

[31] Affinity Chromatography

By PEDRO CUATRECASAS AND CHRISTIAN B. ANFINSEN

General Considerations

Conventional procedures of protein purification are generally based on relatively small differences in the physicochemical properties of the proteins in the mixture. They are frequently laborious and incomplete, and the yields are often low. The selective isolation and purification of enzymes and other biologically important macromolecules by "affinity chromatography" exploits the unique biological property of these proteins or polypeptides to bind ligands specifically and reversibly.¹⁻⁷ The method is related in principle to the use of "immunoabsorbents" introduced as chemically defined materials for chromatographic separation of antibodies by Campbell and his colleagues in 1951⁸ and subsequently employed widely as a standard immunological procedure.⁹ Affinity chromatography, as summarized in the present article, exploits the phenomenon of specific biological interaction in a large variety of protein-ligand systems. A solution containing the macromolecule to be purified is passed through a column containing an insoluble polymer or gel to which a specific competitive inhibitor or other ligand has been covalently attached. Proteins not exhibiting appreciable affinity for the ligand will pass unretarded through the column, whereas those which recognize the inhibitor will be retarded in proportion to the affinity existing under the experimental conditions. The specifically adsorbed protein can be eluted by altering the composition of the solvent so that dissociation occurs.

In principle, specific adsorbents can be used to purify enzymes, antibodies, nucleic acids, cofactor or vitamin-binding proteins, repressor proteins, transport proteins, drug or hormone receptor structures, sulfhydryl group containing proteins, and peptides formed by organic

¹P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U. S.* **61**, 636 (1968).

²P. Cuatrecasas, *J. Biol. Chem.* **245**, 3059 (1970).

³P. Cuatrecasas, in *Biochemical Aspects of Solid State Chemistry*, ed. G. R. Stark. New York, Academic Press. In press.

⁴P. Cuatrecasas, *J. Agr. Food Chem.*, In press (1971).

⁵I. Kato, and C. B. Anfinsen, *J. Biol. Chem.* **244**, 1004 (1969).

⁶P. Cuatrecasas and C. B. Anfinsen, *Annu. Rev. Biochem.*, **40**, In press (1971).

⁷P. Cuatrecasas, *Nature*, **228**, 1327 (1970).

⁸D. H. Campbell, E. Leuscher, and L. S. Lermann, *Proc. Nat. Acad. Sci. U. S.* **37**, 575 (1951).

⁹I. Silman and E. Katchalski, *Annu. Rev. Biochem.* **35**, 873 (1966).

synthesis. Affinity chromatography may also be useful in concentrating dilute solutions of proteins, in removing denatured forms of a purified protein, and in the separation and resolution of protein components resulting from specific chemical modifications of purified proteins. Inherent advantages of this method of purification are the rapidity and ease of a potentially single-step procedure, the rapid separation of the protein to be purified from inhibitors and destructive contaminants, e.g., proteases, and protection from denaturation during purification by active site ligand-stabilization of protein tertiary structure.

Successful application of the methods will depend in large part on how closely the particular experimental conditions chosen permit the ligand interaction to simulate that observed when the components are free in solution. Careful consideration must therefore be given to the nature of the solid matrix, the dependence of the interaction on the structure of the ligand, the means of covalent attachment, and the conditions selected for adsorption and elution. The specific conditions for purification of a particular protein must be highly individualized since they will reflect the uniquely specific biological property of selective interaction with a given ligand. The following general considerations should serve as guidelines in the preparation of adsorbents for affinity chromatography.

Solid Matrix Support. An ideal insoluble support should possess the following properties: (1) It must interact very weakly with proteins in general, to minimize the nonspecific adsorption of proteins. (2) It should exhibit good flow properties which are retained after coupling. (3) It must possess chemical groups which can be activated or modified, under conditions innocuous to the structure of the matrix, to allow the chemical linkage of a variety of ligands. (4) These chemical groups should be abundant in order to allow attainment of a high effective concentration of coupled inhibitor (capacity), so that satisfactory adsorption can be obtained even with protein-inhibitor systems of low affinity. (5) It must be mechanically and chemically stable to the conditions of coupling and to the varying conditions of pH, ionic strength, temperature, and presence of denaturants (e.g., urea, guanidine hydrochloride) which may be needed for adsorption or elution. Such properties also permit repeated use of the specific adsorbent. (6) It should form a very loose, porous network which permits uniform and unimpaired entry and exit of large macromolecules throughout the entire matrix; the gel particles should preferably be uniform, spherical, and rigid. A high degree of porosity is an important consideration for ligand-protein systems of relatively weak affinity (dissociation constant of 10^{-5} M or greater), since the concentration of ligand *freely available* to the protein must be quite high to

permit interactions strong enough to physically retard the downward migration of the protein through the column. This probably explains why certain solid supports, such as cellulose, have been used so successfully as immunoadsorbents but have been of only minimal utility in the purification of enzymes.

The use of hydrophilic cellulose derivatives in the purification of antibodies is described in detail elsewhere in this volume and need not be discussed further here. In some cases cellulose derivatives have been used in the purification of enzymes: (1) aminophenol diazotized to cellulose containing resorcinol residues in ether linkage has been used to purify tyrosinase¹⁰; (2) flavokinase has been specifically adsorbed to carboxymethyl cellulose and to cellulose containing flavin derivatives^{11,12}; (3) avidin has been partially purified with the use of cellulose reacted with biotinyl chloride.¹³ Derivatives of cellulose also have been used to purify nucleotides,¹⁴ complementary strands of nucleic acids,¹⁵ and certain species of transfer RNA.¹⁶ Although cellulose derivatives may be advantageous in specific instances, they are generally much less useful than the agarose derivatives because their fibrous and nonuniform character impedes proper penetration of large protein molecules. Highly hydrophobic polymers, such as polystyrene, display poor communication between the aqueous and solid phases. A technique for the immobilization of organic substances on glass surfaces, described recently by Weetall and Hersh,¹⁷ may be a promising tool for the preparation of specific adsorbents.

Various polysaccharide hydrophilic polymers are very useful solid supports. Commercially available, cross-linked dextran derivatives (Sephadex, Pharmacia) possess most of the desirable features listed above except for their low degree of porosity. For this reason they are relatively ineffective as adsorbents for purification of enzymes of even low molecular weight. However, beaded derivatives of agarose,¹⁸ another polysaccharide polymer, have nearly all the properties of an ideal adsorbent¹⁻⁶ and are commercially available (Pharmacia; Bio-Rad Laboratories). The beaded agarose derivatives have a very loose structure which allows molecules with a molecular weight in the millions to diffuse

¹⁰L. S. Lerman, *Proc. Nat. Acad. Sci. U. S.* **39**, 232 (1953).

¹¹C. Arsenis and D. B. McCormick, *J. Biol. Chem.* **239**, 3093 (1964).

¹²C. Arsenis and D. B. McCormick, *J. Biol. Chem.* **241**, 330 (1966).

¹³D. B. McCormick, *Anal. Biochem.* **13**, 194 (1965).

¹⁴E. G. Sander, D. B. McCormick, and L. D. Wright, *J. Chromatogr.* **21**, 419 (1966).

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¹⁶S. L. Erhan, L. G. Northrup, and F. R. Leach, *Proc. Nat. Acad. Sci. U. S.* **53**, 646 (1965).

¹⁷H. H. Weetall and L. S. Hersh, *Biochim. Biophys. Acta* **185**, 464 (1969).

¹⁸S. Hjertén, *Arch. Biochem. Biophys.* **99**, 446 (1962).

readily through the matrix. These polysaccharides can readily undergo substitution reactions by activation with cyanogen halides,^{19,20} are very stable, and have a moderately high capacity for substitution.^{2,3,7} Synthetic polyacrylamide gels also possess many desirable features and are available commercially in beaded, spherical form, in various pregraded sizes and porosities (Bio-Gel, Bio-Rad Laboratories). Recently described derivatization procedures permit attachment of a variety of ligands and proteins to polyacrylamide beads.^{2,7,21} These beads exhibit uniform physical properties and porosity, and the polyethylene backbone endows them with physical and chemical stability. Preformed beads are available which permit penetration of proteins with molecular weights of about one half million (Bio-Gel P-300). Their porosity, however, is diminished during the chemical modifications required for attachment of ligands, and in this respect the polyacrylamide beads are inferior to those of agarose.^{2,22} The principal advantage of polyacrylamide is that it possesses a very large number of modifiable groups (carboxamide). Thus, highly substituted derivatives may be prepared for use in the purification of enzymes, which exhibit poor affinity for the attached ligand.^{2,22}

Considerations in Selecting a Ligand. The small molecule to be covalently linked to the solid support must be one that displays special and unique affinity for the macromolecule to be purified. It can be a substrate analog inhibitor, effector, cofactor, and, in special cases, substrate. Enzymes requiring two substrates for reaction may be approached by immobilizing one of the substrates, provided sufficiently strong binding is displayed toward that substrate in the absence of the other. Also, a substrate may be used if it binds to the enzyme under some conditions that do not favor catalysis, i.e., in the absence of metal ion, if the pH dependence of K_m and of k_{cat} are different, or at lower temperatures.

The small molecule to be insolubilized must possess chemical groups that can be modified for linkage to the solid support without abolishing or seriously impairing interaction with the complimentary protein. If the strength of interaction of the free complex in solution is very strong, i.e., a K_i of about 1 mM, a decrease in affinity of 3 orders of magnitude upon preparation of the insoluble derivative may still leave an effective and selective adsorbent. The important parameter is the effective experimental affinity—that displayed between the protein in solution and the insolubilized ligand under the experimental conditions chosen. In practice it has been very difficult to prepare such adsorbents for enzyme-

¹⁹J. Porath, R. Axén, and S. Ernbäck, *Nature (London)* 215, 1491 (1967).

²⁰R. Axén, J. Porath, and S. Ernbäck, *Nature (London)* 214, 1302 (1967).

²¹J. K. Inman and H. M. Dintzis, *Biochemistry* 8, 4074 (1969).

²²E. Steers, P. Cuatrecasas, and H. Pollard, *J. Biol. Chem.* 246, 196 (1971).

inhibitor systems whose dissociation constants under optimal conditions in solution are greater than 5 mM.²² It is possible in theory, however, to prepare adequate adsorbents for such systems if a sufficiently large amount of inhibitor can be coupled to the solid support.

It is becoming increasingly clear that, for successful purification by affinity chromatography, the inhibitor groups critical in the interaction with the macromolecule to be purified must be sufficiently distant from the solid matrix to minimize steric interference with the binding process.^{2,4,22} Steric considerations appear to be most important with proteins of high molecular weight. The problem may be approached by preparing an inhibitor with a long hydrocarbon chain, an "arm," attached to it, which can in turn be coupled to the insoluble support. Alternatively, such a hydrocarbon extension "arm" can first be attached to the solid support.²

General Considerations in the Preparation of Specific Adsorbents. The ligand must be coupled to the solid support under mild conditions that are tolerated well by both ligand and matrix. The coupled gel must be washed exhaustively to ensure total removal of the material not covalently bound. In some cases, such as with highly aromatic compounds, e.g., estradiol, complete removal of adsorbed material is very difficult and may require many days of continuous washing. Organic solvents may occasionally be required for this purpose. In rare instances, i.e., Congo red, effective washing can be achieved only by washing the column with large quantities of the protein to be purified, i.e., amyloid, followed by elution to regenerate the column. Whenever possible, radioactivity or other sensitive indicators should be present on the ligand to assist in monitoring the washing procedure. Aqueous dimethyl formamide (50%, v:v) has been useful in accelerating the washing of agarose and polyacrylamide derivatives; both tolerate this solvent well.

