

Protein Purification by Affinity Chromatography

DERIVATIZATIONS OF AGAROSE AND POLYACRYLAMIDE BEADS

(Received for publication, January 28, 1970)

PEDRO CUATRECASAS

From the Laboratory of Chemical Biology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

The preparation of a number of agarose and polyacrylamide bead derivatives useful in the purification of proteins by affinity chromatography is described. These techniques permit (a) the attachment of ligands to the gel through extended hydrocarbon chains which place the ligand at varying distances from the gel matrix backbone; (b) the covalent attachment of ligands to agarose or polyacrylamide gels through amino, carboxyl, phenolic, or imidazole groups of the ligand; and (c) the preparation of adsorbents containing ligands attached by bonds which are susceptible to specific chemical cleavage, thus providing means of removing the intact protein-ligand complex from the affinity adsorbent. It is demonstrated that successful application of affinity chromatography in many cases will critically depend on placing the ligand at a considerable distance from the matrix backbone. Techniques are also described which provide important approaches and considerations in the insolubilization of peptides and proteins to agarose and polyacrylamide.

tions needed to permit satisfactory binding of macromolecules to polymer-insolubilized small molecules. Progress in this area has been handicapped by the lack of solid supports for the attachment of ligands, and by the failure to fix these ligands at a sufficient distance from the matrix backbone.

Successful application of the method (1, 2) requires that the adsorbent have minimal nonspecific interaction with proteins, that it exhibit good flow properties which are retained after coupling, and that it be mechanically and chemically stable to the conditions of coupling and of elution. Ideally, the gel substance should form a very loose, porous network which permits uniform and unimpaired entry and exit of large macromolecules. The gel particles should preferably be of uniform size, spherical, and rigid.

Cross-linked dextran (Sephadex) appears to have many of these desirable features (13, 14). The beaded agarose derivatives (Sephacrose) (15) are even more nearly ideal (1, 2) because of their very loose network (14, 15). A gentle method has been developed for coupling proteins and small molecules to these carbohydrate derivatives by using cyanogen halides (16, 17). Specific agarose adsorbents prepared by this basic procedure (1) have now been used successfully to purify various enzymes (1, 2, 10-12), antibodies (18, 19), chemically synthesized peptides (20, 21), and thyroxine-binding serum protein (22). Accumulating evidence suggests that the insoluble matrices prepared from agarose beads are superior to the insoluble polymers used in earlier studies. Synthetic polyacrylamide gels also appear to be promising as insoluble supports. They are available commercially in beaded, spherical form, in various pregraded sizes and porosities (Biogels). These beads, which lack charged groups, exhibit uniform physical properties and porosity, and the polyethylene backbone endows them with physical and chemical stability. Inman and Dintzis (23) have recently described a number of useful chemical derivatizations of cross-linked polyacrylamide beads. The number of chemically modifiable groups (carboxamide) in polyacrylamide beads is far greater than the number of groups that can be substituted on the agarose granular gels by the cyanogen bromide procedure. Thus, highly substituted derivatives may be prepared for use in the purification of enzymes which exhibit poor affinity for the substituted ligand.

It is becoming increasingly clear that, for successful purification by affinity chromatography, the ligand groups critical in the interaction with the macromolecule to be purified must be sufficiently distant from the backbone of the solid matrix to minimize

The selective isolation and purification of enzymes and other biologically important macromolecules by "affinity chromatography" exploits the unique biological property of these proteins to bind ligands specifically and reversibly (1-3). The protein to be purified is passed through a column containing an insoluble polymer or gel to which a specific competitive inhibitor or 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 to an extent related to the affinity constant under the experimental conditions. The method is related in principle to the use of "immuno-adsorbents" for the purification of antibodies (4). These principles have also been applied to the purification of nucleotides (5), complementary strands of nucleic acids (6), certain species of transfer RNA (7), and enzymes (8, 9) by utilization principally of hydrophobic polystyrene or hydrophilic cellulose polymers for the insoluble supporting matrix. Until recently (1, 10-12) only limited success has been encountered in the application of these techniques to the purification of enzymes (8, 9), perhaps because of the more stringent experimental condi-

steric interference with the binding process (1, 2). This can be accomplished by preparing an inhibitor with a long hydrocarbon chain, an "arm," attached to it, which can in turn be attached to the insoluble support. Alternatively, such a hydrocarbon extension arm can first be attached to the solid support.

The preparation of a number of chemical derivatives of agarose and of polyacrylamide beads will be described here. These derivatives, which are easily and rapidly prepared under mild aqueous conditions, should extend the usefulness of affinity chromatography by providing procedures applicable to cases in which (a) hydrocarbon chains of varying length (arms) need to be interposed between the matrix and the intact inhibitor; (b) an amino group on the ligand is not available; and (c) it is necessary or desirable to remove the intact protein-ligand complex by specific chemical cleavage of the ligand-matrix bond.

