, F. J., In *Polymer Reagents and Catalysts*, Ford, T. E., Ed., American Chemical Society, Washington, D.C., 1986, chap. 3; Laszlo P., *Preparative Chemistry Using Supported Reagents*, Academic Press, Orlando, Fla. 1987.

138. Warshawsky, A., *Angew. Makromol. Chem.*, 109/110, 171, 1982.
139. Warshawsky, A., *Isr. J. Chem.*, 18, 318, 1979.
140. Akelah, A. and Sherrington, D. C., *Chem. Rev.*, 81, 557, 1981.
141. Akelah, A., *Synthesis*, 413, 1981.
142. Fréchet, J. M., *J. Tetrahedron*, 37, 663, 1981.
143. Kraus, M. A. and Patchornik, A., *Macromol. Rev.*, 15, 55, 1980.
144. Manecke, H., and Reuter, P., *Pure Appl. Chem.*, 51, 2313, 1979.
145. Daly, W. H., *Makromol. Chem. Suppl.*, 2, 3, 1979.
146. Rebek, J., Jr., *Tetrahedron*, 35, 723, 1979.
147. Leznoff, C. C., *Acc. Chem. Res.*, 11, 327, 1978.
148. Heitz, W., *Adv. Polym. Sci.*, 23, 1, 1977.
149. Fréchet, M. J. and Farral, M. J., *Chemistry and Properties of Crosslinked Polymers*, Labana, S. S., Eds., Academic Press, New York, 1977, 59.
150. Tsuichida, E. and Nishide, H., *Adv. Polym. Sci.*, 24, 1, 1977.
151. McKillop, A. and Young, D. W., *Synthesis*, 401, 1979.
152. Sigel, H. and Martin, R. B., *Chem. Rev.*, 82, 385, 1982.
153. Pittner, F., Miron, T., Pittner, G., and Wilchek, K. M., *J. Am. Chem. Soc.*, 102, 245, 1980.
154. Jackoby, W. B. and Wilcheck, K. M., *Methods in Enzymology*, Vol. 34, Academic Press, New York, 1974; Jacoby, W. B., Ed., *Enzyme Purification and Related Techniques, Part C, Methods in Enzymology*, Vol. 4, Academic Press, Orlando, Fla., 1984; Suelter, C. N., *Practical Guide to Enzymology*, John Wiley & Sons, New York, 1985.
155. Crowley, J. I. and Rappaport, H., *Acc. Chem. Res.*, 9, 135, 1976.
156. Mosbach, K., Ed., *Methods in Enzymology*, Vol. 44, Academic Press, New York, 1976.
157. Cuatrecasas, P., Wilchek, K. M., and Anfinsen, C. B., *Proc. Natl. Acad. Sci. U.S.A.*, 61, 636, 1968.
158. Cuatrecasas, P., *Proc. Natl. Acad. Sci. U.S.A.*, 63, 450 (1969); *J. Biol. Chem.*, 245, 3059, 1970.
159. Cuatrecasas, P. and Anfinsen, C. B., *Annu. Rev. Biochem.*, 40, 259, 1971.
160. Porath, J. and Kristiansen, T., *The Proteins*, Vol. 1, Neurath, H. and Hill, R. L., Eds., Academic Press, New York, 1975, 95.
161. Lowe, C. R. and Dean, P. D. G., *Affinity Chromatography*, John Wiley & Sons, London, 1974.
162. Gribnau, T. C. J., Visser, J., and Nivard, R. J. F., Eds., *Affinity Chromatography and Related Techniques*, Elsevier, Amsterdam, 1982.
163. Lowe, C. R., *An Introduction to Affinity Chromatography: Laboratory Techniques in Biochemistry and Molecular Biology*, Work, T. S. and Work, E., Eds., North-Holland, Amsterdam, 1979; Lowe, C. R., *Int. J. Biochem.*, 8, 177, 1976; Lowe, C. R., *J. Biotechnol.*, 1, 3, 1984.
163a. Parikh, I. and Cuatrecasas, P., *Chem. Eng. News*, 63, 17, 1985.
163b. Walters, R. R., *Anal. Chem.*, 57, 1099A, 1985.
164a. *Affinity Chromatography, Principles and Methods and Chelating Sepharose 6B for Metal Chelate Affinity Chromatography*, Pharmacia Fine Chemicals, Piscataway, N.J., 1981.