An accurate method for determining the amount of material attached to the solid support should be available. This is preferably done by determining the amount of ligand (by radioactivity, absorbance, amino acid analysis, etc.) released from the substituted matrix by acid or alkaline hydrolysis. Exhaustive digestion with pronase or carboxypeptidase, followed by amino acid analysis, has been used in some cases in which oligopeptides are attached to agarose. It is also possible to determine the radioactive content of the unhydrolyzed solid support by assay in suspension with Cab-O-Sil (Beckman); estimates made in this way are generally low even if appropriate internal standards are used for correcting efficiencies of counting. An alternative means of quantitation is to estimate the amount of ligand not recovered in the final washings. This is less accurate when a very large excess of ligand is added during the coupling

procedure or when appreciable noncovalent adsorption to the solid matrix occurs which demands large volumes of solvent for thorough washing. It is operationally more useful to express the degree of ligand substitution on the solid matrix in terms of concentration, such as micromoles of ligand per milliliter of packed gel, rather than on the basis of dry weight.

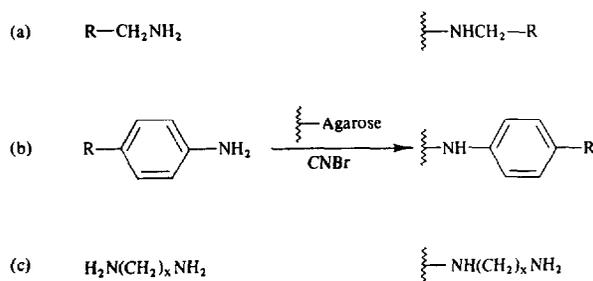
Conditions for Adsorption and Elution of Proteins. The specific conditions for adsorption are dictated by the specific properties of the protein to be purified. Affinity purification need not be restricted to column procedures. In certain circumstances it may be advisable to use batch purification methods. This is the case, for example, when small amounts of protein are to be extracted from very crude or particulate protein mixtures with an adsorbent of very high affinity. Column flow rates in such cases may be severely compromised, and purification can be more expeditiously carried out by adding a slurry of the specific adsorbent to the crude mixture, followed by batchwise washing and elution. In some cases involving very high affinity complexes, such as observed with certain antibody-antigen systems, it is preferable to adsorb the protein to the solid support and to wash extensively while the gel is packed in a column, elute the protein by removing the matrix and incubating in suspension in an appropriate buffer. For reasons of thoroughness of mixing, higher dilution of the insoluble ligand, and easier control of time and temperature, this means of elution requires less drastic conditions and the yields may be higher.

Protein specifically adsorbed to a solid carrier matrix will eventually emerge from the column without altering the properties of the buffer if the affinity for the ligand is not too great. With this type of elution, however, the protein is generally obtained in dilute form. In most cases elution of the protein will require changing the pH, ionic strength, or temperature of the buffer. Dissociation of proteins from very high-affinity adsorbents may require protein denaturants, such as guanidine hydrochloride or urea. Ideal elution of a tightly bound protein should utilize a solvent which causes sufficient alteration in the conformation of the protein to decrease appreciably the affinity of the protein for the ligand but which is not sufficiently severe to completely denature or unfold the protein. The eluted protein should be neutralized, diluted, or dialyzed at once to permit prompt reconstitution of the native protein structure. This may be tested by rechromatographing the purified protein to determine changes in adsorption to the affinity column. Elution of the specifically adsorbed protein can also be achieved with a solution containing a specific inhibitor or substrate. The inhibitor can either be the one that is covalently linked to the matrix (and must be used at higher concentrations), or preferably another, stronger competitive inhibitor.

An alternative method of eluting the specifically bound protein is to selectively cleave the matrix-ligand bond, thus removing the intact ligand-protein complex. Excess ligand can then be removed by dialysis or by gel filtration on Sephadex. This approach can be applied to ligands attached to Sepharose by azo linkage, and by thiol or alcohol ester bonds. These will be discussed under the appropriate sections.

Preparation and Use of Agarose Bead Adsorbents

Of the beaded agarose derivatives commercially available, Sepharose 4B (Pharmacia) is the most useful for affinity chromatography. It is more porous than the 6B derivative, and has considerably greater capacity than the 2B gels. Chemical compounds containing primary aliphatic or aromatic amines can be coupled directly to agarose beads after activation of the latter with cyanogen bromide at alkaline pH (Scheme I). The chemical nature of the intermediate formed by cyanogen halide treatment of polysaccharide derivatives is not known, but the products formed upon coupling with amino compounds appear to be principally derivatives of amino carbonic acid and isourea.²³ Notably, both of these postulated linkage groups retain the basicity of the amino group of the coupled ligand. It must be borne in mind, therefore, that even if the charge on the ligand amino group is known to contribute to the binding interaction, the resultant agarose ligand gel may nevertheless demonstrate considerable binding effectiveness.



SCHEME I

Beaded agarose gels, unlike the cross-linked dextrans, cannot be dried or frozen, since they will shrink severely and essentially irreversibly. Similarly, they will not tolerate many organic solvents. Dimethyl formamide (50%, v:v) and ethylene glycol (50%, v:v) do not adversely affect the structure of these beads. These solvents are quite useful in situations where the compound to be coupled is relatively insoluble in water (e.g., steroids, thyroxine, tryptophan derivatives) since the coupling step

²³J. Porath, *Nature (London)* 218, 834 (1968).

can be carried out in these solvents. Similarly, the final, coupled derivative can be washed with these solvents to remove strongly adsorbed or relatively water-insoluble material.

Agarose beads, both before and after activation and coupling, exhibit very little nonspecific adsorption of proteins provided the ionic strength of the buffer is 0.05 *M* or greater. The coupled, substituted adsorbents of agarose can be stored at 4° in aqueous suspensions with an antibacterial preservative (sodium azide, toluene), for periods of time limited only by the stability of the bound inhibitor or ligand; they should, as mentioned above, not be frozen or dried for storage. Agarose beads tolerate 0.1 *M* NaOH and 1 *N* HCl for at least 2-3 hours at room temperature without detectable adverse alteration of their physical properties, and without cleavage of the covalently linked ligand, if the latter itself does not possess labile bonds. The specific adsorbents formed from these beads can therefore be used repeatedly even after exposure to relatively extreme conditions; the limitation in this respect is more likely determined by the stability of the ligand than of the matrix. Agarose beads are not destroyed by exposure to temperatures as high as 45° for many minutes. They tolerate quite well 6 *M* guanidine·HCl or 7 *M* urea solutions for prolonged periods; a very small amount of dissolution may occur after exposure to 6 *M* guanidine·HCl for 2-3 days at room temperature. These protein denaturants may therefore be used to aid in the elution of specifically bound proteins, for thorough washing of columns in preparation for re-use, and in washing off protein that is tightly adsorbed during the coupling procedure.

*Procedure.*² A given volume of decanted agarose, containing a magnetic stirring bar, is mixed with an equal volume of water and the electrodes of a pH meter are placed in this suspension. The procedure is performed in a well ventilated hood. Finely divided solid cyanogen bromide (50-300 mg per milliliter of packed agarose) is added at once to the stirred suspension, and the pH is immediately raised to 11 with NaOH. The molarity of the NaOH solution will depend on the amounts of agarose and cyanogen bromide added; it should vary between 2 *M* (for 5-10 ml agarose and 1-3 g of cyanogen bromide) and 8 *M* (for 100-200 ml of agarose and 20-30 g of cyanogen bromide). The pH is maintained at 11 by constant manual titration. Temperature is maintained at about 20° by adding pieces of ice as needed. The reaction is complete in 8-12 minutes, as indicated by the cessation of base uptake; no solid CNBr should remain. A large amount of ice is then rapidly added to the suspension, which is transferred quickly to a Büchner funnel (coarse disk) and washed under suction with cold buffer. The buffer should be the same as that which is to be used in the coupling stage, and

the volume of wash should be 10–15 times that of the packed agarose, larger volumes (20–30 times) should be used if a protein is to be coupled. The Büchner funnel, containing the moist, washed agarose is removed from the filtering flask and its outlet is covered tightly with Parafilm. The compound to be coupled, in a volume of cold buffer equal to the volume of packed agarose, is added to the agarose and the suspension is immediately mixed (in the Büchner funnel) with a glass stirring rod. The entire procedure of washing, adding the ligand solution, and mixing should consume less than 90 seconds. It is important that these procedures be performed rapidly and that the temperature be lowered, since the “activated” agarose is unstable. The suspension is transferred from the Büchner funnel to a beaker containing a magnetic mixing bar and is *gently* stirred at 4° for 16–20 hours. Care must be taken to avoid vigorous stirring since Sepharose can be physically disrupted, resulting in material with poor flow rates. The substituted agarose is then washed with large volumes of water and appropriate buffers until it is established with certainty that ligand is no longer being removed.

The quantity of ligand coupled to agarose can be controlled by varying several parameters.² Most important is the amount of ligand added to the activated agarose (Table I). When highly substituted derivatives are desired, the amount of inhibitor added should, if possible, be 20–30 times greater than that which is desired in the final product. For ordinary procedures, 100–150 mg of cyanogen bromide is used per milliliter of packed agarose, but much higher coupling yields can be obtained if this amount is increased to 250–300 mg (Table II). The larger amounts of CNBr should be used only if highly substituted derivatives are desired since greater heat is evolved and longer reaction times

TABLE I
EFFICIENCY OF COUPLING OF 3'-(4-AMINOPHENYLPHOSPHORYL)DEOXYTHYMIDINE 5'-
PHOSPHATE TO AGAROSE, AND CAPACITY OF THE RESULTING ADSORBENT FOR
STAPHYLOCOCCAL NUCLEASE^a

Expt.	μ Moles of inhibitor/ml agarose		Mg of nucelase/ml agarose	
	Added	Coupled	Theoretical ^b	Found
A	4.1	2.3	44	—
B	2.5	1.5	28	8
C	1.5	1.0	19	—
D	0.5	0.3	6	1.2

^aOne hundred milligrams of cyanogen bromide was added per milliliter of packed agarose, and coupling was performed in 0.1 M NaHCO₃, pH 9.0. Data from P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.* 61, 636 (1968).

^bAssuming equimolar binding.

TABLE II
EFFECT OF pH ON THE COUPLING OF [¹⁴C]-ALANINE TO ACTIVATED AGAROSE^a

Conditions for coupling reaction		Alanine coupled
Buffer	pH	(μ moles per ml agarose)
Sodium Citrate, 0.1 M	6.0	4.2
Sodium phosphate, 0.1 M	7.5	8.0
Sodium borate, 0.1 M	8.5	11.0
Sodium borate, 0.1 M	9.5	12.5
Sodium carbonate, 0.1 M	10.5	10.5
Sodium carbonate, 0.1 M	11.5	0.2

^aSixty milliliters of packed Sepharose 4B was mixed with 60 ml of water and treated with 15 mg of cyanogen bromide as described in the text. To the cold, washed activated Sepharose was added 2.2 mmoles of [¹⁴C]alanine (0.1 μ Ci/ μ mole) in 60 ml of cold distilled water, and 20-ml aliquots of the mixed suspension were added rapidly to beakers containing 5 ml of cold 0.5 M buffer of the composition described in the table. The final concentration of alanine was 15 mM. After 24 hours, the suspensions were thoroughly washed, aliquots were hydrolyzed by heating at 110° in 6 N HCl for 24 hours, and the amount of released radioactivity was determined. Data from P. Cuatrecasas, *J. Biol. Chem.* 245, 3059 (1970).

are required. The pH at which the coupling stage is performed will also determine the degree of coupling since it is the unprotonated form of the amino group which is reactive. Compounds containing an α -amino group (pK about 8) will couple optimally at a pH of about 9.5–10.0 (Table II). Higher pH values should not be used, since the stability of the activated Sepharose decreases sharply above pH 10.5 (Table II). Compounds with primary aliphatic aminoalkyl groups, such as the ϵ -amino group of lysine or ethylamine (pK about 10), should be coupled at pH values of about 10, and a large excess of ligand should be added. The most facile coupling occurs with compounds bearing aromatic amines, due to the low pK (about 5) of the amino group; very high coupling efficiencies are obtained at pH values between 8 and 9 (Table I).