METHODS AND RESULTS

Stability and Handling of Agarose Derivatives

Beaded agarose (Sephacrose) gels, unlike the cross-linked dextrans (Sephadex), cannot be dried or frozen, since they will shrink severely and essentially irreversibly. Similarly, they will not tolerate many organic solvents. Dimethylformamide (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 in which the compound to be coupled is relatively insoluble in water (e.g. steroids, thyroxine, tryptophan derivatives) since the coupling step 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. The coupled, substituted adsorbents of Sepharose can be stored at 4° in aqueous suspensions, with antibacterial preservatives, for periods of time limited only by the stability of the bound ligand. Agarose beads tolerate 0.1 M NaOH and 1 M HCl for at least 2 to 3 hours at room temperature without adverse alteration of their physical properties and without cleavage of the linked ligand. These beads can therefore be used repeatedly even after exposure to relatively extreme conditions. Agarose beads also tolerate quite well exposure to 6 M guanidine-HCl or 7 M urea solutions for prolonged periods; slight shrinkage of the gel is observed under these conditions. These protein denaturants may therefore be used to aid in the elution of specifically bound proteins, for thorough washing of columns in preparation for reuse, and in washing off protein that is tightly adsorbed during the coupling procedure.

Coupling Ligands Which Contain an Amino Group to Agarose

Chemical compounds containing primary aliphatic (1, 14, 15) or aromatic amines (1) can be coupled directly to agarose beads after activation of the latter with cyanogen bromide at alkaline pH. The procedure used was modified from that previously described and is presented here in detail.

In a well ventilated hood, a given volume of well washed decanted Sepharose-4B is mixed with an equal volume of water, and finely divided cyanogen bromide (50 to 300 mg per ml of packed Sepharose) is added at once to the stirred suspension. The pH of the suspension is immediately raised to and maintained at 11 with NaOH. The molarity of the NaOH solution will depend on the amounts of Sepharose and cyanogen bromide added; it should vary between 2 M (for 5 to 10 ml of Sepharose and 1 to 3 g of cyanogen bromide) and 8 M (for 100 to 200 ml of Sepharose and

20 to 30 g of cyanogen bromide). The temperature is maintained at about 20° by adding pieces of ice as needed. The reaction is complete in 8 to 12 minutes, as indicated by the cessation of proton release. A large amount of ice is then rapidly added to the suspension, which is transferred quickly to a Buchner funnel (coarse disc) 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 to 15 times that of the packed Sepharose. The ligand to be coupled, in a volume of cold buffer equal to the volume of packed Sepharose, is added to the moist, washed Sepharose and the suspension is immediately mixed (in the Buchner 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" Sepharose is unstable. The suspension is transferred from the Buchner funnel to a beaker containing a magnetic mixing bar and is gently stirred at 4°. Although the reaction is essentially complete in 2 to 3 hours, the mixture is allowed to stand at 4° for 16 to 20 hours to ensure complete loss of reactive agarose groups. The substituted Sepharose 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 Sepharose can in part be controlled by the amount of ligand added to the activated Sepharose (1). When highly substituted derivatives are desired, the amount of ligand added should, if possible, be 20 to 30 times greater than that which is desired in the final product. For ordinary procedures, 100 to 150 mg of cyanogen bromide are used per ml of packed Sepharose (1), but much higher coupling yields can be obtained if this amount is increased to 250 to 300 mg. 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, such as alanine, will couple optimally at a pH of about 9.5 to 10.0 (Table I). Higher pH values should not be used, since the stability of the activated Sepharose decreases sharply above pH 10.5 (Table I). Compounds with primary aliphatic aminoalkyl groups, such as the ϵ -amino group of lysine or ethylamine, 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 of the amino group; very high coupling efficiencies are obtained at pH values between 8 and 9 (1).

The procedures for estimating the amount of ligand coupled to agarose, and determination of the operational capacity for specific protein adsorption, have been described (1, 2). The degree of ligand substitution should be represented as micromoles of ligand bound per ml of packed gel. This means of expression is more useful than those represented on the basis of dry gel weight.

Color Test with Sodium 2,4,6-Trinitrobenzenesulfonate

This simple color test, modified from that described by Inman and Dintzis (23), 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 to 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. 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 rapidly and conveniently estimated from the relative color intensity of the washed gel.

