

Affinity Separation of Enzymes from Mixtures Containing Suspended Solids

Comparisons of Magnetic and Nonmagnetic Techniques

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Abstract

Agarose beads containing immobilized enzymes or affinity ligands have been made magnetically responsive by adsorbing freshly precipitated magnetite on their surface. These beads are used for affinity adsorption of proteins from complex mixtures containing suspended solids. The magnetically responsive beads and the unwanted (diamagnetic) solids are then separated by magnetic filtration. This magnetic adsorption scheme for direct affinity separation of enzymes from mixtures containing suspended solids is compared with a similar, but nonmagnetic, scheme in which the affinity matrix is supported on fiberglass cloth. The enzyme is allowed to adsorb in this matrix, and the matrix is simply removed physically from the suspension to achieve separation from the unwanted solids. The two methods seem comparable in their ability to separate a desired enzymatic activity. The magnetic methods are technically the more complex of the two, but are significantly the more rapid. The efficiency of separation of diamagnetic and ferrimagnetic solids in these biological systems by high gradient magnetic filtration is good.

Index Entries: Affinity separation, of enzymes via magnetic techniques; separation, of enzymes by magnetic affinity techniques; enzymes, separation by magnetic affinity techniques; solids, affinity separation of enzymes from mixtures of suspended; magnetic separation, of enzymes from suspended solids.

[‡]See ref. 1.

Introduction

A ubiquitous problem in biochemical separations is that of isolating soluble proteins from complex mixtures containing suspended solids. This problem is encountered, for example, in the early stages of enzyme separations (where the preparations contain cellular debris), in the recovery of enzymes used as catalysts in organic syntheses and food processing, and in certain types of analytical and diagnostic procedures. Conventional affinity adsorption based on affinity ligands attached, e.g., to gel particles, is only partially effective in these circumstances since reisolation of the affinity ligand-containing matrix from the mixture by filtration or centrifugation commonly leads to extensive contamination by other solid components of the heterogeneous mixtures. We describe here two methods based on magnetic filtration for the affinity separation of proteins from mixtures containing suspended solids, and compare these methods with two others that do not depend on magnetic filtration techniques. The potential advantage of magnetic separation techniques in biochemistry is based on the fact that most biological systems are diamagnetic. Para-, ferro-, or ferrimagnetic components intentionally introduced into biological systems can therefore be manipulated magnetically with relatively little interference from the naturally occurring components.

This work relies on high gradient magnetic filtration (HGMF) for magnetic separations (2–9). This technique provides a method for the rapid isolation of small (1–100 μm) ferro-, ferri-, and paramagnetic particles from suspensions in water (or other diamagnetic media). It effectively traps even weakly magnetic particles in regions of very high magnetic field gradient that surround ferromagnetic filaments placed in a saturating magnetic field, and provides high filtration rates for soft or compressible solids of the type that often clog the face of conventional filters. In practice such magnetic filters often simply consist of steel wool packed in a tube placed between the poles of a magnet.

To utilize HGMF for enzyme separations, we required a simple method of preparing magnetically responsive matrices containing either affinity ligands or immobilized enzymes. Ferrimagnetic matrices containing affinity ligands have been discussed previously (10–12), and magnetic supports for enzymes (10, 13–16), for other catalysts (17, 18), and for cell separations (19–24) have been described. The magnetic methods reported here use functionalized agarose with adsorbed magnetite particles. The nonmagnetic methods are based on PAN [poly(acrylamide-*co*-*N*-acryloxysuccinimide)]—a gel developed previously for enzyme immobilization (25). We have examined and compared four systems.

1. Magnetically responsive agarose beads (prepared by precipitating magnetite on the bead surface) containing affinity ligands. We refer to separations based on this affinity matrix as “magnetic affinity adsorption.”

2. Similar magnetically responsive beads containing immobilized carbonic anhydrase. This immobilized protein is used to adsorb selectively soluble proteins modified by covalent attachment of aryl sulfonamide groups. We have previously described a similar procedure (omitting the magnetic component) as “generalized affinity chromatography” (26). Separations based on

this magnetic matrix will be referred to as "magnetic generalized affinity adsorption."

3. Nonmagnetic polyacrylamide gels, containing affinity ligands, coated as thin films on fiberglass cloth. We will refer to these materials as "affinity sponges" for ease of reference.

4. Nonmagnetic gels, containing immobilized carbonic anhydrase, coated on fiberglass. These materials will be called "generalized affinity sponges."

In all of the methods, the soluble protein of interest adsorbs biospecifically to the gel. The gels containing affinity ligands follow the usual principles of affinity chromatography. Those containing immobilized carbonic anhydrase are useful primarily for the recovery of enzymes used as catalysts in organic synthesis: they use a single, well-defined association (that of carbonic anhydrase for aryl sulfonamides) to adsorb modified proteins. The first two methods rely on HGMF to remove the magnetic affinity or generalized affinity matrices from mixtures containing unwanted suspended solids; the second two use direct physical removal of the fiberglass cloths to separate the attached affinity or generalized affinity matrices from the media.

To evaluate the relative performance of these separation techniques, we have utilized two types of model systems. In one, we measure the efficiency of separation of a soluble enzyme from a well-defined mixture containing suspended nonmagnetic agarose beads (representing contaminating solids) and another soluble protein (representing soluble contaminants). For convenience in the following separations, we label the nonmagnetic beads with an easily assayed enzyme (β -galactosidase or hexokinase) to facilitate detection. In a second model system, we substitute a crude yeast homogenate for the nonmagnetic agarose beads.

Experimental

Materials

Sephacrose 6B-100 (average molecular weight exclusion limit 4×10^6 , particle size in swollen state 40–120 μm), Baker's yeast (YSC-2), ATP, NADP, *o*-nitrophenyl- β -D-galactopyranoside, carbonic anhydrase (bovine), glucose-6-phosphate dehydrogenase (Baker's yeast), hexokinase (Baker's yeast), peroxidase (horseradish), and β -galactosidase (*E. coli*) were purchased from Sigma Chemical Company. Hexokinase- ArSO_2NH_2 (26) and PAN-1000 (25) were prepared as described previously. Water was deionized and distilled using a Corning Model 3B still. All other reagents were purchased from commercial sources and were used without further purification.

High-Gradient Magnetic Filter

The magnetic filter used in this work consisted of ca. 5 g of stainless steel wool (International Steel Wool Corp., fine grade) packed loosely in a glass burette (1-in.

diameter) placed vertically in the pole gap of an electromagnet (Varian V-4004) that possessed a field strength of 10 kG when operated at 3.0 A.

Assay for Carbonic Anhydrase (27)

Water (1.7 mL), 4-nitrophenylacetate solution (1.00 mL, 3 mM) and 300 μL of 0.1 M phosphate buffer, pH 7.0, were added to a quartz cuvette and the rate of change of absorbance at 348 nm measured spectrophotometrically at 26°C. The carbonic anhydrase-containing sample (5–50 μL) was added, the cuvette agitated, and the rate of change of adsorbance again measured. The molar absorptivity at 348 nm (an isobestic point for 4-nitrophenol and 4-nitrophenylate) is $5.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. 4-Nitrophenyl acetate also absorbs slightly at this wavelength ($\epsilon = 0.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). If measurements are made in a 1-cm cuvette, the increase in absorbance at 348 nm divided by 5 gives the concentration (millimolar) of 4-nitrophenol and 4-nitrophenylate anion, independent of pH.

