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PURIFICATION OF TYROSINE AMINOTRANSFERASE BY AFFINITY CHROMATOGRAPHY

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SUMMARY

Tyrosine aminotransferase (L-tyrosine:2-oxoglutarate amino transferase, EC 2.6.1.5) will bind to affinity adsorbents containing covalently bound pyridoxamine phosphate. It can be released by a change of buffers with a resultant 125-fold purification. The enzyme can be further purified by chromatography on Sephadex G-200 to give a product with a specific activity of 400 enzyme units/mg protein. This method apparently copurifies different isozymes of tyrosine aminotransferase.

INTRODUCTION

Tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) is a glucocorticoid-inducible enzyme found in rat liver and hepatoma tissue culture cells. Previous studies have shown it to be a single enzyme of molecular weight 115 000 composed of four apparently identical subunits[1,2]. One mole of enzyme binds 4 moles of pyridoxal phosphate[2] with an apparent K_m of 10^{-8} M[1]. The enzyme also binds pyridoxamine phosphate with an apparent K_m of 10^{-7} M[1]. Because of these favorably low K_m values we felt affinity gels prepared by covalently linking pyridoxamine phosphate to agarose might be useful in purifying the enzyme.

Affinity chromatography[3,4] has been used successfully in the purification of a variety of biologically important macromolecules (reviewed in refs 5 and 6). Columns containing adsorbents to which pyridoxamine phosphate has been covalently attached have been used in the isolation of ribosomes capable of specifically synthesizing tyrosine aminotransferase[7]. In that case the effectiveness of the adsorbents was related to their presumed capacity to interact selectively with tyrosine aminotransferase. This paper describes in detail the preparation of pyridoxamine phosphate–agarose adsorbents and their use in the purification of tyrosine aminotransferase from rat liver and hepatoma tissue culture cells.

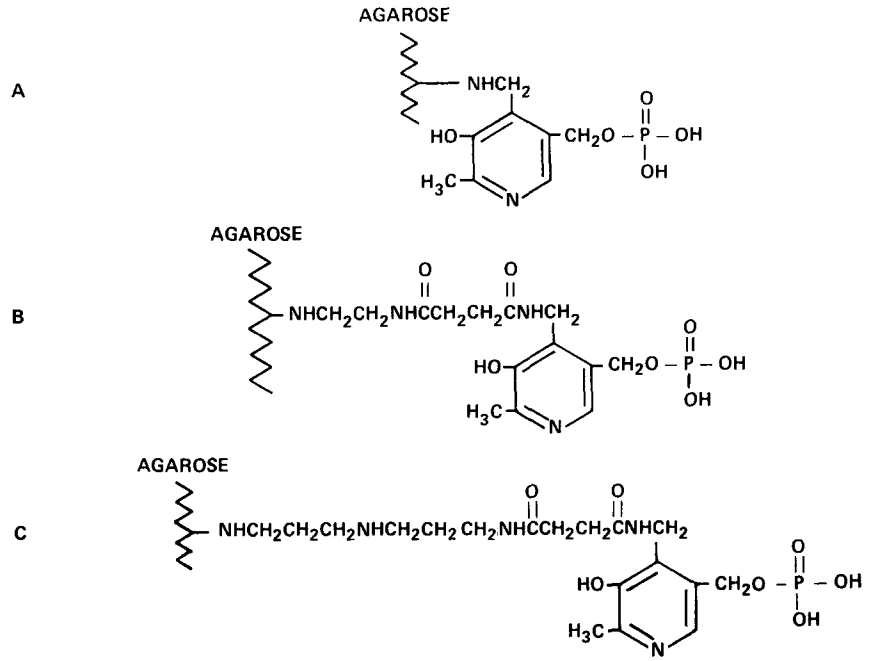

Fig. 1. Affinity chromatographic adsorbents. These were prepared by linking pyridoxamine phosphate to Sepharose 4B through sidearms of increasing length (see Materials and Methods).