164b. Chaiken, I. M., Wilchek, M., and Parikh, I., Eds., *Affinity Chromatography and Biological Recognition*, Academic Press, Orlando, Fla., 1983.
165. Scouten, W. H., *Affinity Chromatography*, John Wiley & Sons, New York, 1981.
165a. Dean, P. D. G., Johnson, W. S., and Midde, F. A., Eds., *Affinity Chromatography: A Practical Approach*, IRL Press, Oxford, 1985.
166. March, S. C., Parikh, I., and Cuatrecasas, P., *Anal. Biochem.*, 60, 149, 1974.
167. Wilchek, M., *Immobilized Biochemicals and Affinity Chromatography*, Dunlap, R. B., Ed., Plenum Press, New York, 1974, 15.
168a. Hoffman-Ostenhof, O., *Affinity Chromatography*, Pergamon Press, New York, 1978.
168b. Turkova, J., *Affinity Chromatography*, Elsevier, Amsterdam, 1978.
168c. Scouten, W. H., Ed., *Solid Phase Biochemistry*, John Wiley & Sons, New York, 1983.
168d. Schoff, H., *Affinity Chromatography*, Marcel Dekker, New York, 1984.
168e. Wilchek, M., Miron, T., and Kohn, J., *Methods Enzymol.*, 104, 3, 1984.
168f. Larsson, P.-O., *Methods Enzymol.*, 104, 212, 1984.
168g. *Fast Affinity Chromatography*, Beckman Instruments, Berkeley, Calif., 1984.
168h. *Chromatography, Electrophoresis, Immunochemistry, Molecular Biology, HLPC*, Bio-Rad Laboratories, Richmond, Calif., 1986, 49.
168i. Pazur, J. H., *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 39, 1981, 405.
168j. Mohr, P. and Pommerning, K., *Affinity Chromatography; Practical and Theoretical Aspects*, Marcel Dekker, New York, 1985.
169. Messing, R. A., *J. Non Cryst. Solids*, 19, 277, 1975.
170. Inman, J. K. and Dintzis, H. M., *Biochemistry*, 8, 4074, 1969.

170a. Muller, A. J. and Carr, P. W., *J. Chromatogr.*, 357, 11, 1986.*
171. Axen, R., Porath, J., and Ernback, S., *Nature (London)*, 214, 1302, 1967; Porath, J. and Ernback, S., *Nature (London)*, 215, 1491, 1967.
172. David, S. G., Harvey, M. J., and Dean, P. D. G., *FEBS Lett.*, 43, 264, 1974.
172a. Lowe, C. R., *Laboratory Techniques in Biochemistry and Molecular Biology*, Work, T. S. and Work, E., Eds., Elsevier, Amsterdam, 1979, 355.
172b. Shimura, K. and Kasai, K-I., *J. Chromatogr.*, 315, 161, 1984; Brandts, P. M., Middelkoop, C. M., Gelsema, W. J., et al., *J. Chromatogr.*, 356, 247, 1986.
173. Axen, R., Myrin, P. A., and Janson, J. C., *Biopolymers*, 9, 401, 1970.
174. Jost, R., Myrin, P. A., and Wilchek, K. M., *Biochem. Biophys. Acta*, 362, 75, 1974.
175. Bethell, G. S., Ayres, J. S., Hancock, W. S., and Hearn, M. T. W., *J. Biol. Chem.*, 254, 1683, 1979.
176. Hearn, M. T. W., Betchell, G. S., Ayres, J. S., and Hancock, W. S., *J. Chromatogr.*, 185, 463, 1979.
177. Lowe, C. R., Harvey, M. J., Craven, D. B., and Dean, P. D. G., *Biochem. J.*, 133, 499, 1973.
178. Hipwell, M. C., Harvey, M. J., and Dean, P. D. G., *FEBS Lett.*, 42, 355, 1974.
179. Wilchek, K. M., *FEBS Lett.*, 33, 70, 1972.
180. Mosbach, K., Guildford, H., Ohlsson, R., and Scott, M., *Biochem. J.*, 127, 625, 1972.
181. Rosengren, J., Pahlman, S., Glad, M., and Hjerten, S., *Biochim. Biophys. Acta*, 412, 51, 1975.
182. O'Carra, P. B. S. and Griffin, T., *Biochem. Soc. Trans.*, 1, 289, 1973.
183. O'Carra, P. B. S. and Griffin, T., *FEBS Lett.*, 43, 169, 1974.
184. Tesser, G. I., Fisch, H. U., and Schnyzer, R., *Helv. Chim. Acta*, 57, 1718, 1978.
185. Hofstee, B. H. J. and Otillo, N. F., *J. Chromatogr.*, 161, 153, 1978.
186. Porath, J. and Sundberg, L., *The Chemistry of Biosurfaces*, Vol. 2, Hair, M., Ed., Marcel Dekker, New York, 1972.
187. Kristiansen, T., *Methods in Enzymology*, Vol. 34, Jakoby, W. B. and Wilchek, K. M., Eds., Academic Press, New York, 1974, 331.