Assay for Hexokinase (28)

To a 3-mL quartz cuvette containing 3.00 mL of a solution (pH 7.6) that was 0.1 M in Hepes buffer, 0.22 M in glucose, and 0.01 M in MgCl_2 , was added 200 μL of a solution that was 40 mM in ATP and 11 mM in NADP. Glucose-6-phosphate dehydrogenase (5 μL of a solution of 1000 u/mL) was added, the cuvette agitated, and the rate of change of absorbance measured at 340 nm and 26°C. The hexokinase-containing sample (5–50 μL) was added, the cuvette agitated, and the rate of change of absorbance again measured at 340 nm. The molar absorptivity of NADH is $6220 \text{ M}^{-1} \text{ cm}^{-1}$ at this wavelength.

Assay for Peroxidase (29)

To a 4-mL quartz cuvette was added 3.00 mL of 0.1 M phosphate buffer (pH 7.0), 50 μL of 18 mM guaiacol solution, and 5–50 μL of the peroxidase-containing sample. The cuvette was equilibrated at 26°C in the spectrophotometer. Hydrogen peroxide (40 μL of an 8mM solution) was added, the cuvette agitated, and the rate of change of absorbance at 436 nm measured. The molar absorptivity used in calculations was $6.39 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Assay for β -Galactosidase (30)

To 1.00 mL of the substrate containing solution (0.75 g *o*-nitrophenyl- β -D-galactopyranoside/L in 0.05 M Tris-HCl buffer, pH 7.5, 0.1 M NaCl, 0.01 M MgCl_2) was added 5–30 μL of the sample. The assay mixture was incubated at room temperature with stirring for 5 min. The reaction was quenched with 1.00 mL of 1.0 M Na_2CO_3 solution, and the absorbance at 420 nm measured. The molar absorptivity of *o*-nitrophenylate ion is $3000 \text{ M}^{-1} \text{ cm}^{-1}$.

Immobilization of Enzymes on Agarose (34)

Carbonic anhydrase (100 mg, 103 U), hexokinase (20 mg, 5000 U), or β -galactosidase (20 mg, 680 U) was dissolved in 20 mL of 0.2 M NaHCO_3 buffer, pH 9.5, and stirred with a magnetic stirrer in a 250-mL beaker equilibrated in an ice

bath. A slurry of 10 mL of Sepharose 6B was washed with 3000 mL of water and 50 mL of 2 M Na_2CO_3 , then filtered with suction using a fritted glass filter. The moist gel was transferred to a 100-mL beaker cooled in an ice bath and equipped with a magnetic stirrer. Water (10 mL) and 2 M Na_2CO_3 (20 mL) were added. While the slurry was stirred rapidly, a solution of 1.6 g of cyanogen bromide (a very toxic material) in 0.8 mL of acetonitrile was added all at once. After 3 min of rapid stirring, the slurry was filtered with suction and washed immediately with 100 mL each of 0.1 M NaHCO_3 (pH 9.5), H_2O , and again with the NaHCO_3 buffer. After the last wash the moist cake was added to the beaker containing the enzyme solution, stirred briefly, and stored at 4°C for 20 h. The immobilized enzymes were packed in columns and washed successively with 100 mL each of 0.1 M phosphate buffer, pH 7.0, 0.1 M phosphate buffer, pH 7.0 and 0.5 M in KCl, and then again with the 0.1 M phosphate buffer, pH 7.0. Activities were: agarose:carbonic anhydrase, 8.4 U/mL gel (82%); agarose:hexokinase, 52 U/mL gel (11%); agarose: β -galactosidase, 59 U/mL gel (86%).

Agarose:ArSO₂NH₂ (33)

Sepharose 6B (16 g of slurry) was washed with water and filtered with suction. The resulting moist sepharose (ca. 10 g) and 14.3 mL of a 70% aq solution of 1,4-butanediol diglycidyl ether were stirred rapidly for 14.5 h with 10 mL of 0.6 M NaOH solution containing 20 mg of NaBH_4 . The gel was collected by filtration under reduced pressure, washed with 1.6 L of water, and transferred to a 200-mL round-bottomed flask. Sulfanilimide (1.14 g, 6.62 mmol) in 35 mL of carbonate buffer, pH 11.0, was added and the suspension stirred for 36 h at 70°C. The gel was filtered and washed with 100 mL each of 0.5 M NaHCO_3 , pH 9.5 and 1.0 M in NaCl, water, 0.05 M glycine·HCl, pH 3.0 and 1.0 M in NaCl, and again with water. The sepharose was packed in a column and washed with 700 mL of 0.05 M glycine·HCl, pH 3.0 and 1 M in NaCl, and finally washed with 800 mL of water.

Fe₃O₄:Agarose:Carbonic Anhydrase and Fe₃O₄:Agarose:ArSO₂NH₂ (17, 18)

Either agarose:carbonic anhydrase or agarose:ArSO₂NH₂ (ca. 10 mL gel) was washed with water. $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.20 g, 1.0 mmol) and 0.54 g (2.0 mmol) of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were dissolved in 25 mL of water and heated to 70°C. NaOH (0.5 g) in 5 mL of water was added with stirring and a black precipitate of magnetite formed. The suspension was allowed to cool to ambient temperature, neutralized with HCl, added to the modified agarose, and stirred for 1 h. The suspension was filtered with suction using a coarse glass frit, which passes the free magnetite, and the gel washed with 1.0 L of water. The resulting brown-colored agarose beads are easily manipulated with a weak bar magnet. The magnetite particles are unobservable by scanning electron microscopy (see Fig. 2).

4-Sulfonamidobenzoyl Chloride

Thionyl chloride (50 mL, 0.685 mol) was added to a suspension of 50.0 g (0.248 mol) of 4-carboxybenzenesulfonamide in 200 mL of dry dioxane under argon. The mixture was heated at reflux for 1.5 h, cooled, and concentrated by rotary evapora-

tion. The resulting pale pink solid was triturated with cold toluene, filtered with suction, and washed with cold toluene. The solid was recrystallized from toluene:dioxane yielding 50 g (93%) of product as off-white flakes, mp 139–142°C, lit. mp 141–143 (31). IR (nujol, cm^{-1}): 3330, 3250, 3100, 1722, 1350, and 1160. ^1H NMR(acetone- d_6 , δ): 8.0–8.5(4H, d of d), 6.8–7.1(2H, b, s).

FG: PAN: ArSO_2NH_2

To 10 mL of a 1.0 M solution of triethylenetetramine (TET, 10 mmol) in acetone stirred vigorously in a 50-mL round-bottomed flask in an ice bath was added dropwise a solution of 1.1 g (5 mmol) of 4-sulfonamidobenzoyl chloride in 10 mL of acetone. The ice bath was removed and the mixture stirred an additional 1.5 h. The precipitate that formed was dissolved by addition of 3 mL of water, the mixture concentrated to a viscous oil by rotary evaporation, then diluted to 10 mL by addition of 0.3 M Hepes buffer, pH 7.5. To a stirred solution of 4 g of PAN-1000 in 15 mL of 0.3 M Hepes, pH 7.5 was added 2.5 mL of the solution prepared above. The mixture was stirred for ca. 15 s, applied to ca. 48 in.² of fiberglass cloth with a rubber roller, and stored in a moist atmosphere for 1.5 h. The resulting FG : PAN : ArSO_2NH_2 was washed several times with 50 mM Hepes buffer [pH 7.5, 50 mM $(\text{NH}_4)_2\text{SO}_4$] and stored in this buffer overnight at 4°C. The affinity sponge was then washed several times with 0.1 M phosphate buffer, pH 7.0, and stored in this buffer at 4°C.

