Separation & Purification Reviews
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597294

Ligand Specific Chromatography

Sheldon W. May[ab]; Oskar R. Zaborsky[b]
[a] Esso Research and Engineering Company, Corporate Research Laboratories, Linden, New Jersey [b] School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia

To cite this Article May, Sheldon W. and Zaborsky, Oskar R.(1974) 'Ligand Specific Chromatography', Separation & Purification Reviews, 3: 1, 1 — 86
To link to this Article: DOI: 10.1080/03602547408068427
URL: http://dx.doi.org/10.1080/03602547408068427

SEPARATION AND PURIFICATION METHODS, 3(1), 1-86 (1974)

LIGAND SPECIFIC CHROMATOGRAPHY

Sheldon W. May*† and Oskar R. Zaborsky*
Corporate Research Laboratories
Esso Research and Engineering Company
Linden, New Jersey 07036

INTRODUCTION

It is well known that many molecules possess the ability to bind specifically and reversibly to other molecules in solution. For example, enzymes form non-covalent Michaelis-complexes with substrates as part of the classical Michaelis-Menten scheme of equation 1. Similarly, antibodies form complexes with their

$$E + S \rightleftharpoons \underset{\text{Complex}}{(ES)} \longrightarrow E + P \qquad (1)$$

respective antigens, and proteins interact with other proteins, with nucleic acids and with carbohydrates. However, this phenomenon is not limited to macromolecules. Through the now familiar Watson-Crick base pairing mechanism, nucleotides interact specifically with each other, and the same is true for a variety of other biological molecules.

This specific binding property forms the basis for a powerful separation technique for which the term "ligand specific chromatography" (LSC) is used in this review. In this technique,

* Address correspondence to either author.

† Present address: School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia, 30332.

a specific adsorbent is prepared by attaching one of the interacting species to a water-insoluble matrix. When a solution containing the other interacting component is passed through the matrix, this component becomes specifically bound to the ligand by virtue of their mutual affinity (Figure 1). In addition, the physicochemical properties of some of the other molecules in the original solution may result in these becoming non-specifically adsorbed to the matrix or to the ligand. The matrix is then washed to remove this non-specifically adsorbed material, and elution of the desired substance is achieved by imposing conditions which dissociate it from the immobilized ligand. Alternatively, an eluant is applied which specifically dissociates the complex, leaving any non-specifically adsorbed material behind on the matrix. In either case, the result is the separation of the desired material from a mixture of often very similar compounds.

Separations based on the same principle can also be accomplished using other methodology. For example, under appropriate conditions the addition of an antibody, A, to a solution containing an antigen, B, can cause precipitation of the AB complex, thereby effecting a separation of B from the other components of the mixture (equation 2). However, in this

$$A + B \rightleftharpoons \underset{\substack{\text{Water Insoluble}\\ \text{Complex}}}{A\cdots B} \qquad (2)$$

review the term "ligand specific chromatography" is used to denote separations accomplished using the water-insoluble matrix technique.

A term which is often used in the literature for this technique is "affinity chromatography". We do not distinguish operationally between ligand specific chromatography and affinity chromatography, but we prefer the former term because it emphasizes the specific nature of the interaction which is

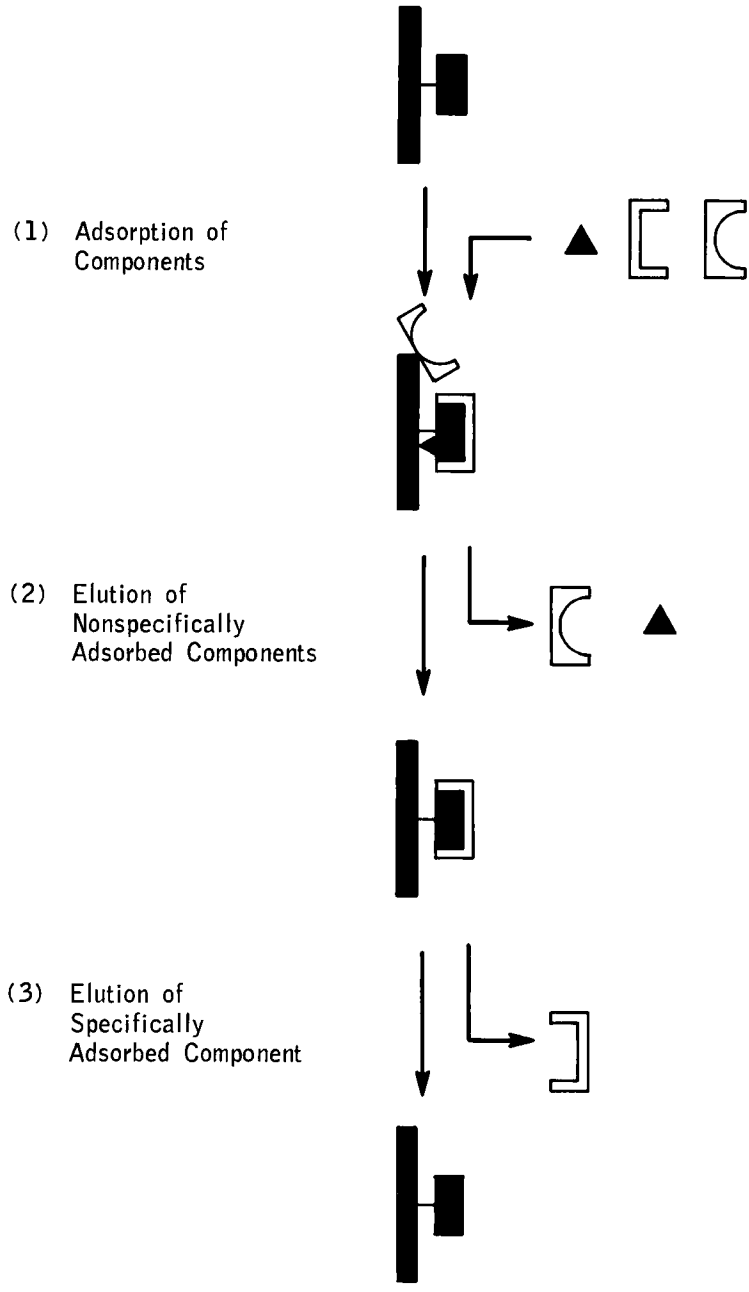


FIGURE 1

Essential steps of Ligand-Specific Chromatography (LSC).

responsible for the separation. Many charged molecules have an affinity for ion-exchange resins, but this affinity is not due to specific binding.

From the rapidly increasing frequency with which papers on this subject are appearing, one gains the impression that LSC is a very recent innovation. However, this is not the case. As early as 1906, Hedin reported the adsorption of trypsin to charcoal[1], and in 1907 he reported the specific adsorption of spleen enzymes onto charcoal and kieselguhr.[2] In 1910, the starch-splitting enzyme, amylase, was specifically adsorbed onto insoluble starch.[3] In 1953, Lerman[4] reported the adsorption of tyrosinase onto a resorcinol-cellulose conjugate, and the elution of the enzyme from the column at pH 9.5. Finally, the specific separation of polynucleotides by virtue of complementary base pairing was achieved in 1962 by several investigators.[5-7]

Several reviews have appeared recently on the use of "affinity chromatography".[8-14] Many of these have dealt extensively with the separation and purification of proteins, and accordingly this review will cover only the very recent literature in this area. It will, however, give a more general view of the use of LSC for the separation and purification of other molecules. In addition, we intend to emphasize the potential of LSC as a powerful tool in mechanistic and structural studies. We have not attempted to make this review comprehensive and certain areas, such as the use of water-insoluble immunoadsorbents for the isolation of antigens and antibodies, have been omitted. Rather, our purpose is to introduce LSC to people who may be unfamiliar with the biochemical literature, in the hope that it will find more general application in non-biological areas.

OPERATIONAL CONSIDERATIONS

Nature of the Support

General Considerations

A "good" support for LSC should possess certain physical and chemical characteristics. The support should be in the form of homogeneous, relatively rigid, porous beads; these allow for good flow properties in column operations. An appropriate porosity permits selective entry of components and also provides for a large effective surface area. A large surface area, in turn, is desirable for obtaining a high degree of modification. The desired porosity of a support can be obtained by adjusting the amount of crosslinking reagent present during the formation of the polymer or by adjusting the concentration of the polymeric component during gel formation.

Ideally, the support should have good mechanical stability and chemical stability toward extremes of pH and temperature, toward organic solvents, and toward denaturants such as urea or guanidine hydrochloride. The support should be highly insoluble in the solvent medium of choice (usually an aqueous buffer) and should have good mechanical and chemical stability under the actual conditions of the separation procedure. A good support should be resistant to microbial attack and should be reusable.

An equally important consideration is the charge of the carrier; it should be neutral in order to minimize nonspecific adsorption of charged contaminants. If the support is charged to any significant extent, the elution of the desired component can become a severe, if not impossible, problem. It is equally important to consider the degree of "hydrophilicity," or conversely "hydrophobicity," of the support. A good support should not destroy the delicate biological activity of

the component to be isolated by drastically and irreversibly altering its structure. For example, a hydrophilic support which is highly "wettable" would be the support of choice for the isolation of soluble proteins.

The support should have certain favorable chemical characteristics. It should be possible to (1) activate the support without destroying its structural integrity, (2) introduce reactive functionalities under mild conditions (meaning usually no extremes of pH or temperature) and (3) achieve a preselected degree of modification. Naturally, the efficiency of the modification reaction (both for activation and for ligand coupling) should be high.

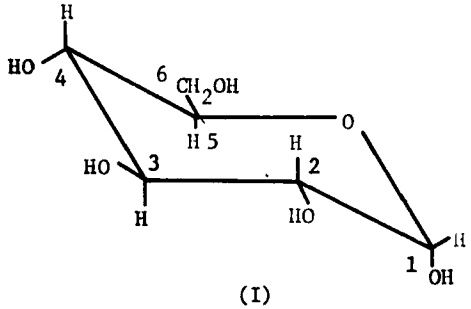
Last, but not least, a good support should be readily available from either supply houses or from simple chemical syntheses. Fortunately, good supports such as crosslinked dextran, agarose and polyacrylamide are commercially available. However, the cost of some of these matrices can become high if large scale isolations are contemplated.

Commonly Employed Supports And The Chemistry of Their Activation and Coupling with Ligands

During the past 15 years several supports possessing varying degrees of the ideal characteristics mentioned in the previous section have been developed and utilized for LSC. A brief survey of two commonly employed matrices and of two that show promise follows. The chemistry discussed in this section is representative of the chemistry of supports not specifically mentioned here.

Crosslinked Dextran (Sephadex)

Sephadex is the registered trademark of Pharmacia Fine Chemicals Inc. for its spherical, gel-beaded, crosslinked dextran. This branched polysaccharide, consisting of glucose units (I) linked predominantly by α-D-1$\rightarrow$6 linkages and to a lesser extent by 1$\rightarrow$3 and 1$\rightarrow$4 linkages, is crosslinked with



(I)

epichlorohydrin to give a three-dimensional network of polymeric chains. Various kinds of Sephadex are available which differ in their swelling capacity--a property dependent on the degree of crosslinking. Thus Sephadex G-10, the most highly crosslinked dextran, consists of particles whose diameter ranges from 40 to 120 μ and has a water regain value of 1.0 $\pm$ 0.1 (g of water/g of dry Sephadex) and a bed volume of 2 to 3 ml/g of dry polysaccharide. Sephadex G-10 can be used for fractionating molecules whose molecular weight is ca. 700. Sephadex G-200, the least crosslinked and most porous dextran, consists of particles whose diameter ranges from 40 to 120 μ and has a water regain value of 20.0 $\pm$ 1.0 and a bed volume of 30 to 40. This gel can fractionate large macromolecules whose molecular weights range from 5,000 to 800,000 for globular proteins and from 1,000 to 200,000 for dextrans. Other Sephadex gels, e.g., G-15, G-25, and G-50, have properties intermediate between those of the G-10 and G-200 gels. Sephadex gels exhibit good flow properties in column operations because of their beaded nature.

Although Sephadex can be regarded as being essentially neutral, there is a small amount of residual negative charge in the purified, crosslinked polysaccharide presumably caused by carboxylic acid groups. This can be eliminated by condensation of these carboxyl groups with glycinamide using a water-soluble carbodiimide.[15]

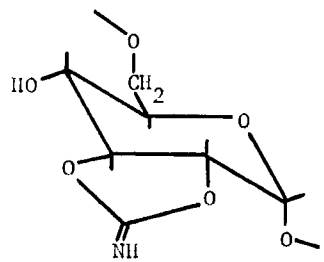
Sephadex gels are stable in salt solutions, buffers, numerous organic solvents, and alkaline and weakly acidic solutions. In strong acids, the glycosidic bonds are hydrolyzed.

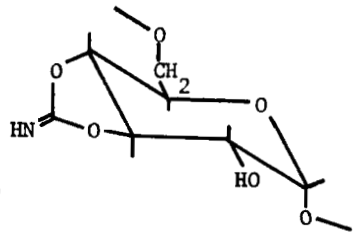
Good thermal stability is also exhibited by these gels, with degradation occurring only above ca. 120°C. Sephadex gels come in a dry form and are swelled in water or in an appropriate buffer. Once swollen, these gels can be stored either in this state (preferably under refrigeration and in the presence of a bacteriocide) or can be returned to a dry form by successive additions of solutions of increasing ethanol concentration. Final drying is achieved at 60 to 80°C.

Excellent literature on the properties and uses of Sephadex gels as well as of other dextrans is provided by Pharmacia Fine Chemicals Inc. (Piscataway, N.J. 08854).

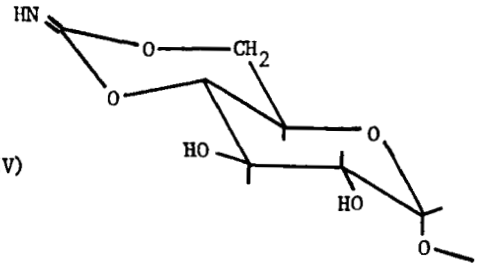
Sephadex gels and other polysaccharide supports are conveniently activated through the cyanogen bromide procedure of Axen, Porath and Ernback.[16] The chemical reactions which are thought to be involved in the activation of the support and the coupling of ligands are given in Figure 2. Studies have been undertaken using model compounds and polysaccharides to test for the existence of the various structures shown in Figure 2, and the results obtained so far are consistent with the proposed reaction sequence.[17-21] Because dextran is a predominantly 1,6-linked polysaccharide, it seems reasonable to assume that for this matrix the 2,3- and 3,4-linked cyclic imidocarbonates are the dominant reactive species (II and III). However, 4,6-linked cyclic imidocarbonates involving the terminal glucose residues of the polymer and imidocarbonates of intermolecularly crosslinked chains may also be formed[20,21] (IV and V).

(II)

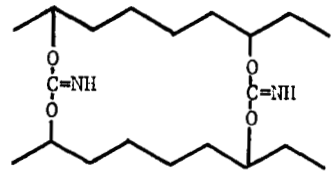




(III)



(IV)



(V)

Activation of Sephadex is achieved by adding CNBr either as a solid or as a solution to a stirred suspension of the dextran gel at ca. 25°C. A constant pH (anywhere from 9.5 to 12) is maintained by addition of sodium hydroxide. The reaction is rapid and usually complete within half an hour. The gel is then rapidly washed with cold buffers and immediately used for covalent coupling of the ligand. The activated product is stable for prolonged time if it is washed briefly with water, dried gradually with acetone, evaporated under vacuum, and stored in the cold.[17]

Coupling of the amino-containing ligands is achieved through contact of the ligand under slightly alkaline conditions (pH 8 to 10) at ca. 5°C for 12 to 24 hr. After coupling, the

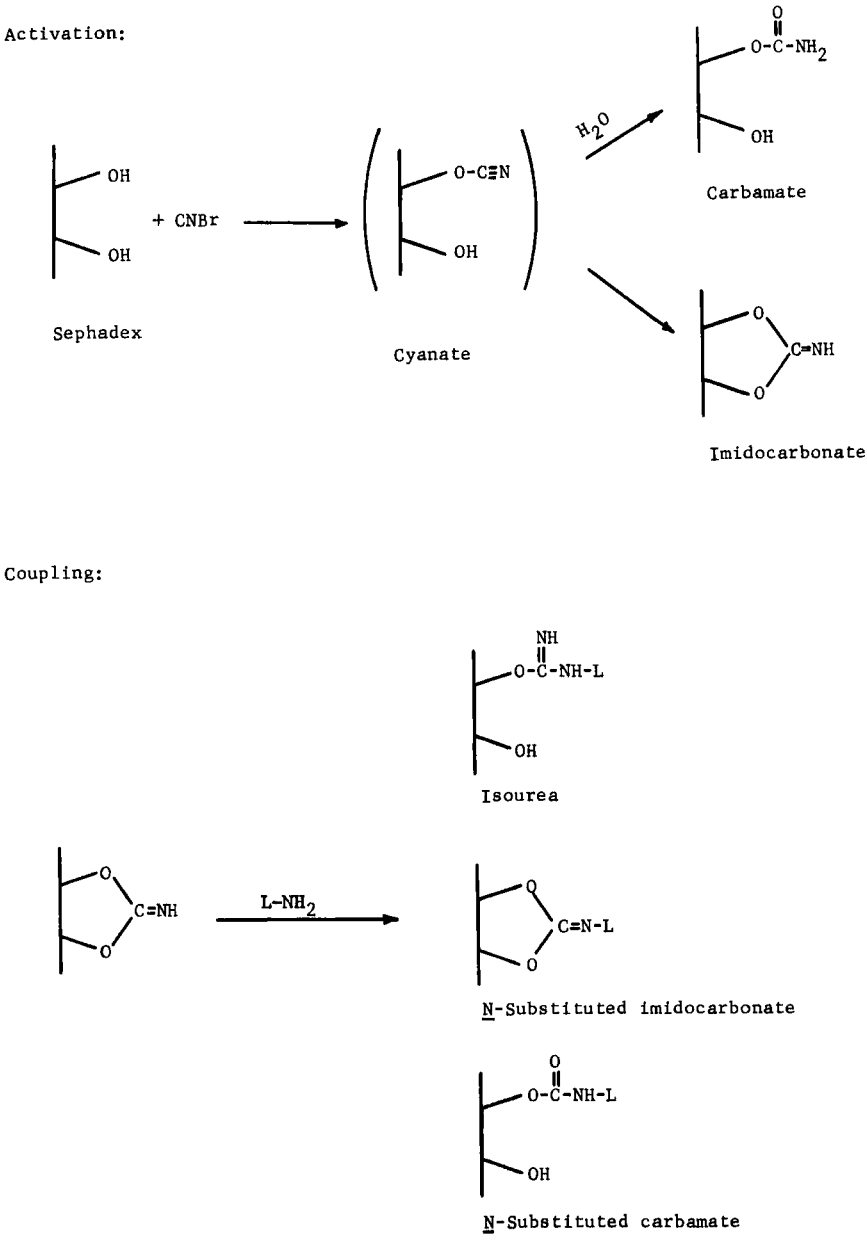


FIGURE 2

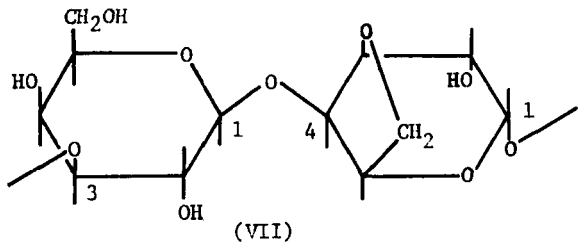
Activation of Sephadex with cyanogen bromide and its coupling with amino-containing ligands.

ligand-containing gels are washed extensively with buffers of different pH and ionic strength in order to completely remove all noncovalently bound ligand.