188. Lowe, C. R. and Dean, P. D. G., *FEBS Lett.*, 14, 313, 1971.
189. Sica, V., Nola, E., Parikh, I., Puca, G. A., and Cuatrecasas, P., *Nature (London) New Biol.*, 244, 36, 1973.
190. Steers, E. J. W., Cuatrecasas, P., and Pollard, H. B., *J. Biol. Chem.*, 246, 196, 1971.
191. Thompson, S. T., Cass, K. H., and Stellwagen, E., *Proc. Natl. Acad. Sci. U.S.A.*, 72, 669, 1975.
192. Turkova, I. P., Hubalkova, O., Krivakova, M., and Coupek, J., *Biochim. Biophys. Acta*, 322, 1, 1973.
193. Yon, R. J., *Biochem. J.*, 137, 127, 1974.
194. Allen, R. H. and Majerus, P. W., *J. Biol. Chem.*, 247, 7709, 1972.
195. Wood, T. and Fletcher, S., *Biochim. Biophys. Acta*, 527, 249, 1978.
196. Deibel, M. and Coleman, M., *J. Biol. Chem.*, 254, 8634, 1979.
197. Lee, C.-Y. and Kaplan, N., *J. Macromol. Sci. Chem.*, A10, 15, 1976.
198. Turkova, J., *J. Chromatogr.*, 91, 267, 1967.
199. Hofstee, B. H. J., *Anal. Biochem.*, 52, 430, 1973.
200. Häckel, R., Hess, B., Lauterborn, W., and Wüster, K.-H., *Hoppe-Seyler's Z. Physiol. Chem.*, 349, 699, 1968.
201. Koppersläger, G., Freyer, R., Diezel, W., and Hofmann, E., *FEBS Lett.*, 1, 137, 1968; *Eur. J. Biochem.*, 22, 40, 1971.
202. Dean, P. D. G. and Watson, D. H., *J. Chromatogr.*, 165, 301, 1979.
203. Fulton, S. P., *Dye-Ligand Chromatography*, Amicon Corporation, Lexington, Mass., 1980.
204. Subramanian, S., *CRC Crit. Rev. Biochem.*, 16, 169, 1984.
205. Lowe, C. R., Small, D. A. P., and Atkinson, A., *Int. J. Biochem.*, 13, 33, 1981.
206. Lowe, C. R., Hans, M., Spibey, N., and Drabble, W. T., *Anal. Biochem.*, 104, 23, 1980.
207. Fulton, S. P. and Carlson, E. R., *Am. Lab.*, 12, 55, 1980.
208. Bruton, C. J. and Atkinson, A., *Nucleic Acid Res.*, 7, 1579, 1979.
209. Watson, D. H., Harvey, M. J., and Dean, P. D. G., *Biochem. J.*, 173, 591, 1978.
210. Burgett, M. and Greenley, L., *Am. Lab.*, 9, 74, 1977.
211. Baird, J. K., Sherwood, R. F., Carr, R. J. G., and Atkinson, A., *FEBS Lett.*, 70, 61, 1976.
212. Mandel, P., Okazaki, H., and Niedergang, C., *FEBS Lett.*, 84, 331, 1977.
213. Easterday, R. L. and Easterday, I. M., *Adv. Exp. Med. Biol.*, 42, 123, 1974.
214. Edwards, R. A. and Woody, R. W., *Biochemistry*, 18, 5197, 1979.
215. Angal, S. and Dean, P. D. G., *Biochem. J.*, 167, 301, 1977.

* On quantitative affinity chromatography: increased versatility of the technique for studies of ligand binding, see Bergman, D. A. and Winzor, D. J., *Anal. Biochem.*, 153, 380, 1986.