FG: PAN: Carbonic Anhydrase

PAN 1000 (3.00 g) was dissolved in 12 mL of 0.3 M Hepes buffer, pH 7.5, in a 25-mL beaker. With stirring, 2.25 mL of 0.5 M aq. triethylenetetramine (TET) was added. After ca. 15 s, a solution of 500 mg of carbonic anhydrase in 2 mL of 0.3 M Hepes buffer, pH 7.5, was added, the solution stirred an additional 10 s, and applied to ca. 40 in.² of fiberglass cloth with a rubber roller. The resulting FG: PAN: carbonic anhydrase was stored in a moist atmosphere for 1 h, washed several times with 50 mM Hepes buffer [pH 7.5, 50 mM in $(\text{NH}_4)_2\text{SO}_4$] and stored at 4°C in this buffer overnight. It was then washed several times with 0.1 M phosphate buffer, pH 7.0, and stored in this buffer at 4°C.

Magnetic Separation of Fe_3O_4 : Agarose: Carbonic Anhydrase from Suspension

A suspension (15 mL) consisting of agarose:hexokinase (ca. 5 mL of gel, 80.6 U), 5 mL of Fe_3O_4 :agarose:carbonic anhydrase (27.3 U), and peroxidase (ca. 1 mg, 132 U) in 1 mL phosphate buffer, pH 7.0 was prepared. The peroxidase, carbonic anhydrase, and hexokinase activities were assayed. The suspension was diluted to 100 mL with 1 mM phosphate buffer, pH 7.0, passed through the magnetic filter with a flow rate of 90 mL min^{-1} , and washed with additional phosphate buffer. The suspension passed by the magnetic filter was filtered through a glass frit and the fraction passed by this filter assayed for each of the enzymatic activities. The material retained by the conventional filter was resuspended in 10

mL of 0.1 M phosphate buffer and assayed for each of the enzymes. The magnetic filter was flushed by turning off the electromagnet and pouring 50 mL of 1 mM phosphate buffer, pH 7.0, through it. This suspension was also assayed for each of the enzymatic activities.

Magnetic Affinity Adsorption of Carbonic Anhydrase from a Mixture Containing Suspended Solids

Agarose:hexokinase (5 mL of gel, 54.6 U) was suspended in 0.1 M phosphate buffer, pH 7.0, containing peroxidase (ca. 3 mg, 343 U) and carbonic anhydrase (ca. 37 mg, 35.7 U). The resulting 25.0-mL suspension was assayed for each of the enzymatic activities. Fe_3O_4 :agarose: ArSO_2NH_2 (ca. 10 mL of gel) was added and the suspension stirred for 1 h. The suspension was then subjected to consecutive magnetic and conventional filtrations and the fractions assayed as in the previous experiment. In this case, however, the magnetically responsive suspension collected from the magnetic filter was packed in a column and washed with 50 mL of 0.1 M phosphate buffer, pH 7.0. The carbonic anhydrase was then eluted with 50 mL of 0.1 M acetate buffer, pH 5.15 and 1.0 M in NaCl. The resulting solution was dialyzed twice against 2 L of 1 mM phosphate buffer, pH 7.0, and then assayed.

Magnetic Generalized Affinity Adsorption of Hexokinase~ ArSO_2NH_2 from a Mixture Containing Suspended Solids

A slurry of agarose: β -galactosidase (20 mL, 114 U) was added to a solution of peroxidase (ca. 3 mg, 292 U) and hexokinase~ ArSO_2NH_2 (22.8 U) in 8 mL of 1 mM phosphate buffer, pH 7.0, and the resulting suspension was assayed for each of the enzymatic activities. Fe_3O_4 :agarose: carbonic anhydrase (ca. 7 mL of gel) and 30 mL of 0.1 M phosphate buffer, pH 7.0, were added. The suspension was stirred for 1 h at room temperature, then subjected to consecutive magnetic and conventional filtrations. The magnetically responsive suspension flushed from the magnetic filter was packed in a column, washed with 50 mL of 0.1 M phosphate buffer (pH 7.0), eluted with 50 mL of 0.1 M acetate buffer (pH 5.15, 1 M NaCl), and then assayed.

Adsorption of Carbonic Anhydrase from a Mixture Containing Suspended Solids Using the Affinity Sponge

Agarose:hexokinase (ca. 5 mL gel, 126 U) was suspended in 95 mL of 0.01 M phosphate buffer, pH 7.0, containing carbonic anhydrase (ca. 50 mg, 43.9 U) and peroxidase (ca. 3 mg, 172 U). The resulting suspension was stirred for 8 h at 4°C in a 500-mL crystallizing dish containing the affinity sponge, FG : PAN : ArSO_2NH_2 (ca. 48 in²). The sponge was removed, washed briefly with 100 mL of 0.01 M phosphate buffer, pH 7.0, and stirred for 8 h at 4°C in another 500-mL crystallizing dish containing 100 mL of 0.1 M acetate buffer (pH 5.15, 1 M NaCl). The sponge was removed and 26 mL of the solution dialyzed several times against 4 L of 1 mM phosphate buffer, pH 7.0. The original suspension, the wash solution, and the dialyzed solution were assayed for each of the three enzymatic activities.

Adsorption of Hexokinase~ArSO₂NH₂ from a Mixture Containing Suspended Solids Using the Generalized Affinity Sponge

Agarose:β-galactosidase (ca. 5 mL gel, 151 U) was suspended in 95 mL of 0.01 M phosphate buffer, pH 7.0, containing hexokinase~ArSO₂NH₂ (64.0 U) and peroxidase (ca. 3 mg, 167 U). The resulting suspension was stirred for 8 h at 4°C with the generalized affinity sponge (FG: PAN: carbonic anhydrase, ca. 40 in²) in a 500-mL crystallizing dish. The sponge was withdrawn, washed briefly with 100 mL of 0.01 M phosphate buffer, pH 7.0, and stirred for 8 h at 4°C in another 500-mL crystallizing dish containing 100 mL of 0.1 M acetate buffer (pH 5.15, 1 M NaCl). The sponge was removed and the original suspension, wash solution, and eluant assayed for each of the enzymatic activities.

Affinity Adsorption of Carbonic Anhydrase from a Suspension of Sonicated Yeast

A suspension was prepared by sonicating 20 g of Bakers yeast (Sigma YSC-2) in 250 mL of 0.01 M phosphate buffer, pH 7.0, and adding ca. 80 mg of carbonic anhydrase (94 U). The suspension was separated into two 100-mL aliquots, and the carbonic anhydrase was adsorbed from these with either the magnetic affinity matrix or the affinity sponge, using the procedures described previously.

Results

Magnetic Affinity and Generalized Affinity Matrices

Aromatic sulfonamides bind carbonic anhydrase strongly ($K_i = 10^{-6}M$ – $10^{-8}M$), are easily manipulated chemically, and are readily available (32). The methods used to prepare magnetic matrices based on this affinity system are illustrated in Fig. 1.