The coupling reaction shown in Figure 2 is specific for compounds containing amino groups. The use of a diamine under excess conditions permits the attachment of a "spacer" ligand to which other functionalities can be added (see Figure 3, and also the section on the Nature of the Ligand).

Agarose (Sepharose and Bio-Gel A)

Agarose is a linear polysaccharide consisting of alternating β-D-galactose and 3,6-anhydro-α-L-galactose residues connected through the 1- and 4-positions (VII). Beaded agarose gels are available from Pharmacia Fine Chemicals Inc. (Sepharose) and from Bio-Rad Laboratories (Bio-Gel A) in a variety of exclusion limits and mesh sizes. Unlike the Sephadex gels, agarose gels are not chemically crosslinked. The exclusion limit is dependent on the percentage of agarose in the gel. Thus 10% agarose Bio-Gel A has a fractionation range from ca. 10,000 to 500,000 while the corresponding 1% agarose gel has a fractionation range from ca. 1,000,000 to 150,000,000. Bio-Gel A gels are available as 1,2,4,6,8 and 10% agarose; Sepharose gels are available as 2,4 and 6% agarose. The gels are supplied as aqueous suspensions. The particle size of the Sepharose gels in the dry state ranges from ca. 50 to 250 μ in diameter.

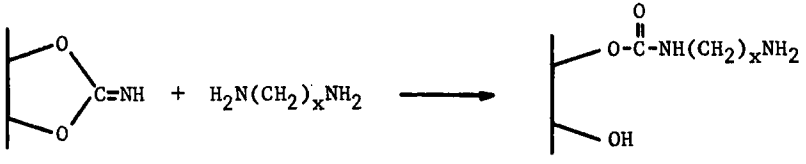


(VII)

β-D-galactopyranosyl residue

3,6-anhydro-α-L-galactopyranosyl residue

Coupling:



(VI)

Derivatization:

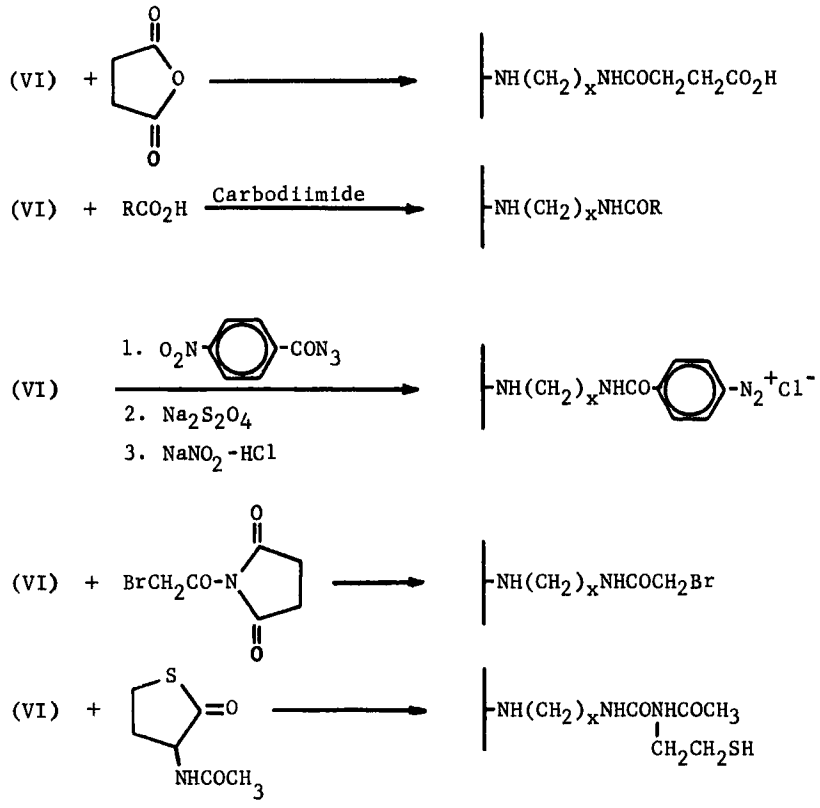


FIGURE 3

Coupling of activated Sephadex with diamines and further derivatization of the aminoalkyl supports.

Sepharose gels, unlike Sephadex gels, cannot be dried with many organic solvents or lyophilized because these treatments usually lead to irreversible shrinkage. However, agarose is compatible with certain mixed solvent systems, such as 50% DMF or 50% ethylene glycol, or with 100% dioxane. Indeed, lyophilization of beads after thorough washing with dioxane leads to a dried powder that swells immediately when reintroduced into aqueous solutions.

Although it is recommended by the manufacturer that the pH of a Sepharose suspension should not exceed 9 nor fall below 4 for any extended period of time, agarose beads can tolerate 0.1 M NaOH or 1 M HCl for at least 2 to 3 hours at room temperature without adverse alteration of their physical properties.[22] Sepharose gels can also tolerate exposure to 6 M guanidine hydrochloride or 7 M urea for prolonged periods, but a slight shrinkage of the gels is observed under these conditions. The gels and the ligand-bound derivatives are normally stored in aqueous suspension at ca. 5°C in the presence of an appropriate bacteriocide. Temperatures above 40°C should be avoided.

To date, Sepharose 4B has been the most widely used support for LSC. Its appropriate porosity and good flow behavior make it an almost ideal support. Excellent literature on the properties and uses of Sepharose can be obtained from Pharmacia Fine Chemicals Inc. (Piscataway, N.J. 08854). Cyanogen bromide activated Sepharose 4B, the aminohexyl derivative, and the carboxyl derivative derived from 6-aminohexanoic acid are commercially available. A brochure describing in detail the use of activated Sepharose 4B is also provided by this company. A larger number of ligand-bound agarose derivatives are available from Affitron Corp. (Costa Mesa, Calif. 92626).

The activation procedure of choice for agarose gels is the cyanogen bromide method of Axen, Porath and Ernback[17]

described in the previous section. Agarose, unlike Sephadex, does not have vicinal hydroxyl groups and the activated product must be assumed to contain only cyclic 4,6-linked and intermolecularly crosslinked imidocarbonate groups.[21] The chemistry of ligand coupling discussed for the Sephadex support is equally applicable to the agarose gels. The formed imidocarbonate reacts with primary-amino-containing ligands[23] to give the products indicated in Figure 2. A variation of the coupling step using cyanogen bromide-activated agarose gels was introduced recently by Cuatrecasas and Parikh.[24] This method is based on the formation of the N-hydroxysuccinimide esters of succinylated aminoalkyl agarose derivatives (Figure 4). Coupling of the succinylated aminoalkyl derivative with N-hydroxysuccinimide and a carbodiimide is accomplished in dioxane, and the resulting active esters are then conveniently stored in the dry solvent at room temperature over molecular sieves or can be lyophilized to a powder form. Some advantages of this coupling procedure are the following. (1) A long spacer arm is inserted. (2) Ligands such as amino acids, which contain more than one functional group, can be coupled without using complicated blocking and deblocking procedures. (3) The synthesis of the active esters and coupling of the amino-containing ligands can be accomplished under mild conditions (aqueous solvent, 5°C, neutral pH). (4) The activated derivative is stable and can be stored at room temperature for several months.

Cuatrecasas and Parikh made an important observation about the stability of these ligand-substituted agarose derivatives at room temperature. They noted that the derivatives had rather limited stability and they showed that this was due to instability of the bonds formed in the cyanogen bromide linkage step and not of those in the amide linkage. "Ligand leakage" from cyanogen bromide-activated gels, as well as from other supports, is an important consideration too often neglected.

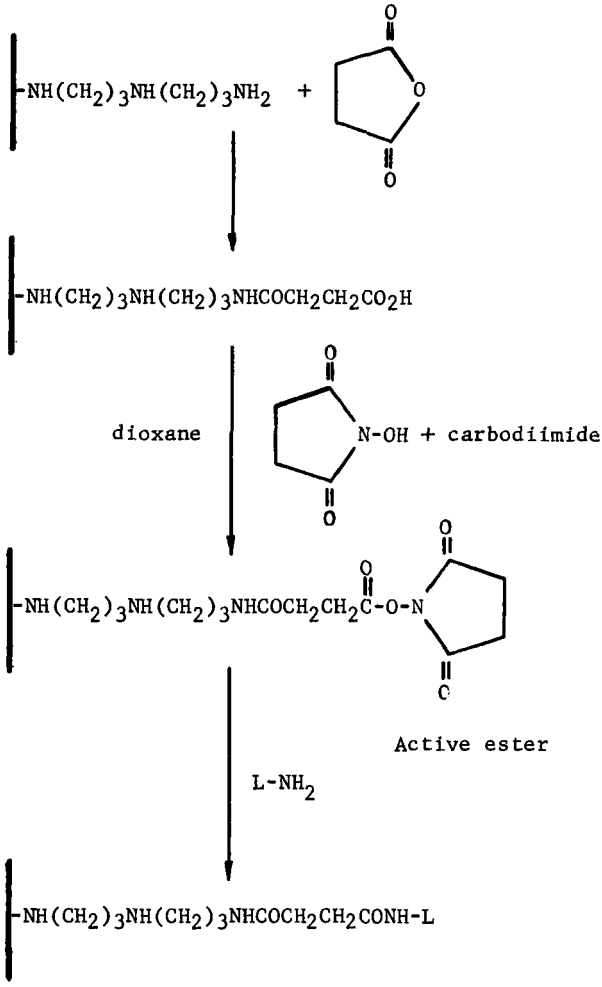
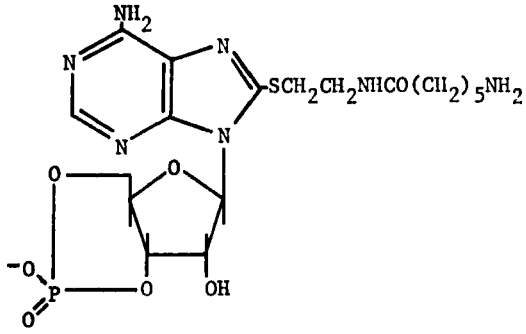


FIGURE 4

Preparation of N-hydroxysuccinimide ester derivative of agarose and its coupling with amino-containing ligands.

Leakage has also been observed by Tesser et al.[25] for a cyclic adenosine 3':5'-monophosphate, c-AMP, analog (VIII) attached to agarose. The c-AMP-Sepharose

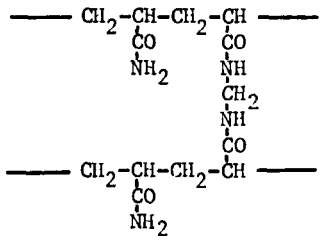
(VIII)



conjugate prepared at pH 10.2 contained about 2 to 5 μmoles of VIII per ml of suspension and was "reasonably stable" below ca. pH 5. However, at all higher pH's, a c-AMP-containing soluble product was continuously released. The exact identity of the released compound was not ascertained because of its low quantity (pmolar range) but it probably arose from the breakdown of the cyanogen bromide-introduced bond and not any bond in the c-AMP ligand. Ligand VIII is very stable in neutral and alkaline solutions; not even the slightest deterioration could be detected by chromatography and electrophoresis after standing for three weeks in 1 M ammonia at 20°C. In contrast to these reports of lability, no "significant free" glucagon was observed in the column effluent of a glucagon-agarose column.[26] The detection limits of the procedures used for glucagon determinations were about 10^{-10} M glucagon. Thus, the evidence to date does not conclusively establish whether or not "ligand leakage" is a general phenomenon, and more definitive studies are needed.

Crosslinked Polyacrylamide Gels

Promising supports for LSC developed recently by Inman and Dintzis[27] are based on the chemical modification of preformed polyacrylamide beads. Polyacrylamide (IX) offers the advantages of enhanced chemical stability by virtue of its polyethylene backbone structure, and of a statistically uniform



(IX)

physical state and porosity which result from its formation as a crosslinked synthetic polymer. The beads are available in spherical form as Bio-Gel P from Bio-Rad Laboratories (Richmond, Calif. 94804) in pregraded sizes and porosities. The separation of macromolecules up to 500,000 is permitted with the most porous beads.

Although the carboxamide side groups of polyacrylamide are chemically stable and resistant to hydrolysis in the pH range between 1 and 10, these groups are readily exchanged by other amino-containing compounds. The conversion of these amide side groups into the aminoethyl and the hydrazide derivatives is accomplished under the conditions given in equations 3 and 4. The resulting derivatives can then be modified further to

$$\text{-CONH}_2 + \text{H}_2\text{NCH}_2\text{CH}_2\text{NH}_2 \longrightarrow \text{-CONHCH}_2\text{CH}_2\text{NH}_2 + \text{NH}_3 \qquad (3)$$

Polyacrylamide — Aminoethyl Derivative

$$\text{-CONH}_2 + \text{H}_2\text{NNH}_2 \longrightarrow \text{-CONHNH}_2 + \text{NH}_3 \qquad (4)$$

Hydrazide

give a variety of ligand-containing supports similar to those attainable from aminoalkyl Sephadex and agarose. A distinct advantage of the polyacrylamide supports is the very large

number of carboxamide groups per g of matrix which are available for chemical modification. However, some reservations about the use of polyacrylamide beads for LSC have been expressed recently by Cautrecasas.[13,28] The rather limited porosity range presently available in these supports precludes the effective isolation of large macromolecules.

The aminoethyl and hydrazide derivatives of Bio-Gel P are available from Bio-Rad Laboratories.

Aminoalkyl Glass

Aminoalkyl glass, a support introduced recently by Corning Glass Works and distributed by Pierce Chemical Company (Rockford, Ill. 61105), is a glass derivative whose preparation is given in equation 5. The glass usually employed is in the form of porous beads which have typically a pore diameter of 2000 Å and are 200 mesh in size. Porous glass beads offer the

$$\text{Si-OH} + (EtO)_3Si\text{-}CH_2CH_2CH_2NH_2 \longrightarrow \text{Si-O-Si}\text{-}CH_2CH_2CH_2NH_2 \quad (5)$$

Aminoalkyl glass

advantages of being very rigid, resistant to microbial attack and impervious to most aqueous and organic solvent systems. One should, however, observe the recommended guidelines offered by the manufacturer as to the use of these glass supports, for they are "soluble" to some extent at extremes of pH where severe ligand leakage can be encountered. Other disadvantages of glass are its propensity for nonspecific adsorption and the limited porosity range of the beads available at the moment. Cuatrecasas[13] also claims that the flow rates of columns packed with such glass beads are prohibitively low, and that the tight packing of the beads results in mechanical trapping of particulate materials that causes significant problems on passing

large samples through a column. Although column procedures are difficult, batchwise methods can be used successfully provided that the system displays a high degree of affinity. The use of glass supports for LSC has been reported recently by Robinson et al.[29] and by Cuatrecasas and co-workers.[13,28]

Literature on the properties and uses of porous glass beads and of the aminoalkyl derivatives is available from Corning (Corning Biological Products Dept., Medfield, Mass. 02052) and from the Pierce Chemical Co.

Nature of the Ligand

A consideration of the principle upon which LSC is based leads one to the conclusion that any potential ligand should possess the ability to bind specifically, strongly and reversibly to the substance which is to be isolated. The reasons for this seem obvious--specific binding minimizes co-adsorption of impurities, strong binding minimizes spontaneous leakage of adsorbed material from the column, and reversible binding insures that the desired material can be recovered from the column under appropriate elution conditions. In practice, however, less-than-ideal ligands can often be used quite successfully.

The degree of binding specificity required for a given separation is clearly a function of the type of impurities present in the original mixture, and will thus vary from one procedure to the next. A relatively nonspecific ligand can often be used successfully if the elution procedure is capable of selectively removing only the desired substance from among the various compounds which bind to the column. For example, columns of immobilized NADH, a cofactor which is capable of binding to a large number of different enzymes, have been used in the separation of several NADH-dependent enzymes. Sophisticated techniques have been developed to accomplish selective elutions from columns of this rather nonspecific ligand[30-33] (see page 75).

Strength of binding is expressed by the value of the dissociation constant, K_d, for the complex between the ligand and the soluble component (equation 6). In general, a low

$$\text{ligand} + \text{component} \rightleftharpoons [\text{complex}] \qquad (6)$$

$$K_d = \frac{[\text{ligand}][\text{component}]}{[\text{complex}]}$$

dissociation constant is desirable since it leads to efficient adsorption onto the matrix, and conditions can usually be found to dissociate even the tightest of complexes in order to accomplish elution. It should be kept in mind that it is the operational value of K_d, i.e., the affinity of the immobilized ligand for the soluble species, and not the value of K_d measured when both species are in free solution, which is important in LSC. Since the former value can in principle be substantially greater than the latter, it is generally advisable to choose a ligand which exhibits a high degree of affinity for the desired component in free solution.

It is impossible to state precisely what the K_d of an "ideal" ligand should be. In practice, ligands of various affinities can be accommodated by appropriately adjusting the adsorption and/or elution conditions. However, Cuatrecasas[9] has pointed out that it has been very difficult to successfully use inhibitor-based adsorbants for the purification of enzymes when the dissociation constants of the inhibitors in free solution are greater than 5 mM, unless the ligands are attached to the matrix by long arms (see below). Alternatively, it is theoretically possible to employ adsorbants with such "weak" ligands if very high loading of the ligands onto the supports can be achieved.

"Irreversible" ligands, i.e., those which form covalent bonds with the substance being isolated, can also be used successfully. Ashani and Wilson[34] isolated acetylcholinesterase

by using a conjugate of 2-aminoethyl-*p*-nitrophenyl methylphosphonate and Sepharose (Figure 5). The latter forms a covalent phosphoryl-enzyme derivative with acetylcholinesterase, and thereby retains the enzyme on the column. Elution was accomplished by adding various nucleophiles which cleave the phosphoryl-enzyme bond. However, several problems were encountered in this procedure. The column is incapable of separating other serine esterases from the desired acetylcholinesterase, since all serine esterases would be expected to bind to the column in an identical fashion. Furthermore, there is evidence that some spontaneous release of the inhibited enzyme from the column occurs during the procedure. Also, the purity of the enzyme finally obtained was not firmly established by these investigators.

Using the same approach, Blumberg and Strominger[35] covalently bound penicillin binding proteins from *Bacillus subtilis* to a conjugate of Sepharose and 6-aminopencillanic acid, and subsequently eluted the proteins with hydroxylamine. One of the penicillin binding components is D-alanine carboxypeptidase, which comprises only one percent of the total membrane protein. Yet, these investigators succeeded in obtaining this protein in pure form and in 50-75% yield using a slight modification of this "covalent" LSC procedure.