216. Böhme, H.-J., Kopperschlager, G., Schulz, J., and Hofmann, E., *J. Chromatogr.*, 69, 209, 1972.
217. Stellwagen, E., *Acc. Chem. Res.*, 10, 92, 1977.
218. Thompson, S. and Stellwagen, E., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 361, 1976.*
219. Jankowski, W., von Muenchhausen, W., Sulkowski, E., and Carter, W., *Biochemistry*, 15, 5182, 1976.
220. DeMayer-Guignard, J., Tang, M., and Demayer, E., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3787, 1977.
221. Stockton, J., Pearson, A., West, L., and Turner, A. J., *Biochem. Soc. Trans.*, 6, 200, 1978.
222. Turner, A. J. and Hryszko, J., *Biochim. Biophys. Acta*, 613, 256, 1980.
223. Bouriotis, V. and Dean, P. D. G., *J. Chromatogr.*, 206, 521, 1981.
224. Clonis, Y. D., Goldfinch, M. J., and Lowe, C. R., *Biochem. J.*, 197, 203, 1981.
225. Glazor, A. N., *Proc. Natl. Acad. Sci. U.S.A.*, 65, 1057, 1970.
226. Clonis, Y. D. and Lowe, C. R., *Biochem. J.*, 191, 247, 1980.
227. Clonis, Y. D. and Lowe, C. R., *Biochim. Biophys. Acta*, 659, 86, 1981.
228. Hughes, P., Lowe, C. R., and Sherwood, R. F., *Biochim. Biophys. Acta*, 700, 90, 1982.
229. Apps, D. K. and Gleed, C. D., *Biochem. J.*, 159, 441, 1976.
229a. Jeans, E. R. A., Marshall, P. J., and Lowe, C. R., *Trends Biotechnol.*, 3, 267, 1985.
230. Takeo, K. and Nakamura, S., *Arch. Biochem. Biophys.*, 153, 1, 1972.
231. Ticha, M., Horejsi, V., and Barthova, J., *Biochim. Biophys. Acta*, 534, 58, 1978.
232. Horejsi, V., *Colloq. L'Inserm Affinity Chromatogr.*, 534, 58, 1979.
233. Horejsi, V., *J. Chromatogr.*, 178, 1, 1979; Dely, Z., Ed., *Electrophoresis, A Survey of Techniques and Applications*, Elsevier, Amsterdam, 1983.
234. Dahlbery, H. E., Dingman, C. W., and Peacock, A. C., *J. Mol. Biol.*, 41, 139, 1969.
235. Johnson, S. J., Metcalf, E. C., and Dean, P. D. G., *Anal. Biochem.*, 109, 63, 1980.
236. Bog-Hansen, T. C., *Anal. Biochem.*, 56, 480, 1973.
237. Murphy, R. F., Buchanan, K. D., and Elmore, D. T., *Biochim. Biophys. Acta*, 303, 118, 1973; Laurell, C. B. and McKay, E. J., *Methods in Enzymology*, Vol. 73, Langone, J. J. and van Vunakis, H., Eds., Academic Press, New York, 1980, 339.
238. Hearn, M. T. W., Bethhell, G. S., Ayers, J. S., and Hancock, W. S., *J. Chromatogr.*, 185, 463, 1979.
239. Anfinsen, C. B., Rose, S., Corley, L., and Gurari-Rotman, D., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 3139, 1974.
240. Berg, K., Heron, I., and Hamilton, R., *Scand. J. Immun.*, 8, 429, 1978.
241. Kieda, C., Delmonte, F., and Monsigny, M., *FEBS Lett.*, 76, 257, 1977.
242. Chen, J. K., Jankowski, W. J., O'Malley, J. A., Sulkowski, E., and Carter, W. A., *J. Virol.*, 19, 425, 1976.
243. Berg, K., Osther, K., and Heron, I., *Interferon and Interferon Inducers, Clinical Applications*, Stringfellow, D. A., Ed., Marcel Dekker, New York, 1981, chap. 1.