Sulfanilamide was coupled to agarose beads through a diglycidyl ether spacer arm using a modification of a literature procedure (33). Carbonic anhydrase was coupled to BrCN-activated agarose by conventional methods (34, 35). These functionalized matrices were made ferrimagnetic using a modification of a procedure developed earlier for the preparation of magnetically recoverable catalyst supports (17, 18). A magnetite colloid was prepared by reaction of a 1:2 aqueous mixture of Fe(II) and Fe(III) with sodium hydroxide. When this colloid was neutralized and treated with an aqueous suspension of agarose beads (either functionalized or not), the agarose and a part of the magnetite became tightly coupled. The magnetite particles are unobservable in a scanning electron micrograph of the resulting brown beads (Fig. 2). The agarose:magnetite composite can be separated from unconjugated magnetite by filtration on a coarse glass frit: the beads are retained, while the unconjugated magnetite colloid passes through the filter. We have not explored the chemistry of the attachment of magnetite to agarose, but it appears to involve some combination of physical entrapment of magnetite particles in the agarose gel and replacement of magnetite surface oxide groups by hydroxyl functions (proba-

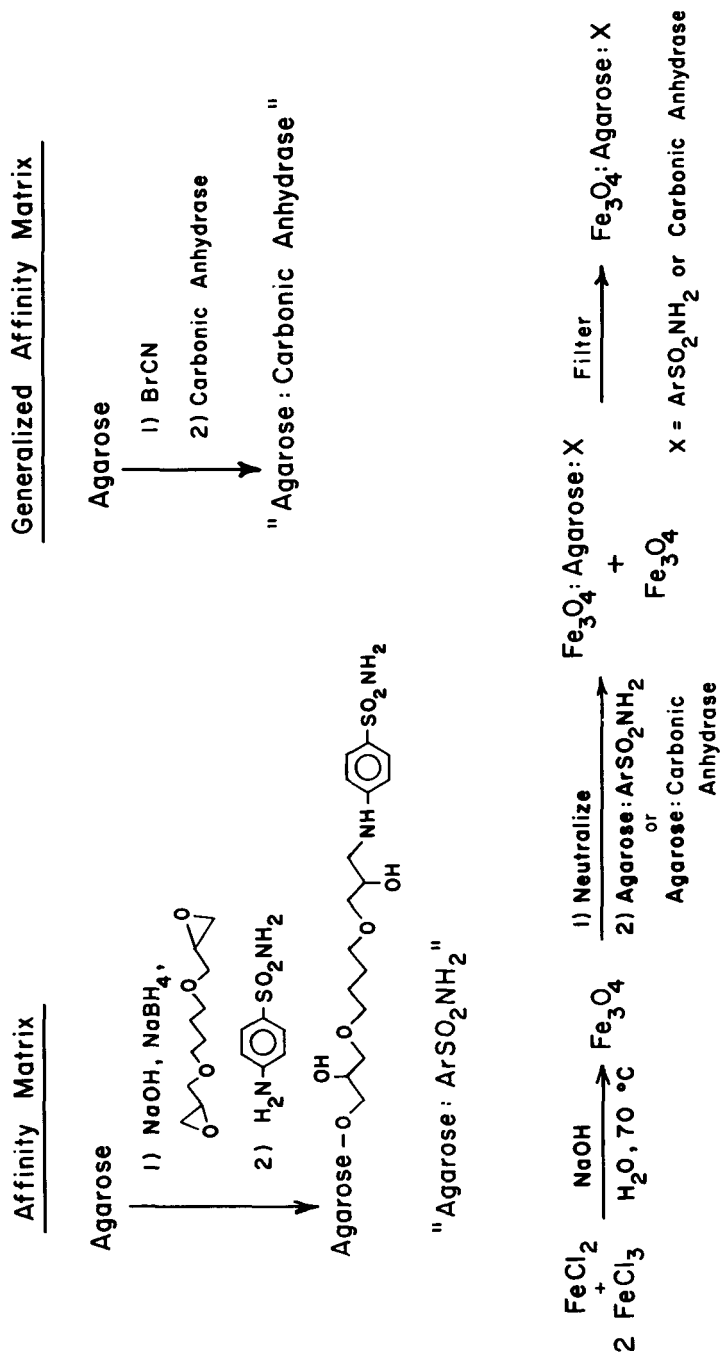


Fig. 1. Preparation of magnetic affinity and generalized affinity matrices.
 Fe₃O₄ : agarose represents agarose beads having magnetite particles adsorbed on their surface.

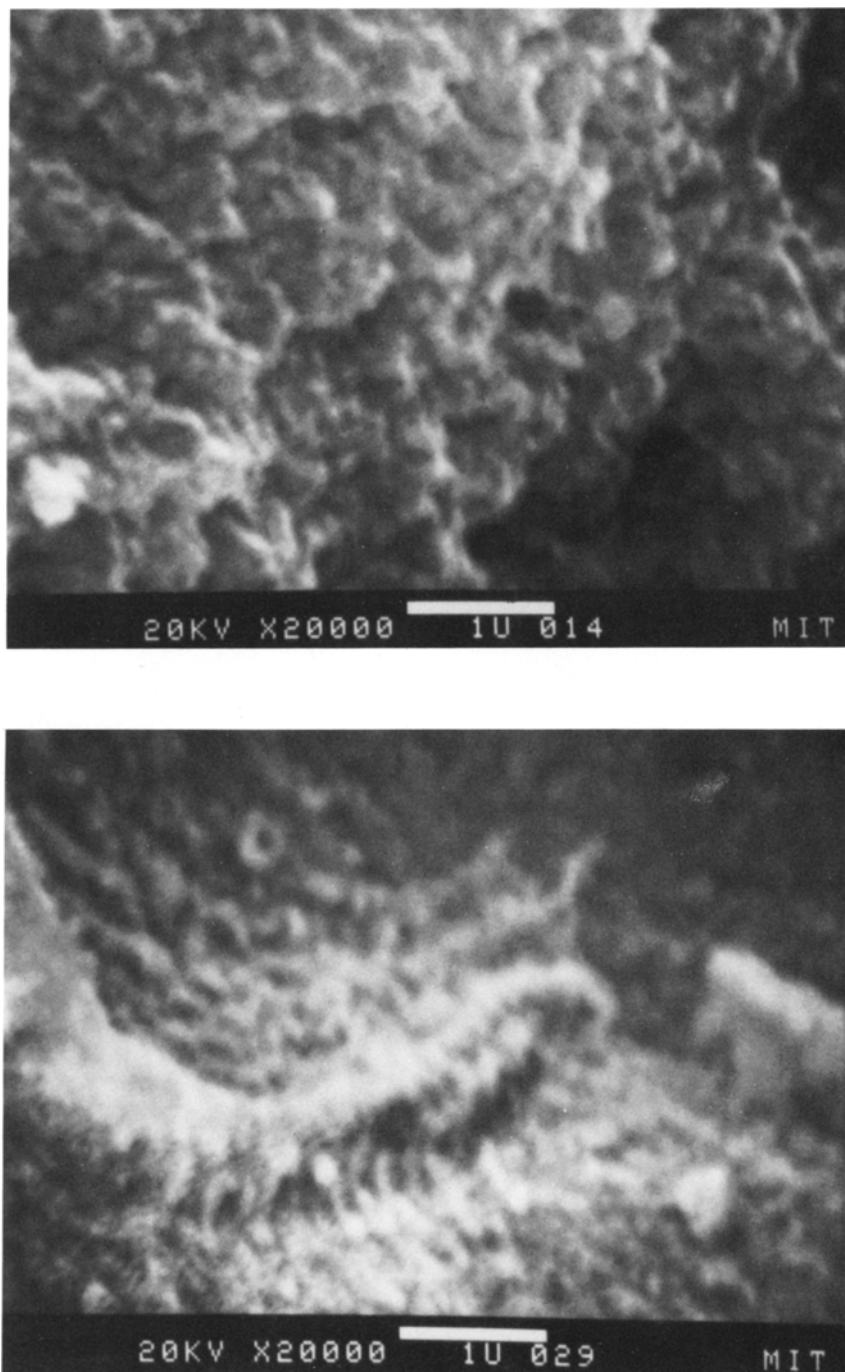


Fig. 2. Scanning electron micrographs of agarose beads (top) and Fe_3O_4 on agarose beads (bottom). The bar labeled 1U at the bottom of each micrograph is a 1- μm calibration. No difference between magnetic and nonmagnetic beads is observed at lower magnification.

bly chelating) from the polysaccharide gel. In any event, the attachment is firm: after the initial preparation, very little magnetite is lost from the beads on subsequent manipulation. The method also appears to be sufficiently gentle for use with at least some biochemical systems: no decrease in carbonic anhydrase activity was detected upon deposition of magnetite on agarose containing immobilized carbonic anhydrase.