It has been noted time and again in the literature that it is often desirable to insert an "arm" or "spacer"--usually a hydrocarbon chain--between the ligand and the insoluble matrix in order to maximize the efficiency of the LSC column. The arm presumably functions by extending the ligand away from the relatively restricted environment of the matrix, thereby increasing its freedom of motion. This effect would be expected to be especially important when the ligand is to interact with a large macromolecule, *e.g.*, an enzyme, since such interactions should be sterically hindered when the ligand is in close proximity to a bulky matrix. Unfavorable interactions of this

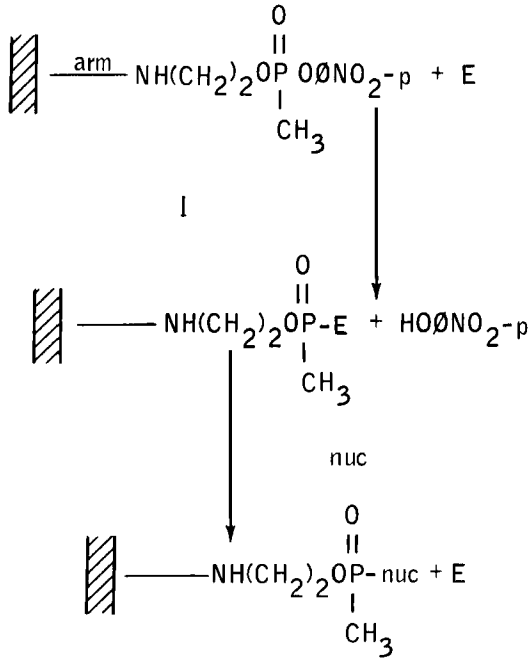


FIGURE 5

Isolation of acetylcholinesterase using covalent LSC procedure (from Ashani and Wilson, ref. 34).

type increase the "operational" value of K_d and thus may cause ligands which in free solution have relatively low affinity for the macromolecule to be totally ineffective in LSC procedures.

That arm extensions are indeed most important when the ligand has a relatively weak affinity for the compound being purified is illustrated by experiments quoted by Cuatrecasas.[22] In studies on the purification of bacterial β-galactosidase using conjugates of Sepharose and the relatively weak ($K_I \approx 10^{-3}$M) competitive inhibitor p-aminophenyl-β-D-thiogalactopyranoside, the enzyme did not bind to a column containing the ligand directly attached to the Sepharose. Only by interposing an arm prepared by succinylating the 3,3'-diaminodipropylamine derivative of Sepharose between the matrix and the ligand, could strong

adsorption of the enzyme be achieved. Similar results were obtained during the purification of muscle glycerol 3-phosphate dehydrogenase and hepatoma tyrosine aminotransferase. In contrast, deoxythymidine 5'-p-aminophenyl phosphate 3'-phosphate, a relatively strong ($K_I \approx 10^{-6}$M) competitive inhibitor of staphylococcal nuclease, effectively adsorbs the enzyme even when attached directly to Sepharose, although the total binding capacity of the gel is increased when an arm is present.

Other specific examples of the dramatic effect which arms of appropriate length often have in LSC procedures abound, and many of these are cited in later sections of this review. However, for the purpose of illustration, a few examples from the recent literature deserve mention. Lefkowitz et al.[36] purified the cardiac β-adrenergic receptor protein using a norepinephrine-Sepharose conjugate with an unusually long arm. Sepharose was reacted sequentially with 3,3'-diaminodipropylamine, succinic anhydride, 3,3'-diaminodipropylamine, and succinic anhydride and finally with norepinephrine to give an adsorbent with an arm 30 Å in length. Cuatrecasas[37] synthesized a series of eight insulin-Sepharose derivatives for use in the purification of the insulin receptor protein of liver cell membranes, and found that those derivatives in which an arm separates the ligand from the matrix were most effective. Similarly, Whiteley et al.[38] tested the effectiveness of a series of amethopterin-Sepharose derivatives in the purification of dihydrofolate reductases, and obtained best results when the adsorbent contained a diaminohexane spacer group.

Can an extension arm be so long that it defeats its purpose? It has often been observed that increasing the length of an arm beyond a certain point results in no incremental improvement in the performance of the LSC column. (See, for example, staphylococcal nuclease purification described in ref. 22.) An example of an arm which has a detrimental effect on a purification procedure is provided by the work of Cardinaud and Holguin.[39] These investigators attempted the purification

of nucleoside deoxyribosyltransferase using various immobilized purine derivatives and found that an n-hexylamine arm prevented the enzyme from binding to the LSC column. They ascribed this to the flexibility of the hydrocarbon chain which allows it to fold back on itself and bring the ligand too close to the matrix. This explanation is supported by their observation that a rigid arm, m-phenylenediamine, was highly effective in increasing the binding capacity of the column.

Finally, it should be noted that Er-el et al.[40] have recently reported that hydrocarbon chains attached to Sepharose are able to bind proteins, even when no "specific" ligand is attached to the matrix. For example, phosphorylase b was retained very tightly by a conjugate of Sepharose and hexylamine, and the tightness of this binding decreased as the hydrocarbon chain length was decreased. On the other hand, several other proteins (e.g., lysozyme, bovine serum albumin) did not bind to a 4 carbon long alkyl-Sepharose derivative which reversibly bound phosphorylase b. These findings must be taken into account in designing LSC columns, since they indicate that the very hydrocarbon chains often used as arms may contribute significantly to the binding of proteins to the matrix, presumably through hydrophobic interactions. Although this may enhance the capacity of the LSC column to bind the desired material, it may just as easily contribute to the binding of undesired impurities via nonspecific interactions with the hydrocarbon chain.

Adsorption and Elution

As mentioned above, the interactions which are responsible for the specific binding of molecules to the ligand on an LSC column may be either "reversible" or "irreversible" in nature. (In this context, the term reversible binding denotes interactions which do not result in the formation of a covalent chemical bond.) Examples of reversible interactions

commonly important are enzyme-inhibitor or enzyme substrate, antibody-antigen, protein-protein, protein-carbohydrate and protein-nucleic acid interactions. In the purification of nucleic acids and nucleoproteins, advantage is often taken of the strong interaction of these molecules with each other through the familiar base-pairing mechanism (see Nucleic Acids Section). The successful use of ligands which interact irreversibly--i.e., through formation of a covalent bond--with the molecule being purified, depends on the availability of a satisfactory procedure for subsequent elution of the desired substance from the column. Specific examples of LSC procedures using irreversible ligands are described in references 34 and 35.

It is clear that the extent of purification which is achieved in any LSC procedure depends directly upon the degree of specificity inherent in the particular adsorption and elution procedures employed. In practice, satisfactory results are often obtained when only one of the two steps is highly specific. For example, when a crude mixture is contacted with a highly specific ligand-matrix conjugate, the desired material will be adsorbed to a far greater extent than any of the other components of the original mixture. Thus, the subsequent elution step can be accomplished using a strongly dissociating, rather non-selective medium. On the other hand, ligands often contain charged groups which are capable of nonspecifically binding ionic substances through ion-exchange-type interactions. In such cases, elution procedures must be employed which distinguish between material bound ionically and material bound through specific affinity for the ligand. Of course, an LSC procedure which utilizes both specific adsorption and specific elution steps would be expected to give an exceptionally high degree of purification.

Some of these considerations are illustrated by reports in the recent literature. One example is the work of Robert-Gero and Waller[41] who purified methionyl-tRNA synthetase using a methionine-Sepharose conjugate with a 6-carbon-long

alkyl arm. Adsorption of the crude enzyme preparation was accomplished in the presence of 0.2 M potassium phosphate in order to minimize retention of the desired enzyme by ion exchange interactions with the charged free amino groups of the ligand. (Despite this precaution, a large amount of the bulk protein from the starting mixture was retained on the column under these conditions, presumably because of ion-exchange interactions.) When highly substituted Sepharose was used (12 μmole methionine/ml gel), methionyl-tRNA synthetase was strongly adsorbed onto the column and was subsequently eluted with buffer containing 20 mM methionine. With less-highly substituted Sepharose, the enzyme was merely retarded on the column and thereby separated from the bulk protein, but good results were not obtained unless some preliminary purification procedures were carried out prior to the LSC step.

As mentioned in the preceding section, extension arms are often coupled to the matrix prior to the attachment of the ligand. These arms commonly terminate with free amino or carboxyl functionalities in order to facilitate the subsequent chemical attachment of the ligand. However, this attachment step is seldom 100% efficient, and the final matrix will contain a certain number of charged amino or carboxyl groups at the end of unreacted arms. In order to avoid complicating ion-exchange interactions, these functionalities can be chemically blocked prior to starting the LSC procedure. For example, acetic anhydride can be used to acetylate unreacted amino groups.

An entirely different type of nonspecific adsorption is encountered when cofactors are used as ligands, since these molecules are capable of binding to a large number of different proteins. Nevertheless, Mosbach and co-workers[30-33] have successfully used columns of immobilized NAD and AMP in the purification of a variety of enzymes. The advantage in using such ligands is that the same columns can be used for the purification of a variety of enzymes, thus avoiding the necessity of synthesizing a new ligand-matrix conjugate for each enzyme. On the other

hand, highly specific elution procedures must be used in order to ensure that the desired protein is separated from the large number of different molecules which bind to the column. Among the elution procedures developed by Mosbach and co-workers are the use of pulses of oxidized or reduced cofactors at appropriate concentrations and the use of linear gradients of NADH. In both cases, since the adsorbed enzymes differ in their affinities toward NADH or NAD they are selectively eluted at different concentrations of these cofactors.

A powerful technique in the design of selective adsorption and elution procedures is to utilize the ability of certain enzymes to form ternary complexes with substrates and cofactors. Thus, if two such enzymes are both adsorbed onto the same column, they can be separately eluted by first applying an eluant containing cofactor plus a substrate of one enzyme and subsequently applying an eluant containing the same cofactor plus a substrate of the other enzyme. Each enzyme will elute only when both the appropriate substrate and cofactor are present in solution, allowing formation of a ternary enzyme-substrate-cofactor complex. The details of such procedures are considered in the Mechanistic Section of this review.

In principle, compounds which are reversibly bound to an LSC column can eventually be eluted by the starting buffer if their affinity for the ligand is not exceptionally strong. However, they would be obtained in dilute form if this type of elution were employed, and it is therefore necessary to alter some experimental variable in the elution procedure. Any substance or environmental condition which affects the binding of the desired molecule to an immobilized ligand can be used for this purpose. For example, if an enzyme binds to the ligand only in the presence of oxygen, elution can be easily effected by excluding oxygen from the buffer. Similarly, advantage can be taken of changes in pH, temperature, ionic strength, or solvent composition, and specific examples of such procedures are considered throughout this review. In practice, care must be taken

to avoid environmental extremes which permanently denature or decompose either the substance which is being isolated or the immobilized ligand.

It should be kept in mind that the specialized techniques and practices of conventional chromatography are equally applicable to LSC procedures. For example, care must be taken to swell the matrix properly, to pack the column correctly, and to apply the sample carefully. A standard chromatographic "trick" often useful in LSC for the elution of very strongly adsorbed material, is to physically remove the upper layer of the matrix and to mix this in a container with a large volume of an appropriate buffer.

PROTEINS

Table I gives a partial list of proteins isolated by LSC during 1972. Most of the proteins are enzymes, but this technique is being used increasingly for the purification of other kinds of proteins as well. The general procedures used in most of these investigations are described in the section on Operational Considerations and in the reviews mentioned in the Introduction, and they will not be considered in this section. There were, however, several studies reported in 1972 which deserve special mention. The selectivity achieved in some procedures was of such magnitude that it was possible to separate active from inactive molecules, chemically modified from native forms, and different molecular species of an enzyme (isoenzymes) from each other. The few examples chosen for illustration exemplify the potential of LSC and the cleverness of certain investigators.

The separation of fully functional xanthine oxidase ($\geq$95%) from the nonfunctional enzyme was achieved by Edmondson *et al.*[46] on a Sepharose column using 3-(1-H-pyrazolo(3,4-d)-

TABLE I

PARTIAL LIST OF PROTEINS ISOLATED BY LSC DURING 1972

Protein	Ligand	Support	Reference
	Oxidoreductases		
Dihydrofolate reductase	Amethopterin	Sepharose, various aminoalkyl derivatives	38
Glyceraldehyde 3-phosphate dehydrogenase	NAD	Sepharose, hexanoyl derivative	30,31
Glyceraldehyde 3-phosphate dehydrogenase	N^6-(6-Aminohexyl)-AMP	Sepharose	30,31
Lactate dehydrogenase	NAD	Sepharose, hexanoyl derivative	30,31
Lactate dehydrogenase	N^6-(6-Aminohexyl)-AMP	Sepharose	30,31
Lactate dehydrogenase	N^6-(6-Aminohexyl)-AMP	Sepharose	33
Lipoxygenase, soybean	Linoleic acid	Agarose, aminoethyl derivative	42
NAD-dependent dehydrogenases	NAD	Sepharose, hexanoyl derivative	32 (See also 33)

Protein	Ligand	Support	Reference
NADP-dependent dehydrogenases	NADP	Sepharose, hexanoyl derivatives	32
NADP-dependent dehydrogenases	N^6-(6-Aminohexyl)-AMP	Sepharose	32
NADP-dependent dehydrogenases	5'-AMP	Sepharose, hexanoyl derivative	32
Oestradiol-17-β-dehydrogenase	Oestrone	Sepharose, hexanoyl or undecanoyl derivative	43
Oestradiol-17-β-dehydrogenase	Oestrone hemisuccinate	Sepharose, aminoethyl derivative	43
Tetrahydrofolate dehydrogenase	Methotrexate	Sepharose, aminoethyl derivative	44,45
Xanthine oxidase	3-(1-H-Pyrazolo(3,4-d)-pyrimidin-4-ylamino)-1-propyl-6-aminohexanoate	Sepharose	46
	Transferases		
Anthranilate 5-phosphoribosylpyrophosphate phosphoribosyl transferase	Anthranilic acid	Sepharose, aminohexyl succinyl derivative	47

DNA polymerase I	fd DNA	Agarose	48
DNA polymerase I and II	Calf thymus DNA	Agarose	48
Nucleoside deoxyribosyl-transferase	6-(p-Aminobenzylamino)-purine	Sepharose, m-amino-phenyl derivative	39
Phosphofructokinase	Cibacron blue F3G-A	Sephadex	49
T4 Polynucleotide kinase	Calf thymus DNA	Agarose	48
Pyruvate kinase	Cibacron blue F3G-A	Sephadex	50
Ribonuclease, tobacco	5'-(4-Aminophenylphos-phoryl)guanosine-2'(3')-monophosphate	Sepharose	51
Ribonuclease, tobacco	Guanosine 2'(3')-mono-phosphate	Sepharose, hexanoyl derivative	51
RNA-dependent DNA poly-merase (from murine leukemia virus)	Antipolymerase (antibody)	Sepharose	52
RNA polymerase	Calf thymus DNA	Agarose	48

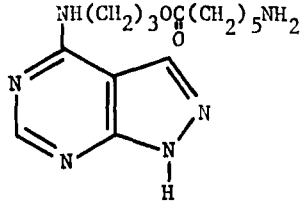
Protein	Ligand	Support	Reference
RNA polymerase	fd DNA	Agarose	48
UDP-galactose: N-acetyl-glucosamine galactosyl-transferase	UDP-hexanolamine followed by 6-amino-1-hexyl-2-acetamido-2-deoxy-β-D-glucopyranoside or α-lactalbumin	Sepharose	53
	Hydrolases		
Acetylcholinesterase	1-Methyl-9-[N^{γ}-(ε-amino-caproyl)-γ-aminopropyl-amino]acridinium bromide hydrobromide	Sepharose	54
Acetylcholinesterase	d-Tubocurarine	Sepharose, extended diazotized p-amino-benzoyl derivative	55
Acetylcholinesterase	2-Aminoethyl p-nitrophenyl methylphosphonate	Sepharose, aminopentyl succinyl derivative	34
Acetylcholinesterase	ε-Aminocaproyl choline	Sepharose, 3,3'-di-aminodipropylamine succinyl derivative	56

Acetylcholinesterase	p-Aminophenyltrimethylammonium bromide, mono- and di-6-aminocaproyl derivatives	Sepharose	57
Anhydro-chymotrypsin	Lima bean inhibitor	Sepharose	58
Collagenase	Calf skin collagen	PAG extrapment	59
Exonuclease III	Calf thymus DNA	Agarose	48
β-Galactosidase	p-Aminophenyl-β-D-thiogalactopyranoside	Sepharose	60
Kallikreins	Guanidated trypsin-kallikrein inhibitor	Cellulose, carboxymethyl derivative	61
Trypsin	Mixture of arginyl peptides	Sepharose	62
	Lyases		
Carbonic anhydrase	Sulfanilamide	Sephadex or Sepharose	63

Protein	Ligand	Support	Reference
	Ligases		
Methionyl-tRNA synthetase	Methionine	Sepharose, aminohexyl derivative	41
Thymidylate synthetase	6-*p*-Aminobenzamidohexyl-2'-deoxyuridyllic acid	Sepharose	64
	Other Proteins		
Acetylcholine receptor	[*N*-(ε-Aminohexanoyl)-3-aminopropyl]trimethyl ammonium bromide	Sepharose	65
Acetylcholine receptor	"CT5263" (Acetylcholine analog)	Sepharose, aminohexyl-*N*-acetyl-homocysteinyl derivative	66
Acetylcholine receptor	*Naja Naja Siamensis* neurotoxin	Sepharose, epichlorohydrin crosslinked	67
β-Adrenergic receptor protein	Norepinephrine	Sepharose, $(3,3'\text{-diaminodipropylamine succinyl})_2$ derivative	36

Folate-binding protein	Folic acid	Sepharose, aminohexyl derivative	68
L-Fucose binding proteins	N-(ε-Aminocaproyl)-β L-fucopyranosylamine	Sepharose	69
Insulin receptor protein	Insulin	Sepharose, various 3,3'-diaminodipropyl-amine succinyl derivatives	37
Long-acting thyroid stimulator (LATS)	LATS binding protein (4S Thyroid protein)	Sepharose	70
Penicillin binding proteins (or D-Alanine carboxy-peptidase)	6-Aminopenicillanic acid	Sepharose	35
Protein mediator (for enhancement of DNA-dependent RNA synthesis)	2,4-Dichlorophenoxyacetic acid	Agarose	71
Soybean agglutinin	N-(ε-aminocaproyl)-β-D-galactopyranosylamine	Sepharose	72
Vitamin B_{12} binding proteins	Vitamin B_{12}	Sepharose, 3,3'-diamino-dipropylamine derivative	73

pyrimidin-4-ylamino)-1-propyl-6-aminohexanoate, X, as the ligand. Both the functional and nonfunctional forms of the



(X)

enzyme possess the full complement of the oxidation-reduction components--molybdenum, iron, FAD, and acid-labile sulfur. However, nonfunctional xanthine oxidase does not possess the cyanolyzable persulfide group, i.e., the persulfide which is removable upon treatment of the enzyme with cyanide. The nonfunctional enzyme can be partially reactivated by incubation with sodium sulfide. Resolution of these two species was elegantly accomplished in the following manner. The 4-substituted pyrazolo(3,4-d)pyrimidine attached to Sepharose was first converted to its 6-hydroxy derivative by incubation at room temperature with xanthine oxidase. Then, a column of the pyrimidine-Sepharose conjugate was made anaerobic by equilibration under nitrogen with 0.01 M $Na_2S_2O_4$ in 0.1 M pyrophosphate, pH 8.5. Approximately 0.6 μmole of salicylate-free xanthine oxidase was then reduced with dithionite and applied to the column. The flow was stopped and the charged column was equilibrated overnight at room temperature to allow sufficient time for complex formation with active enzyme. Low activity, nonfunctional enzyme was subsequently eluted with dithionite-containing buffer.