244. Hattori, T. and Furusaka, C., *J. Biochem.*, 50, 312, 1961.
245. Zvyaginstesev, D. G., *Mikrobiologiya*, 31, 339, 1971.
246. Kennedy, J. F., *Nature (London)*, 261, 242, 1972.
247. Kennedy, J. F., Barker, S. A., and Zamir, A., *Antimicrob. Agents Chemother.*, 6, 777, 1974.
248. Hanisch, W. H., Pamela, A. D., and Rickard, S., *Biotechnol. Bioeng.*, 20, 95, 1978..
249. Vijayalakshmi, M., Marcipar, A., Segard, E., and Broun, G. B., *Ann. N.Y. Acad. Sci.*, 326, 249, 1979.
250. Fatiadi, A. J., *J. Am. Chem. Soc.*, 100, 2586, 1978.
251. Fatiadi, A. J., *J. Org. Chem.*, 45, 1338, 1980.
252. Fatiadi, A. J., *Oxocarbons*, West, R., Ed., Academic Press, New York, 1980, 59.
253. Fatiadi, A. J., unpublished.
254. Porath, J., *J. Chromatogr.*, 159, 13, 1978.
255. Porath, J., *Pure Appl. Chem.*, 51, 1549, 1979.
256. Fatiadi, A. J. and Alexander, L., unpublished results.
257. Porath, J., Carlsson, J., Olsson, I., and Belfrage, G., *Nature (London)*, 258, 598, 1975.
258. Porath, J., Asperg, K., Drevin, H., and Axen, R., *J. Chromatogr.*, 86, 53, 1973.
259. Sundberg, L. and Porath, J., *J. Chromatogr.*, 90, 87, 1974.
259a. Maier, M., Austen, K. F., and Spragg, J., *Anal. Biochem.*, 134, 336, 1983; Shriner, C. L. and Brautigan, D. L., *J. Biol. Chem.*, 259, 1383, 1984.
260. Porath, J. and Belew, M., *Affinity Chromatography and Biological Recognition*, Chaiken, I. M., Wilchek, M., and Parikh, I., Eds., Academic Press, New York, 1983, 173.

* On reactive triazine dyes, their usefulness and limitations in protein purification, see recent overview, Quadri, F., *Trends Biotechnol.*, 3, 7, 1985.

260a. Hemdan, E. S. and Porath, J., *J. Chromatogr.*, 323, 255, 1985.
260b. Hemdan, E. S. and Porath, J., *J. Chromatogr.*, 323, 265, 1985.
260c. Hemdan, E. S. and Porath, J., *J. Chromatogr.*, 323, 247, 1985.
260d. Rassi, Z. E. and Horvath, C., *J. Chromatogr.*, 359, 241, 1986.
260e. Kato, Y., Nakamura, K. and Hashimoto, T., *J. Chromatogr.*, 354, 511, 1986.
260f. Sulkowski, E., *Treands Biotechnol.*, 3, 1, 1985.
261. Lönnerdal, B., Carlsson, J., and Porath, J., *FEBS Lett.*, 75, 89, 1977.
262. Sottrup-Jensen, L., Petersen, T. E., and Magnusson, S., *FEBS Lett.*, 121, 275, 1980.
263. Borth, W., Menzel, E. J., Salzer, M., and Steffen, C., *Clin. Chim. Acta*, 117, 219, 1981.
264. Hudson, N. W. and Koo, P. H., *Biochim. Biophys. Acta*, 704, 290, 1982.
265. Imber, M. J., Pizzo, S. V., Johnson, W. J., and Adams, D. O., *J. Biol. Chem.*, 257, 5129, 1982.
266. Kurecki, T., Kress, L. F., and Laskowski, M., *Anal. Biochem.*, 99, 415, 1979.
267. Lebreton, J. P., *FEBS Lett.*, 80, 351, 1977.
268. Lebreton, J. P., Joisel, F., and Rault, J. P., *J. Clin. Invest.*, 64, 1118, 1979.
269. Rijken, D. C., Wijngaards, G., DeJong, M. Z., and Welbergen, J., *Biochim. Biophys. Acta*, 580, 140, 1979.
270. Rijken, D. C. and Collen, D., *J. Biol. Chem.*, 256, 7035, 1981.
270a. Andersson, L., *J. Chromatogr.*, 315, 167, 1984.
271. Cawston, T. E. and Tyler, J. A., *Biochem. J.*, 183, 647, 1979.
272. Ohkubo, I., Kondo, T., and Taniguchi, N., *Biochim. Biophys. Acta*, 616, 89, 1980.
273. Torres, A. R., Peterson, E. A., Evans, W. H., Mage, M. G., and Wilson, S. M., *Biochim. Biophys. Acta*, 576, 385, 1979.