Preliminary Experiments—Separation of Diamagnetic and Ferrimagnetic Agarose Beads

We began our work by examining the efficiency of separation of components in a model biochemical system consisting of peroxidase (as a representative soluble protein contaminant), hexokinase covalently bonded to agarose beads (agarose:hexokinase, an insoluble and diamagnetic component), and carbonic anhydrase covalently bonded to agarose beads having attached magnetite particles (Fe_3O_4 :agarose:carbonic anhydrase, an insoluble ferrimagnetic component). These enzymes were chosen for their availability and the ease with which each can be assayed in the presence of the others: they were intended primarily to provide labels for each of the three phases. Figure 3 summarizes results.

Magnetic filtrations were carried out using a magnetic filter constructed simply by packing stainless steel wool loosely into a 1-in diameter glass burette, and placing the burette in a magnetic field of approximately 10 kG (generated by a

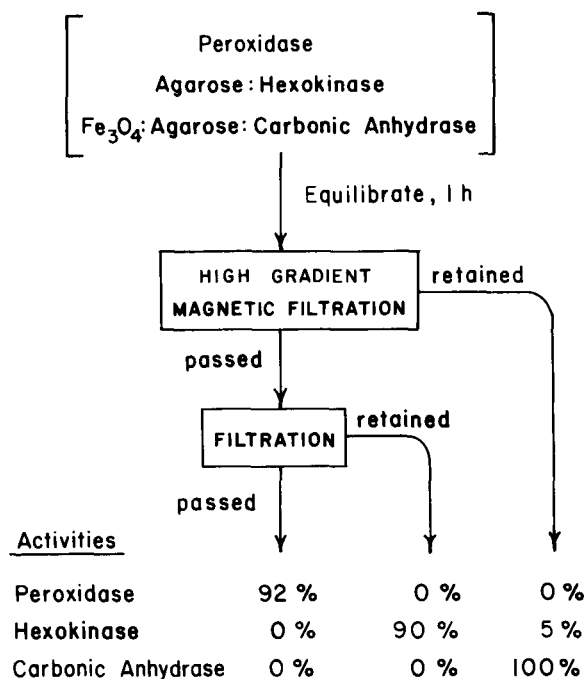


Fig. 3. Magnetic separation of soluble, insoluble diamagnetic, and insoluble ferrimagnetic components.

small electromagnet). The suspension containing the diamagnetic and ferromagnetic agarose beads (phosphate buffer, 1 mM, pH 7) was passed through the filter rapidly (90 mL min^{-1}), and the material retained on the filter washed with phosphate buffer. This magnetically responsive material was collected by turning off the electromagnet and flushing the filter with additional phosphate buffer. The suspension collected from the magnetic filter was assayed for each of the enzymatic activities originally present. The numbers in Fig. 3 represent recoveries of enzymatic activity, based on the activity of the original suspension before filtration. Thus, the recovery of carbonic anhydrase activity in the magnetic material retained in the magnetic filter was quantitative; the only contaminant was 5% of the hexokinase activity originally present conjugated to the diamagnetic agarose beads.

The material that passed through the magnetic filter was subjected to conventional filtration, and the fractions retained on the filter and passed through it were analyzed. As expected, the peroxidase was found in good yield in the solution (92% of the original activity), and the hexokinase was found in the material retained on the filter (90% of the original activity). There was no contamination of either the peroxidase-containing fraction or the agarose:hexokinase fraction by other enzymatic activities.

Magnetically responsive, functionalized agarose beads are thus easily prepared and stable, and are separated rapidly and cleanly from diamagnetic insoluble and soluble materials using a high-gradient magnetic filter of very simple design.

Magnetic Affinity Adsorption

To demonstrate magnetic affinity adsorption, a suspension was prepared containing four components: two soluble enzymes (peroxidase and carbonic anhydrase), the magnetic affinity matrix for carbonic anhydrase (Fe_3O_4 : agarose: ArSO_2NH_2) described previously, and a diamagnetic solid (agarose:hexokinase, intended to model insoluble components in a crude biological preparation). The mixture was allowed to equilibrate with stirring for 1 h, then subjected to successive magnetic and conventional filtrations, using procedures analogous to those described in the preceding experiment (Fig. 4). The major part of the carbonic anhydrase (83%) was recovered by elution from the particles retained in the magnetic filter; 8% was found in solution with the peroxidase. The material retained on the conventional filter contained 93% of the hexokinase activity.

Magnetic Generalized Affinity Adsorption

An example of magnetic generalized affinity adsorption is outlined in Fig. 5. Hexokinase-sulfonamide conjugate was prepared, as reported previously, (26) by reaction of hexokinase with the reagent **1** in the presence of ADP and glucose (to protect the enzyme active site).

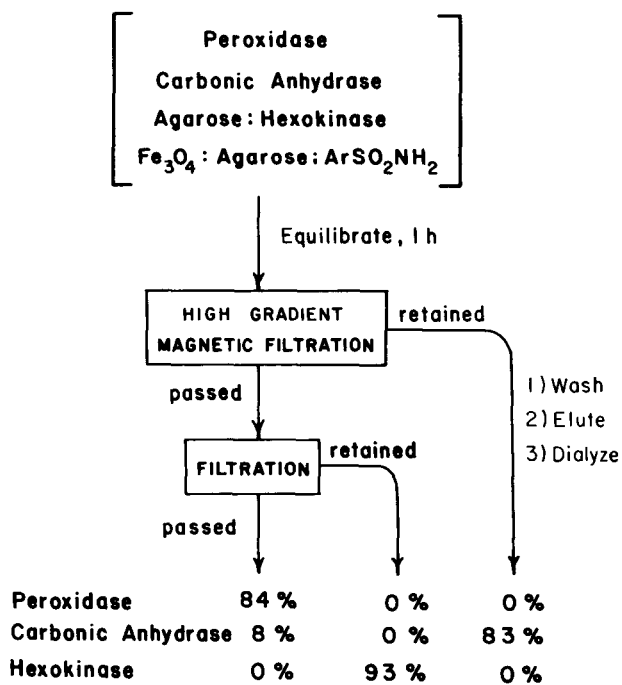


Fig. 4. Model magnetic affinity separation. (Elution with 0.1 M acetate, 1 M NaCl, pH 5.15.)