ω-Aminoalkyl Derivatives of Agarose Beads

Aliphatic diamine compounds, such as ethylene diamine, may be substituted directly to Sepharose by the cyanogen bromide procedure described above. 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 Sepharose 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. The following procedure has been used to prepare aminoethyl agarose. To an equal volume of a Sepharose-4B water suspension are added 250 mg of cyanogen bromide per ml of packed gel, and the reaction is performed as described above. To the washed, activated Sepharose is added an equal volume of cold distilled water containing 2 mmoles of ethylene diamine for each milliliter of Sepharose, previously titrated to pH 10 with 6 N HCl. 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 groups per ml of Sepharose. Different ω -aminoalkyl derivatives can be prepared in a similar way by using other diamine compounds, of the class, $NH_2(CH_2)_xNH_2$.

Coupling Carboxylic Acid Ligands to Aminoethyl Agarose

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

1. *Preparation of Estradiol-Sepharose*—Three hundred milligrams of 3-O-succinyl-³H-estradiol are added in 40 ml of dimethylformamide to 40 ml of packed aminoethyl Sepharose-4B (2 μ moles of aminoethyl groups per ml). The dimethylformamide is needed to solubilize estradiol. The pH of this suspension is brought to 4.7 with 1 N HCl. Five hundred milligrams (2.6 mmole) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, dissolved in 3 ml of water, are added over a 5-min period, and the reaction is allowed to proceed at room temperature for 20 hours. The substituted Sepharose is then washed continuously, while packed in a column, with 50% aqueous dimethylformamide until radioactivity is absent from the effluent; the volume of wash required is about 10 liters, over a 3- to 5-day period. The completeness of washing is checked by collecting 200 ml of effluent wash, lyophilizing, adding 2 ml of dimethylformamide, and determining radioactivity. About 0.5 μ mole of ³H-estradiol is covalently bound per ml of Sepharose.

This estradiol-Sepharose derivative is very effective in extracting estradiol binding proteins from human serum and from calf

TABLE I

Effect of pH on the coupling of ¹⁴C-alanine to activated sepharose

Packed Sepharose-4B, 60 ml, was mixed with 60 ml of water and treated with 15 g of cyanogen bromide as described in the text. To the cold, washed activated Sepharose were added 2.2 mmoles of ¹⁴C-L-alanine (0.1 μ Ci per μ mole) in 60 ml of cold distilled water, and 20-ml samples of the mixed suspension were added rapidly to beakers containing 5 ml of cold 0.5 M buffer of composition described in the table. The final concentration of alanine was 0.015 M. 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 ¹⁴C-alanine released was determined.

Conditions for coupling reaction	pH	Alanine coupled
		μ moles per ml Sepharose
0.1 M sodium citrate	6.0	4.2
0.1 M sodium phosphate	7.5	8.0
0.1 M sodium borate	8.5	11.0
0.1 M sodium borate	9.5	12.5
0.1 M sodium carbonate	10.5	10.5
0.1 M sodium carbonate	11.5	0.2

uterus.¹ It is possible to elute strongly adsorbed serum estradiol-binding protein by cleaving the estradiol-Sepharose bond with mild base or with neutral hydroxylamine (1 N), thus avoiding the use of protein denaturants.

2. *Preparation of Organomercurial-Sepharose for Separation of -SH Proteins*—Thirty-five milliliters of packed aminoethyl-Sepharose (capacity, 10 μ moles per ml) are suspended in a total volume of 60 ml of 40% dimethylformamide; 2 mmoles of sodium *p*-chloromercuribenzoate are added; the pH is adjusted to 4.8; and 5 mmoles of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide are added. After reaction at room temperature for 18 hours the substituted Sepharose is washed with 8 liters of 0.1 M NaHCO₃, pH 8.8, over an 8-hour period. Complete reaction of the agarose amino groups is confirmed by the yellow color obtained with the sodium trinitrobenzenesulfonate color test. Judged by the uptake of ¹⁴C-cysteine, about 8 μ moles of organomercurial are coupled per ml of agarose. A small column (0.5 × 2 cm) containing this derivative can bind 40 to 50 mg of horse hemoglobin per ml of packed gel in 0.05 M sodium phosphate buffer, pH 7.5. 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 (24), 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 0.5 M cysteine 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 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 Sepharose beads (*i.e.* drying, dioxane), and the stability of the final derivative.

Preparation of Bromoacetyl Sepharose Derivatives

Bromoacetamidoethyl-Sepharose can be prepared easily in mild aqueous conditions by treating aminoethyl-Sepharose-4B

¹ P. Cuatrecasas and G. Puca, unpublished.

with *O*-bromoacetyl-*N*-hydroxysuccinimide. This derivative of Sepharose 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. The following procedure can be used to prepare such derivatives. In 8 ml of dioxane are dissolved 1.0 mmole of bromoacetic acid and 1.2 mmole of *N*-hydroxysuccinimide (25). To this solution, 1.1 mmoles of dicyclohexylcarbodiimide are added. After 70 min 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 ml) in a total volume of 50 ml at pH 7.5 in 0.1 M sodium phosphate buffer. After 30 min, Sepharose 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 sodium trinitrobenzenesulfonate color test. Reaction of this bromoacetamidoethyl-Sepharose gel with a competitive inhibitor of staphylococcal nuclease, 0.01 M pdTp-aminophenyl (26), 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 ml of packed Sepharose. A similar reaction with ³H-5'-GMP resulted in the covalent attachment of 0.2 μmole per ml. Such bromoacetyl-Sepharose derivatives have also been used to insolubilize ³H-estradiol, by alkylation at the C3 —OH of the A ring, for use in the purification of serum and uterine estradiol-binding proteins.¹ 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 pH values or lower temperatures.