The column was then washed aerobically with cold 0.1 M pyrophosphate, pH 8.5, containing 1 mM salicylate and 0.2 mM EDTA. The function of the salicylate is to interfere

with the complex forming properties of the derivatized Sepharose column. A great enhancement in color intensity of the reddish brown band of bound xanthine oxidase became apparent as the iron and flavin chromophores reoxidized. Flow was stopped and the column allowed to stand in the cold for 3 to 4 days to permit the slow reoxidation of reduced enzyme-bound molybdenum and the accompanying release of xanthine oxidase from the gel. Final elution of the column with the same buffer gave a reddish brown enzyme fraction of very high activity (Figure 6). This resolution of the xanthine oxidase provides conclusive evidence for the existence of nonfunctional enzyme in preparations containing stoichiometric amounts of molybdenum, FAD, iron, and acid-labile sulfur.

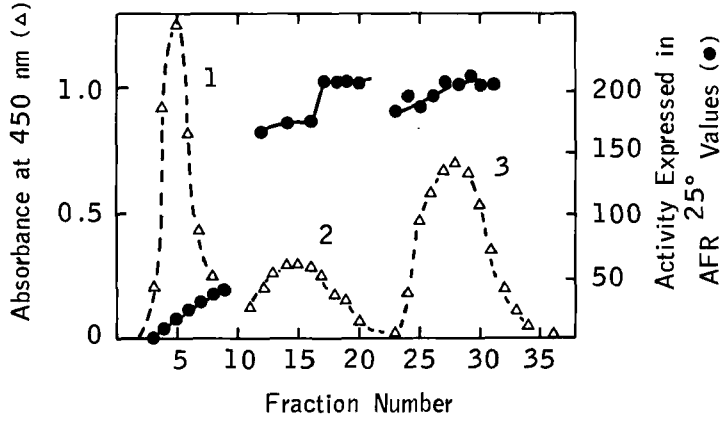
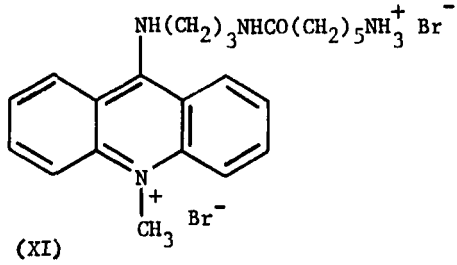


FIGURE 6

Chromatography of functional and nonfunctional xanthine oxidase on the pyrazolo(3,4-d)pyrimidine-Sepharose column. Peak 1 was eluted after a 16 h incubation in the column in 0.1 M pyrophosphate, pH 8.5, containing 0.01 M $Na_2S_2O_4$. Peak 2 was eluted 24 h after washing the column with oxygen-saturated 0.1 M pyrophosphate, pH 8.5, containing 1 mM salicylate and 0.2 mM EDTA. Peak 3 was eluted 72 h after the elution of peak 2. Xanthine-oxygen reductase activity was measured spectrophotometrically at 295 nm at 25° and expressed as $AFR^{25°}$ values (enzymic activity per weight of protein determined by flavin measurement at 450 nm) (from Edmonson et al., ref. 46).

The purification of acetylcholinesterase using LSC is being pursued actively and two such studies illustrate the highly selective isolations attainable. Acetylcholinesterase from the electric organ of electric eel is present in salt extracts of tissue as three main components, which can be distinguished by their sedimentation coefficients (about 18, 14 and 8S) on sucrose-gradient centrifugation at high ionic strength. The 18S and 14S components, which comprise about 90% of the total acetylcholinesterase activity, aggregate at low ionic strength. At high ionic strength, for example in 1 M NaCl, the 14S and 18S forms are not aggregated.

Dudai et al.[54] isolated the 14S and 18S forms from a crude extract by using 1-methyl-9-[N^{γ}-(ε-aminocaproyl)-γ-aminopropylamino]acridinium bromide hydrobromide, XI, bound to Sepharose. The key behind the successful separation is that



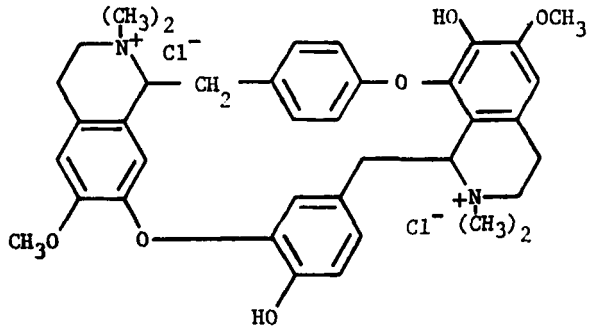
(XI)

the N-methylacridinium ion is a powerful inhibitor of the enzyme even in 1 M NaCl. Separate samples of the purified 14S and 18S components were obtained from the single chromatographic elution peak of the LSC separation, by sucrose gradient centrifugation at high ionic strength. Previous attempts to purify the native forms of acetylcholinesterase using the inhibitor [N-(ε-aminopropyl)-p-aminophenyl]-trimethylammonium bromide hydrobromide, XII, covalently linked to Sepharose

(XII)

failed. With ligand XII at low ionic strength the forms are poorly adsorbed on the resin, and appear, when adsorbed, to be associated with other components. At high ionic strength, where the forms are not aggregated, they are also not adsorbed.

Two interesting and clever separations of acetylcholinesterase species from bovine erythrocytes were accomplished by Jung and Belleau[55] with d-tubocurarine, XIII, bound



(XIII)

to Sepharose. The principle behind the successful separations is the fact that this enzyme possesses allosteric binding sites.

In the first procedure, crude acetylcholinesterase was adsorbed onto the derivatized Sepharose column in the presence of the allosteric effector decamethonium bromide (at a concentration of 0.5 mM in 0.01 M NaCl). Elution of the

column with 0.01 M NaCl in the presence of 0.5 M decamethonium bromide gave a peak that corresponded to 80% of applied activity. The balance of 20% was then eluted with a stronger NaCl solution (0.15 M). The first fraction was dialyzed and reapplied to the column. Washing with 0.01 M NaCl removed some inactive proteins, and subsequent elution with 0.15 M NaCl removed all activity from the column. The K_m and V_{max} values of the purified enzyme (with acetylcholine as substrate) were not significantly different from those of the crude enzyme. However, the purified form was inhibited noncompetitively by decamethonium bromide while the crude enzyme was inhibited competitively by this compound. The interpretation of this behavior given by the investigators was that the bound allosteric site reagent, d-tubocurarine, profoundly alters the molecular structure of the enzyme oligomers or it removes a constituent controlling the binding properties and quaternary structure of the enzyme.

In a second separation procedure, Jung and Belleau fractionated crude acetylcholinesterase into two subspecies on the d-tubocurarine-Sepharose column using increasing concentrations of the effector, β-D-methylsuccinyldicholine iodide, as the eluant. The effector was also present during the application of the enzyme onto the column (Figure 7). The two species, isolated in a ratio of about 1:1 and accounting for 90% of the initial activity, showed single sharp bands on disc gel electrophoresis and exhibited readily distinguishable regulatory properties (based on their kinetic behavior toward methanesulfonyl fluoride). It is to be emphasized that in contrast to these results, when acetylcholinesterase was initially adsorbed on the tubocurarine-Sepharose column in the absence of either effector, it was "hardly elutable" by the same compounds. The presence of decamethonium bromide or β-D-methylsuccinyldicholine iodide on the enzyme presumably alters the quaternary structure in such a manner that the affinity of the curare-binding site (or sites) for the regulatory center is drastically modified.

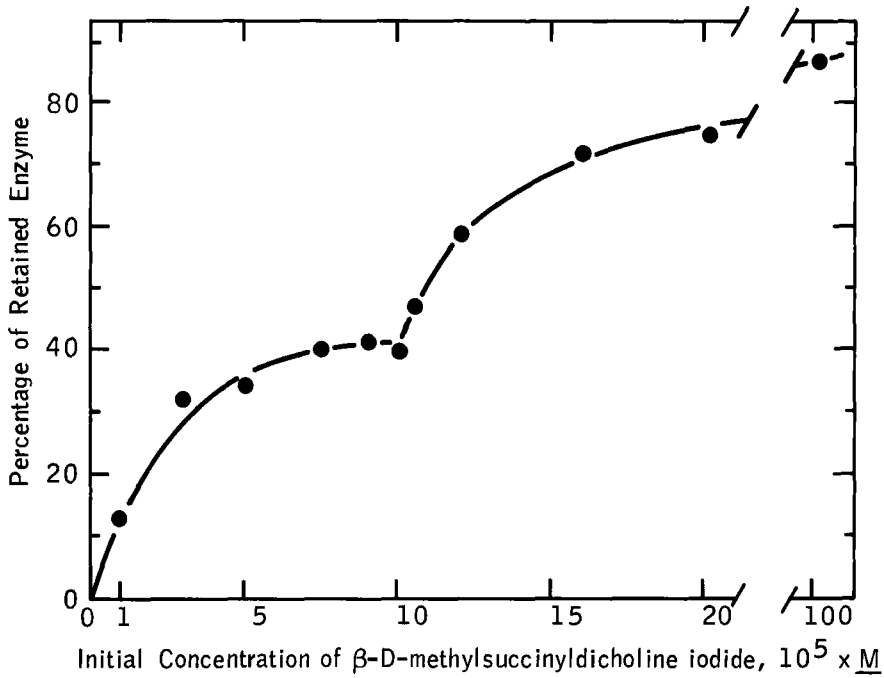


FIGURE 7

Chromatography of acetylcholinesterase on a tubocurarine-Sepharose column with increasing concentrations of β-D-methyl-succinyldicholine iodide. Each point represents the percentage of enzyme retained on the column at each initial concentration of the effector after washing with the same solution until no more activity was elutable (from Jung and Belleau, ref. 55).

The LSC separation of carbonic anhydrase isoenzymes and of a species with a chemically modified active site was reported by Falkbring et al.[63] Sulfanilamide coupled to Sephadex was used to separate human erythrocyte carbonic anhydrase B from C, two isoenzymes obtained from a hemolysate of red blood cells which differ in catalytic behavior and in sensitivity to sulfonamide inhibitors. A good separation of native carbonic anhydrase B and chemically modified B (obtained by treatment of the enzyme with bromoacetate) was also achieved with the same derivatized Sephadex. Bromoacetate selectively

alkylates an active site histidine residue to give the carboxymethyl derivative and this modification alters the catalytic and inhibitor binding properties of the enzyme. A modified enzyme preparation containing 0.51 equivalents of carboxymethylhistidine, eluted first with 0.1 M Tris-sulfate buffer, pH 8, and then with 0.1 M Tris-sulfate buffer containing 0.5 M NaI, gave two separate elution peaks. The protein of the first peak (the modified enzyme) contained 0.72 equivalents of carboxymethylhistidine while the protein of the second peak (the native enzyme) contained only trace amounts (ca. 0.03 equivalents) of modified histidine (Figure 8).

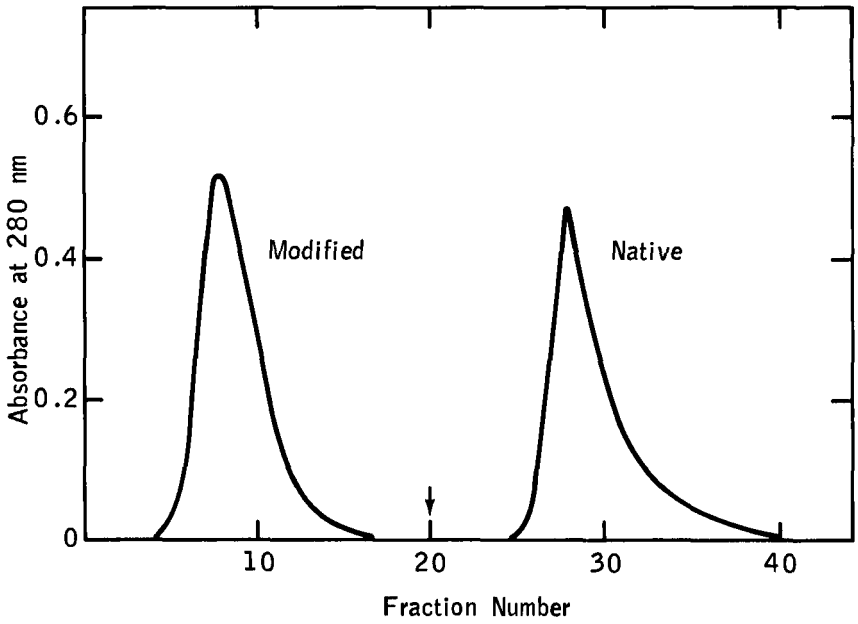


FIGURE 8

Chromatography of bromoacetate-modified and native human carbonic anhydrase B on the sulfanilamide-Sephadex column. Elution with 0.1 M Tris-sulfate buffer, pH 8, and then with 0.1 M Tris-sulfate, pH 8, containing 0.5 M NaI (arrow indicates second elution system) (from Falkbring et al., ref. 63).

PEPTIDES

Specific chemical modification is one of the most powerful tools available for investigating the structure and catalytic activity of proteins (for reviews see references 74 and 75). In this procedure, the native protein is first treated with a chemical reagent specific for certain amino acid residues, and then hydrolyzed to give a mixture of peptides. The peptide containing the particular amino acid residue(s) modified is then isolated and identified (Figure 9). For several reasons, it is this isolation procedure which often presents the major difficulty in such studies. First, the modification reaction is usually not totally selective and several different modified residues are often obtained in various yields. Furthermore, digestion of even a specifically modified protein will give a mixture of peptides of varying length, all containing the modified residue. Finally, conventional methods for the isolation of peptides are laborious and inefficient. Thus, the recent development of the use of LSC for the isolation of specifically modified peptides by several investigators at the Weizmann Institute of Science is particularly timely and significant.

The first reports of this application of LSC were those of Givol et al.[76] and of Wilchek,[77] which describe the isolation of labeled peptides derived from affinity-labeled enzymes or antibodies. The principle behind these studies is illustrated in Figure 10 for the case of an enzyme. A substrate analog which possesses a chemically reactive group is mixed with the enzyme, and by virtue of its structural similarity to a substrate, is bound specifically at the active site in the classical reversible fashion. Once bound, the chemically reactive group reacts covalently with a residue at or near the active site, thereby effecting the "affinity label." Thus, when the modified enzyme is subsequently digested, one of the peptides obtained is covalently labeled with the chemical

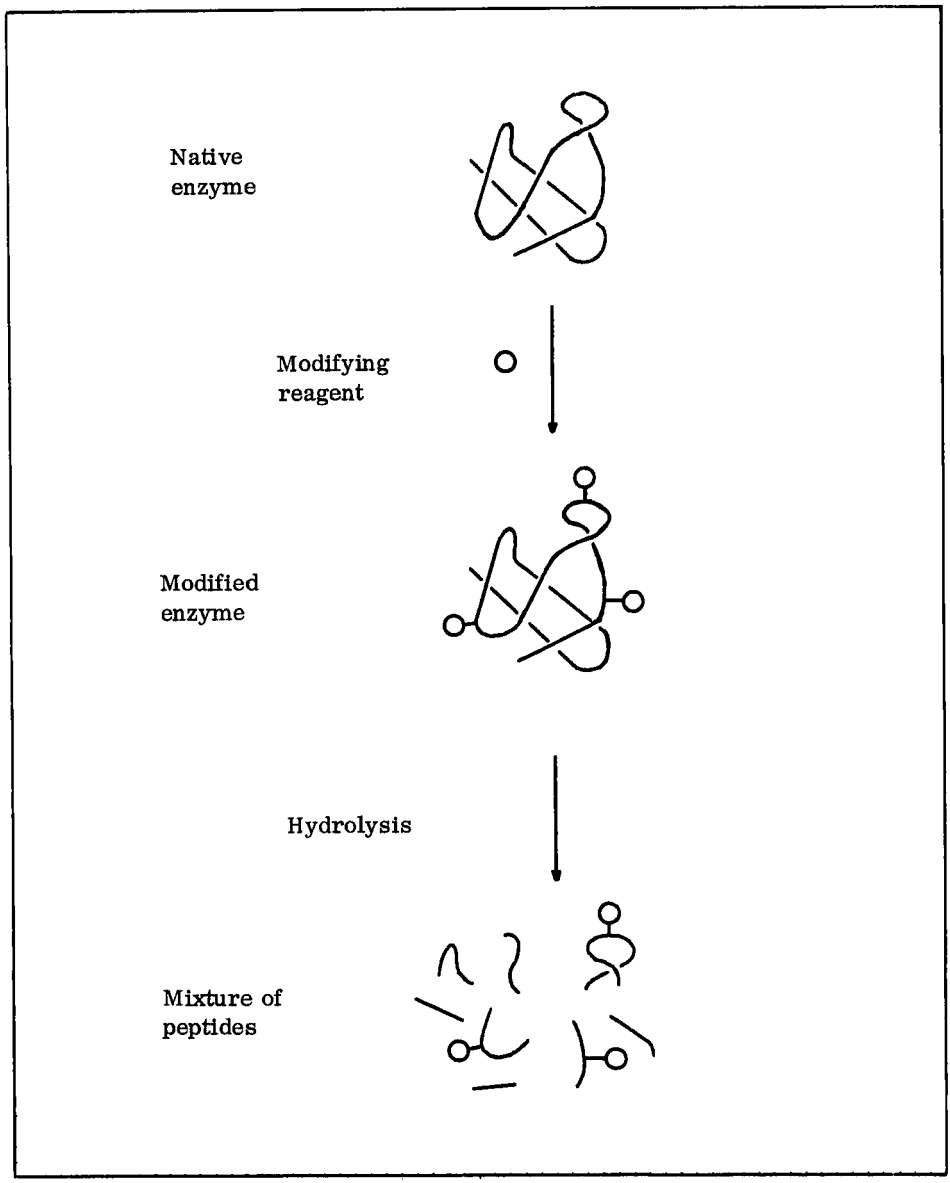


FIGURE 9

Chemical modification of a protein followed by hydrolysis to give a mixture of peptides.

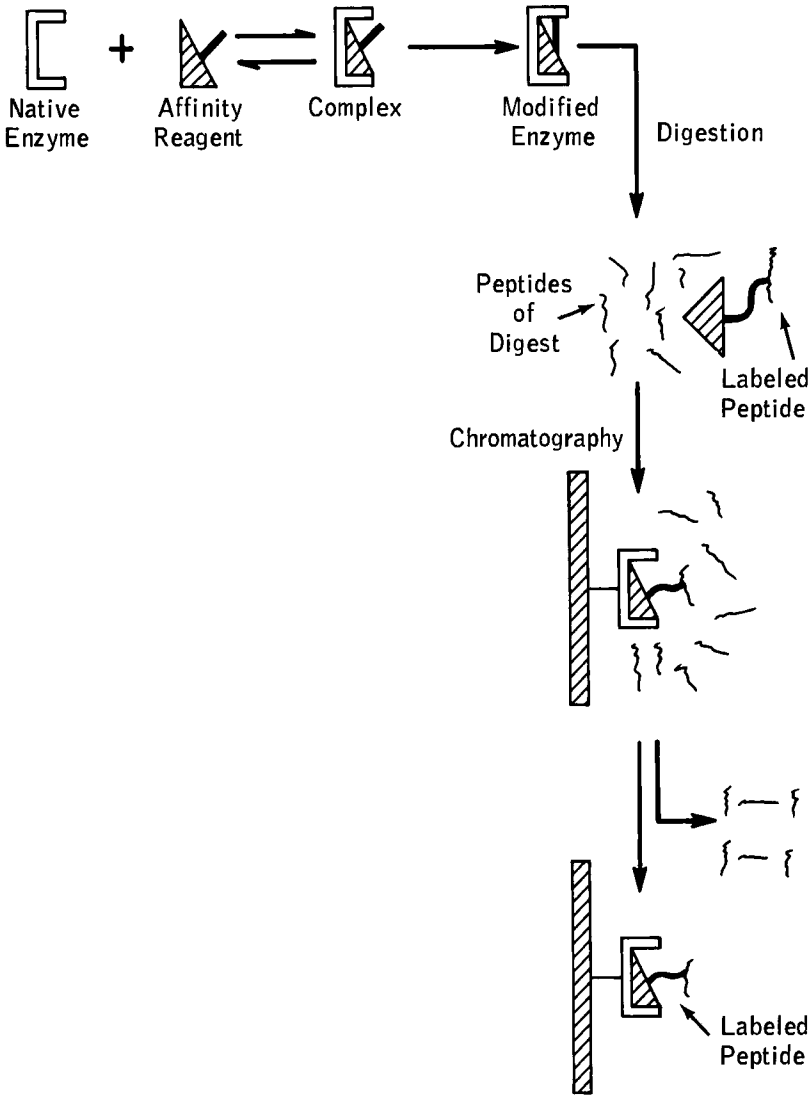


FIGURE 10

Isolation of an affinity labeled peptide.

reagent, which still possesses its strong structural similarity to a substrate of the original enzyme. If the native enzyme is then attached to a solid support and the entire digest of the modified enzyme passed over it, the labeled peptide will be specifically retained on the column and thereby separated from all other peptides. Subsequent elution of the column under conditions which dissociate the enzyme-substrate analog complex gives the labeled peptide--isolated in essentially one step.