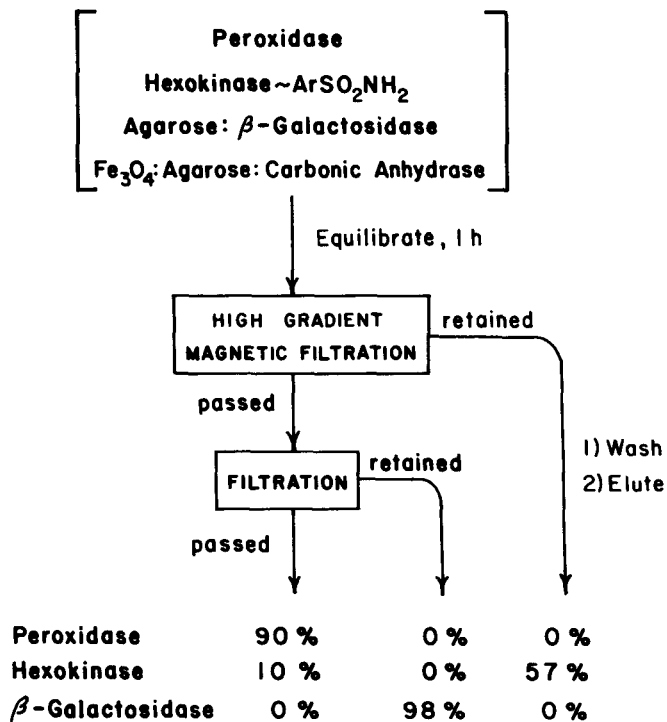
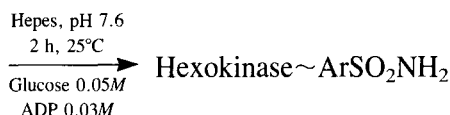
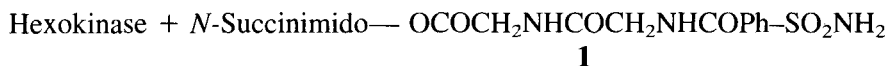


Fig. 5. Model magnetic generalized affinity separation. (Elution with 0.1 M acetate, 1 M NaCl, pH 5.15.)



The magnetic generalized affinity matrix (Fe_3O_4 :agarose:carbonic anhydrase) was equilibrated for 1 h with a mixture of the two soluble enzymes peroxidase and hexokinase-sulfonamide conjugate (hexokinase $\sim\text{ArSO}_2\text{NH}_2$), and a diamagnetic solid (agarose: β -galactosidase). The components were separated by successive magnetic and conventional filtrations, as in previous experiments. Recovery of the hexokinase $\sim\text{ArSO}_2\text{NH}_2$ was only modest (57%). We believe this low recovery results from problems with the affinity separation and not inefficiency of the magnetic filtration, but several attempts to improve the efficiency of the separation were unsuccessful.

Affinity and Generalized Affinity Sponges

In principle, all that is required to carry out affinity separations of proteins from mixtures containing suspended solids is an affinity matrix with physical properties sufficiently distinct from those of the suspended solids that the two types of solids can be easily separated. Magnetically responsive affinity matrices provide one solution to this problem. A second is an affinity gel connected to a support having the size and mechanical properties required for it to be conveniently inserted into and removed from the suspension of interest. The preparation of affinity gels of these types ("affinity sponges") is outlined in Fig. 6. These sponges are prepared by crosslinking poly(acrylamide-*co*-*N*-acryloxysuccinimide) (PAN), as a thin gel film on a fiberglass cloth support. Affinity ligands or enzymes are covalently attached through the active ester groups of PAN during the crosslinking step. The gel is porous to macromolecules and its capacity for enzyme binding is not limited by superficial surface area—in principle, the entire gel volume is utilized.

In the preparation of gels based on PAN, an α,ω -diamine (commonly triethylenetetramine, TET) is used as a crosslinking agent. We have also used TET as a spacer arm to separate the affinity ligand from the polymer backbone. Incorporation of a spacer arm between affinity ligand and matrix is generally considered desirable in affinity chromatography to relieve steric restrictions imposed by the matrix. The preparation of ligands incorporating spacer arms often requires a synthesis involving reaction of the affinity ligand with a large excess of the α,ω -difunctional moiety intended to become the spacer arm, followed by separation of the desired modified affinity ligand from the excess of the spacer moiety. This procedure is uneconomical and inconvenient. We have simply allowed the affinity ligand (here an aryl sulfonamide) to couple with excess TET, and used the crude reaction mixture, without purification, in the crosslinking reaction which forms the gel. This procedure should be readily generalizable to other affinity systems. In practice, PAN, TET, and the affinity ligand (or carbonic anhydrase, for enzyme-containing gels) are mixed in aqueous solution, and then spread rapidly over the

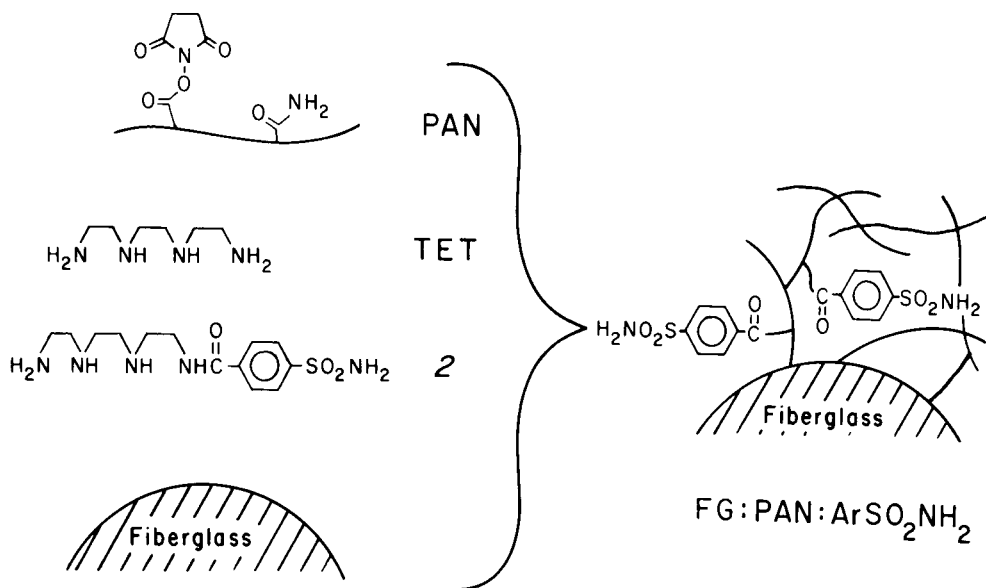


Fig. 6. Preparation of an affinity sponge supported on fiberglass. PAN is poly(acrylamide-co-N-acryloxysuccinimide), and the polymer backbones are represented schematically by curved lines. TET is triethylenetetramine.

fiberglass cloth. The polymer gels to a soft, resilient film over the cloth backing: the thickness of the film is ca. 0.5 mm. The gel-coated fiberglass is then washed and inserted into the solution or suspension from which the enzyme is to be adsorbed.

Rate of Adsorption of Proteins by the Affinity Sponge

The rate of disappearance of carbonic anhydrase activity from a solution in the presence of the affinity sponge, FG: PAN: ArSO₂NH₂, is plotted in Fig. 7. The relatively slow rate of disappearance of activity may be due to slow diffusion of enzyme through the gel. This experiment suggests, in agreement with previous studies of PAN gels, that the interior of the gel is accessible to proteins by diffusion, and emphasizes a significant disadvantage of these sponges: that is, because diffusion is a relatively slow process, enzyme separations using these materials take more time to perform than magnetic affinity separations, in which particle sizes are relatively small and diffusion times fairly short.