Preparation of Succinylaminoethyl-Sepharose

This derivative is prepared by treating aminoethyl-Sepharose with succinic anhydride in aqueous media at pH 6.0. One millimole of succinic anhydride is added per ml of packed aminoethyl-Sepharose (capacity, 8 μmoles per 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 groups occurs, as evidenced by the sodium trinitrobenzenesulfonate 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. The conditions for this reaction are identical with those described above for coupling carboxylic acid ligands to ω-aminoalkyl gels.

Preparation of *p*-Aminobenzamidoethyl-Sepharose for Coupling Compounds via Azo Linkage

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. Aminoethyl-Sepharose, in 0.2 M sodium borate, pH 9.3, and 40% dimethylformamide (v/v), is treated for 1 hour at room temperature with 0.07 M *p*-nitrobenzoyl azide (Eastman). The substitution is complete, as judged by the loss of color reaction with sodium trinitrobenzenesulfonate. The *p*-nitroben-

zamidoethyl-Sepharose is washed extensively with 50% dimethylformamide and then reduced by reaction for 40 min at 40° with 0.1 M sodium dithionite in 0.5 M NaHCO₃, pH 8.5. The effectiveness of this procedure is demonstrated by the red-orange color produced upon reaction with sodium trinitrobenzenesulfonate. The washed *p*-aminobenzamidoethyl-Sepharose derivative, in 0.5 N HCl, can be diazotized by treating for 7 min at 4° with sodium nitrite (0.1 M). This diazonium-Sepharose derivative can 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 10 (phenolic); this reaction is allowed to proceed for about 8 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.

An important advantage of the azo-linked ligand derivatives of Sepharose 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.

Preparation of Tyrosyl-Sepharose for Coupling Diazonium Compounds

A peptide containing a COOH-terminal tyrosine residue can be attached to Sepharose 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. The ligand thus can extend a good distance from the matrix backbone. The azotization procedure is performed as follows. One hundred micromoles of ligand are dissolved in 1.5 ml of 1.5 N HCl and placed in an ice bath; 700 μmoles of NaNO₂, in 0.5 ml of water, are added over a 1-min period to the stirred ligand solution. After 7 to 8 min the entire mixture, without further purification, is added to a suspension, in an ice bath, of tyrosyl-Sepharose containing 0.2 M Na₂CO₃. The pH is adjusted rapidly to 9.4. After 3 hours the Sepharose suspension is transferred to a Buchner funnel and washed. If dimethylformamide 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 containing Sepharose-Gly-Gly-Tyr to which the competitive inhibitor, pdTp-aminophenyl (26), is attached in azo linkage.

Preparation of Sulfhydryl Agarose Derivatives

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 (27). Five grams of *N*-acetylhomocysteine thiolactone are added to a cold (4°) suspension consisting of an equal volume (50 ml) of aminoethyl Sepharose-4B and 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. 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-Sepharose with 0.05 M iodoacetamide in 0.5 M NaHCO₃, pH 8.0, for 15 min at room temperature, followed by washing with distilled water. This confirms that complete substitution of the amino-Sepharose groups has occurred.

The degree of sulfhydryl substitution can be conveniently determined by reacting the thiol-Sepharose with 5,5'-dithiobis(2-nitrobenzoic acid) (28) at pH 8, followed by centrifugation and determination of the absorbance at 412 m μ in the supernatant. By this procedure, the derivative described above contained 0.58 μ mole of sulfhydryl per ml of packed Sepharose. Reaction with 0.01 M ¹⁴C-iodoacetamide in 0.1 M NaHCO₃, pH 8.0, for 15 min at room temperature, resulted in uptake of 0.65 μ mole of radioactivity per ml of Sepharose. To determine whether disulfide bond formation had occurred during the preparation or washing steps, a sample of the sulfhydryl Sepharose was treated with 0.1 M mercaptoethanol in 0.5 M Tris·HCl buffer, pH 8.0, for 1 hour at room temperature, followed by washing with 0.1 N NaCl. The sulfhydryl content, determined with 5,5'-dithiobis(2-nitrobenzoic acid), was unchanged.