It is possible, in some cases, to increase the amount of ligand coupled to agarose by repeating the activation and coupling procedures of the already substituted material, provided the inhibitor is stable for about 10 minutes at pH 11.

Quantitation of the amount of amino acid or protein coupled to agarose is best determined by hydrolyzing a lyophilized aliquot of material in 6 N HCl, 110°, for 24 hours. After hydrolysis, the brown-black suspension is filtered twice by passing through a Pasteur pipette containing glass wool, or through a Millipore filter; the solution is dried and its composition is determined with an amino acid analyzer.

The operational capacity for specific adsorption of the agarose adsorbents¹ (Table I) can be tested by passing slowly an amount of en-

zyme or protein in excess of the theoretical capacity through a sample of adsorbent packed in a column, washing with buffer until negligible protein emerges in the effluent, and eluting (Fig. 1). Alternatively, small samples of preferably pure protein can be added successively to the column until detectable protein or enzymatic activity emerges in the effluent.¹ The total amount added, or that eluted, is considered the operational capacity.

*Examples 1: Purification of Staphylococcal Nuclease with a Competitive Inhibitor.*¹ The inhibitor of staphylococcal nuclease, 3'-(4-aminophenylphosphoryl)deoxythymidine 5'-phosphate (pdTp-aminophenyl) is an ideal ligand for preparation of specific agarose adsorbents because it is a strong competitive inhibitor (K_i , 10^{-6} M), its 3'-phosphodiester bond is not cleaved by the enzyme, it is stable at pH values of 5-10, the

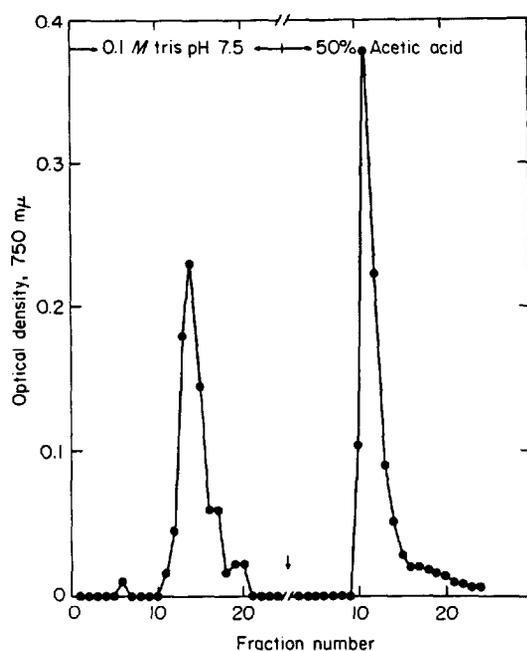


FIG. 1. Determination of the operational capacity of a column of agarose-RNase-S-protein (0.8×19 cm) for RNase-S-peptide. The column having a bed volume of 9.6 ml and containing 27 mg of bound S-protein on the basis of amino acid analysis, was washed with 200 ml of 0.1 M Tris-HCl buffer, pH 7.5. It was then perfused with 3.8 ml of an aqueous solution containing 4.2 mg of S-peptide. After thorough washing with buffer (0.1 M Tris-HCl, pH 7.5) bound S-peptide (2.3 mg) was eluted with 50% acetic acid. Since 27 mg of S-protein should combine, theoretically, with approximately 5 mg of S-peptide, it may be calculated that the binding efficiency of the immobilized S-protein was about 45%. Data from I. Kato and C. B. Anfinsen, *J. Biol. Chem.* 244, 1004 (1969).

pK of the aromatic amino group is low, and the amino group is relatively distant from the basic structural unit (pTp-X) recognized by the enzymatic binding site.²⁴ This inhibitor couples to agarose with high efficiency, and the resulting adsorbent has a high capacity for the enzyme (Table I). Columns containing this specific adsorbent completely and strongly adsorb samples of pure and crude nuclease (Fig. 2). Virtually no enzyme escapes from such columns with washes exceeding 50 times the column bed volume if the amount of protein applied does not exceed 30% of the operational capacity. Elution is accomplished by washing with solutions of low pH (acetic acid, pH 3) or high pH (NH_4OH , pH 11). The eluted protein emerges in a very small volume, and the material can be lyophilized directly.

If very crude enzyme solutions with total protein concentration greater than 20 mg/ml are passed through such columns, some of the enzyme escapes, probably because of interaction with the major protein fraction. Rechromatography usually will allow complete removal of the residual

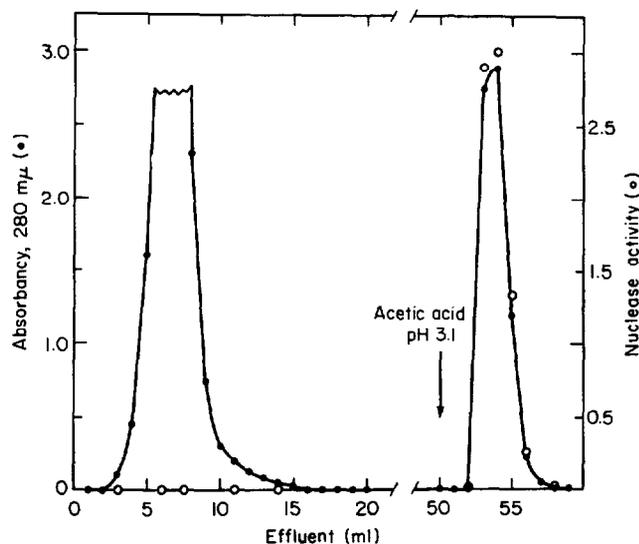


FIG. 2. Purification of staphylococcal nuclease by affinity adsorption chromatography on a nuclease-specific agarose column (0.8×5 cm) (sample B, Table I.). The column was equilibrated with 50 mM borate buffer, pH 8.0, containing 10 mM CaCl_2 . Approximately 40 mg of partially purified material containing about 8 mg of nuclease was applied in 3.2 ml of the same buffer. After 50 ml of buffer had passed through the column, 0.1 M acetic acid was added to elute the enzyme. Nuclease, 8.2 mg, and all the original activity was recovered. The flow rate was about 70 ml per hour. Data from P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.* 61, 636 (1968).

²⁴P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Biochemistry* 8, 2277 (1969).

enzyme. Very dilute solutions of enzyme should be passed through these columns at moderately slow flow rates. The columns can be used repeatedly, and over protracted periods, without detectable loss of effectiveness. These affinity adsorbents can also be used in the separation of active and inactive nuclease derivatives from samples subjected to chemical modification²⁵⁻²⁷ (as will be described), and they are effective in stopping enzymatic reactions.¹

*Example 2: Purification of α -Chymotrypsin with an Inhibitor Containing an "Arm."*¹ An agarose adsorbent containing a relatively weak competitive inhibitor, D-tryptophan methyl ester, (K_i , 10^{-4} M) is relatively ineffective in adsorbing α -chymotrypsin even though 10 μ moles of inhibitor are present per milliliter of agarose (Fig. 3B). Only a slight retardation of the enzyme is observed. However, by interposing a 6-carbon chain between the same inhibitor and the agarose matrix, dramatically stronger adsorption of the enzyme occurs (Fig. 3C). This illustrates the marked steric interference that results when the ligand is attached too closely to the supporting gel. DFP-treated α -chymotrypsin (Fig. 3D), chymotrypsinogen, pancreatic ribonuclease, subtilisin, and trypsin are not adsorbed by this specific affinity adsorbent. Greater than 90% recovery is obtained on elution with distilled water titrated to pH 3 with acetic acid. It is also possible to elute the protein with a 0.018 M solution of the competitive inhibitor, β -phenylpropionamide (K_i , 7 mM).

*Example 3: Purification of DAHP Synthetase with an Effector.*²⁸ An agarose adsorbent containing L-tyrosine (3.2 μ moles/ml), an effector of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthetase (K_i , 5×10^{-5} M), was capable of separating this enzyme from a crude protein mixture (Fig. 4). As with α -chymotrypsin, the enzyme is merely retarded, and it is not necessary to change the nature of the buffer to obtain elution. Nevertheless, a very useful derivative is obtained which separates the weakly adsorbed enzyme from the bulk of the protein and from a related enzyme.

*Example 4: Batch Purification of a Vitamin-Binding Protein.*²⁹ An early example of affinity chromatography was the purification of avidin on biotin-cellulose.¹³ A much more effective adsorbent for avidin can be prepared by coupling a derivative of biotin containing a 6-carbon chain, biocytin (ϵ -N-biotinyl-L-lysine), to Sepharose. The lysyl portion serves as an "arm" to hold the essential structural portion of the vitamin, the

²⁵P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *J. Biol. Chem.* **244**, 4316 (1969).

²⁶P. Cuatrecasas, *J. Biol. Chem.* **245**, 574 (1970).

²⁷H. Taniuchi and C. B. Anfinsen, *J. Biol. Chem.* **244**, 3864 (1969).

²⁸W. C. Chan and M. Takahashi, *Biochem. Biophys. Res. Commun.* **37**, 272 (1969).

²⁹P. Cuatrecasas and M. Wilchek, *Biochem. Biophys. Res. Commun.* **33**, 235 (1968).