Affinity Adsorption of Carbonic Anhydrase with the Affinity Sponge

The use of the affinity sponge, FG: PAN: ArSO₂NH₂, for the adsorption of carbonic anhydrase directly from suspension is summarized in Fig. 8. A suspension containing the soluble enzymes carbonic anhydrase and peroxidase, agarose-immobilized hexokinase (agarose:hexokinase), and the affinity sponge (FG: PAN: ArSO₂NH₂) was stirred for 8 h at 4°C. The affinity sponge was then withdrawn from the suspension, washed with phosphate buffer, and stored for 8 h

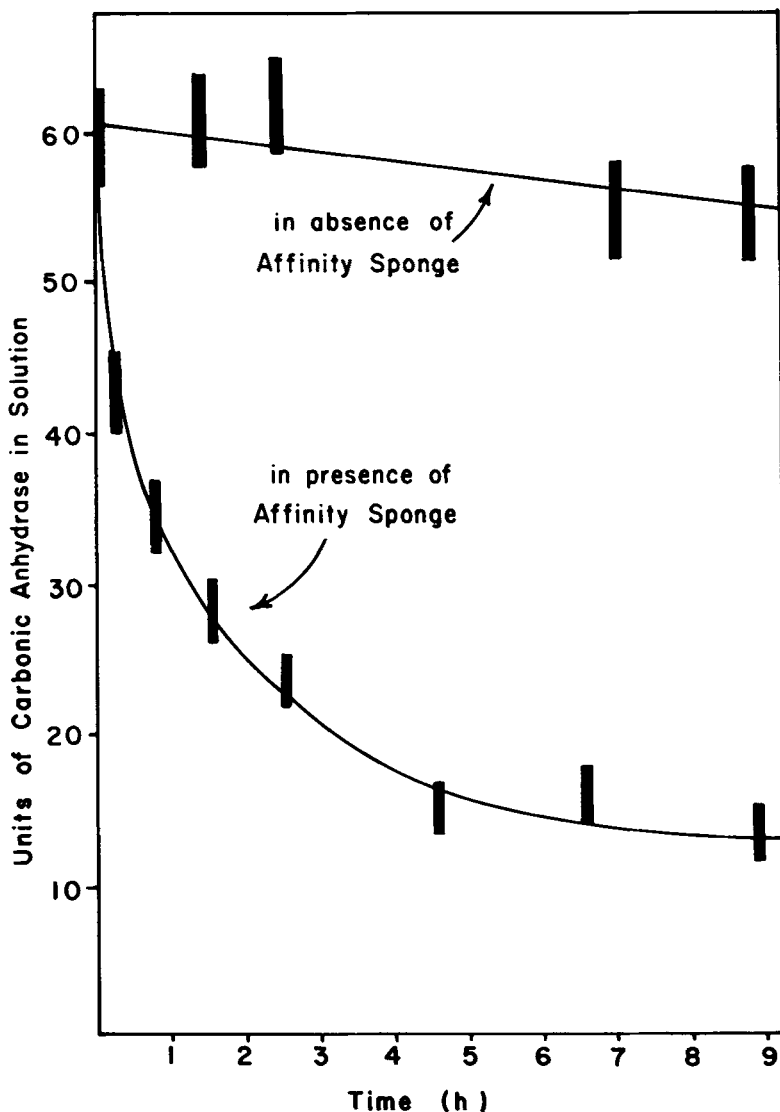


Fig. 7. Rate of disappearance of carbonic anhydrase activity from solution in the presence of an affinity sponge.

at 4°C in the stirred eluting buffer. The resulting solution was dialyzed and assayed for enzymatic activities. The carbonic anhydrase was separated cleanly from peroxidase and hexokinase, but its recovery was only 50%. Attempts to increase the fraction of the carbonic anhydrase recovered by utilizing smaller amounts in the original suspension were unsuccessful. We suspected the possibility that the positive charges on the TET spacer arm were decreasing affinity of the ligand for carbonic anhydrase. In independent solution experiments, however, the K_i of the inhibitor 2 (Fig. 6) was determined to be $6.9 \times 10^{-7} M$. This value is comparable to that of benzenesulfonamide itself, $4.6 \times 10^{-7} M$ (27). The relatively low recovery of carbonic anhydrase by this method thus remains unexplained.

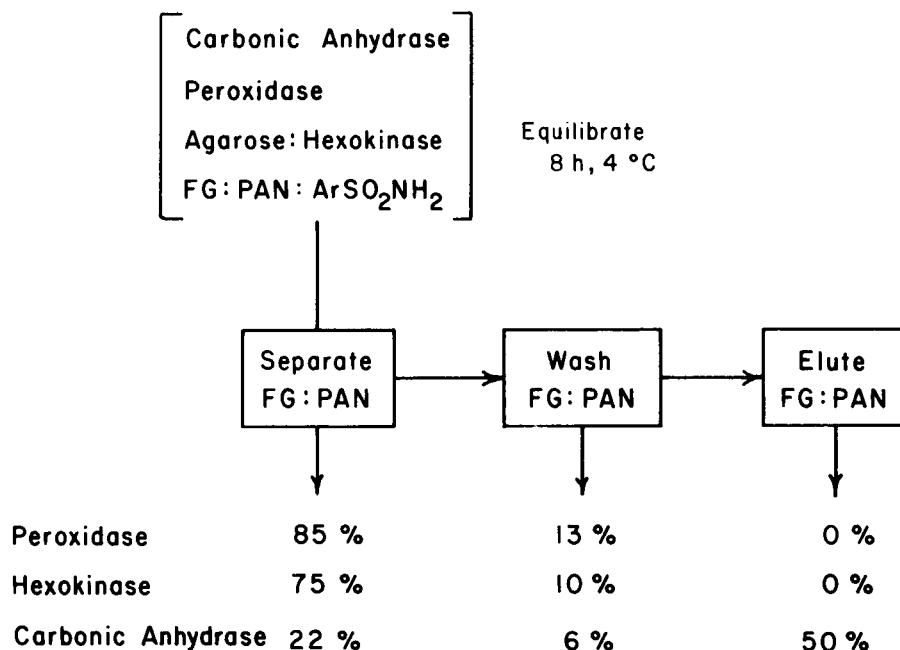


Fig. 8. Adsorption of carbonic anhydrase from a solution containing suspended solids. (Washing solution: 0.01 M phosphate, pH 7.0; elution: 0.1 M acetate, 1 M NaCl, pH 5.15.)

Generalized Affinity Adsorption of Hexokinase~ArSO₂NH₂ with the Generalized Affinity Sponge

The use of the generalized affinity sponge (FG: PAN: carbonic anhydrase) for the separation of hexokinase-sulfonamide conjugate directly from a suspension is illustrated in Fig. 9. The suspension contained the soluble enzymes peroxidase and hexokinase~ArSO₂NH₂, as well as suspended agarose:β-galactosidase. Separation of hexokinase~ArSO₂NH₂ was accomplished cleanly.

Separation of Carbonic Anhydrase from Sonicated Yeast Suspension

To examine the relative efficiency of magnetic affinity matrices and affinity sponges for the separation of carbonic anhydrase from a mixture that more closely resembled an actual biochemical preparation, we prepared a suitable test system by sonicating yeast in phosphate buffer and adding carbonic anhydrase. The reisolation of carbonic anhydrase from this suspension using both the magnetic affinity matrix and the affinity sponge is summarized in Figs. 10 and 11, respectively. Magnetic affinity separation resulted in the isolation of 50% of the original carbonic anhydrase activity in the solution eluted from the magnetic affinity matrix. The suspension retained 44% of its original carbonic anhydrase activity. The affinity sponge was slightly less effective, with 45% of the carbonic anhydrase activity found in the solution eluted from the sponge, 36% remaining in the suspension, and 7% in the wash solution.