Ligands or proteins which 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

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 min. Thus, it is possible by specific chemical cleavage to remove the intact ligand-protein complex from an adsorbent containing the specifically bound protein.

N-Acetylglycine (0.2 M) is added to 40 ml of packed sulfhydryl Sepharose (containing 0.7 μ mole of thiol per ml of packed Sepharose) 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 to 12 hours at room temperature. The Sepharose 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), is about 0.03 μ mole per ml of Sepharose. All the sulfhydryl groups remaining free on the Sepharose are masked by treatment with 0.02 M iodoacetamide for 20 min 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.¹

Preparation of Derivatives of Polyacrylamide Beads

The procedures for polyacrylamide derivatization are basically those described by Inman and Dintzis (23). These involve conversion of the carboxamide side groups to hydrazide groups, which in turn are converted with nitrous acid into acyl azide derivatives. The latter react rapidly with compounds having

aliphatic or aromatic primary amino groups without intermediate washings or transfers. Fifty milliliters of the packed hydrazide polymer, prepared as previously described (23), are washed, suspended in 100 ml of 0.3 N HCl, and placed in an ice bath. Ten milliliters of 1.0 M NaNO₂ are added rapidly under vigorous magnetic stirring. After 90 sec the amine is added 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 and 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 that of the hydrazide content of the gel.

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*-aminobenzamidoethyl derivatives can be prepared by procedures identical with those described above. Satisfactory affinity adsorbents have been prepared for the purification of staphylococcal nuclease by many of these procedures.

DISCUSSION

The importance of interposing a hydrocarbon chain between the ligand and the agarose backbone was suggested by the relative ineffectiveness of Sepharose-bound D-tryptophan methyl ester, compared to ϵ -aminocaproyl-D-tryptophan methyl ester, in the purification of α -chymotrypsin (1). This report describes several procedures for the preparation of gels which contain such hydrocarbon extensions ("arms") of varying length. Fig. 1 illustrates several derivatives of agarose and polyacrylamide which contain a competitive inhibitor of staphylococcal nuclease, pT_p-aminophenyl, attached to the solid matrix in various ways. Although the affinity adsorbent which contains the ligand attached *directly* to the matrix (Fig. 1A) is quite effective in selectively extracting the enzyme from dilute aqueous solutions, the protein-binding capacity of the gel can clearly be increased by placing the ligand at some distance from the matrix (Table II). However, extending the position of the ligand farther than in the derivative shown in Fig. 1B does not result in a further increase in binding capacity.

The ligand used in the above examples for the purification of staphylococcal nuclease, pT_p-aminophenyl, is a relatively strong competitive inhibitor (K_i about 10⁻⁶ M) of this enzyme (29). The importance of the arm extension is much more dramatic in situations which involve ligand-protein interactions of low affinity. This is clearly illustrated in studies of the purification of bacterial β -galactosidase with agarose derivatives containing a relatively weak competitive inhibitor (K_i about 10⁻⁸ M), *p*-aminophenyl- β -D-thiogalactopyranoside (Fig. 2).² No binding of enzyme was observed with derivatives of agarose (or polyacrylamide) which contained the ligand attached directly to the matrix (Fig. 2A), even though gel derivatives were prepared which contained as much as 14 μ moles of inhibitor per ml of packed gel. By placing the inhibitor at a moderate distance from the solid support (Fig. 2B) the β -galactosidase activity emerges slightly behind the major protein breakthrough in column chromatography experiments. However, agarose derivatives obtained after insertion of a very long arm between the

² E. Steers, H. Pollard, and P. Cuatrecasas, manuscript in preparation.