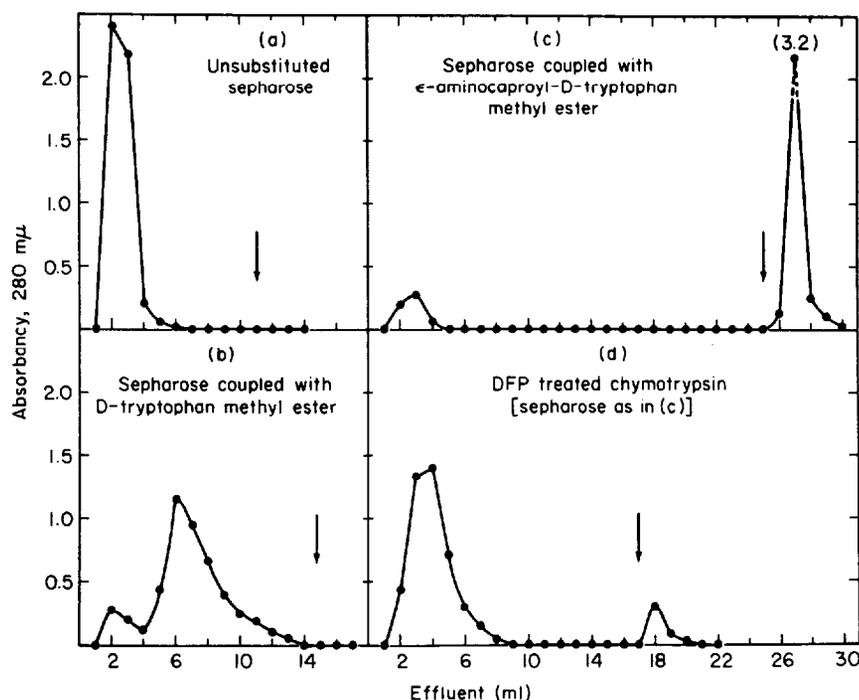


FIG. 3. Affinity chromatography of α -chymotrypsin on inhibitor-agarose columns. The columns (0.5×5 cm) were equilibrated and run with 50 mM Tris-HCl buffer, pH 8.0. Each sample (2.5 mg) was applied in 0.5 ml of the same buffer. Fractions of 1 ml were collected at a flow rate of about 40 ml per hour at room temperature. α -Chymotrypsin was eluted with 0.1 M acetic acid, pH 3.0 (arrows). Peaks preceding the arrows in B, C, and D were devoid of enzyme activity. In preparing the agarose adsorbent, 65 μ moles of inhibitor was added per milliliter of Sepharose, 10 μ moles of which was found to be covalently attached, yielding an effective concentration of inhibitor, in the column, of about 10 mM. Data from P. Cuatrecasas, M. Wilchek, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.* 61, 636 (1968).

ureido and thiophan rings, at a good distance from the matrix. Adsorbents containing very small amounts (0.02 μ mole per milliliter of agarose) of this compound which binds very tightly to avidin (K_i , 10^{-15} M) can be used to purify, in a batchwise manner, avidin from crude egg white in a single step (Table III). The binding of avidin is so strong that elution must be performed with 6 M guanidine-HCl, pH 1.5.

Example 5: Purification of Thyroxine-Binding Protein from Serum. Columns containing agarose with *N*-(6-aminocaproyl)thyroxine,³⁰ or thyroxine^{30,31} itself, remove most of the thyroxine-binding globulin from serum. This

³⁰R. A. Pages, H. J. Cahnmann, and J. Robbins, personal communication (1969).

³¹J. Pensky and J. S. Marshall, *Arch. Biochem. Biophys.* 135, 304 (1969).

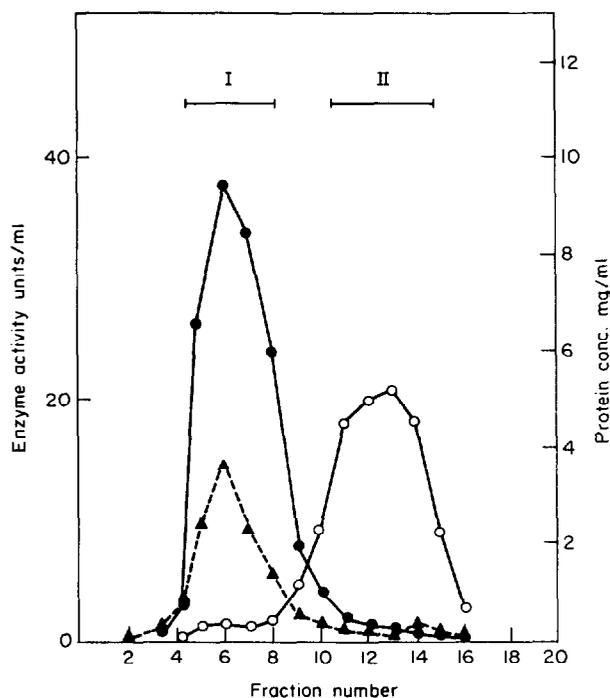


FIG. 4. Purification of tyrosine-sensitive 3-deoxy-D-arabinoheptulosonate 7-phosphate synthetase by affinity chromatography. Chromatography of crude yeast extract on tyrosine-Sepharose column (0.6 × 15.5 cm), equilibrated with 0.2 M sodium phosphate buffer, pH 6.5 containing CuSO_4 (0.1 mM) and PMSF (0.1 mM). The sample consisted of 2 ml of crude extract, and 1-ml fractions were collected. ○—○, Tyrosine-sensitive DAHP synthetase; ●—●, phenylalanine-sensitive DAHP synthetase; —△—, protein concentration. Pooled fractions are indicated (I and II). Data from W. W. C. Chan and M. Takahashi, *Biochem. Biophys. Res. Commun.* 37, 272 (1969).

protein, which has a dissociation constant of about 10^{-10} M for thyroxine and which is present at very low concentrations in serum (10–20 $\mu\text{g}/\text{ml}$), remains strongly adsorbed to the agarose after the column is washed with 0.1 M NaHCO_3 buffer, pH 9.0, it is eluted with 4 mM NaOH, pH 11.4.³⁰ Although not pure, the eluted protein is enriched considerably by this single-step procedure. Sepharose-4B, activated with 100 mg of cyanogen bromide per milliliter of Sepharose by the previously described procedures, is coupled with thyroxine or aminocaproyl-thyroxine (3 μmoles per milliliter of Sepharose) in 50% (v:v) ethylene glycol (to enhance thyroxine solubility), 50 mM NaHCO_3 , pH 9.6; about 1.8 μmoles of thyroxine derivative is coupled per milliliter of agarose. Adsorbed [^{125}I]thyroxine is completely removed by washing with 20 volumes of the ethylene glycol buffer followed by 20 volumes of 0.1 M NaHCO_3 , pH

TABLE III
PURIFICATION OF AVIDIN FROM EGG WHITE BY BIOCYTIN-AGAROSE CHROMATOGRAPHY

Sample	Protein (mg)	Avidin (mg)	Specific activity (μg biotin per mg protein)	Purification factor
Egg white	8000	2.2	0.0037	—
Washings of Sepharose	8000	0.2	0.0003	—
Elution from Sepharose	1.5 ^a	1.6	14.6	4000

^aBased on $E_{1\text{ cm}}^{0.1\%}$ of 1.57 (M. D. Melamed and N. M. Green, *Biochem. J.* 89, 591 (1963)). Two milliliters of biocytin-Sepharose were added to 25 ml of 0.2 M NaHCO₃, pH 8.7, containing 8 g of crude, dried egg white. After a few minutes the solution was centrifuged at 20,000 *g* for 10 minutes. The Sepharose was suspended in bicarbonate buffer and poured onto a 1 × 5 cm column. The column was washed with about 25 ml of 3 M guanidine-HCl, pH 4.5, and the avidin was eluted with 6 M guanidine-HCl at pH 1.5. Avidin concentration was determined by addition of biotin ¹⁴C, followed by dialysis for 24 hours at room temperature against large volumes of 0.1 M NaHCO₃ at pH 8.2; it was assumed that 13.8 μg of biotin are bound per milligram of avidin. Data from P. Cuatrecasas and M. Wilchek, *Biochem. Biophys. Res. Commun.* 33, 235 (1968).

9.0. These studies indicated that ethylene glycol (50%, v:v), but not dimethyl sulfoxide, which was also investigated, is well tolerated by agarose.

*Example 6: Resolution of Chemically Modified Enzyme Mixtures.*²⁵⁻²⁷ Studies of the functional effects of chemical modifications of purified enzymes frequently reveal incomplete loss of enzymatic activity. It is often difficult to determine whether this results from an altered protein possessing diminished catalytic properties or to residual native enzyme. In the latter case, separation of the active native and the catalytically inert proteins may not be possible. In certain cases, both problems may be resolved by using affinity adsorbents. Modification of staphylococcal nuclease by attachment of a single molecule of an affinity labeling reagent through an azo linkage to an active site tyrosyl residue, resulted in loss of 83% of the enzyme activity.²⁶ Chromatography of this enzyme solution on a nuclease-specific agarose column revealed that the residual activity was due entirely to a 20% contamination of native enzyme; complete resolution of the two components was possible by affinity chromatography (Fig. 5).²⁶ Similar separation was obtained with partially active preparations of staphylococcal nuclease modified with bromoacetamidophenyl affinity labeling reagents,²⁵ and residual native nuclease could be separated from an inactive peptide fragment obtained by specific tryptic cleavage.²⁷

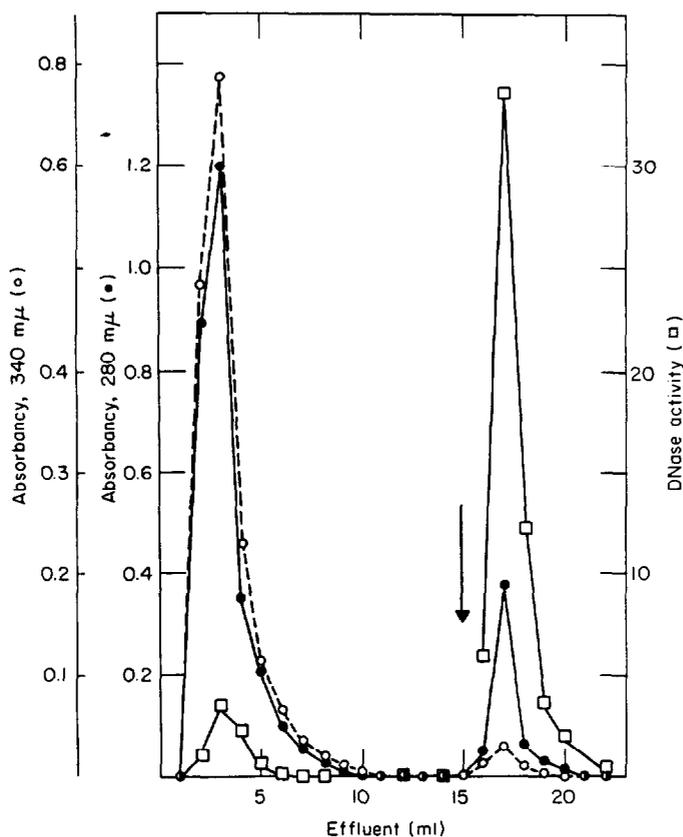


FIG. 5. Affinity chromatography on specific agarose column (Table I, Fig. 2) of staphylococcal nuclease treated with a 1.7-fold molar excess a diazonium labeling reagent derived from pdTp-aminophenyl. About 3 mg of chemically modified nuclease, containing 17% of the DNase activity of the native enzyme, were applied to a 0.5×7 cm column which contained Sepharose 4B conjugated with the inhibitor, pdTp-aminophenyl ($0.8 \mu\text{mole}$ per milliliter of Sepharose). The column was equilibrated and developed with 50 mM borate buffer, pH 8.0, and 10 mM CaCl_2 . The bound enzyme was eluted with NH_4OH , pH 11 (arrow). The small amount of enzymatic activity present in the early, unretarded peak could be removed by rechromatography of this peak through the same column. The specific activity of the small amount of protein adsorbing strongly to the column was identical with that of native nuclease. Data from P. Cuatrecasas, *J. Biol. Chem.* 245, 574 (1970).