Using this principle, Givol et al.[76] succeeded in isolating a uridine diphosphate (PUDP)-labeled peptide from bovine pancreatic ribonuclease (RNase) and a dinitrophenyl (DNP)-labeled peptide from an antibody to DNP. In both cases the chromatographic step was accomplished using the native RNase or antibody coupled to Sepharose 4B through the CNBr activation procedure, and the labeled peptides were eluted with 0.8 M NH_4OH (for RNase) or 20% formic acid (for the antibody). The modified peptide from RNase was recovered in 60% yield and freed from minor impurities by paper electrophesis. Analysis revealed that this peptide was identical to the PUDP-peptide of RNase previously isolated by several steps of chromatography and electrophoresis. After one cycle, the capacity of the RNase column to bind the PUDP peptide had decreased by 20%. Similarly, the DNP-labeled peptide from the antibody to DNP was recovered in 75% yield, and was identified after paper electrophoresis as a lysyl peptide of twelve residues containing a DNP-labeled tyrosine. The capacity of the antibody column to bind DNP decreased 40% after one cycle of adsorption and elution.

It is noteworthy that in both of the above cases it was necessary to carry out a final step of electrophoresis before the eluted peptide could be obtained in pure form. Apparently, some of the protein attached to the Sepharose had been released under the elution conditions, a fact which might account for the losses in column capacity observed after one

cycle. This problem seems especially significant when the compound to be purified is itself a peptide or protein because the removal of impurities which are structurally similar to the desired material is often not a simple task. Another consideration is that Givol et al.[76] noticed a relatively rapid decrease in the specific binding capacity of the Sepharose-antibody column, unless the coupling of the antibody to the Sepharose was carried out in the presence of a "protector"--a low-affinity analog of the hapten. However, despite these limitations, the advantages of the simple and rapid LSC procedure over the laborious standard procedures for peptide isolation are readily apparent.

Wilchek[77] reported the success of a similar approach for the isolation of affinity-labeled paptides from staphylococcal nuclease. The labeled peptide from the enzyme modified with the bromoacetyl derivative of deoxythymidine-3'-p-aminophenylphosphate-5'-phosphate, XIV, was obtained in 80% yield and was identical to material previously isolated by several steps of chromatography, electrophoresis and radioautograms. It is very interesting to note that while the peptide labeled with XIV was selectively bound by the nuclease-Sepharose column, that labeled with bromoacetyl-p-aminophenylphosphate, XV, emerged together with the other peptides in the digest (Figure 11). This is due to the fact that XV, which lacks the thymidine ring, has a poor affinity for the nuclease, and the result underscores the specific binding principle responsible for the separation. Only those ligands having a high affinity for the protein will be specifically absorbed on the protein-Sepharose column. More recently, Wilchek[78] reported the isolation of the heme peptide of cytochrome c using bovine or human serum albumin-Sepharose columns. This procedure takes advantage of the propensity of these albumins to bind the heme peptide.

Although the use of native protein-Sepharose columns is a powerful tool for the isolation of labeled peptides, its

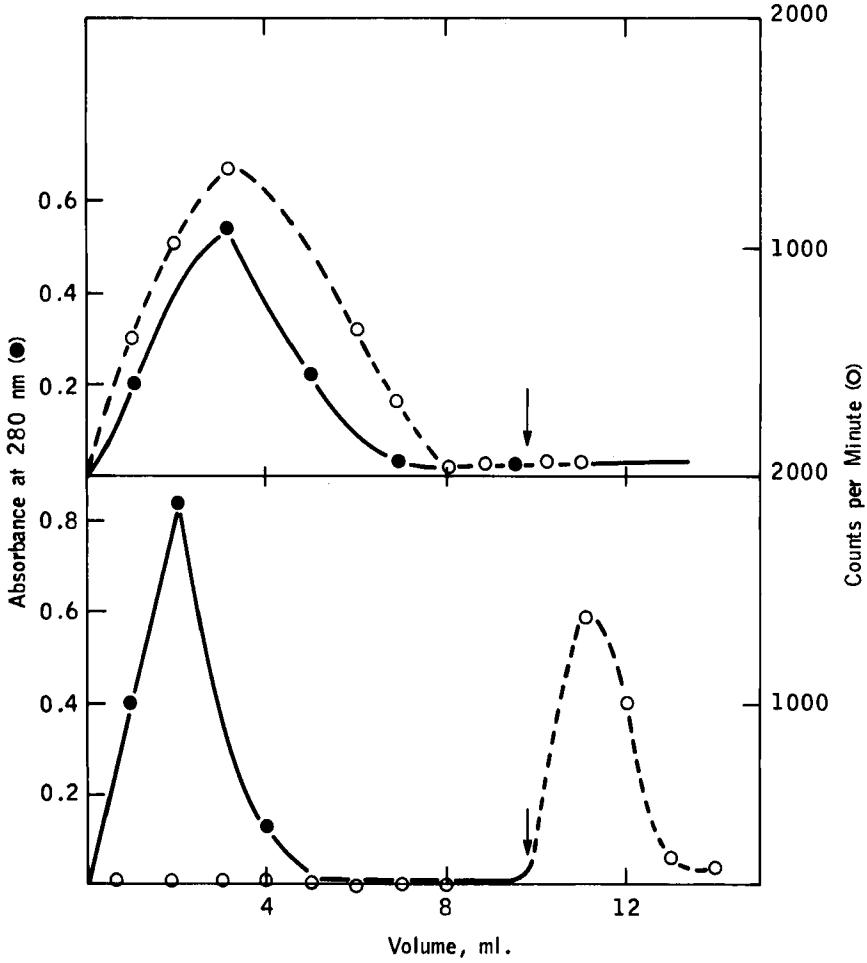
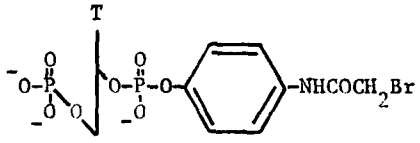
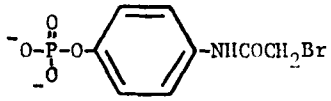


FIGURE 11

Elution of labeled peptides from a staphylococcal nuclease-Sepharose column. The top profile was obtained for the peptide labeled with XV and the bottom profile for that labeled with XIV. Tryptic digests of the modified enzyme were applied in 0.05 M borate, pH 8.0, containing 10 mM $CaCl_2$. At the point indicated by the arrow, NH_4OH, pH 11.0, was applied to the column (from Wilchek, ref. 77).



(XIV)



(XV)

application is limited to cases of affinity labeling. In contrast, antibody-Sepharose columns can, in principle, be used for the isolation of a large variety of labeled peptides, since specific antibodies with high affinity can be raised against almost any small molecule. The only requirement is that the portion of the modifying reagent recognizable to the antibody must not be so structurally altered in the labeled peptide so as to preclude its specific binding to the antibody column.

As mentioned above, Givol et al.[76] first reported the use of such columns for the isolation of a DNP-labeled peptide from the antibody itself, and the Weizmann Institute investigators have since improved this technique and extended it to a variety of other peptides. Wilchek et al.[79] reported the isolation of arsanilazotyrosyl peptides from arsanilazo-N-succinylcarboxypeptidase A using an anti-arsanilazo antibody-Sepharose column, and of the DNP-lysyl peptide from mono-DNP-ribonuclease using an anti-DNP antibody-Sepharose column. The arsanilazo-peptides were eluted with 1 M NH_4OH, after which the column capacity had decreased 15-20%. However, no further

decrease in capacity was observed after the second cycle and the column was used repeatedly six times. The DNP-peptides were eluted with 6 M guanidine HCl, after which the antibody-Sepharose column had lost most of its capacity and could not be reused. In addition, the guanidine-HCl treatment released a small amount of antibody from the column, causing difficulty in the purification of the desired peptide. These results are similar to those of Givol et al.,[76] quoted above, and illustrate some of the difficulties encountered in the use of insoluble antibody columns.

In an extension of this work, Helman and Givol[80] prepared antibodies to 3-nitrotyrosine, and purified them on a nitro-γ-globulin-Sepharose column. They then successfully used the purified antibodies, coupled to Sepharose, for the isolation of nitrotyrosine-containing-peptides from nitrated lysozyme. In this case, the nitrotyrosine hapten is part of the peptide chain itself and not a small molecule introduced by chemical modification of an amino acid residue. Elution was accomplished with 1 M ammonia and the column could be used 10 times without significant loss in capacity. A somewhat different approach is that of Bustin and Givol[81] who isolated the same modified peptides from nitrated lysozyme by converting the nitrotyrosine residues to DNP-aminotyrosine. Readily available anti-DNP antibodies were then attached to Sepharose and used for the isolation of the modified peptides. Thus, appropriate further modification of an already modified residue can allow isolation of the desired peptide on a more readily available antibody column.

Very recently, Wilchek and Miron[82] reported the successful isolation of tryptophan-containing peptides by first modifying the tryptophan residue with 2,4-dinitrophenylsulfenyl chloride, and then using the familiar anti-DNP antibody-Sepharose column in the chromatographic step. Ten percent formic acid was used for the elution, and the authors claim

that this treatment does not release attached antibodies from the column. Thus, no contaminating protein emerges along with the desired material, and the procedure is an example of a true one-step isolation of a modified peptide using LSC.

CARBOHYDRATES AND GLYCOPROTEINS

A number of polysaccharides and glycoproteins have been purified by LSC using concanavalin A (Con A) as the ligand. This phytohomaglutinin, isolated from the jack bean Canavalia ensiformis, has been purified to homogeneity, and its structure and chemistry have been characterized quite extensively.[83-85] A good introductory review by Sharon and Lis[83] of this and other lectins has recently appeared.

Con A is a globular protein that exists in solution largely as dimers below pH 6 and as tetramers above pH 7, the molecular weight of the monomeric unit being 25,500. The tentative sequence of the 238 amino acid residues in this protein has been determined mainly by chemical means but partially on the basis of X-ray crystallographic data.[84] Con A is also a metallo-protein, requiring Mn^{+2} and Ca^{+2} for saccharide binding. The calcium ion binds only after the manganese ion site is occupied, and occupancy of both metal sites is essential for saccharide binding. Both metal binding sites are approximately 20 Å from the saccharide binding site, a deep pocket in the molecule the inner portion of which is occupied by hydrophobic residues. Con A exhibits a high degree of specificity toward polysaccharides and glycoproteins having terminal nonreducing α-D-glucopyranosyl, α-D-mannopyranosyl, β-D-fructofuranosyl, or α-D-arabinofuranosyl residues.[86] Low molecular weight saccharides also interact with Con A and are used commonly for the elution of polysaccharides. The values of the binding constants, K_a, of methyl α-D-mannopyranoside and methyl α-D-glycopyranoside, determined by equilibrium dialysis

measurements, are 1.4 x 10^4 and 0.3 x 10^4 liter/mole, respectively.[87] Contrary to most other lectins, Con A itself is not a glycoprotein.

Most often, Con A has been immobilized by covalent attachment to Sepharose through the cyanogen bromide procedure,[88-90] but insolubilization has also been achieved by intermolecular crosslinking with glutaraldehyde[91,92] and by copolymerization with L-leucine-N-carboxyanhydride.[88]

The interactions of polysaccharides and glycoproteins with various water-insoluble derivatives of Con A reveal further aspects of its specificity and its potential for purification purposes. Lloyd[88] observed strong binding of Dextran B1355 and yeast mannan to the insoluble conjugates of Con A with poly-L-leucine or Sepharose. For the poly-L-leucine derivative, the degree of dextran binding was dependent on the ratio of reagents used in the copolymerization. The maximum weight of Dextran B1355 absorbed(520 μg/mg of Con A conjugate) occurred at a weight ratio of 1 (weight of L-leucine-N-carboxyanhydride to weight of Con A); lower or higher ratios showed decreased dextran adsorption (395 and 345 μg/mg Con A conjugate at ratios of 0.5 and 2.0, respectively). The efficiency of binding relative to that of soluble Con A (42%) was also highest at a reagent ratio of 1. Analogous results were obtained with the yeast mannan except that the adsorption capacity of both insoluble Con A preparations was lower by a factor of about five. For example, the maximum binding of mannan to the polypeptide conjugate obtained at a reagent ratio of 2 was 83 μg/mg Con A conjugate.

Both Dextran B1355 and mannan are highly branched polysaccharides. On the contrary, Dextran B512-F, a polysaccharide which has a very low degree of branching and precipitates only at high concentrations of native soluble Con A, was not removed from solution by the water-insoluble conjugates to any significant extent. This selectivity of Con A

indicates that specificity is dependent not only on the presence of appropriate terminal groups in the polysaccharide but also on the degree of branching.

Other systems examined by Lloyd included hog blood group substance and a galactomannan from the yeast Cladosporium werneckii. His separations of hog blood group substance by LSC and by a precipitation method are especially illustrative of the advantages and disadvantages of each of these processes. Precipitation of hog blood group substance with Con A gave two fractions. The supernatant fraction did not precipitate with Con A and retained its blood group activity. The precipitated fraction was both blood group and Con A active (comparable to the original blood group substance in its precipitating capacity). However, a decided disadvantage of the precipitation method is the considerable difficulty encountered in reisolating pure fractions from the system.

In the column operation using a poly-L-leucyl-Con A conjugate, two fractions were again obtained. One of these was adsorbed on the column, while the other was not. When the isolated fractions were tested with soluble Con A, the non-adsorbed fraction still retained some ability to precipitate with Con A while the adsorbed fraction was only slightly better than the original mixture in Con A activity. Column overloading and insufficient equilibration (to form the ligand-component complex) were eliminated as possible factors for this finding by appropriate controls. Thus, in comparison to the precipitation method, the LSC method gave a fraction enriched in Con A activity but failed to give a fraction which did not precipitate with the lectin.

In Lloyd's studies, elution of adsorbed carbohydrates was accomplished with methyl α-D-mannopyranoside. The Sepharose-Con A conjugate could be reused after washing with about 200 volumes of 1 M NaCl. After use at room temperature, the insoluble lectin retained about two-thirds of its activity; after use at 4°, essentially all the activity was retained.

Any column operation using poly-L-leucyl Con A must also employ an inert support such as Biogel.

Other polysaccharides and glycoproteins shown to interact specifically with insolubilized Con A include rabbit liver glycogen,[91] immunoglobulin M,[91,92] Dextran B-1355-S,[91] glycoproteins from human serum,[89,92] glucose oxidase,[92] peroxidase,[92] and several glycoprotein hormones (human chorionic gonadotrophin, luteinizing hormone, and follicle stimulating hormone).[90]

The studies of Dufau et al.[90] on the interaction of hormones with Con A-Sepharose conjugates illustrate the potential value of LSC in the purification of glycoprotein hormones. Human chorionic gonadotrophin from blood and urine was recovered in good yield and exhibited enhanced binding potency after purification. When partially purified preparations (Pregnyl, obtained from Organon) were subjected to LSC, purifications of up to 3-fold were observed. It was suggested by these workers that the most useful applications of insolubilized Con A may lie in the analysis of glycoprotein hormones in small samples of plasma or urine and in the bulk fractionation, at early stages, of crude tissue or urinary extracts prior to further purification. The ability of antibody-bound human chorionic gonadotrophin to be adsorbed by insolubilized Con A was also noted. This interaction (shown in equation 7) confirms the fact that

$$\text{Glycoprotein}\cdots\text{Antibody} + \left|\text{-Con A}\right. \qquad \left|\text{-Con A}\cdots\text{Glycoprotein}\cdots\text{Antibody}\right. \tag{7}$$

carbohydrate residues are not involved in the immunological activity of the hormone. In this as well as in other studies,[91]

the specificity of insolubilized Con A toward carbohydrate-containing substances was the same as exhibited by unmodified, soluble Con A. It is interesting to point out that Con A itself may be purified conveniently from the jack bean by LSC using Sephadex (a crosslinked dextran); elution is accomplished with either D-glucose or a buffer of low pH.[88,93,94]

An example of the isolation of a glycoprotein without the use of Con A was reported by Warecka et al.[95] Human brain-specific alpha$_2$-glycoprotein was partially purified by means of a series of Sepharose immunoadsorbents prepared from cyanogen bromide-activated agarose and brain extract, a mixture of liver extract and human serum, and purified brain-specific antibodies. In addition, a second brain-specific protein was found along with the glycoprotein. This component of the system had not been detected previously by testing the crude brain extracts with anti-brain serum. Its appearance only after the purification procedure suggests that this new component was enriched by LSC.

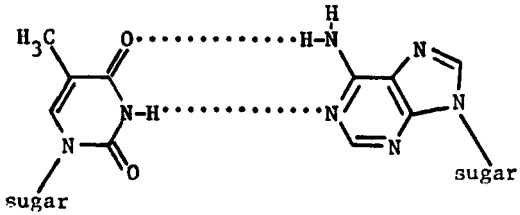
NUCLEIC ACIDS AND COMPONENTS

A considerable amount of work has been reported on the isolation and purification of nucleosides, nucleotides, and polynucleotides (both natural and synthetic) through the use of LSC. In fact, this kind of chromatography was employed successfully as early as 1962.[5-7] For the separation of these compounds, nucleotides, polyribonucleotides, polydeoxyribonucleotides, or proteins have been used as ligands. The affinity of nucleic acids for these ligands is due to either complementary base pairing or specific nucleic acid-protein interactions.

Complementary base pairing is the interaction of purine and pyrimidine bases of nucleosides, nucleotides, and polynucleotides to form hydrogen bonded bridged structures

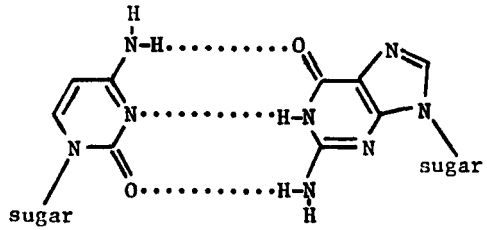
such as those given below for adenosine (A) and thymidine (T) (structure XVI) and for cytidine (C) and guanosine (G) (structure (XVII). This association is also responsible for the double helical structure of nucleic acids first proposed by Watson and Crick. The elution of nucleic acids (or components) from adsorbents containing complementary nucleic acids (or component nucleotides) is achieved by disrupting this favorable base pairing. Destabilization of hydrogen bonding in nucleic acids can be achieved by (1) lowering the ionic strength, (2) increasing the concentration of organic solvents such as formamide and dimethylformamide, or (3) increasing the temperature. Commonly employed

Thymidine Adenosine



(XVI)

Cytidine Guanosine



(XVII)

water-insoluble supports for the immobilization of polynucleotides or their components are cellulose and the phosphorylated derivative of this polysaccharide.