274. Borrebaeck, C. A. K., Lönnerdal, B., and Etzler, M. E., *FEBS Lett.*, 130, 194, 1981.
275. Conlon, M. and Murphy, R. F., *Biochem. Soc. Trans.*, 4, 860, 1976.
276. Hansson, H. and Kagedal, L., *J. Chromatogr.*, 215, 333, 1981.
277. Salier, J. P., Martin, J. P., Lambin, P., McPhee, H., and Hochstrasser, K., *Anal. Biochem.*, 109, 273, 1980.
278. Yoshimoto, M. and Laskowski, M., Sr., *Prep. Biochem.*, 12, 235, 1982.
279. Gozdzicka-Jozefiak, A. J. and Augustyniak, D., *J. Chromatogr.*, 131, 91, 1977.
280. Ryley, H. C., *Biochem. Biophys. Res. Commun.*, 89, 871, 1979.
281. Kikuchi, H. and Watanabe, M., *Anal. Biochem.*, 115, 109, 1981.
282. Takeda, A., Fukuyama, K., Ohtani, O., and Epstein, W. L., *Biol. Trace Elem. Res.*, 3, 317, 1981.
283. Khazaeli, M. B. and Mitra, R. S., *Appl. Environ. Microbiol.*, 41, 46, 1981.
284. Redinbaugh, M. G. and Campbell, W. H., *Plant Physiol.*, 71, 205, 1983.
285. Knight, E., Jr., *J. Pharmac. Ther. A.*, 2, 439, 1978.
286. Edy, V. G., Billiau, A., and DeSomer, P., *J. Biol. Chem.*, 252, 5934, 1977.
287. Bollin, E., Jr. and Sulkowski, E., *Arch. Virol.*, 58, 149, 1978.
288. Chadha, K. C., Grob, P. M., Mikulski, A. J., Davis, L. R., Jr., and Sulkowski, E., *J. Gen. Virol.*, 43, 701, 1979.
289. Sulkowski, E., Vastola, K., and Oleszek, D., *Affinity Chromatography and Related Techniques*, Gribnau, T. C. J., Visser, J., and Nivard, R. J. F., Eds., Veldhoven, The Netherlands, 1982, 313.
290. Ferreira, P. C. P., Paucher, M., and Golgher, R. R., *Arch. Virol.*, 68, 27, 1981.
291. Yonehara, S., Yanase, Y., Sano, T., Imai, M., Nakasawa, S., and Mori, H., *J. Biol. Chem.*, 256, 3770, 1981.
292. Heine, J. W., van Damme, J., DeLay, M., Billiau, A., and DeSomer, P., *J. Gen. Virol. (London)*, 54, 47, 1981.
293. Berg, K. and Heron, I., *J. Gen. Virol. (London)*, 50, 441, 1981.
294. Berg, K. and Heron, I., *Scand. J. Immunol.*, 11, 489, 1980.
295. Thacore, H. R., Mount, D. T., and Chadha, K. C., *J. Interferon Res.*, 2, 401, 1982.
296. Scully, M. F. and Kakkar, V. V., *Biochim. Biophys. Acta*, 700, 130, 1982.
297. George, S. T. and Balasubramanian, A. S., *Biochem. J.*, 209, 471, 1983.
298. Hubert, P. and Porath, J., *J. Chromatogr.*, 198, 247, 1980.
299. Hubert, P. and Porath, J., *J. Chromatogr.*, 206, 164, 1981.
300. Coulet, P. R., Carlsson, J., and Porath, J., *Biotechnol. Bioeng.*, 23, 663, 1981.
301. Anderson, D. J. and Walters, R. R., *Affinity Chromatography and Biological Recognition*, Proc. 5th Int. Symp., Chaiken, I. M., Wilcheck, M., and Parikh, I., Eds., Academic Press, Orlando, Fla., 1983, 265.
302. Small, D. A. P., Atkinson, T., and Lowe, C. R., *Affinity Chromatography and Biological Recognition*, Proc. 5th Int. Symp., Chaiken, I. M., Wilcheck, M., and Parikh, I., Eds., Academic Press, Orlando, Fla., 1983, 267.
303. Vijayalakshmi, M. A., *Affinity Chromatography and Biological Recognition*, Proc. 5th Int. Symp., Chaiken, I. M., Wilcheck, M., and Parikh, I., Eds., Academic Press, Orlando, Fla., 1983, 269.

304. Rokugawa, K., Fujishima, T., Kuninaka, A., and Yoshino, H., *J. Ferment. Technol.*, 58, 509, 1980.
304a. Weetall, H. H., *Trends Biotechnol.*, 3, 276, 1985.