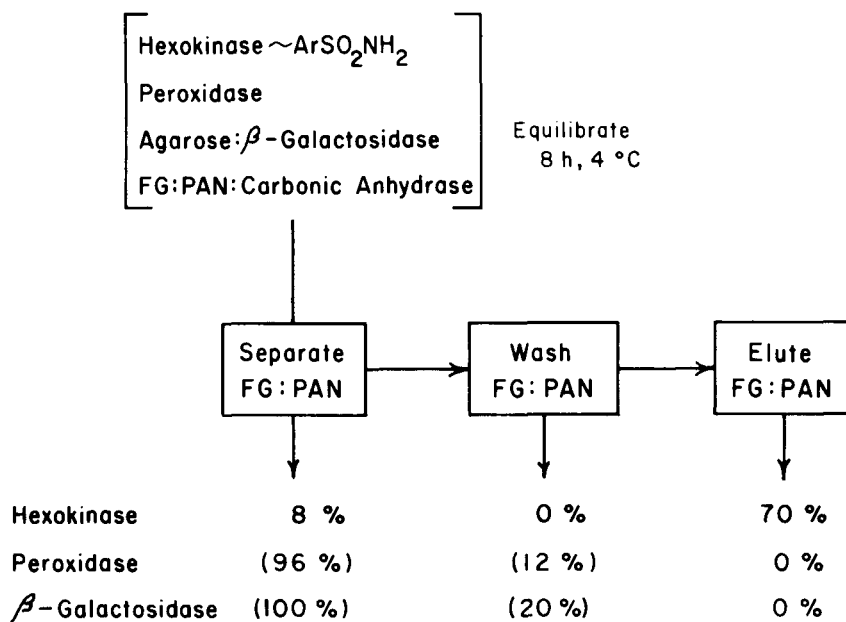


Fig. 9. Adsorption of hexokinase \sim ArSO_2NH_2 from a solution containing suspended solids. (Washing solution: 0.01 *M* phosphate, pH 7.0; elution: 0.1 *M* acetate, 1 *M* NaCl, pH 5.15.) *The precision of the enzymatic assays is ca. \pm 5% for soluble enzymes and \pm 10% for immobilized enzymes. The "120%" recovery of agarose : β -galactosidase in this experiment may reflect a systematic error.

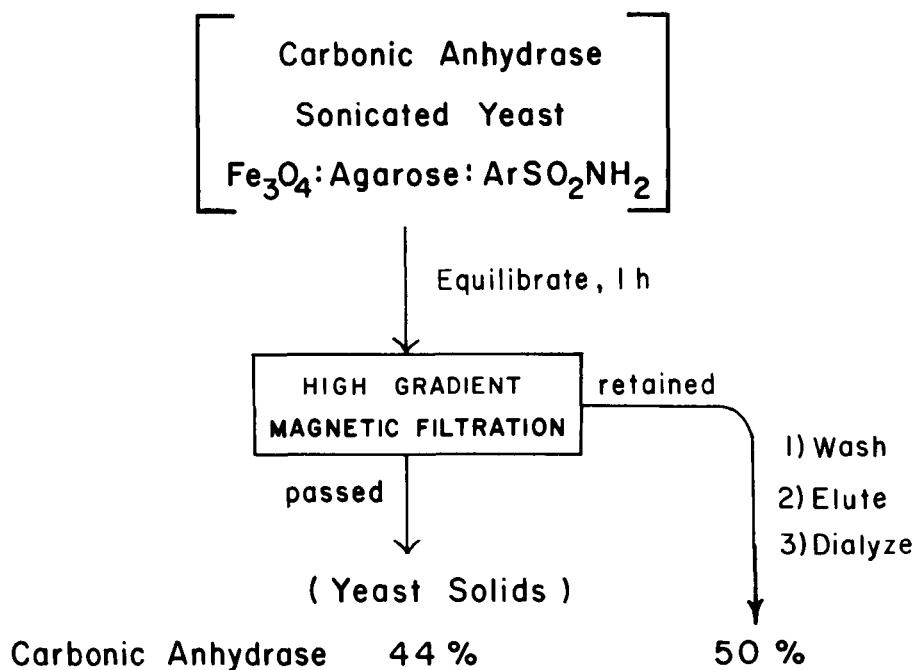


Fig. 10. Magnetic affinity separation of carbonic anhydrase from yeast suspension. (Elution: 0.1 *M* acetate, 1 *M* NaCl, pH 5.15.)

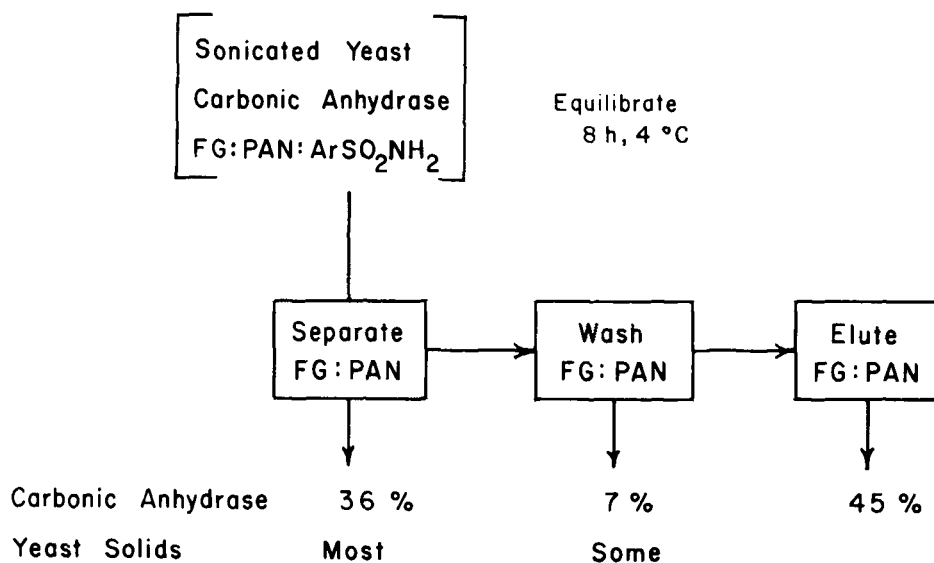


Fig. 11. Separation of carbonic anhydrase from yeast suspension using an affinity sponge. (Washing solution: 0.01 M phosphate, pH 7.0; elution: 0.1 M acetate, 1 M NaCl, pH 5.15.)

Summary

We draw several conclusions from this work. First, relatively clean separation of ferrimagnetic solids from biochemical systems is practical using high gradient magnetic filtration. The features of this filtration method that make it attractive in biochemical applications are that it is rapid (especially in filtration of soft or sticky materials that might clog conventional filters), it is applicable to relatively small (several micrometers) particles, and it is selective for one (ferrimagnetic) solid in the presence of other (diamagnetic) solids. Thus, in principle it might be possible to use this method for protein isolation directly from crude cell homogenates, or from active fermentations, or to recover immobilized enzymes added to systems containing suspended solids.

The procedure used here to prepare the magnetic agarose beads is a very simple one experimentally, and works better than might reasonably be expected. It is certainly less expensive and complex than procedures based on preformed ferrofluids (12, 15), but seems to yield materials in which the connection between the agarose and the magnetite is quite durable. We have not, however, tested these systems for stability in the presence of strong chelating agents or materials (e.g., thiols) having a high affinity for transition metals. We believe that this technique should be applicable to the preparation of other ferrimagnetic gel-magnetite conjugates.

Our comparisons of magnetic and nonmagnetic affinity adsorption procedures suggests that they are comparable in their practicality. The magnetic methods are somewhat more complex technically, but because the magnetic beads are small, the kinetics of adsorption seem better than those characterizing the macroscopic fiberglass-supported gel films.

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