Example 7: Other Enzymes. Papain has been purified with an agarose adsorbent to which glycyglycyl-(*o*-benzyl)-L-tyrosine-L-arginine had been coupled by the cyanogen bromide procedure described earlier.³² Bovine pancreatic ribonuclease can be purified with an agarose adsorbent

³²S. Blumberg, I. Schechter, and A. Berger, *Israel J. Chem.* 7, 125 p (1969).

containing 5'-(4-aminophenylphosphoryl)uridine-2'(3')-phosphate, prepared by the cyanogen bromide method.³³

Preparation of and Coupling to Modified Agarose Beads

A number of stable chemical derivatives of agarose beads, which can be rapidly and easily prepared under mild aqueous conditions,^{2,3,4,7} will be presented in order to provide greater versatility to the general method. Since selective adsorbents must be tailored to the special characteristics of the individual protein, and to the equally stringent ligand requirements of that protein, it is useful to have alternative methods of attaching ligands to agarose. In many cases it is much easier to modify the matrix support than the ligand. More specifically, these derivatizations provide procedures especially applicable to cases in which (1) an amino group on the ligand is not available or its synthesis is difficult; (2) hydrocarbon chains of varying length need to be interposed between the matrix and the ligand; and (3) only the mildest eluting conditions, i.e., neutral pH, absence of protein denaturants, are tolerated by the adsorbed enzyme. In the last case, the protein ligand complex is removed intact by specific cleavage of the ligand matrix bond. Some of the derivatives described below are now available commercially (Affitron Corp.).

ω -Aminoalkyl Derivatives of Agarose Beads.^{2,7}

Aliphatic diamine compounds, such as ethylenediamine, may be substituted directly to agarose by the cyanogen bromide procedure described earlier (Scheme I, C). Although on theoretical grounds cross-linking might be expected to occur, in practice very little, if any, can be detected. This is probably due to the large excess of diamine added during the coupling stage, so that the amino groups on the agarose must compete with a very large excess of amino groups free in solution. It is possible, therefore, to prepare agarose derivatives having free amino groups which extend a good distance from the solid matrix, depending on the nature of the group, $-(CH_2)_x$. The ω -aminoalkyl groups can be used, in turn, for attachment of other functional groups, ligands, or proteins.

Procedure—Preparation of Aminoethyl Agarose. To an equal volume of a Sepharose-4B water suspension is added 250 mg of cyanogen bromide per milliliter of packed gel, and the reaction is performed as described above. The washed, activated Sepharose is suspended in an equal volume of cold distilled water containing 2 mmoles of ethylenediamine per

³³M. Wilchek and M. Gorecki, *Eur. J. Biochem.* 11, 491 (1969).

milliliter of Sepharose; the pH of this solution is adjusted to 10.0 with 6 N HCl before it is added to the Sepharose. After reaction for 16 hours at 4°, the gel is washed with large volumes of distilled water. This treatment results in a derivative having about 12 μ moles of aminoethyl group per milliliter of Sepharose. Different ω -aminoalkyl derivatives can be prepared in a similar way by using other diamines. 3,3'-Diaminodipropyl amine has been one of the most useful for a variety of purposes.^{2,3,22}

Color Test with Sodium 2, 4, 6-Trinitrobenzenesulfonate (TNBS)²

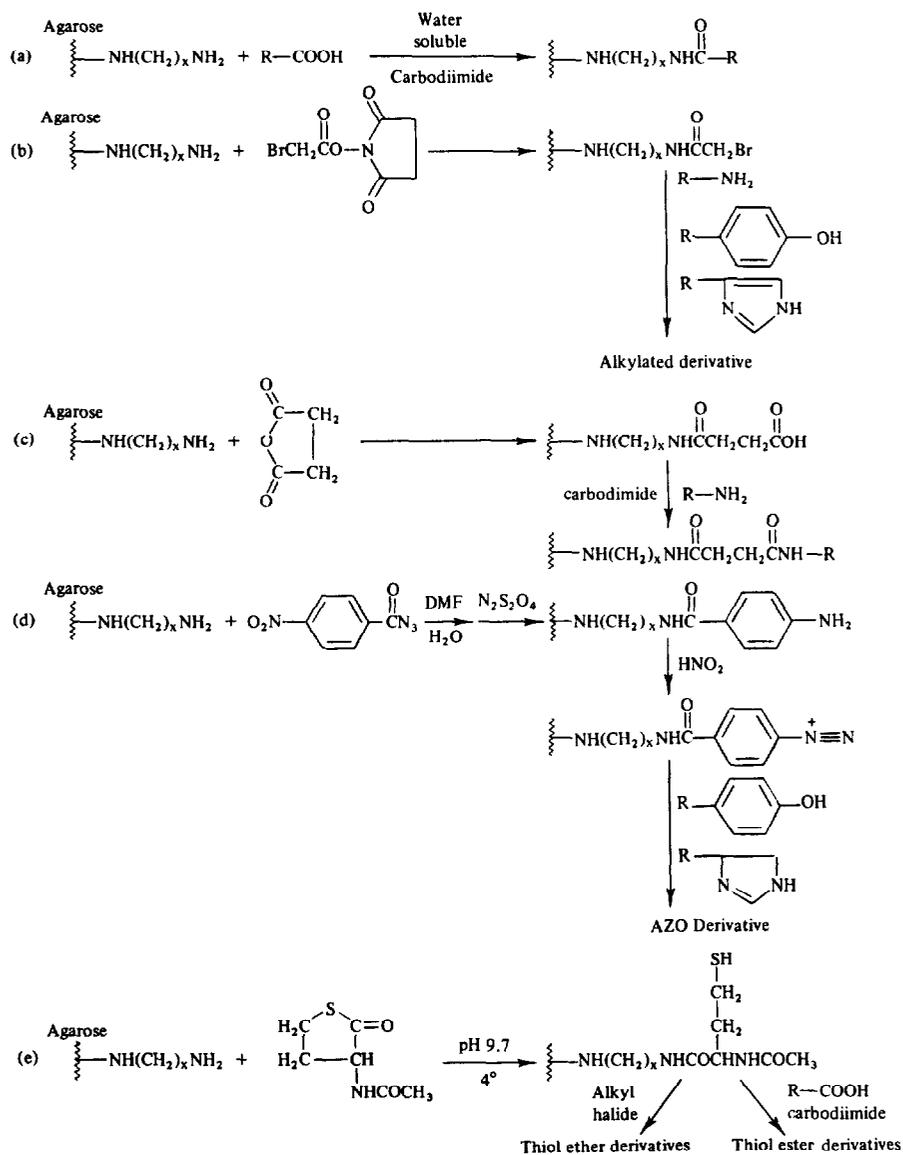
This simple color test, modified² from that described by Inman and Dintzis,²¹ is very useful in following the course of the various agarose and polyacrylamide derivatizations to be described. One milliliter of saturated sodium borate is added to a slurry (0.2-0.5 ml, in distilled water) of the agarose or polyacrylamide gel. Three drops of a 3% aqueous solution of sodium 2, 4, 6-trinitrobenzenesulfonate are added; the stock solution may be stored, in a brown glass bottle, for at least 2 weeks. At room temperature the color reaction of the gel beads is complete within 2 hours. The following color products are formed with various derivatives: unsubstituted agarose or polyacrylamide beads, yellow; derivatives containing primary aliphatic amines, orange; derivatives containing primary aromatic amines, red-orange; unsubstituted hydrazide derivatives, deep red; carboxylic acid and bromoacetyl derivatives, yellow. The degree of substitution of amino gel derivatives by carboxylic acid ligands, and of hydrazide gels by amino group containing ligands, can be conveniently estimated from the relative color intensity of the washed, coupled gel.

Coupling Carboxylic Acid Ligands to Aminoethyl Agarose.^{2,7}

Ligands containing free carboxyl groups may be coupled directly to the ω -aminoalkyl-Sepharose derivatives described above with a water-soluble carbodiimide (Scheme II, A). Two examples of such reactions are presented below.

Procedure a: Preparation of Estradiol-Sepharose.³⁴ Three hundred milligrams of 3-O-succinyl-[³H]estradiol (prepared by reacting [³H]-estradiol with succinic anhydride in 50% dimethyl formamide-pyridine; specific activity, 0.3 μ Ci/mole) is added in 40 ml of dimethyl formamide to 40 ml of packed aminoethyl Sepharose-4B (2 μ moles of aminoethyl groups per milliliter). The dimethyl formamide is required to solubilize estradiol. The pH of this suspension is brought to 4.7 with 1 N HCl. Five hundred milligrams (2.6 mmoles) of 1-ethyl-3-(3-dimethylamino-

³⁴P. Cuatrecasas and G. A. Puca, unpublished observations (1970).



SCHEME II

propyl) carbodiimide, dissolved in 3 ml of water, is added over a 5-minute period and the reaction is allowed to proceed at room temperature for 20 hours. The substituted agarose is then washed continuously, while packed in a column or on a Büchler funnel with 50% aqueous dimethyl formamide until radioactivity is absent from the effluent. The

completeness of washing is checked by collecting 200 ml of effluent wash, lyophilizing, adding 2 ml of dimethyl formamide, and determining radioactivity. Before use, it is essential to incubate the agarose in an albumin buffer (3%, v/v) to determine if there is release of substances into the medium which are capable of inhibiting the binding of ^3H estradiol to the uterine binding protein, this is an excellent way of detecting non-covalently bound estradiol. About 0.5 μmole of [^3H]-estradiol are covalently bound to the agarose matrix. This is determined by counting the Sepharose in Cab-O-Sil, or by hydrolyzing the ester bond by exposure to 0.1 *N* NaOH for 60 minutes at room temperature, followed by centrifugation and estimating radioactivity in the supernatant fluid. With such derivatives it is possible to elute strongly adsorbed serum estradiol binding protein by cleaving the estradiol agarose bond with mild base, thus avoiding the use of protein denaturants. This estradiol-agarose derivative is very effective in extracting estradiol binding proteins from serum and from calf uterine extracts.³⁴ Estradiol has been attached to polyvinyl and cellulose polymers by different procedures.³⁵

Procedure b: Preparation of Organomercurial-agarose for Separation of SH-proteins.^{2,7} Packed aminoethyl-Sepharose, 35 ml (capacity, 10 $\mu\text{moles/ml}$) is suspended in a total volume of 60 ml of 40% dimethyl formamide; 2 mmoles of sodium *p*-chloromercuribenzoate is added; the pH is adjusted to 4.8; and 5 mmoles of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide are added. The pH is maintained at 4.8 by continuous titration with 0.1 *N* NaOH for one hour. After reaction at room temperature for another 18 hours, the substituted agarose is washed with 4 liters of 0.1 *M* NaHCO_3 , pH 8.8, over an 8-hour period. Complete substitution of the agarose amino groups is confirmed by the TNBS color test, which yields a yellow gel. The derivative can bind 40–50 mg of horse hemoglobin per milliliter of packed gel. The effectiveness of the binding, compared to that of a derivative prepared by attaching a bifunctional organomercurial to SH-Sephadex G25 by much more complicated procedures,³⁶ is reflected in the fact that solutions of low pH (2.7, acetic acid) or complexing agents, such as EDTA (0.1 *M*), remove the protein only very slowly. The most effective elution is achieved by passing a 50 *mM* cysteine or dithiothreitol solution into the column and stopping the flow for 1 or 2 hours before collecting the effluent. Advantages of the present procedures include the ease of

³⁵B. Vonderhaar and G. C. Mueller, *Biochim. Biophys. Acta* 176, 626 (1969).

³⁶L. Eldjarn and E. Jellum, *Acta Chem. Scand.* 17, 2610 (1963).

preparation, the use of agarose with its inherently large capacity to bind large proteins by permitting their diffusion throughout the mesh, the circumvention of conditions which irreversibly destroy the agarose beads, i.e., drying and dioxane, and the stability of the final derivative.

Preparation of Bromoacetyl Agarose Derivatives.^{2,7}

Bromoacetamidoethyl-Agarose can be prepared under mild aqueous conditions by treating aminoethyl-Sepharose-4B with *O*-bromoacetyl-*N*-hydroxysuccinimide (Scheme II, B). This derivative of agarose can react with primary aliphatic or aromatic amines as well as with imidazole and phenolic compounds. Additionally, proteins readily couple to bromoacetamidoethyl-Sepharose, forming insoluble derivatives in which the protein is located at some distance from the solid support.