The nucleic acid-protein interactions may be due to hydrogen bonds, ionic bonds, van der Waals attractive forces,

etc. These specific and selective interactions can be disrupted by increasing the ionic strength of the eluant. Sepharose has generally been used as the water-insoluble support for the coupling of nucleic acid-binding proteins. Only a few examples are given here to illustrate the potential of LSC with these classes of compounds.

The separation of nucleosides and nucleotides with an oligothymidine-cellulose conjugate, $p(T)_n$-cellulose, prepared by the reaction of thymidine 5'-phosphate with cellulose and N,N'-dicyclohexylcarbodiimide, was investigated by Sander et al.[96] Figure 12 shows the chromatographic elution patterns for a mixture of nucleosides and nucleotides. The better retention of the nucleosides compared with that of the nucleotides was suggested to be due to electrostatic repulsion between the phosphate groups of the nucleotides and those of the bound oligothymidylate. The slight retardation of the guanosine and guanosine 5'-phosphate was interpreted to be a reflection of the greater basicity of these compounds. As expected, adenosine and adenosine 5'-phosphate were preferentially retained on the column. Similarly, Saxinger et al.[97] examined the interaction of nucleotides with amino acids immobilized on polyvinylamine gel particles and Fish et al.[98,99] reported the adsorption of cyclic adenosine 3':5'-monophosphate (c-AMP) with immobilized cyclic AMP-binding proteins.

A number of excellent studies which clearly demonstrate the potential of LSC for the separation of oligo- and polynucleotides have been reported. Gilham, a leading advocate of the application of LSC to nucleic acids, showed the complete and quantitative separation of the hexanucleotides of thymidine, $p(T)_6$, and deoxyadenosine, $p(dA)_6$, on a column of oligothymidine-cellulose.[6] Figure 13 shows the results obtained when a mixture of $p(T)_6$ and $p(dA)_6$ in 1 M NaCl solution is adsorbed onto a column of $p(T)_n$-cellulose conjugate (where n can be as high as 20) and eluted by changing the temperature of the column. The thymidine oligomer was eluted at the solvent point

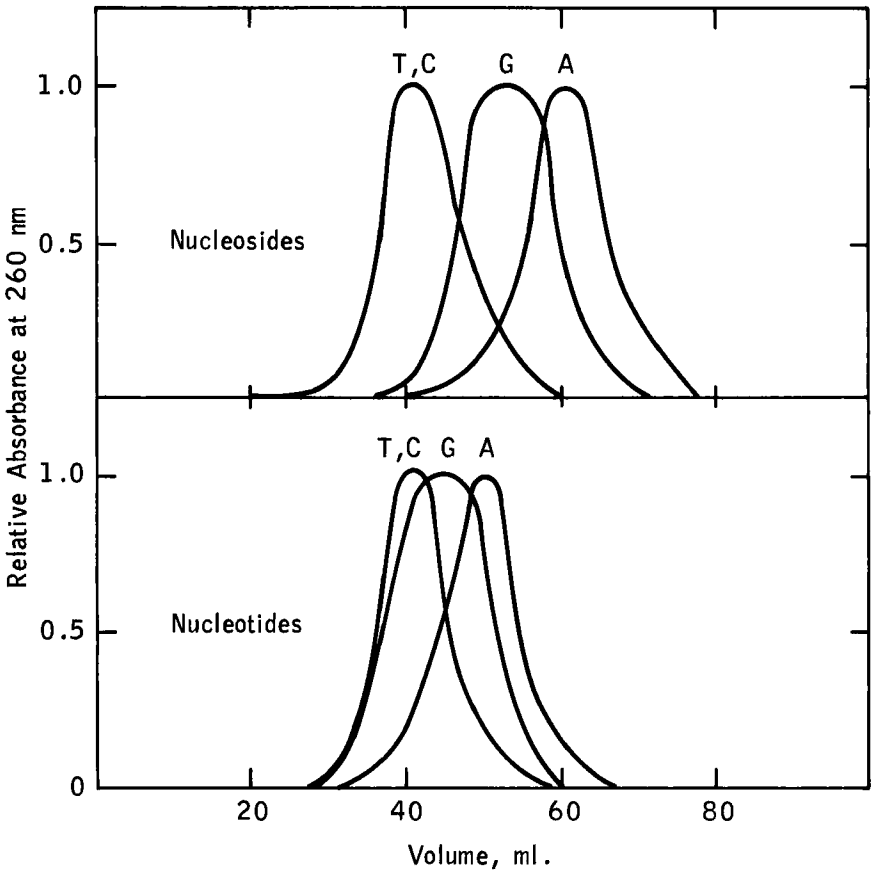


FIGURE 12

Chromatography of nucleosides and mononucleotides on a $p(T)_n$-cellulose column. Elution with 1 M NaCl in 0.01 M sodium phosphate buffer, pH 7, at 5°C (from Sander et al., ref. 96).

at 4° while the deoxyadenosine oligomer was retained. The dependency of the temperature of elution on the number of consecutive deoxyadenosine residues in an oligonucleotide was also investigated.[100] A mixture of the deoxyadenosine tri-, tetra-, penta-, hexa-, and heptanucleotides applied to an oligothymidine-cellulose column (a conjugate having chains up to 12 nucleotides long) showed the elution pattern given in Fig-

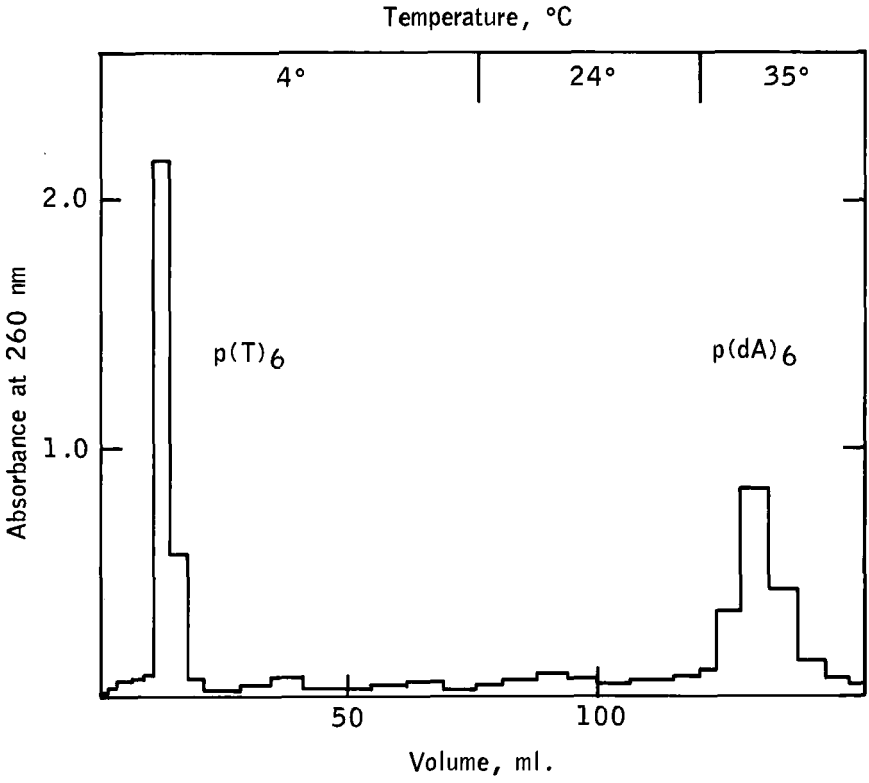


FIGURE 13

Chromatography of $p(T)_6$ and $p(dA)_6$ on a $p(T)_n$-cellulose column (chain length n can be as high as 20). Thermal elution with 1 M NaCl in 0.01 M sodium phosphate buffer, pH 7 (from Gilham, ref. 6).

ure 14 when the column was subjected to a stepwise change in temperature. The small unidentified peaks are presumed to be impurities introduced by the reactions used for syntheses of the oligonucleotides. The separation of oligouridines, oligo (U), (where n is from 4 to 15) on a column of oligo $p(dA)_n$-cellulose (where n is a number up to eight)[100] and of oligonucleotides derived from the ribonucleic acid of bromegrass mosaic virus on a column of $p(T)_n$-cellulose[101] was also demonstrated. The latter investigation also revealed the

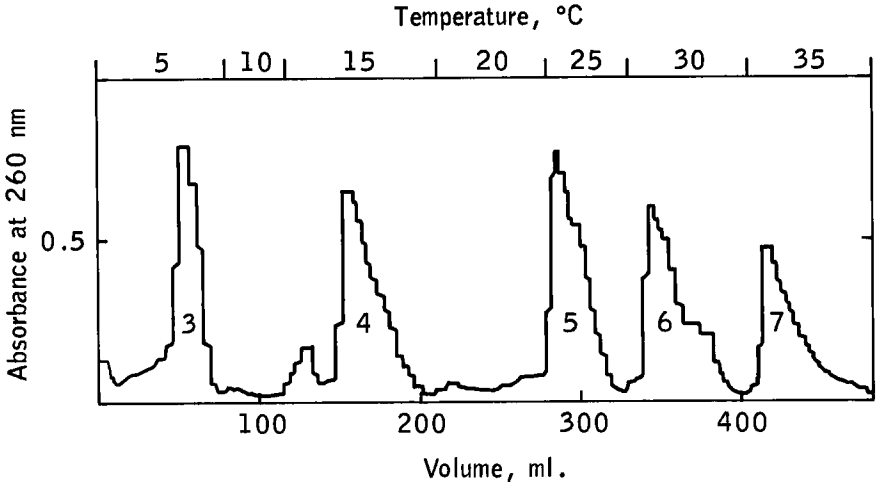


FIGURE 14

Chromatography of $p(dA)_n$ (chain length n from 3 to 7) on a $p(T)_n$-cellulose column (chain length n can be as high as 12). Thermal elution with 1 M NaCl in 0.01 M sodium phosphate buffer, pH 7 (from Gilham, ref. 100).

potential of oligonucleotide-cellulose conjugates for estimating the type and size of the consecutive adenosine sequences in oligonucleotides.

Astell and Smith[102] extended the early work of Gilham and colleagues and recently reported the results of their fine study on the separation of $p(dA)_n$ (where n is from 6 to 11) on $p(dT)_9$-cellulose. Their results indicated that when the length of the deoxyadenosine nucleotide is equal to, or shorter than, that of the cellulose bound $p(dT)_9$, oligomers differing in length by 2 nucleotide residues are completely resolved on a 9 mm diam. x 50 mm column, while oligomers differing in length by only 1 nucleotide are less completely resolved. However, use of a longer column (150 mm) of $p(dT)_9$-cellulose with a ratio of bound oligomer to complementary oligomer of 20:1 gave complete resolution. If the Tm^c (the temperature of the midpoint of a peak of oligonucleotide eluted from the oligonucleotide-cellulose column) for each oligomer is plotted vs.

the length of the oligomer (Fig. 15), a linear relationship exists between the two up to the point where the length of the $p(dA)_n$ equals the length of the cellulose-bound $p(dT)_n$. These data were interpreted by Astell and Smith to indicate that the entire oligonucleotide attached to the cellulose is capable of hydrogen bonding with a complementary sequence. The increase in Tm^c after the length of the free oligonucleotide exceeds that of the bound oligonucleotide might be due to the extended stacking of the purine bases. The possibility that the increased stability was due to the participation of adjacent bound oligonucleotides in the binding of a single oligonucleotide chain was discounted on the basis of the rather low degree of loading obtained (0.3-1 μmole of bound oligomer/g

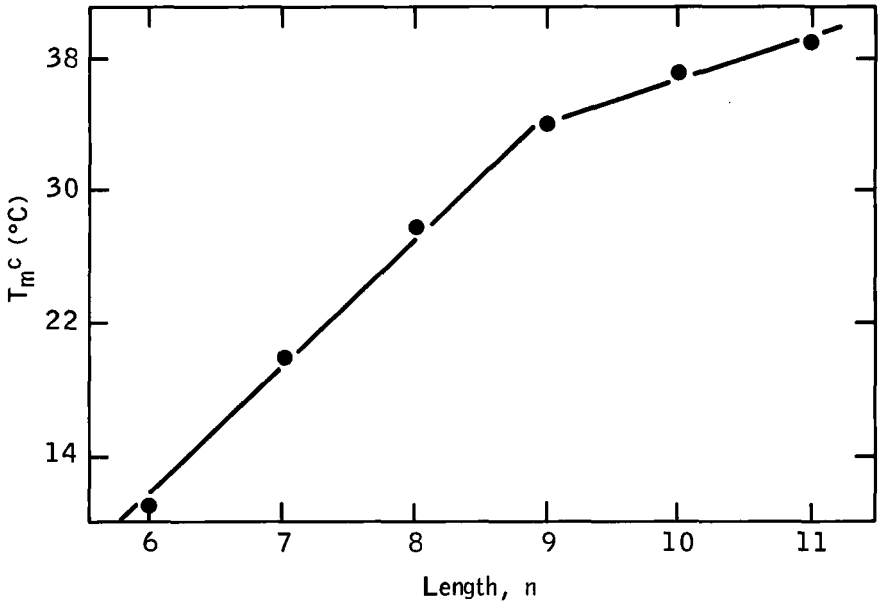


FIGURE 15

Relationship of T_m^c vs. chain length of $p(dA)_n$ on a $p(dT)_9$-cellulose column. Thermal elution with 1 M NaCl in 0.01 M sodium phosphate buffer, pH 7 (from Astell and Smith, ref. 102).

of cellulose). This degree of modification would mean that fewer than one glucose residues in 6000 are substituted.

Chromatography of ribooligonucleotides (the series $r(A)_n$ where n=6 to 9) and dephosphorylated deoxyribonucleotides (the series $d(A)_n$ where n=6 to 9) on the same $p(dT)_9$-cellulose column revealed that ribooligonucleotides elute 4-9° lower than the corresponding deoxyribooligomers and that the phosphorylated $p(dA)_n$ series elutes approximately 1-2° lower than the dephosphorylated series $d(A)_n$. The latter finding suggests that even at a very high salt concentration (1 M NaCl), the 5'-phosphate group on the oligonucleotide is capable of reducing the stability of the hybrid structure. According to these workers, the Tm^c of a particular complementary oligomer was quite reproducible ($\pm$ 0.5°) within a given batch of oligonucleotide-cellulose conjugate. However, variability did exist between different preparations.

Synthetic polyribonucleotides have also been shown to selectively interact with complementary oligonucleotide- or polynucleotide-cellulose conjugates.[6,96,103-105]

Certainly one of the most important uses of LSC in nucleic acid chemistry is for the isolation and purification of such macromolecules as messenger RNA, transfer RNA, ribosomal RNA, and DNA from various sources. A large variety of ligands ranging in size and complexity have been employed, and the chemistry of coupling and the experimental conditions used for elution have varied equally. Table II gives a brief summation of the macromolecules isolated by LSC.

In concluding this section, it should be mentioned that immobilized oligo- and polynucleotides can also be used for the isolation of proteins (for a leading review see ref. 106) and for mechanistic studies. For example, an oligomer of deoxyribothymidylate bound to cellulose was used as a primer and a template for *E. coli* DNA polymerase, as a template for RNA polymerase, and as an initiator for the calf thymus terminal deoxynucleotyltransferase.[107]

MISCELLANEOUS SUBSTANCES

The use of an anti-insulin globulin-Sepharose conjugate for the specific binding of the hormone, insulin, has been described by Akanuma et al.[115] In preliminary studies using purified, labeled insulin, it was established that the hormone was specifically retained on a column of the conjugate at pH 8.2 to 8.4, and that it could be eluted sharply with 1 M acetic acid. After one cycle, the column was equilibrated with buffer and reused without any apparent loss in its insulin-binding capacity. This procedure was then used successfully for the specific extraction of insulin from crude dog pancreatic vein serum. As before, above pH 8 the unadsorbed fraction contained mainly serum proteins, while insulin was retained on the column, and then eluted with 1 M acetic acid.

Using the same approach, Gospodarowicz[116] employed an antibody-Sepharose column for the isolation of luteinizing hormone from horse or fetal calf serum and from extracts of ovine pituitary glands. The ovine hormone was eluted with 6 M guanidine-HCl, pH 1.5, and was shown to be homogeneous by several criteria. However, the elution conditions caused dissociation of the isolated hormone into its two subunits, and biological activity was recovered only after recombination of the subunits through dialysis. The final preparation possessed 50% of the biological activity of native ovine luteinizing hormone. Similarly, glucagon has been purified from pig ileum extracts using an antibody-Sepharose column and 4.25 M ethanol in 4 mM HCl as the eluant.[117]

Guyda and Friesen[118] used LSC to separate growth hormone activity from prolactin activity in monkey pituitary fractions. This was accomplished by coupling antibodies for human placental lactogen to CNBr-activated Sepharose and passing monkey pituitary incubation media or tissue homogenates over a small column (1.0 cm) of the conjugate. The column removed more than 99% of the growth hormone activity from the unadsorbed

TABLE II

NUCLEIC ACIDS ISOLATED BY LSC

Nucleic Acid Isolated	Source	Ligand	Support	Reference
DNA	*B. subtilis*	Ribosomal RNA	Methylated cellulose	108
DNA	*E. coli*	16S Ribosomal RNA	Sepharose, caproyl hydrazine derivative	104
mRNA	Polysomes of KB-cells	Polyuridylic acid	Sepharose	109
mRNA, rabbit globin	Ribosomal RNA in crude polysomal extracts	Oligodeoxythymidine	Cellulose	110
mRNA, T4-specific	*E. coli* RNA	DNA, T4-wild type	Acetylated phosphocellulose	4
mRNA of $T4r^+$ phage	*E. coli* RNA	DNA of $T4r^+$ wild type and of mutant phage r1273	Nitrocellulose	111

rII-RNA (complementary to the rII region of the T4 genome)	*E. coli* RNA	DNA from the T4 mutants r1272 and r^+	Acetylated phosphocellulose	4
Ribosomal RNA (16S and 23S)	*E. coli*	Ribosomal proteins	Sepharose	112
tRNA	*E. coli* RNA	Oligothymidine	Cellulose	5
Isoleucyl-tRNA	*E. coli* RNA	Isoleucyl-tRNA synthetase	Sepharose	113
Lysyl-tRNA	*E. coli* RNA	Polydeoxyadenosine	Cellulose	114
Phenylalanyl-tRNA	*E. coli* RNA	Polythymidine	Cellulose	114

fraction, while the prolactin activity of these fractions was undiminished, and even slightly increased. This procedure provides the first method for obtaining primate pituitary preparations free of growth hormone, but with significant prolactin activity. It also provides strong evidence against the view that in primates only one hormone is responsible for both growth hormone and prolactin activities.

It is interesting to note that Guyda and Friesen apparently made no attempt to isolate the presumably bound growth hormone from the Sepharose-antibody column. Thus, by the strictest criteria, they did not demonstrate the success of the LSC procedure; inactivation of the growth hormone on passage through the column could also account for their results. It is entirely possible that recovery of biologically active hormones from antibody-Sepharose columns may present considerable difficulty, as evidenced by the results of Gospodarowicz. Clearly, additional investigations are needed to clarify this point.