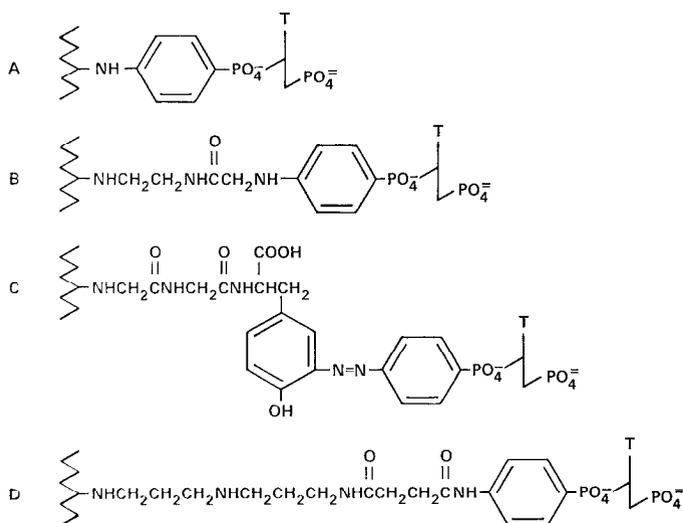


FIG. 1. Specific staphylococcal nuclease affinity chromatographic adsorbents prepared by attaching the competitive inhibitor, pdTp-aminophenyl (26, 29), to various derivatives of Sepharose-4B or Bio-Gel P-300 by the procedures described in the text. In A the inhibitor was attached *directly* to agarose, after activation of the gel with cyanogen bromide, or to polyacrylamide via the acyl azide step. In B, ethylenediamine was reacted with cyanogen bromide-activated Sepharose, or with the acyl azide polyacrylamide derivative. This amino gel derivatate was then reacted with *N*-hydroxysuccinimide ester of bromoacetic acid to form the bromoacetyl derivative; the latter was then treated with the inhibitor. In C, the tripeptide, Gly-Gly-Tyr, was attached by the α -amino group to agarose or polyacrylamide by the cyanogen bromide or acyl azide procedure, respectively; this gel was then reacted with the diazonium derivative of the inhibitor. In D, 3,3'-diaminodipropylamine was attached to the gel matrix by the cyanogen bromide or acyl azide step. The succinyl derivative, obtained after treating the gel with succinic anhydride in aqueous media, was then coupled with the inhibitor with a water-soluble carbodiimide. The jagged vertical lines represent the agarose or polyacrylamide backbone.

agarose matrix and ligand (Fig. 2C) adsorb β -galactosidase from various sources very strongly.²

Similarly, totally ineffective adsorbents for muscle glycerol 3-phosphate dehydrogenase,³ and for hepatoma tyrosine aminotransferase,⁴ result when the respective ligands are attached directly to the agarose backbone. In contrast, specific and useful adsorption of these enzymes occurs when the same ligands are linked to agarose derivatives containing long arms of the type described in this report. The interposition of such hydrocarbon arms between gel and ligand appears to be most important for interacting systems of low affinity, and in cases involving multi-subunit or high molecular weight proteins. The dramatic effects of increasing distance observed in some of these cases may be in part explained by relief of steric restrictions imposed by the matrix backbone, and perhaps by the increased flexibility and mobility of the ligand as it protrudes farther into the solvent.

As discussed earlier, the principal theoretical advantage of polyacrylamide beads over agarose beads is that a much higher degree of ligand substitution is possible with the former. However, the considerably lower porosity of currently available polyacrylamide beads may limit their use in the affinity chromatography of very large proteins. Furthermore, some shrinkage

³ T. P. Fondy, P. D. Holokan, and K. Mahajan, personal communication.

⁴ J. D. Miller, E. B. Thompson, and P. Cuatrecasas, manuscript in preparation.

TABLE II
Capacity of columns containing various agarose and polyacrylamide adsorbents for staphylococcal nuclease

Derivative ^a	Capacity ^b
	mg of nuclease/ml of gel
Agarose A	2
Agarose B	8
Agarose C	8
Agarose D	10
Polyacrylamide A	0.6
Polyacrylamide B	2
Polyacrylamide C	3

^a These refer to those depicted in Fig. 1.

^b Derivatives were diluted with unsubstituted gel to obtain 2 μ moles of ligand per ml of packed gel; the theoretical capacity for staphylococcal nuclease is therefore about 40 mg per ml. The capacity for enzyme and the experimental conditions have been described previously (1).

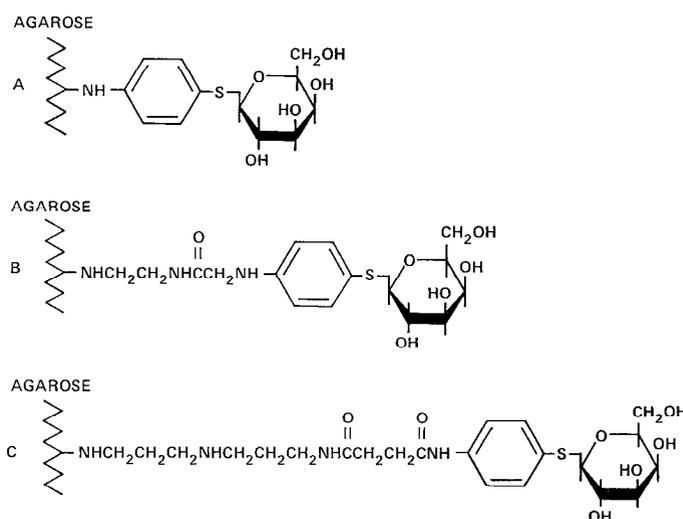


FIG. 2. Affinity chromatographic adsorbents prepared by attaching a weak competitive inhibitor of bacterial β -galactosidase, *p*-aminophenyl- β -D-thiogalactopyranoside, to Sepharose-4B through extensions of varying length. The ligand was attached directly to cyanogen bromide-activated Sepharose (A), to the bromoacetyl derivative (B), and to the succinylated 3,3'-diaminodipropylamine derivative (C), respectively, as described in the text and in the legend to Fig. 1. Columns containing these gels exhibit no β -galactosidase binding (Derivative A), only a slight retardation in the chromatographic migration of the enzyme (Derivative B), and very strong binding of the protein (Derivative C).²

and decrease in porosity of the gels occurs during formation of the acyl azide intermediate. 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. Only the most porous beads (Bio-Gel P-300) have been found effective for the purification of staphylococcal nuclease, a protein with a molecular weight of 17,000.