Procedure. In 8 ml of dioxane are dissolved 1.0 mmole of bromoacetic acid and 1.2 mmole of *N*-hydroxysuccinimide. To this solution, 1.1 mmole of dicyclohexylcarbodiimide is added. After 70 minutes, dicyclohexylurea is removed by filtration, and the entire filtrate, or crystalline bromoacetyl-*N*-hydroxysuccinimide ester, is added, without further purification, to a suspension, at 4°, which contains 20 ml of packed aminoethyl-Sepharose (2 μmoles of amino groups per milliliter) in a total volume of 50 ml at pH 7.5 in 0.1 M sodium phosphate. After 30 minutes, the product is washed with 2 liters of cold 0.1 N NaCl. Quantitative reaction of the amino groups occurs as shown by the loss of orange color with the TNBS test. The bromoacetamidoethyl-Sepharose gel can be stored at 4° as a suspension in distilled water (pH about 5.5), or can be treated with a primary amine, R-NH₂ (Scheme II, B). Reaction with 10 mM pdTp-aminophenyl as a 50% (v:v) suspension in 0.1 M NaHCO₃, pH 8.5, for 3 days at room temperature, followed by reaction for 24 hours at room temperature with 0.2 M 2-aminoethanol to mask unreacted bromoacetyl groups, results in attachment of 0.8 μmole of inhibitor per milliliter of packed agarose. A similar reaction with [³H]5'-GMP resulted in the covalent attachment of 0.2 μmoles/ml. Such bromoacetyl-Sepharose derivatives have also been used to insolubilize [³H]estradiol for use in the purification of serum and uterine estradiol binding proteins.³⁴ Proteins can be similarly attached by reacting in 0.1 M NaHCO₃, pH 9.0, for 2 days at room temperature, or for longer periods at lower temperatures.

Preparation of Succinylaminoethyl-Agarose^{2,7}

This derivative is prepared by treating aminoethyl-Sepharose with succinic anhydride in aqueous media at pH 6.0 (Scheme II, C). One

millimole of succinic anhydride is added per milliliter of packed aminoethyl-Sepharose (capacity, 8 μ moles/ml), in an equal volume of distilled water at 4°, and the pH is raised to and maintained at 6.0 by titrating with 20% NaOH. When no further change in pH occurs, the suspension is left for 5 more hours at 4°. Complete reaction of the amino group occurs as shown by the TNBS color reaction. Compounds containing primary amino groups can be coupled at pH 5 to such carboxyl containing Sepharose derivatives in the presence of the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, by the same procedures described above.

*Example: Purification of Bacterial β -Galactosidase.*²² The very "long" diamine derivative, 3, 3' -diaminodipropylamine, was coupled to Sepharose (about 10 μ moles/ml of gel) by the procedure described for the preparation of aminoethylagarose. The succinyl derivative was then prepared as described above. One millimole of *p*-aminophenyl- β -D-thiogalactopyranoside is added to 30 ml of the succinyl gel suspended in 50 ml of distilled water. The pH is adjusted to 4.7, and 7.5 mmoles of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 3 ml of water, are added dropwise over a 5-minute period. The pH is maintained at 4.7 for 1 hour by titrating with 0.1 N NaOH. The suspension is stirred at room temperature for another 16 hours, then washed with about 12 liters 0.1 N NaCl over an 8-hour period. The adsorbent, prepared in this way, binds β -galactosidase from several bacterial sources very strongly; elution of the protein from such a column is achieved by passage of a buffer having a pH of 10, or with a substrate-containing buffer. It is notable that gel derivatives prepared by attaching the same ligand directly to agarose, or by the diazotization procedure (described below), were ineffective in binding the enzyme. This compound, which is a very weak competitive inhibitor with a K_i of about 5 mM, must be attached to the solid matrix backbone by a rather long "arm."

Preparation of p-Aminobenzamidoethyl-Agarose for Coupling Compounds via Azo Linkage.^{2,7}

Diazonium derivatives of agarose, which are capable of reacting with phenolic and histidyl compounds, can be prepared under mild aqueous conditions from *p*-aminobenzamidoethyl-Sepharose (Scheme II, D). The azo-substituted agarose gels retain the properties of good flow rate, porosity, capacity, and stability, all of which are necessary for effective affinity chromatography.

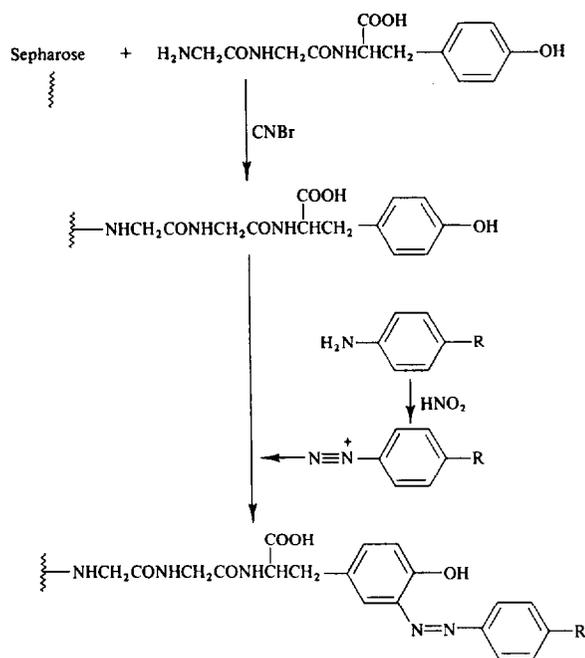
Procedure. Aminoethyl-Sepharose, in 0.2 M sodium borate, pH 9.3, and 40% dimethyl formamide (v:v), is treated for 4 hours at room temperature with 70 mM *p*-nitrobenzoyl azide (Eastman). The sub-

stitution is complete, as judged by the loss of color reaction with TNBS. The *p*-nitrobenzamidoethyl-Sepharose is washed extensively with 50% dimethyl formamide and is reduced by reaction for 60 minutes at 40° with 0.2 *M* sodium dithionite in 0.5 *M* NaHCO₃ at pH 8.5. The *p*-aminobenzamidoethyl-Sepharose derivative, in 0.5 *N* HCl, can be diazotized by treating for 7 minutes at 4° with 0.1 *M* sodium nitrite. This diazonium-Sepharose derivative may be utilized at once, without further washing, by adding the phenolic, histidyl, or protein component to be coupled in a strong buffer such as saturated sodium borate, and adjusting the pH with NaOH to 8 (histidyl) or 9.2 (phenolic); the reaction is allowed to proceed for about 16 hours at 4°. Strong tertiary amines, such as triethylamine, should not be used as buffers during the azotization reaction since they can react with the diazonium intermediate. Agarose derivatives containing estradiol in azo linkage at C-2 and C-4 of the A ring remove estradiol binding proteins from human serum and from calf uterine extracts.³⁴

An important advantage of the azo-linked ligand derivatives of agarose or of polyacrylamide beads is that passage of a solution of 0.1 *M* sodium dithionite in 0.2 *M* sodium borate at pH 9, through such columns causes rapid and complete release of the bound inhibitor by reducing the azo bond. This allows easy and accurate estimation of the quantity of inhibitor bound to the gel. Of greater importance, the procedure permits elution of the intact protein-inhibitor complex under relatively mild conditions. For example, serum estradiol binding protein is denatured irreversibly by exposure to pH 3 or 11.5, and by low concentrations of guanidine hydrochloride (3 *M*) or urea (4 *M*). The protein, which binds estradiol very tightly (K_i about 10^{-9} *M*), can be removed in active form from the agarose-estradiol gel by reductive cleavage of the azo link with dithionite, but not with buffers of low pH or with guanidine·HCl.³⁴

Preparation of Tyrosyl-Agarose for Coupling Diazotized Compounds^{2,7}

A tripeptide containing a COOH-terminal tyrosine residue can be attached to agarose by the cyanogen bromide procedure described earlier. Ligands containing an aromatic amine, which can be diazotized, can then be coupled in high yield through azo linkages to the tyrosyl moiety (Scheme III). The ligand thus extends considerably from the matrix backbone. An example of such a coupling procedure which has been used to insolubilize deoxythymidine 5'-phosphate 3'-*p*-aminophenylphosphate (for staphylococcal nuclease) and *p*-aminophenyl- β -D-thiogalactoside (for β -galactosidase), and which is applicable to many other compounds is the following: 100 μ moles of inhibitor is



dissolved in 1.5 ml of 1.5 *N* HCl, and placed in an ice bath; 700 μ moles of NaNO₂, in 0.5 ml of water, is added over a 1-minute period to the stirred ligand solution. After 7-8 minutes the entire mixture, without further purification, is added to a rapidly mixing suspension, in an ice bath, of tyrosyl-Sepharose containing 0.2 *M* Na₂CO₃. The pH is rapidly adjusted to 9.4. After 3 hours the agarose suspension is transferred to a Büchner funnel and washed. If dimethyl formamide is to be used in the reaction mixture, the buffer should be sodium borate in order to prevent precipitation of the salt. Staphylococcal nuclease is readily purified in one step by passage through a column of such a derivative containing the competitive inhibitor, pdTp-aminophenyl, in azo linkage. Congo red dye has also been attached to Sepharose in this manner; small amounts of amyloid protein are agarose adsorbed to this matrix after passage of crude material dissolved in 4 *M* guanidine·HCl at pH 6; elution is achieved with 6 *M* guanidine·HCl at pH 3. These ligand-protein complexes can be removed by treating the gel with sodium dithionite, as described earlier for other gels containing azo-linked ligands.

Preparation of Sulfhydryl Agarose Derivatives²

Thiol groups can be introduced into ω -aminoalkyl agarose derivatives

by reaction with homocysteine thiolactones by using procedures similar to those used for thiolation of proteins (Scheme II, E).^{37,38} Five grams of *N*-acetylhomocysteine thiolactone is added to a cold (4°) suspension consisting of an equal volume (50 ml) of aminoethyl Sepharose 4-B and of 1.0 M NaHCO₃, pH 9.7. The suspension is stirred gently for 24 hours at 4°, then washed with 8 liters of 0.1 N NaCl. The agarose derivative is then incubated in 0.05 M dithiothreitol, 0.5 M Tris·HCl buffer, pH 8.0, for 30 minutes at room temperature. The suspension is washed with 2 liters of 0.1 M sodium acetate buffer, pH 5.0. At this stage a strong red-brown color is obtained with the sodium trinitrobenzenesulfonate color test. The color produced with this reagent is completely lost after treating a sample of the thiol agarose with 50 mM iodoacetamide in 0.5 M NaHCO₃, pH 8.0, for 15 minutes at room temperature, followed by washing with distilled water. This confirms that complete substitution of the amino-agarose groups has occurred. The derivative can be stored for many months as an aqueous suspension at 4° provided that it is treated, before use, with dithiothreitol as described above.

The degree of sulfhydryl substitution can be conveniently determined by reacting the thiol-agarose with 5,5'-dithio-bis-(2-nitrobenzoic acid)³⁹ at pH 8, followed by centrifugation and determination of the absorbancy at 412 m μ in the supernatant. By this procedure, the derivative described above contains 0.58 μ mole of sulfhydryl per milliliter of packed agarose. Reaction with 10 mM [¹⁴C]iodoacetamide in 0.1 M NaHCO₃, pH 8.0, for 15 minutes at room temperature, results in uptake of 0.65 μ mole of radioactivity per milliliter of agarose.