304b. Porath, J., Olin, B., and Granstrand, B., *Arch. Biochem. Biophys.*, 225, 543, 1983.
304c. Ramadan, N. and Porath, J., *J. Chromatogr.*, 321, 81, 1985.
304d. Ramadan, N. and Porath, J., *J. Chromatogr.*, 321, 105, 1985.
304e. Ramadan, N. and Porath, J., *J. Chromatogr.*, 321, 93, 1985.
304f. Andersson, L. and Porath, J., *Anal. Biochem.*, 154, 250, 1986.
305. Lönnerdal, B. and Keen, C. L., *J. Appl. Biochem.*, 4, 203, 1982.
306. *Chelating Sepharose 6B for Metal Chelate Affinity Chromatography*, Tech. Bull., Pharmacia Fine Chemicals, Piscataway, N.J., 1983.
307. Marguerie, G., Chagniel, G., and Suscillon, M., *Biochim. Biophys. Acta*, 490, 94, 1977.
308. Porath, J., Olin, B., and Granstrand, B., *Arch. Biochem. Biophys.*, 225, 543, 1983.
309. Hagfeldt, E., *Stability Constants of Metal-Ion Complexes, Part A, Inorganic Ligands, Part B, Organic Ligands*, Pergamon Press, New York, 1982.
310. Irving, H. and Williams, R. J. P., *Nature (London)*, 162, 746, 1948.
311. Sillen, L. G. and Marteu, A. E., *Stability Constants of Metal-Ion Complexes*, Chemical Society Special Publications, London, 1964, 17.
312. Tanabe, Y. and Sugano, S., *J. Phys. Soc. Jpn.*, 9, 753, 1964.
313. Coleman, J. E. and Vallee, B. L., *J. Biol. Chem.*, 235, 390, 1960.
314. Cohen, S. R. and Wilson, I. B., *Biochemistry*, 5, 904, 1966.
315. Cotton, F. A. and Wilkinson, G., *Advanced Inorganic Chemistry*, 4th ed., John Wiley & Sons, New York, 1980, 107.
316. Pearson, R. G., *J. Am. Chem. Soc.*, 85, 3533, 1963.
317. Pearson, R. G., *Hard and Soft Acids and Bases*, Dowden, Hutchinson, Ross, Stroudsberg, 1973.
318. Ho, T.-L., *Hard and Soft Acids and Bases, Principles in Organic Chemistry*, Academic Press, New York, 1977.
319. Bertini, I., Drago, R. S., and Luchinat, C., *Coordination Chemistry of Metalloenzymes, The Role of Metals in Reaction Involving Water, Dioxygen and Related Species*, D. Reidel, Boston, 1983, 1.
320. Hare, C. R., *Spectroscopy and Structure of Metal Chelate Compounds*, Nakamoto, K. and McCarthy, P. J., Eds., John Wiley & Sons, New York, 1968, 76.
321. Warshel, A., *Acc. Chem. Res.*, 14, 284, 1981.
322. Jardetzky, O., *Acc. Chem. Res.*, 14, 291, 1981.
323. Jardetzky, O. and Roberts, G. C., *NMR in Molecular Biology*, Academic Press, New York, 1981.
324. Pincus, M. R., *Acc. Chem. Res.*, 14, 299, 1981.
325. Kaptein, R., *NMR Spectroscopy in Molecular Biology*, Pullman, E., Ed., D. Reidel, Dordrecht, The Netherlands, 1978, 211.
326. Scheek, R. M., Stob, S., Boelens, R., Dijkstra, K., and Kaptein, R., *J. Am. Chem. Soc.*, 107, 705, 1985.
327. Griffey, R. H., Jarema, M. A., Kunz, S., Rosevear, R. R., and Redfield, A. G., *J. Am. Chem. Soc.*, 107, 711, 1985.
328. Yamauchi, O., Tsujide, K., and Odani, A., *J. Am. Chem. Soc.*, 107, 659, 1985.
329. Meares, C. F. and Wensel, T. G., *Acc. Chem. Res.*, 17, 202, 1984.
330. Rosset, R., *Bull. Soc. Chim. Fr.*, II, 1845, 1964.
331. Cheminat, A., Benezra, C., and Fréchet, J. M. J., *J. Chem. Res. (S)*, 148, 1982; *J. Am. Chem. Res. (M)*, 1618, 1982.*
332. Margolis, S. A., Fatiadi, A. J., Alexander, L. A., and Reeder, D. J., Federation Proceedings, Abstract N° 20842, April 21—25, Anaheim, California, 1985.