MATERIALS AND METHODS

Pyridoxamine-agarose derivatives

Pyridoxamine phosphate was coupled directly to CNBr-activated agarose (Fig. 1A) by procedures previously described[3]. In the activation step, 250 mg of CNBr were used per ml of packed Sepharose 4B. The concentration of pyridoxamine phosphate in the coupling step was 20 mM, in 0.2 M $NaHCO_3$ buffer (pH 9.8). Approximately 10 μmoles of pyridoxamine were coupled per ml of packed agarose as determined by the amount of pyridoxamine phosphate recovered (by ultraviolet spectroscopy) in the washes.

Pyridoxamine phosphate was linked to the agarose backbone through either one of two hydrocarbon "arms" (Figs 1B and 1C) according to recently developed derivatization procedures[3]. To prepare the "short arm" gel (Fig. 1B), aminoethyl agarose was synthesized by reacting Sepharose 4B, previously activated with cyanogen bromide (250 mg per ml of agarose), with 0.3 M ethylenediamine in 0.2 M $NaHCO_3$ (pH 9.8). About 14 μmoles of amino groups were present per ml of the packed, washed agarose derivative. The ω-aminoalkyl agarose was succinylated by treatment with succinic anhydride[3]. Pyridoxamine phosphate was coupled to the succinylaminoethyl agarose with a water soluble carbodiimide[3]. 100 mg of pyridoxamine phosphate were added to a suspension containing 20 ml of derivatized agarose in a total volume of 40 ml of distilled water. The pH was adjusted to 4.7 and 1 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, dissolved in 3 ml of water, was added rapidly. The reaction was

maintained at constant pH by monitoring for 2 h with a pH meter and the suspensions were permitted to stir gently for another 3 h. The final adsorbent was washed with 8 l of 0.1 M NaCl over a 6-h period to remove non-covalently bound pyridoxamine. Approximately 9 μmoles of pyridoxamine were coupled per ml of packed agarose.

Pyridoxamine phosphate was also linked to agarose by an even longer "arm" (Fig. 1C) by reacting cyanogen bromide activated Sepharose 4B with the diamino compound, 3,3'-diaminodipropylamine. Succinylation and coupling of pyridoxamine phosphate were done as described above. The final, washed derivative contained 3 μmoles of pyridoxamine phosphate per ml of packed agarose.

Before use each derivative was washed with at least 1 bed volume of 1.0% (w/v) bovine serum albumin in Tris–KCl–$MgCl_2$ buffer (0.05 M Tris–HCl, pH 7.6, 0.025 M KCl and 0.005 M $MgCl_2$), followed by an equal volume of 0.1 M NaOH and then Tris–KCl–$MgCl_2$ buffer until the pH of the eluate became 7.6. Columns were regenerated after use by washing with 0.1 M NaOH followed by Tris–KCl–$MgCl_2$ buffer.

Hepatoma tissue culture cell tyrosine aminotransferase

Hepatoma tissue culture cells were grown in suspension culture in Swimm's 77 medium as previously described[1]. The cells were harvested by brief centrifugation at 500 × *g*, and were washed twice in Tris–KCl–$MgCl_2$ buffer by resuspension and centrifugation. After swelling for 10 min at 4 °C in a volume of Tris–KCl–$MgCl_2$ buffer equal to one-half the packed cell volume, the cells were homogenized in a Teflon–glass homogenizer. The homogenate was centrifuged at 100 000 × *g* for 45 min. The resulting supernatant fluid was used in the experiments indicated in the text.

Rat liver tyrosine aminotransferase

Male Sprague–Dawley rats (150 to 250 g) were induced by intraperitoneal steroid injection as previously described[1]. 8 h later, the livers were excised, chopped into small bits and homogenized in an equal volume of 0.1 M KCl and 0.001 M EDTA in a lucite–glass homogenizer. The homogenate was centrifuged at 10 000 × *g* for 20 min, the supernatant fluid was reserved, and the pellet was rehomogenized in an equal volume of 0.1 M KCl and 0.001 M EDTA and recentrifuged. The supernatant fluids were combined and used as described in the text. This procedure consistently gave an enzyme preparation with a specific activity of 0.6 enzyme unit/mg protein (1 enzyme unit = 10^{-6} moles *p*-hydroxyphenylpyruvate/min at 37 °C).