It is sometimes difficult or impossible to elute proteins which are strongly adsorbed to affinity columns without resorting to the use of extreme conditions of pH, or of denaturants (guanidine-HCl, urea), which may irreversibly destroy the biological function of the protein. In such cases it may be of value to remove the intact protein-ligand complex from the solid support. Three of the derivatization products described in this report

are readily adapted to specific chemical cleavage of the ligand-gel bond under relatively mild conditions. The azo-bonded derivatives can be cleaved, as described in the appropriate section, by reduction with sodium dithionite at pH 8.5. This has been of considerable value in studies of the purification of serum estradiol-binding protein with derivatives of Sepharose to which estradiol is attached by the azo linkage described in this report.⁵ The serum estradiol-binding protein is denatured irreversibly by exposure to pH 3 or 11.5, and by low concentrations of guanidine·HCl (3 M) or urea (4 M). The protein, which binds estradiol very tightly (K_d about 10^{-9} M), can be removed in active form from the Sepharose-estradiol gel by reductive cleavage of the azo link with dithionite.⁵ Carboxylic acid ester derivatives of various ligands have also been linked to agarose by the procedures described in this report. Such bonds can be cleaved readily by short periods of exposure to pH 11.5 at 4°. Ligands attached to agarose by thiol esters, as described in this report, can be similarly cleaved by short exposure to alkaline pH or by treatment with neutral hydroxylamine. These procedures present alternative ways of removing intact ligand-protein complexes from insoluble supports.

Agarose derivatives which contain covalently attached proteins or peptides can be useful in the purification of antibodies (11, 19) or in the resolution of peptide mixtures obtained by organic synthesis (20, 21). In addition to attaching proteins or peptides directly through their amino groups to agarose by the cyanogen bromide procedure (16, 17, 20, 21, 30, 31), it is possible to use the bromoacetyl, diazonium, or sulfhydryl agarose derivatives described in this report. Proteins attached in the latter fashion will extend some distance from the matrix backbone by an arm, which may be very useful in overcoming steric difficulties when interactions with other macromolecules are being studied. For example, the study of the interaction of certain insolubilized proteins or polypeptides with intact cells or cell structures may best be achieved with such derivatives.

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 (2, 31). This will increase the probability that the attached macromolecule will retain its native tertiary structure, and its properties may more nearly resemble those of the native protein in solution. Proteins react with cyanogen bromide-activated Sepharose through the unprotonated form of their free amino groups. Since most proteins are richly endowed with lysyl residues, most of which are exposed to solvent, it is likely that such molecules will have multiple points of attachment to the resin when the coupling reaction is done at pH 9.5 or higher, as is usually the case. This problem may be circumvented by carrying out the coupling procedure at a less favorable pH. For example, it has been demonstrated that, if antibodies are coupled to Sepharose at pH 6.0 to 6.5, the resultant immunoadsorbent has a much greater capacity for antigen than that which is prepared by performing the coupling procedure at pH 9.5.⁶ Adsorbents containing a high concentration of protein can still be prepared at the lower pH values if a large amount of

cyanogen bromide (250 mg per ml of packed gel) is used for activation, and if a relatively high concentration of protein (10 mg per ml) is used in the coupling step.

Acknowledgment—The technical assistance of Mrs. Juanita Eldridge is gratefully acknowledged.