An example of a hormone isolation procedure based on LSC which does not make use of an immobilized antibody column is provided by the work of Pradelles et al.[119] In this procedure, isotopically labeled vasopressin, a polypeptide hormone, was specifically adsorbed onto a column of immobilized neurophysins. The latter are a family of small carrier proteins which are known to form noncovalent complexes with the hormone, and are thus suitable for use as specific ligands. Elution was accomplished with 0.1 N formic acid, and the recovered material exhibited both the biochemical characteristics and biological activity of native vasopressin. The isolation of glycoprotein hormones using LSC is discussed in the Carbohydrates and Glycoproteins Section.

There have been several reports in the literature on the use of immobilized antibodies for the specific isolation of viruses. For example, Ladipo and deZoeten[120] used antibodies which had been insolubilized by crosslinking with glutaraldehyde to purify tobacco ringspot virus from crude sap. A batch pro-

cedure was used, and the virus was dissociated from the antibody-virus complex with 0.1 M glycine-HCl buffer, pH 2.8. The recovered material exhibited the ultra-violet spectrum typical of nucleoproteins, and possessed 80 to 85% of the infectivity of virus purified by a previously reported procedure. When a column procedure was used the virus was effectively adsorbed, but all attempts to elute it from the column were unsuccessful. Similarly, Galvez[121] used immobilized antibodies to specifically adsorb plant viruses, which were subsequently eluted with glycine-HCl buffer, pH 3.0.

An example of the use of LSC for the isolation of cells is provided by the work of Henry et al.[122] Crude cell suspensions from spleens of unimmunized mice were passed through columns of polyacrylamide beads to which azophenyl-β-lactoside groups had been covalently attached. The column was washed free of unbound cells, and then eluted with buffer containing p-aminophenyl-β-lactoside. In eight separate experiments, only 1000-4000 cells were recovered during the specific elution procedure for every 10^8 cells passed through the column. Yet, when 6000 recovered cells were tested for anti-azophenyl-β-lactoside activity, they gave an immune response equivalent to that produced by 2×10^7 unfractionated spleen cells. This result provides strong support for the clonal selection theory of antibody formation, which holds that unimmunized vertebrates contain many different populations of lymphocytes with various antibody-like receptors on their surfaces. A given antigen thus stimulates only those lymphocytes with the corresponding receptor specificity to proliferate, and these then give rise to the formation of numerous antibodies specific toward the original antigen. It is interesting to note in this connection that when mice were first primed with the azophenyl-β-lactoside antigen, application of the procedure of Henry and co-workers gave rise to the isolation of 50,000-200,000 purified cells for every 10^8 cells passed through the column.

A clever approach to the isolation of ribosomes using LSC has been reported by Miller et al.[123] These workers attempted to isolate the ribosomes engaged in the synthesis of the enzyme, tyrosine aminotransterase (TAT), from hepatoma tissue-culture cells, and to utilize the isolated ribosomes for the cell-free synthesis of TAT. They first established that columns of a conjugate between Sepharose and the TAT inhibitor, pyridoxamine phosphate, can selectively remove TAT from crude cell homogenates. Since TAT is thought to consist of four identical subunits each capable of binding one molecule of pyridoxal phosphate, they reasoned that an almost completed TAT subunit still attached to a polysome might also bind to the Sepharose-pyridoxamine phosphate column, and thereby act as a "hook" to "fish out" the entire protein-synthesizing system. Accordingly, crude ribosomal preparations were passed over the column and the bound material was eluted at pH 4.0 and examined. Immunoprecipitation data and assays for cell-free protein synthesizing activity indicated that ribosomes capable of synthesizing TAT had indeed been partially purified by the procedure.

MECHANISTIC AND STRUCTURAL STUDIES

The great potential of LSC for the isolation and purification of a variety of compounds is readily apparent from the foregoing sections of this review. During the past few years, the method has been greatly developed and widely applied and there is no doubt that it is well on its way to becoming a standard laboratory procedure. What has not been quite so widely recognized is the potential of LSC as a powerful tool in kinetic, mechanistic, and structural studies of macromolecules. Indeed, it may be in these latter areas, which are certainly no less significant than the former, that the greatest potential for innovative and elegant work lies. Here the investigator must bring together information and insight gleaned from studies

using a variety of other techniques in order to utilize LSC in a meaningful way. Some examples of these uses of LSC are presented in this section.

The use of LSC as an adjunct to classical kinetic studies is beautifully illustrated by the work of Akanuma et al.[124] The principle behind this work is presented in Figure 16, which illustrates the kinetic forms for competitive, non-competitive and uncompetitive inhibition of an enzyme, E. In pure competitive inhibition, the binding of the substrate, S, and the binding of the inhibitor, I, are mutually exclusive processes, and an (ESI) complex is not formed. In pure non-

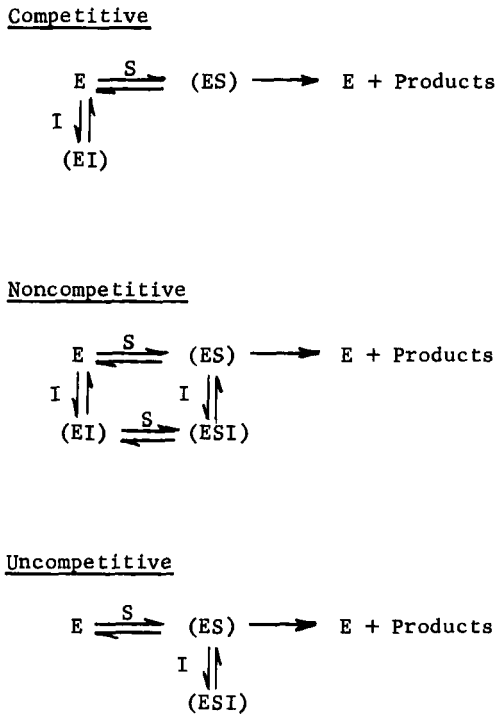


FIGURE 16

Kinetic forms of competitive, non-competitive and uncompetitive inhibition.

competitive inhibition, S and I interact with completely independent sites on E, and the binding of one has no effect on the binding of the other. Thus, (ES), (EI) and (ESI) complexes can all be formed. Finally, in pure uncompetitive inhibition, I binds only to the (ES) complex, not to the free enzyme, and thus an (ESI), but not an (EI), complex is formed. (It should be noted that inhibition which is of mixed kinetic form can also occur).

It follows from the above that if a substrate or substrate analog were covalently attached to an insoluble matrix, and the conjugate then contacted with a solution of enzyme, the subsequent addition of an inhibitor could have one of three possible effects on the migration of the enzyme through a column of the conjugate. (1) If the inhibitor is competitive, the addition of I will result in the formation of (EI) at the expense of (ES), thus increasing the fraction of enzyme in the mobile phase and reducing the extent of retardation of the enzyme on the column. (2) If the inhibitor is noncompetitive, the addition of I will have no effect on the affinity of the enzyme for the insoluble substrate and the mobility of the enzyme through the column will not be affected. (3) If the inhibitor is uncompetitive, the addition of I will result in the formation of (ESI) complexes in addition to (ES) complexes and the retardation of the enzyme on the column will be increased. Thus, the form of the elution profile of the enzyme in the presence of the inhibitor provides a diagnostic test for the kinetic form of the inhibition.

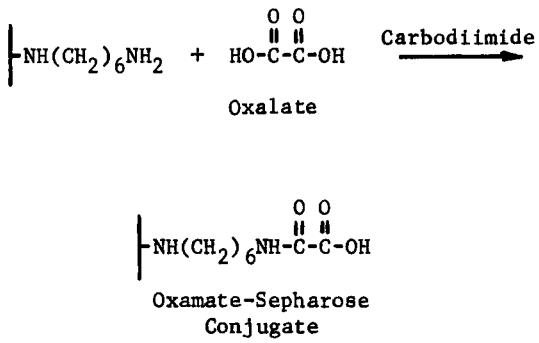
Akanuma *et al.*[124] first coupled ε-aminocaproyl-D-arginine to the azide derivative of carboxymethyl Sephadex (CM-Sephadex) to give an arginine-containing matrix. Carboxypeptidase B (CPB) was then adsorbed on a column of the matrix. Control experiments indicated that the enzyme was specifically adsorbed to the column by virtue of its affinity for the acyl-D-arginine group, which is commonly found in substrates of CPB such as benzoylglycyl-L-arginine. When a buffer containing

ε-aminocaproic acid, a competitive inhibitor with respect to benzoylglycyl-L-arginine, was added, the enzyme dissociated from the column. In contrast, CPB was not significantly retained on a column prepared from the conjugate of CM-Sephadex and ε-aminocaproyl-D-phenylalanine, unless a small amount (ca. 20 μM) of ε-aminocaproic acid was present in the buffer. Under these conditions, the enzyme was tightly adsorbed onto the column, and it was necessary to decrease the concentration of ε-aminocaproic acid to about 0.1 μM in order to elute the enzyme from the column. Alternatively, CPB, cooperatively adsorbed to the phenylalanine-Sephadex column in the presence of ε-aminocaproic acid, could be eluted if β-phenylpropionic acid, a phenylalanine analog, was added to the eluant.

These results were interpreted as follows: CPB possesses at least two separate binding sites--one for basic and one for aromatic or hydrophobic amino acids--which are capable of mutual interaction. Thus, ε-aminocaproic acid, an analog of the basic amino acid, lysine, acts as a competitive inhibitor with respect to immobilized arginine since the two molecules compete for the "basic" binding site on the enzyme. On the other hand, ε-aminocaproic acid, CPB and immobilized phenylalanine cooperatively form a ternary complex which enhances the adsorption of the enzyme onto the column. This is possible since the basic lysine analog and the aromatic phenylalanine ligand cooperatively interact with different sites on the enzyme giving rise to the behavior expected for uncompetitive inhibition. This conclusion is in agreement with kinetic evidence that ε-aminocaproic acid is an uncompetitive inhibitor of the CPB-catalyzed hydrolysis of carbobenzoxyglycyl-L-phenylalanine. Similarly, adsorbed CPB was eluted from the phenylalanine matrix by a solution containing ε-aminocaproic acid and β-phenylpropionic acid, due to the formation of a ternary complex in the mobile phase.

The use of LSC for the purification of multisubstrate enzymes and as a tool in the study of their catalytic mecha-

nisms is nicely illustrated by the recent work of O'Carra and Barry.[125] These authors studied the interaction of lactate dehydrogenase (LDH) with the oxamate-Sepharose derivative shown in equation 8, and the principle behind their work is as follows.



(8)

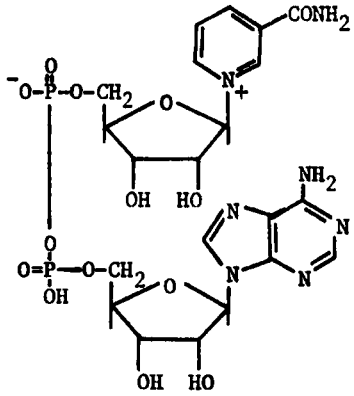
The catalytic mechanism which is widely accepted for the reaction catalyzed by LDH is shown in Figure 17. This mechanism is of the "compulsory-ordered" type, in which NADH must bind before the substrate, pyruvate, can interact with the enzyme. The competitive inhibitor, oxamate, which is a structural analog of pyruvate, also binds to the active site of LDH only after the binding of NADH. It is therefore expected that LDH will be specifically adsorbed by an NADH-Sepharose matrix, but not by a pyruvate-Sepharose or an oxamate-Sepharose conjugate. However, LDH would be expected to bind to the latter conjugates in the presence of soluble NADH, since the LDH·NADH complex formed in solution should have a strong affinity for the immobilized pyruvate or oxamate ligand.

These predictions were borne out by the experimental results obtained by O'Carra and Barry.[125] In the presence of 0.5 M NaCl, which prevented non-specific binding of charged molecules to the anionic matrix, LDH was completely unretarded on the oxamate-Sepharose column. However, in the presence of NADH at concentrations as low as 10 μM, LDH was strongly re-

tained on the column. Subsequent omission of the nucleotide caused immediate elution of the enzyme. This "positive" adsorption and "negative" elution with NADH affords a very high degree of specificity to the procedure, since only those proteins having dual affinity for both NADH and oxamate in a compulsory ordered manner would respond to the procedure. For example, a protein which could bind either ligand in a random fashion would remain adsorbed on the column when NADH was suddenly omitted from the eluant. Similarly, elution by omitting NADH rather than by including a high concentration of soluble oxamate in the eluant leaves behind proteins which have affinity only for oxamate. This high degree of specificity is illustrated by the success of O'Carra and Barry in isolating pure LDH from crude extracts of human placenta in 98 to 100% yield using only the one LSC step.

The above results are clearly in accord with the catalytic mechanism for LDH illustrated in Figure 17, and they emphasize the utility of LSC as an adjunct to kinetic investigations in the study of reaction mechanisms. Two further pieces of mechanistic information obtained in the above investigation are the following. (1) It was found that LDH binds to immobilized oxamate in the presence of $NAD^{\oplus}$, and that this binding is effectively reversed in the presence of soluble pyruvate due to the direct competition between pyruvate and immobilized oxamate. This result confirms the formation of a ternary $LDH \cdot NAD^{\oplus} \cdot PYR$ complex which is responsible for substrate inhibition at high pyruvate concentrations (Figure 17). (2) There is no retardation of LDH on the oxamate column in the presence of high concentrations of AMP or ADP, even though these molecules correspond to the "AMP half" of NADH (see XVIII), which is known to be responsible for binding the cofactor to LDH.[126] This implies that the nicotinamide end of NADH is responsible for generating the binding site for oxamate or pyruvate, and that the compulsory binding order to LDH is:

"AMP half" of NADH, followed by "nicotinamide half," followed by pyruvate (or oxamate).



Nicotinamide half

AMP half

(XVIII)

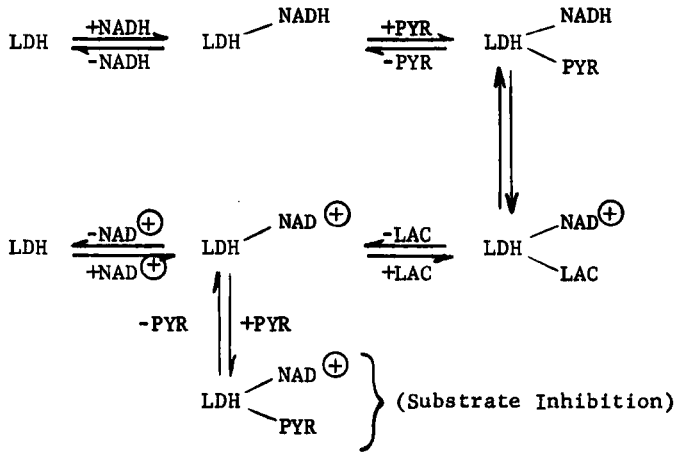


FIGURE 17

Catalytic mechanism for the reaction catalyzed by LDH. (PYR = pyrvate; LAC = lactate.)

Using a similar approach, Mawal et al.[127] studied the mobility of galactosyltransferase on an α-lactalbumin-Sepharose column in the presence of various combinations of reactants, in order to determine which of the various enzyme-reactant complexes are capable of interacting with α-lactalbumin. As in the studies described above, the expectation was that the passage of the enzyme through the column would be retarded when interaction with the immobilized ligand occurred. In general, the results obtained were in agreement with the conclusions which had been reached from earlier kinetic studies. However, evidence was also obtained for the existence of certain dead-end complexes which had not been detected in kinetic investigations. Mawal and co-workers reasoned that these arose as a consequence of the high effective concentration of α-lactalbumin on the matrix (57.2 mg per ml of settled Sepharose), which caused a shift in the appropriate equilibria, thereby favoring formation of the complexes. Obviously, such considerations must be kept in mind when comparing results obtained from kinetic studies with those obtained using LSC.

A different approach to the purification of pyridine nucleotide-dependent enzymes is that of Lowe and Dean[128] and of Mosbach and co-workers.[30-33] These investigators have developed columns of immobilized cofactors, e.g., $NAD^{\oplus}$ and AMP, for use in LSC, and they claim that such columns have the advantage of being generally useful in the purification of a large number of different enzymes. The problem is, of course, that in any one purification procedure a number of different proteins in the crude starting mixture will bind to the LSC column, and one must devise procedures for selectively eluting the desired material (see Adsorption and Elution Section).

An example of how the phenomenon of ternary complex formation can be exploited for the elution of enzymes from columns of immobilized cofactors is provided by the work of Ohlsson et al.[33] A mixture of yeast alcohol dehydrogenase

(YADH) and LDH was applied to an AMP-Sepharose column, and both enzymes were readily adsorbed due to their common affinity for the "AMP-half" of NADH (see above). Application of a mixture of $NAD^{\oplus}$ and hydroxylamine caused elution of only YADH due to formation of a ternary complex between the enzyme, $NAD^{\oplus}$, and the competitive inhibitor, hydroxylamine. Subsequent application of a mixture of $NAD^{\oplus}$ and pyruvate caused elution of LDH, due to formation of the ternary LDH·$NAD^{\oplus}$·PYR complex (Figure 17). In addition, by observing which combinations of substances eluted these enzymes from the column, Ohlsson and co-workers were able to draw conclusions about the ability of certain compounds to form complexes with the enzymes.

Wilchek *et al.*[129] have reported an elegant use of LSC in the study of c-AMP-dependent protein kinases. These enzymes are now recognized as indispensable links in the mechanism by which c-AMP exerts its effect as a "second messenger" in a variety of intracellular processes. It is generally accepted that c-AMP activates the protein kinase by binding to a regulatory subunit, thus causing dissociation of the protein and release of the active catalytic subunit (Figure 18).

In Wilchek's experiments, a c-AMP derivative was attached to Sepharose via the CNBr procedure, and crude preparations of rat parotid protein kinase or rabbit skeletal muscle protein kinase were passed through the column. The column effluent contained a protein which exhibited protein-kinase activity even in the absence of c-AMP and which had no detectable ability to bind c-AMP. Apparently, the native enzyme became dissociated on passage through the column--the regulatory subunit remaining bound to the immobilized c-AMP and the catalytic subunit passing through with the eluant. Unfortunately, all attempts to recover the presumably bound regulatory subunit from the column were unsuccessful even though a variety of compounds capable of binding to native protein kinase were included in the eluant.

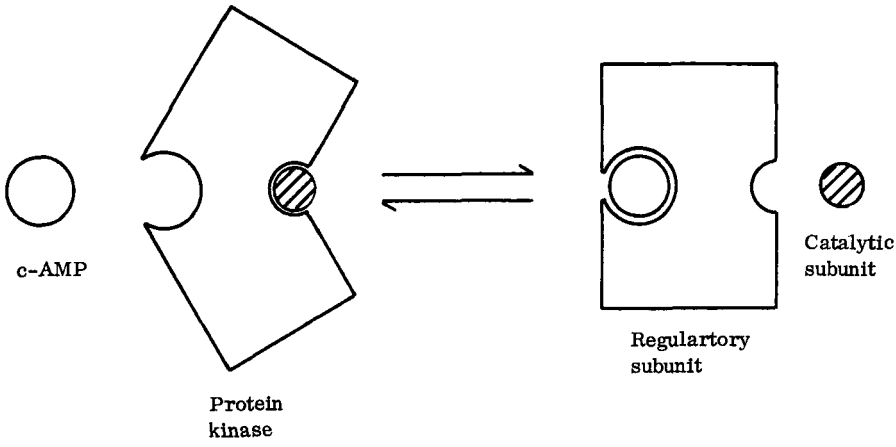


FIGURE 18

Schematic representation of the dissociation of a c-AMP-dependent protein kinase in the presence of c-AMP.