Ligands or proteins that contain alkyl halides can be readily coupled to sulfhydryl agarose derivatives through stable thioether bonds. The reactivity of the thiol gels with heavy metals may be of special value in affinity chromatography. Furthermore, agarose derivatives containing free sulfhydryl groups can be used to couple ligands by thiol ester linkage, as described below.

*Coupling Ligands to Agarose by Thiol Ester Bonds.*² Ligands containing a free carboxyl group can be coupled to sulfhydryl-agarose with water soluble carbodiimides. Although the thiol ester bonds thus formed are stable at neutral pH, cleavage is readily achieved by exposure for a few minutes to pH 11.5, or by treatment with 1 N hydroxylamine for about 30 minutes. Thus, it is possible by specific chemical cleavage to remove the intact ligand-protein complex from an adsorbent containing the specifically bound protein.

³⁷R. Benesch and R. E. Benesch, *J. Amer. Chem. Soc.* **78**, 1597 (1956).

³⁸R. Benesch and R. E. Benesch, *Proc. Nat. Acad. Sci. U. S. A.* **44**, 848 (1958).

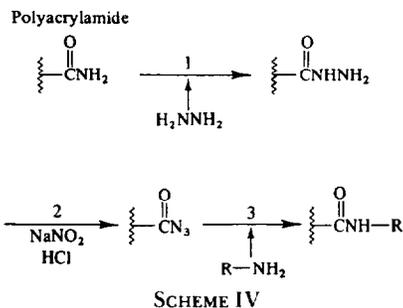
³⁹G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).

N-Acetylglycine (0.2 *M*) is added to 40 ml of packed sulfhydryl-agarose (containing 0.7 μ mole of thiol per milliliter of packed agarose) adjusted to 80 ml with distilled water. The pH is adjusted to 4.7, and 1.5 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, dissolved in 3 ml of water, is added. The pH is maintained at 4.7 for 1 hour by continuous titration. The suspension is allowed to stir gently for 8–12 hours at room temperature. The agarose is then washed with 2 liters of 0.1 *N* NaCl. At this stage virtually no color is obtained with the sodium trinitrobenzene sulfonate color test, and the sulfhydryl content, determined with 5,5'-dithiobis-(2-nitrobenzoic acid),³⁹ is about 0.03 μ mole per milliliter of Sepharose. All the sulfhydryl groups remaining free on the Sepharose are masked by treatment with 0.02 *M* iodoacetamide for 20 minutes at room temperature in 0.2 *M* NaHCO₃, pH 8.0. 3-*O*-Succinylestradiol has been coupled to sulfhydryl-Sepharose by these procedures. The resulting adsorbent is very effective in binding estradiol binding proteins from serum and uterine extracts.³⁴

Preparation of Derivatives of Polyacrylamide Beads

As discussed earlier, the principal advantage of polyacrylamide beads over agarose is that a much higher degree of substitution is possible with the former. However, the considerably lower porosity of currently available beads may limit their use in the affinity chromatography of very large proteins. Another consideration is that some shrinkage of the gels occurs during formation of the acyl azide intermediate²; shrinkage would be expected to lead to decreased porosity. Thus, it may be possible to prepare an adsorbent containing a very high concentration of ligand, much of which is inaccessible to the protein to be purified.²² Only the most porous beads, Bio-Gel P-300 (Bio-Rad Labs), have been found effective for the purification of staphylococcal nuclease, a protein with a molecular weight of 17,000. The procedures, based on those described by Inman and Dintzis,²¹ described below, involve conversion of the carboxamide side groups to hydrazide groups which in turn are converted with nitrous acid into azyl azide derivatives, thereby allowing ready reaction with aliphatic and aromatic primary amines.

Preparation of Hydrazide Derivatives (Scheme IV, 1).^{2,21} Swollen polyacrylamide beads, in a volume of water equal to 1.5 times the value of the hydrated gel and containing 3–6 *M* hydrazine hydrate are heated in a constant temperature bath to between 45° and 50°. The procedure is carried out under constant stirring in a stoppered vessel in a hood. The time required for reaction is a function of the degree of derivatization desired; the amount of substitution varies linearly for at least an 8-hour period (Fig. 6). Thereafter, the hydrazide-acrylamide



derivative is washed in a hood with large volumes of 0.1 M NaCl until the washes are free of hydrazine as determined by the TNBS color reaction. Bio-Gel P-300 derivatives are conveniently washed by repeated decantation using large volumes of saline, rather than on a Büchner funnel with its slower flow rate. Approximately 30 μ moles of hydrazide per milliliter of packed resin (Bio-Gel P-300) are formed after treatment for 3 hours at 47° in 3 M hydrazine. Greater substi-

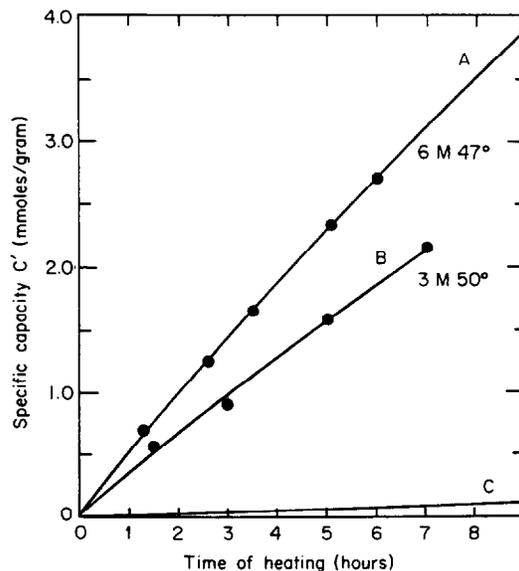


FIG. 6. Time course of the reaction between polyacrylamide Bio-Gel P-60, 100-200 Mesh) and aqueous hydrazine. Molar concentrations of hydrazine and temperature are indicated. Curve C is the average time course of carboxyl group formation during the runs for curves A and B. Specific capacity, C' , refers to mmoles of hydrazide group found on the amount of derivative produced from 1 g of the original dry polyacrylamide. Similar curves are to be expected for Bio-Gel beads other than P-60. Data from J. K. Inman and H. M. Dintzis, *Biochemistry* 8, 4074 (1969).

tution on Bio-Gel P-300 is not recommended because the physical properties of the beads are adversely affected. The polyacrylamide-hydrazide derivatives may be stored in aqueous media in the presence of a preservative, such as sodium azide or toluene, for at least 5 months.

Coupling of Primary Amines via the Azyl Azide Procedure (Scheme IV, 2, 3).^{3,18} Primary aliphatic or aromatic amines can be coupled via the azyl azide derivative without intermediate washings or transfers. The hydrazide polymer (packed volume, 50 ml) is washed, and suspended in twice its volume of 0.3N HCl and the entire suspension is placed in an ice bath. Ten milliliter of 1.0 M NaNO₂ are added rapidly under vigorous magnetic stirring. After 90 seconds, the amine is added rapidly in about 20 ml of cold 0.2 M Na₂CO₃, and the pH is raised to 9.4 with 4 M NaOH. After reaction for 2 hours at 4°, the suspension is washed with distilled water and suspended for 5 hours in 2 M NH₄Cl-1 M NH₄OH, pH 8.8, in order to convert unreacted hydrazide to the carboxamide form. If a high degree of substitution is desired, the amine should be added in amounts 10- to 20-fold greater than that of the hydrazide content of the gel.

*Other Polyacrylamide Derivatizations.*² In general, the specific polyacrylamide adsorbents are more useful when the ligand is attached at some distance from the matrix. For this purpose ω -aminoalkyl, bromoacetamidoethyl, Gly-Gly-Tyr, and *p*-aminobenzamido ethyl derivatives can be prepared by procedures identical to those described earlier. Very satisfactory affinity adsorbents have been prepared for the purification of staphylococcal nuclease by many of these procedures. A derivative obtained by coupling pdTp-aminophenyl directly to Bio-Gel P-300 via the acyl azide step, which contained 12 μ moles of inhibitor per milliliter of gel, effectively adsorbed 22 mg of nuclease per milliliter of resin from a crude enzyme preparation. Enzyme was recovered by elution with NH₄OH at pH 11. Other ligands which have been coupled to polyacrylamide beads include Congo red, *p*-aminophenyl- β -D-thiogalactopyranoside, 5'-GMP, estradiol, adenosine 3', 5'-cyclic monophosphate, and several amino acids.

Coupling of Proteins and Peptides to Agarose for Affinity Chromatography

The previously described desirable features of agarose, particularly its porosity, permit the preparation of insoluble protein derivatives which can be used to exploit reversible macromolecular interactions. In addition to attaching proteins or peptides to agarose directly by the cyanogen bromide procedure, it is possible to use the bromoacetyl or the diazonium derivatives described earlier. Proteins coupled to such gels extend some distance from the matrix backbone by an "arm." This may

be important in decreasing steric difficulties when interactions with other macromolecules are being studied. For example, purification or isolation of intact cells or particulate cell structures by insolubilized proteins may best be achieved with such derivatives.³

An important consideration in the covalent attachment of a biologically active protein to an insoluble support is that the protein should be attached to the matrix by the fewest possible bonds. This will enhance the probability that the attached macromolecule will retain its native tertiary structure. Proteins react with cyanogen bromide-activated agarose through the unprotonated form of their free amino groups. Since most proteins are richly endowed with lysyl residues, which are exposed to the solvent, it is likely that such molecules will have multiple points of attachment to the resin when the coupling reaction is performed at pH 9 or higher. The number of linkage points and the resultant biologic activity of the insolubilized protein can be controlled by manipulating the pH of the coupling reaction. Proteins having few amino groups, e.g., insulin, can be preferentially attached to agarose through the α -amino group by coupling at a relatively low pH (5.0) and high ionic strength.⁴⁰ Proteins with a large number of lysyl residues, and any protein of high molecular weight, yield much more efficient adsorbents when coupled to agarose at pH values in the neighborhood of 6.0–6.5 (see example 2, Fig. 7).^{2,3} Only a small fraction of the lysyl residues, and the α -amino groups, will be significantly nonionized and thus capable of reaction with the cyanogen bromide-activated agarose.

In spite of the low pH, adsorbents containing a large amount of protein can still be obtained by the procedures described above by (1) using agarose activated with relatively large amounts of cyanogen bromide (250 mg per milliliter of packed Sepharose), (2) performing the coupling procedure at pH 6, and (3) using high protein concentrations during the coupling reaction.^{2,3}

Because of its greater porosity, Sepharose 2B should be used for studies involving the isolation of particulate structures, such as cells, cell membranes, or viruses. Protein-agarose derivatives are quite stable when stored in suspension at 4°, and it is generally possible to use them repeatedly.

It should be stressed that when the biological activity of insoluble ligands or proteins is to be studied, special care must be taken to ensure *complete* removal of adsorbed, noncovalently bound material. This may require long and tedious washing procedures. It is recommended that, whenever possible, insolubilized protein derivatives be washed ex-

⁴⁰P. Cuatrecasas, *Proc. Nat. Acad. Sci. U. S.* 63, 450 (1969).