333. Agnew, N. H., *J. Polym. Sci.*, 14, 2819, 1976.
334. Hallensleben, M. L. and Wurm, H., *Angew. Chem.*, 88, 192, 1976.
335. Fréchet, J. M., Farral, M. J., and Nyens, L. J., *J. Macromol. Sci. Chem.*, 11, 507, 1977.
336. Johar, Y., Zupan, M., and Sket, B., *J. Chem. Soc. Perkin Trans.*, 1, 2059, 1982.
337. Fréchet, J. M., Warnock, J., and Farral, M. J., *J. Org. Chem.*, 43, 2618, 1978.
338. Menger, F. M., Shinozaki, H., and Lee, H. C., *J. Org. Chem.*, 45, 2724, 1980.
339. Kamat, P. V., Fox, M. A., and Fatiadi, A. J., *J. Am. Chem. Soc.*, 106, 1191, 1984.
340. Kamat, P. V. and Fox, M. A., *Chem. Phys. Lett.*, 92, 595, 1982.
341. Nishide, H. and Tsuchida, E., *Makromol. Chem.*, 177, 2295, 1976.
342. Nishikawa, H. and Tsuchida, E., *J. Phys. Chem.*, 79, 2072, 1975.
343. Tsuchida, E. and Nishide, H., Proc. IUPAC Int. Symp. on Macromolecules, Vol. 4, Florence, Italy, 1980, 147.

* On a recent discussion of Chelating Sorbents in Analytical Chemistry, see Myasoedova, G. V. and Savvin, S. B., *CRC Crit. Rev. Anal. Chem.*, 17, 1, 1986.

4. Chanda, M., O'Driscoll, K. F., and Rempel, G. L., *J. Mol. Catalys.*, 7, 389, 1980.
5. Meinders, H. C. and Challa, G., *J. Mol. Catal.*, 7, 32, 1980; Tsuchida, E., Kaneko, M., and Nishide, H., *Makromol. Chem.*, 164, 203, 1973.
5. Clear, J. M., Kelly, J. M., O'Connell, C. M., and Vos, J. G., *J. Chem. Res. (S)*, 260 (1981); *J. Chem. Res. (M)*, 3039, 1981.
7. Fletcher, R. A. and Fatiadi, A. J., *Polym. Commun.*, 26, 270, 1985.
8. Hodgson, D. J., *Prog. Inorg. Chem.*, 19, 173, 1975.
9. Couglin, P. K. and Lippard, S. J., *J. Am. Chem. Soc.*, 103, 3228, 1981.
0. Drew, M. G. E., McCann, M., and Nelson, S. M., *J. Chem. Soc. Dalton Trans.*, 1868, 1981.
1. Karlin, K. D., Hayes, J. C., and Zubieta, J., *Copper Coordination Chemistry: Biochemical and Inorganic Perspectives*, Karlin, K. D. and Zubieta, J., Eds., Adenine Press, Guilderland, N.Y., 1982, 457; McKee, V., Dadgegian, J. V., Bau, R., and Reed, C. A., *J. Am. Chem. Soc.*, 103, 7000, 1983.
2. Meinders, H. C., van Bolhuis, F., and Challa, G., *J. Mol. Catal.*, 5, 225, 1979.
3. Etz, E. and Fatiadi, A. J., unpublished results.
4. Karlin, K. D. and Zubieta, J., Eds., *Biological and Inorganic Copper Chemistry*, Vol. 1 and 2, Adenine Press, Guilderland, N.Y., 1986.
5. Horowitz, E., Tryon, M., Christensen, R. G., and Perros, T. P., *J. Appl. Polym. Sci.*, 9, 2321, 1965.
6. Sasson, A., Biotechnologies, Challenges and Promises, UNESCO, Paris, 1984.
7. Xavier, A. V., Ed., *Frontiers in Bioinorganic Chemistry*, VCH Verlagsgesellschaft, Weinheim, West Germany, 1986; Jacobsson, S., Jamison, A., and Rothman, H., *The Biological Challenge*, Cambridge University Press, London, 1986.
8. Walker, J. M. and Gingold, E. B., Eds., *Molecular Biology and Biotechnology*, Royal Society of Chemistry, London, 1985.
9. Warren, D. C., *Anal. Chem.*, 56, 1528, 1984.