These results of Wilchek et al.[129] are of great significance, since they demonstrate the potential of LSC as a powerful tool in the study of protein structure. In addition, it is clear that the method may often have great practical value. For example, the c-AMP-Sepharose column is a simple and highly effective agent for the isolation of free catalytic subunits from c-AMP-dependent protein kinase. Indeed, when one considers the studies summarized in this section, it seems likely that one of the more important applications of LSC will be its use as a tool for furthering our understanding of the structure and function of macromolecules.

ADDENDUM

The literature search for this review was completed late in 1972. Since the anticipated publication date for this volume is mid 1974, a few words are in order about some of the developments in the field of LSC which have occurred over the past year or so.

Shaltiel and Er-el[130] have extended the earlier work of Er-el et al[40] on the binding of proteins to hydrocarbon chains attached to Sepharose. A series of homologous ω-aminoalkyl derivatives of Sepharose with carbon chains of varying length were prepared and used for the purification of glycogen synthetase and glycogen phosphorylase. This work underscores the fact that the widely used hydrocarbon extension "arms" can cause binding of undesired proteins to an LSC column through hydrophobic interactions. The authors therefore suggest that LSC conjugates should be prepared by first linking a side chain to the specific ligand and then attaching the elongated ligand to the agarose bead, rather than the alternative procedure of coating the beads with hydrocarbon arms prior to the attachment of the specific ligand. This precaution should minimize the number of "dangling arms" on the LSC column which contribute to hydrophobic binding. It should be kept in mind that in certain cases hydrophobic hydrocarbon-coated agarose beads may be extremely useful, e.g. in the purification of lipophilic membrane-bound proteins. In addition, Hofstee[131] has demonstrated that hydrocarbon-substituted agarose can be successfully used for the immobilization of enzymes.

Among the substances recently reported to have been isolated by LSC are oestradiol receptor proteins[132], wheat germ agglutinin[133], serum albumin[134], mouse interferon[135] and Escherichia coli ribonuclease[136]. Whitney[137] has reported the separation of chemically modified carbonic anhydrase B from the native enzyme using LSC, and Berg and Prockop[138] isolated protocollagen proline hydroxylase by binding the enzyme to a conjugate of Sepharose and a peptide substrate, and then specifically eluting the enzyme with a solution containing a high concentration of a second peptide substrate of lower affinity.

The list of commercially-available materials designed for use in LSC procedures continues to expand. Affitron Corporation (Costa Mesa, Ca., 92626), Bio-Rad Laboratories (Richmond, Ca., 94804), Miles Laboratories (Kankankee, Il., 60901) and Pharmacia

Fine Chemicals (Piscataway, N.J. 08854) have all expanded their lines of "affinity chromatographic" products, and descriptive literature is available upon request. The increased availability of such products is a sure sign that LSC is well on its way to becoming a standard laboratory technique.

REFERENCES

1. S. G. Hedin, Biochem. J., I, 484 (1906).
2. S. G. Hedin, Biochem. J., II, 112 (1907).
3. E. Sarkstein, Biochem Z., 24, 210 (1910).
4. L. S. Lerman, Proc. Nat. Acad. Sci. U.S., 39, 232 (1953).
5. E. K. F. Bautz and B. D. Hall, Proc. Nat. Acad. Sci. U.S., 48, 400 (1962).
6. P. T. Gilham, J. Am. Chem. Soc., 84, 1311 (1962).
7. A. J. Adler and A. Rich, J. Am. Chem. Soc., 84, 3977 (1962).
8. P. Cuatrecasas and C. B. Anfinsen, Ann. Rev. Biochem., 40, 259 (1971).
9. P. Cuatrecasas, in "Biochemical Aspects of Reactions on Solid Supports," G. R. Stark, ed., Academic Press, N.Y., 79 (1971).
10. P. Cuatrecasas and C. B. Anfinsen, in "Methods in Enzymology," Vol. 22, W. Jakoby, ed., Academic Press, N.Y., 345 (1971).
11. F. Friedberg, Chromatog. Rev., 14, 121 (1971).
12. R. H. Reiner and A. Walch, Chromatographia, 4, 578 (1971).
13. P. Cuatrecasas, in "Advances in Enzymology," Vol. 36, A. Meister, ed., Interscience, N.Y., 29 (1972).
14. J. Porath and L. Sundberg in "The Chemistry of Biosurfaces," Vol. 2, M. Hare, ed., Marcel Dekker. N.Y., 633 (1972).
15. H.-C. Chen, L. C. Craig, and E. Stoner, Biochemistry, 11, 3559 (1972).

16. R. Axen, J. Porath, and S. Ernback, Nature, 214, 1302 (1967).

17. R. Axen and S. Ernback, Eur. J. Biochem., 18, 351 (1971).

18. R. Axen and P. Vretblad, Acta Chem. Scand., 25, 2711 (1971).

19. G. J. Bartling, H. D. Brown, L. J. Forrester, M. T. Koes, A. N. Mather, and R. D. Stasin, Biotechnol. Bioeng., 14, 1039 (1972).

20. L. Kagedal and S. Akerstrom, Acta Chem. Scand., 24, 1601 (1970).

21. L. Ahrgren, L. Kagedal, and S. Akerstrom, Acta Chem. Scand., 26, 285 (1972).

22. P. Cuatrecasas, J. Biol. Chem., 245, 3059 (1970).

23. P. Cuatrecasas, Nature, 228, 1327 (1970).

24. P. Cuatrecasas and I. Parikh, Biochemistry, 11, 2291 (1972).

25. G. I. Tesser, H.-U. Fisch, and R. Schwyzer, FEBS Lett., 23, 56 (1972).

26. F. Krug, B. Desbuquois, and P. Cuatrecasas, Nature, New Biol., 234, 268 (1971).

27. J. K. Inman and H. M. Dintzis, Biochemistry, 8, 4074 (1969).

28. E. Steers, Jr., P. Cuatrecasas, and H. B. Pollard, J. Biol. Chem., 246, 196 (1971).

29. P. J. Robinson, P. Dunnill, and M. D. Lilly, Biochim. Biophys. Acta, 242, 659 (1971).

30. K. Mosbach, H. Guilford, R. Ohlsson, and M. Scott, Biochem. J., 127, 625 (1972).

31. K. Mosbach, H. Guilford, P.-O. Larsson, R. Ohlsson, and M. Scott, Biochem. J., 125, 20 (1972).

32. C. R. Lowe, K. Mosbach, and P. D. G. Dean, Biochem. Biophys. Res. Commun., 48, 1004 (1972).

33. R. Ohlsson, P. Brodelius, and K. Mosbach, FEBS Lett., 25, 234 (1972).

34. Y. Ashani and I. B. Wilson, Biochim. Biophys. Acta, 276, 317 (1972).

35. P. M. Blumberg and J. L. Strominger, Proc. Nat. Acad. Sci. U.S., 69, 3751 (1972).

36. R. J. Lefkowitz, E. Haber, and D. O'Hara, Proc. Nat. Acad. Sci. U.S., 69, 2828 (1972).

37. P. Cuatrecasas, Proc. Nat. Acad. Sci. U.S., 69, 1277 (1972).

38. J. M. Whiteley, R. C. Jackson, G. P. Mell, J. H. Drais, and F. M. Huennekens, Arch. Biochem. Biophys., 150, 15 (1972).

39. R. Cardinand and J. Holguin, Biochem. J., 127, 30p (1972).

40. Z. Er-el, Y. Zaidenzaig and S. Shaltiel, Biochem. Biophys. Res. Commun., 49, 383 (1972).

41. M. Robert-Gero and J. P. Waller, Eur. J. Biochem., 31, 315 (1972).

42. S. Grossman, M. Trop, S. Yaroni, and M. Wilchek, Biochim. Biophys. Acta, 289, 77 (1972).

43. J. C. Nicolas, M. Pons, B. Descomps, and A. Crastes de Paulet, FEBS Lett., 23, 175 (1972).

44. P. L. Chello, A. R. Cashmore, S. A. Jacobs and J. R. Bertino, Biochim. Biophys. Acta, 268, 30 (1972).

45. J. Gauldie and B. L. Hillcoat, Biochim. Biophys. Acta, 268, 35 (1972).

46. D. Edmondson, V. Massey, G. Palmer, L. M. Beacham, III, and G. B. Elion, J. Biol. Chem., 247, 1597 (1972).

47. S. L. Marcus and E. Balbinder, Biochem. Biophys. Res. Commun., 47, 438 (1972).

48. H. Schaller, C. Nűsslein, F. J. Bonhoeffer, C. Kurz, and I. Nietzschmann, Eur. J. Biochem., 26, 474 (1972).

49. H.-J. Bőhme, G. Kopperschläger, J. Schulz, and E. Hofmann, J. Chromatogr., 69, 209 (1972).

50. P. Rőschlau and B. Hess, Hoppe-Seyler's Z. Physiol. Chem., 353, 441 (1972).

51. L. Jervis, Biochem. J., 127, 29p (1972).

52. D. M. Livingston, E. M. Scolnick, W. P. Parks, and G. J. Todaro, Proc. Nat. Acad. Sci. U.S., 69, 393 (1972).

53. R. Barker, K. W. Olsen, J. H. Shaper and R. L. Hill, J. Biol. Chem., 247, 7135 (1972).

54. Y. Dudai, I. Silman, M. Shinitzky, and S. Blumberg, Proc. Nat. Acad. Sci. U.S., 69, 2400 (1972).

55. M. J. Jung and B. Belleau, Mol. Pharmacol., 6, 589 (1972).

56. R. Schwyzer and J. Frank, Helv. Chim. Acta, 55, 2678 (1972).

57. T. L. Rosenberry, H. W. Chang and Y. T. Chen, J. Biol. Chem., 247, 1555 (1972).

58. H. Ako, C. A. Ryan, and R. J. Foster, Biochem. Biophys. Res. Commun., 46, 1639 (1972).

59. Y. Nagai and H. Hori, Biochim. Biophys. Acta, 263, 564 (1972).

60. P. J. Robinson, P. Dunnill and M. D. Lilly, Biochim. Biophys. Acta, 285, 28 (1972).

61. H. Fritz and B. Főry-Brey, Hoppe-Seyler's Z. Physiol. Chem., 353, 901 (1972).

62. K. Kasai and S. Ishii, J. Biochem. (Tokyo), 71, 363 (1972).

63. S. O. Falkbring, P. O. Göthe, and P. O. Nyman, FEBS Lett., 24, 229 (1972).

64. P. V. Danenberg, R. J. Langenbach, and C. Heidelberger, Biochem. Biophys. Res. Commun., 49, 1029 (1972).

65. J. Schmidt and M. A. Raftery, Biochem. Biophys. Res. Commun., 49, 572 (1972).

66. R. W. Olsen, J. C. Meunier and J. P. Changeux, FEBS Lett., 28, 96 (1972).

67. E. Karlsson, E. Heilbronn, and L. Widlund, FEBS Lett., 28, 107 (1972).

68. D. N. Salter, J. E. Ford, K. J. Scott, and P. Andrews, FEBS Lett., 20, 302 (1972).

69. S. Blumberg, J. Hildesheim, J. Yariv, and K. J. Wilson, Biochim. Biophys. Acta, 264, 171 (1972).

70. B. R. Smith, J. Endocr., 52, 229 (1972).

71. M. A. Yenis, Biochem. J., 124, 29p (1972).

72. J. A. Gordon, S. Blumberg, H. Lis, and N. Sharon, FEBS Lett., 24, 193 (1972).

73. R. H. Allen and P. W. Majerus, J. Biol. Chem., 247, 7695 (1972).

74. B. L. Vallee and J. F. Riordan, Ann. Rev. Biochem., 38, 733 (1969).

75. S. J. Singer, Advan. Protein Chem., 22, 1 (1967).

76. D. Givol, Y. Weinstein, M. Gorecki, and M. Wilchek, Biochem. Biophys. Res. Commun., 38, 825 (1970).

77. M. Wilchek, FEBS Lett., 7, 161 (1970).

78. M. Wilchek, Anal. Biochem., 49, 572 (1972).

79. M. Wilchek, V. Bocchini, M. Becker, and D. Givol, Biochemistry, 10, 2828 (1971).

80. M. Helman and D. Givol, Biochem. J., 125, 971 (1971).

81. M. Bustin and D. Givol, Biochim. Biophys. Acta, 263, 459 (1972).

82. M. Wilchek and T. Miron, Biochim. Biophys. Acta, 278, 7 (1972).

83. N. Sharon and H. Lis, Science, 177, 949 (1972).

84. G. M. Edelman, B. A. Cunningham, G. N. Reeke, Jr., J. N. Becker, M. J. Waxdal, and J. L. Wang, Proc. Nat. Acad. Sci. U.S., 69, 2580 (1972).

85. G. Gachelin, L. Goldstein, D. Hofnung, and A. J. Kalb, Eur. J. Biochem., 30, 155 (1972).

86. R. D. Poretz and I. J. Goldstein, Biochemistry, 9, 2890 (1970).

87. L. L. So and I. J. Goldstein, Biochim. Biophys. Acta, 165, 398 (1968).

88. K. O. Lloyd, Arch. Biochem. Biophys., 137, 460 (1970).

89. K. Aspberg and J. Porath, Acta Chem. Scand., 24, 1839 (1970).

90. M. L. Dufau, T. Tsuruhara, and K. J. Catt, Biochim. Biophys. Acta, 278, 281 (1972).

91. E. H. Donnelly and I. J. Goldstein, Biochem. J., 118, 679 (1970).

92. S. Avrameas, C. R. Acad. Sci., Ser. D, 270, 2205 (1970).

93. B. B. L. Agrawal and I. J. Goldstein, Biochim. Biophys. Acta, 147, 262 (1967).

94. M. D. J. Olson and I. E. Liener, Biochemistry, 6, 105 (1967).

95. K. Warecka, H. J. Moller, H.-M. Vogel, and I. Tripatzis, J. Neurochem., 19, 719 (1972).

96. E. G. Sander, D. B. McCormick, and L. D. Wright, J. Chromatogr., 21, 419 (1966).

97. C. Saxinger, C. Ponnamperuma, and C. Woese, Nature, New Biol., 234, 172 (1971).

98. H.-U. Fisch, V. Pliska, and R. Schwyzer, Eur. J. Biochem., 30, 1 (1972).

99. H.-U. Fisch, V. Pliska, and R. Schwyzer, Experientia, 28, 630 (1972).

100. P. T. Gilham, J. Am. Chem. Soc., 86, 4982 (1964).

101. P. T. Gilham and W. E. Robinson, J. Am. Chem. Soc., 86, 4985 (1964).

102. C. R. Astell and M. Smith, Biochemistry, 11, 4114 (1972).

103. A. F. Wagner, R. L. Bugianesi, and T. Y. Shen, Biochem. Biophys. Res. Commun., 45, 184 (1971).

104. D. L. Robberson and N. Davidson, Biochemistry, 11, 533 (1972).

105. W. C. Saxinger, C. Ponnamperuma, and D. Gillespie, Proc. Nat. Acad. Sci. U.S., 69, 2975 (1972).

106. B. Alberts and G. Herrick in "Methods in Enzymol.", Vol. 21, L. Grossman and K. Moldave, ed., Academic Press, N.Y., 198 (1972).

107. T. M. Jovin and A. Kornberg, J. Biol. Chem., 243, 250 (1968).

108. I. Smith, H. Smith, and S. Pifko, Anal. Biochem., 48, 27 (1972).

109. U. Lindberg and T. Persson, Eur. J. Biochem., 31, 246 (1972).

110. H. Aviv and P. Leder, Proc. Nat. Acad. Sci. U.S., 69, 1408 (1972).

111. E. K. F. Bautz and E. Reilly, Science, 151, 328 (1966).

112. L. Gyenge, V. A. Spiridonova, and A. A. Bogdanov, FEBS Lett., 20, 209 (1972).

113. J. Denberg and M. DeLuca, Proc. Nat. Acad. Sci. U.S., 67, 1057 (1970).

114. S. Erhan, L. G. Northrup, and F. R. Leach, Proc. Nat. Acad. Sci. U.S., 53, 646 (1965).

115. Y. Akanuma, T. Kuzuya, M. Hayashi, T. Ide, and N. Kuzuya, Biochem. Biophys. Res. Commun., 38, 947 (1970).

116. D. Gospodarowicz, J. Biol. Chem., 247, 6491 (1972).

117. R. F. Murphy, D. T. Elmore, and K. D. Buchanan, Biochem. J., 125, 61p (1971).

118. H. J. Guyda and H. G. Friesen, Biochem. Biophys. Res. Commun., 42, 1068 (1971).

119. P. Pradelles, J. L. Morgat, P. Fromageot, M. Camier, D. Bonne, P. Cohen, J. Bockaert and S. Jard, FEBS Lett., 26, 189 (1972).

120. J. L. Ladipo and G. A. de Zoeten, Virology, 46, 567 (1971).

121. G. E. Galvez, Virology, 28, 171 (1966).

122. C. Henry, J. Kimura, and L. Wolfsy, Proc. Nat. Acad. Sci. U.S., 69, 34 (1972).

123. J. V. Miller, Jr., P. Cuatrecasas and E. B. Thompson, Proc. Nat. Acad. Sci. U.S., 68, 1014 (1971).

124. H. Akanuma, A. Kasuga, T. Akanuma and Y. Makoto, Biochem. Biophys. Res. Commun., 45, 27 (1971).

125. P. O'Carra and S. Barry, FEBS Lett., 21, 281 (1972).

126. A. McPherson, J. Mol. Biol., 51, 39 (1970).

127. R. Mawal, J. F. Morrison, and K. E. Ebher, J. Biol. Chem., 246, 7106 (1971).

128. C. R. Lowe and P. D. G. Dean, FEBS Lett., 14, 313 (1971).

129. M. Wilchek, Y. Salomon, M. Lowe, and Z. Selinger, Biochem. Biophys. Res. Commun., 45, 1177 (1971).

130. S. Shaltiel and Z. Er-el, Proc. Nat Acad. Sci. U.S., 70, 778 (1973).

131. B. H. J. Hofstee, Biochem. Biophys. Res. Commun., 53, 1137 (1973).

132. V. Sica, E. Nola, I. Parikh, G. A. Puca, and P. Cuatrecasas, Nature New Biology, 244, 36 (1973).

133. R. Lotan, A. E. S. Gussin, H. Lis, and N. Sharon, Biochem. Biophys. Res. Commun., 52, 656 (1973).

134. T. Peters, Jr., H. Taniuchi, and C. B. Anfinsen, Jr., J. Biol. Chem., 248, 2447 (1973).

135. J. D. Sipe, J. de Maeyer-Guignard, B. Fauconnier, and E. de Maeyer, Proc. Nat. Acad. Sci. U.S., 70, 1037 (1973).

136. S. C. Weatherford, L. S. Weisberg, D. T. Achord, and D. Apirion, Biochem. Biophys. Res. Commun., 49, 1307 (1972).

137. P. L. Whitney, J. Biol. Chem., 248, 2785 (1973).

138. R. A. Berg and D. J. Prockop, J. Biol. Chem., 248, 1175 (1973).