REFERENCES

1. CUATRECASAS, P., WILCHEK, M., AND ANFINSEN, C. B., *Proc. Nat. Acad. Sci. U. S. A.*, **61**, 636 (1968).
2. CUATRECASAS, P., AND ANFINSEN, C. B., in S. P. COLOWICK AND N. O. KAPLAN, *Methods in enzymology*, Vol. 21, Academic Press, New York, in press.
3. JERINA, D. M., AND CUATRECASAS, P., *Proceedings of the IV International Congress of Pharmacology, Basel, Switzerland, July, 1969*, Vol. 1, P. Schwabe and Company Publishers, Basel, in press.
4. SILMAN, I., AND KATCHALSKI, E., *Annu. Rev. Biochem.*, **35**, 873 (1966).
5. SANDER, E. G., MCCORMICK, D. B., AND WRIGHT, L. D., *J. Chromatogr.*, **21**, 419 (1966).
6. BAUTZ, E. K. F., AND HOLT, B. D., *Proc. Nat. Acad. Sci. U. S. A.*, **48**, 400 (1962).
7. ERHAN, S. L., NORTHRUP, L. G., AND LEACH, F. R., *Proc. Nat. Acad. Sci. U. S. A.*, **53**, 646 (1965).
8. LERMAN, L. S., *Proc. Nat. Acad. Sci. U. S. A.*, **39**, 232 (1953).
9. ARSENI, C., AND MCCORMICK, D. B., *J. Biol. Chem.*, **239**, 3093 (1964); **241**, 330 (1966).
10. CUATRECASAS, P., AND WILCHEK, M., *Biochem. Biophys. Res. Commun.*, **33**, 235 (1968).
11. CHAN, W. C., AND TAKAHASHI, M., *Biochem. Biophys. Res. Commun.*, **37**, 272 (1969).
12. CHUA, G. K., AND BUSHUK, W., *Biochem. Biophys. Res. Commun.*, **37**, 545 (1969).
13. PORATH, J., AND FLODIN, P., *Nature*, **183**, 1657 (1959).
14. PORATH, J., *Nature*, **218**, 834 (1968).
15. HJERTEN, S., *Arch. Biochem. Biophys.*, **99**, 446 (1962).
16. PORATH, J., AXEN, R., AND ERNBACK, S., *Nature*, **215**, 1491 (1967).
17. AXEN, R., PORATH, J., AND ERNBACK, S., *Nature*, **214**, 1302 (1967).
18. CUATRECASAS, P., *Biochem. Biophys. Res. Commun.*, **35**, 531 (1969).
19. WOFSY, L., AND BURR, B., *J. Immunol.*, **103**, 380 (1969).
20. KATO, I., AND ANFINSEN, C. B., *J. Biol. Chem.*, **244**, 1004 (1969).
21. ONTJES, D., AND ANFINSEN, C. B., *J. Biol. Chem.*, **244**, 6316 (1969).
22. PENSKY, J., AND MARSHALL, J. S., *Arch. Biochem. Biophys.*, **135**, 304 (1969).
23. INMAN, J. K., AND DINTZIS, H. M., *Biochemistry*, **8**, 4074 (1969).
24. ELDJARN, L., AND JELLUM, E., *Acta Chem. Scand.*, **17**, 2610 (1963).
25. CUATRECASAS, P., WILCHEK, M., AND ANFINSEN, C. B., *J. Biol. Chem.*, **244**, 4316 (1969).
26. CUATRECASAS, P., TANIUCHI, H., AND ANFINSEN, C. B., *Brookhaven Symposia in Biology*, **21**, 172 (1968).
27. BENESCH, R., AND BENESCH, R. E., *J. Amer. Chem. Soc.*, **78**, 1597 (1956); *Proc. Nat. Acad. Sci. U. S. A.*, **44**, 848 (1958).
28. ELLMAN, G. L., *Arch. Biochem. Biophys.*, **82**, 70 (1959).
29. CUATRECASAS, P., WILCHEK, M., AND ANFINSEN, C. B., *Biochemistry*, **8**, 2277 (1969).
30. WIDE, L., AND PORATH, J., *Biochim. Biophys. Acta*, **130**, 257 (1966).
31. CUATRECASAS, P., *Proc. Nat. Acad. Sci. U. S. A.*, **63**, 450 (1969).

⁵ P. Cuatrecasas, unpublished.

⁶ For example, sheep antiporcine insulin antibodies, purified with insulin-Sepharose (18), have been linked in nearly quantitative yield to Sepharose-4B at pH 6.5 (0.2 M sodium citrate); 40 mg of protein were added to 5 ml of packed Sepharose which had been activated with 1.5 g of cyanogen bromide, in a total volume of 10 ml. This derivative was capable of binding approximately

80% of the theoretical capacity for insulin. In contrast, an immunoglobulin-Sepharose derivative prepared in an identical manner except that the pH of the coupling step was 9.5 could bind only 7% of the theoretical insulin capacity. Since the total protein content of both derivatives is the same, the latter derivative must contain immunoglobulin which is incapable of effectively binding antigen (P. Cuatrecasas, unpublished).

**Protein Purification by Affinity Chromatography: DERIVATIZATIONS OF
AGAROSE AND POLYACRYLAMIDE BEADS**

Pedro Cuatrecasas

J. Biol. Chem. 1970, 245:3059-3065.

Access the most updated version of this article at <http://www.jbc.org/content/245/12/3059>

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
<http://www.jbc.org/content/245/12/3059.full.html#ref-list-1>