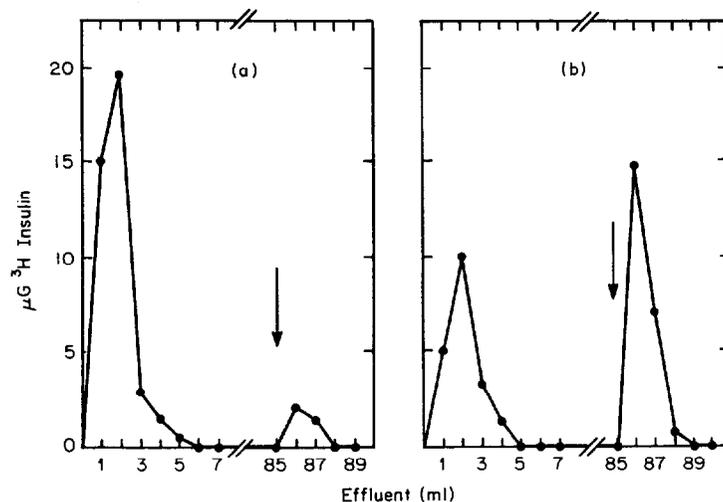


FIG. 7. Affinity chromatography patterns of ^3H insulin on columns containing purified antiporcine insulin sheep immunoglobulin which had been coupled by the cyanogen bromide procedure to agarose at pH 9 (A) and at pH 6.5 (B). Forty milligrams of purified γ -globulin, in 10 ml of 0.2 M sodium citrate, pH 6.5, or 8 ml of 0.2 M sodium bicarbonate, pH 9.0, were added to 5 ml of Sepharose 4B activated with 300 mg of cyanogen bromide per milliliter of gel. Based on the recovery of absorbancy ($280\text{ m}\mu$) in the washes, about 90–95% of the protein was coupled in both cases. The specific adsorbent was washed with 20 times its volume of 6 M guanidine-HCl before use. ^3H Acetyl insulin, 40 μg , in 1 ml of 50 mM borate buffer at pH 8.5 containing 0.1 N NaCl, and 0.5% bovine albumin, was applied to a $12 \times 0.6\text{ cm}$ column. The column was then washed with 85 ml of the above buffer, and elution of bound insulin was accomplished with 6 M guanidine-HCl (arrow). The theoretical capacity for insulin binding, based on the amount of antibody covalently linked and on the capacity of the antibody to bind insulin in solution, was about 30 μg for both adsorbents. Column A adsorbed 2 μg of insulin, or 7% of its theoretical capacity, whereas column B adsorbed 23 μg , or 77% its theoretical capacity.

tensively with 6 M guanidine-HCl. To further ensure that the biological function being measured is due to material covalently attached, the effect of varying the concentration of the insoluble derivative should be studied, and the possible release of the material must be examined after incubation in buffers containing high concentrations of albumin or other substances that simulate the biological environment.⁴⁰

Example 1: Purification of Antibodies. Protein-agarose and haptene-agarose derivatives appear to be nearly ideally suited for use as immunosorbents.^{41–43} For example, porcine insulin linked to agarose 2B through its single lysyl residue (B-29) can completely remove insulin antibodies from crude mixtures.⁴¹ The very strong binding of antibody by this

⁴¹P. Cuatrecasas, *Biochem. Biophys. Res. Commun.* **35**, 531 (1969).

⁴²L. Wofsy and B. Burr, *J. Immunol.* **103**, 380 (1969).

⁴³G. Omenn, D. Ontjes, and C. B. Anfinsen, *Nature (London)* **225**, 189 (1970).

derivative is reflected by the need to use strong acid conditions (1 *N* HCl) to achieve elution of the major fraction of the antibody from the column. Recovery of antibody applied to such a column approaches 80%. Large-scale purification of sheep antiporcine insulin is readily achieved with these adsorbents. A column containing 20 ml of packed insulin-Sepharose described in Fig. 7 removed all the insulin antibody present in 200 ml of crude immune sheep serum. The combined washes of equilibrating buffer (500 ml) and of 0.1 *M* sodium acetate, pH 5.5 (1 liter), contained only 5% of the total antibody applied to the column. About 65% (150 mg) of the total insulin antibody applied was eluted sharply with acetic acid at pH 2.8 in a volume of 29 ml. Subsequent elution with 6 *M* guanidine·HCl removed about 15% of the antibody applied.

*Example 2: Immunoglobulin-Sepharose for Purification of Antigens.*⁴⁴ Purified insulin antibodies, obtained by affinity chromatography as described above, can in turn be covalently attached to agarose to prepare derivatives capable of isolating the corresponding antigen and other immunologically cross-reacting proteins. The studies on anti-insulin shown in Fig. 7 illustrate the importance of attaching large proteins by few linkage points. The immunoglobulin coupled to activated agarose at pH 9 could adsorb only 7% of its theoretical capacity for insulin, whereas that coupled at pH 6.5 could bind 77% of its theoretical capacity for insulin. Since the total protein content of both derivatives is the same, the former derivative must contain immunoglobulin which is incapable of effectively binding antigen.

Example 3: Polypeptide Hormones and Isolation of Cellular Receptor Structures. Insolubilized ligands or proteins can also be used to study interactions of various molecules with intact cells, and to explore membrane phenomena. Although the macromolecules involved were not attached covalently to the supporting matrix, the recent studies of Wigzell and Andersson⁴⁵ on the fractionation of immunologically active cells on columns of glass or plastic beads to which antigen coatings were tightly adsorbed indicate the power of the approach. Similar studies on the immunoadsorption of cells on reticulated polyester polyurethane foam-coated with antibodies have been reported by Evans, Wage, and Peterson.⁴⁶

Insulin-agarose derivatives are capable of stimulating several different biological properties of intact isolated fat cells nearly as effectively as native insulin (Fig. 8).⁴⁰ Columns prepared with these insulin-agarose derivatives, in contrast to those with unsubstituted

⁴⁴P. Buatrecasas, unpublished observations (1970).

⁴⁵H. Wigzell and B. Andersson, *J. Exp. Med.* 129, 23 (1969).

⁴⁶W. H. Evans, M. G. Wage, and E. A. Peterson, *J. Immunol.* 102, 899 (1969).

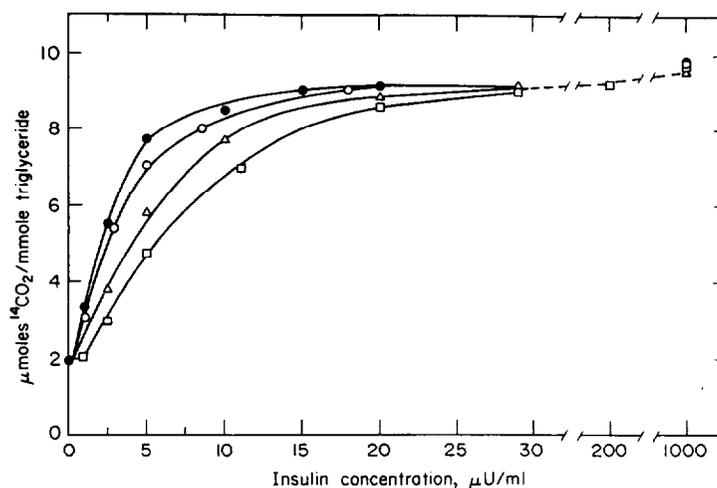


FIG. 8. Effect of insulin-agarose concentration on the oxidation of $[U^{14}C]$ glucose by isolated adipose cells. Fat cells (3–8 μ moles of triglyceride) were incubated for 2 hours at 37° in 2 ml of medium containing 0.5 mM $[U^{14}C]$ (0.1 μ Ci/ μ mole). The data presented are for native insulin (●), insulin coupled to Sepharose 2B (at pH 9) through lysine B29 (○), insulin coupled (at pH 5) through phenylalanine B1 (Δ), and insulin acetylated at positions A1 and B1 and coupled to agarose by lysine B29. Data from P. Cuatrecasas, *Proc. Nat. Acad. Sci. U. S.* **63**, 450 (1969).

agarose, bind intact fat cell "ghosts" very strongly.⁴⁴ The latter can be specifically eluted intact from the insulin-agarose columns with concentrated insulin solutions. These studies indicate that such insoluble hormone derivatives interact very effectively with membrane structures, and thus are promising tools for the isolation of "receptor" proteins (hormone, drug, etc.).

*Example 4: Purification of Histidyl-tRNA with Enzyme-Sepharose.*⁴⁷ Five milliliters of packed Sepharose 4B, activated with 1.3 g of cyanogen bromide, is reacted with 1.8 mg of purified histidyl-tRNA-synthetase (lacking other acyl tRNA-synthetase activities) in 50 mM phosphate buffer, pH 6.5. Virtually all the protein is coupled to Sepharose, since the washes contain essentially no absorbance at 280 $m\mu$ and only 1–3% of the enzyme activity is present. Columns containing this adsorbent strongly adsorb histidyl-tRNA, which can be eluted with 1 M NaCl (Fig. 9).

Example 5: Purification of Peptides Prepared by Organic Synthesis. A polypeptide corresponding to the amino acid sequence from residue 6 through 47 in staphylococcal nuclease (149 amino acids), obtained by organic synthesis, can be purified by passing the crude synthetic poly-

⁴⁷F. Blasi and R. Goldberger, personal communication (1969).

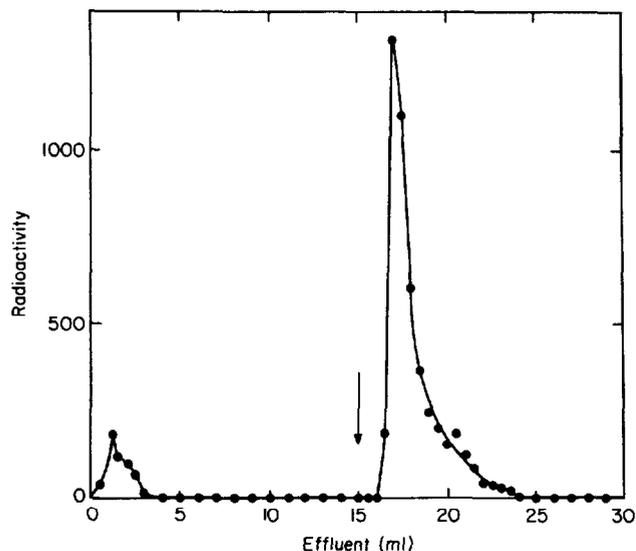


FIG. 9. Purification of histidyl-tRNA on a 0.6×8 cm column containing agarose coupled with purified histidyl-tRNA-synthetase (0.36 mg/ml Sepharose). The column was equilibrated with 66 mM potassium cacodylate, pH 7.0, containing 8 mM reduced glutathione and 10 mM $MgCl_2$. About 0.4 optical density unit of [3H]histidyl-tRNA was applied to the column in 0.2 ml of the above buffer. Elution was obtained with 3.0 M NaCl, acetic acid pH 3; it is also possible to elute with 1 N NaCl. Data from F. Blasi and R. Goldberger, unpublished.

peptide mixture through a column containing a Sepharose-linked native peptide which comprises residues 49-149.⁴⁸ In solution these two peptides separately have no discernible tertiary structure or enzymatic activity, but upon mixing there is regeneration of enzymatic activity. Advantage of the affinity of these two peptides is taken to obtain a "functional" purification of the synthetic peptide. In a similar manner, it is possible to purify synthetic RNase-S-peptide derivatives on agarose columns containing RNase-S-protein (Fig. 1).⁵ The specific activity of the purified peptide is increased by this procedure, but the chemical and catalytic properties of the purified materials suggest the presence of closely related synthetic side products which bind tightly to the S-protein conjugate but which yield enzymatically inactive complexes with RNase-S-protein.

⁴⁸D. Ontjes and C. B. Anfinsen, *J. Biol. Chem.* 244, 6316 (1969).