Assays

Tyrosine aminotransferase activity was determined by the Diamondstone[9] assay, modified as previously described[1]. Protein was estimated by the method of Lowry *et al.*[10] using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Stacking gels were prepared by a modification of the method of Laemmli[11] using Tris–H_2SO_4 buffer (pH 6.8) without sodium dodecylsulfate and the lower gels were prepared by the method of Davis[12] using Tris–HCl buffer (pH 8.8). 50-μl samples in 15% sucrose were applied, and the samples were electrophoresed at 2.5 mA/tube at room temperature. The buffer used was 0.05 M Tris–0.38 M glycine (pH 8.3) and $2 \cdot 10^{-4}$

M pyridoxal phosphate with bromphenol blue as tracking dye. Proteins were stained with 0.1% Coomasie blue in 10% trichloroacetic acid and were destained in 7% acetic acid. Tyrosine aminotransferase activity was located on the gels by the histochemical stain previously described[2] except that nitro blue tetrazolium[13] replaced *p*-iodonitrotetrazolium violet.

Materials

Pyridoxal phosphate, α-ketoglutarate and tyrosine were obtained from Calbiochem (Rockville, Md.), pyridoxamine phosphate from Sigma Chemical Co. (St. Louis, Mo), CNBr, ethylenediamine and 3,3'-diaminodipropylamine from Eastman Organic Chemicals (Rochester, N.Y.), succinic anhydride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide from Pierce Chemical Co. and gel electrophoresis materials from Canalco (Rockville, Md.). Mixed ^{3}H-labeled amino acids (1 mCi/ml) were obtained from Schwartz Bioresearch (Orangeburg, N.Y.). Dexamethasone phosphate was a gift from Merck Sharpe and Dohme (Baltimore, Md.).

RESULTS

Binding of tyrosine aminotransferase

The binding of tyrosine aminotransferase to pyridoxamine phosphate columns is a function both of the gel derivative used and the physical state of the enzyme. It has been found for several other enzymes that binding to an adsorbent can be augmented if the ligand is placed some distance from the matrix backbone by a molecular sidearm[3–5,14]. This phenomenon is also observed with tyrosine aminotransferase adsorbents (Table I). In this case, rat liver tyrosine aminotransferase was passed through columns of each derivative until the enzyme specific activity in the eluate equaled that of the starting material. The maximum, or saturating amount of enzyme which was bound by each derivative was determined by subtracting the total amount of enzyme

TABLE I

SATURATION BINDING OF RAT LIVER TYROSINE AMINOTRANSFERASE TO PYRIDOXAMINE PHOSPHATE COLUMNS

For Derivative A, 4 ml of 100 000 × *g* rat liver supernatant were passed through a 5-ml column. The 53 units of tyrosine aminotransferase activity applied were quantitatively recovered in the eluate. For Derivative B, 250 ml of the 100 000 × *g* rat liver supernatant were passed through a 60-ml column over a 12-h period. Of 3850 total enzyme units applied, 3100 appeared in the eluate, and of the 750 enzyme units which appeared to have bound, 570 were eluted from the column with the same buffer used in Table III. 200 ml of the same homogenate were passed through a 21-ml column of Derivative C over a 12-h period. Of the 3080 enzyme units applied, 2120 appeared in the eluate, and of the 960 presumably bound units 730 were eluted from the column with the same buffer as used in Table IV. Calculation of moles of tyrosine aminotransferase is based on the highest reported specific activity of 500 enzyme units /mg and on a molecular weight of 115 000[2].

Derivative	*Enzyme bound to adsorbent*	
	units/ml of gel	*moles/mole of ligand*
Pyridoxamine-*P* (A)	0	0
Pyridoxamine-*P* Short arm (B)	9.5	$2.0 \cdot 10^{-5}$
Pyridoxamine-*P* Long arm (C)	35	$2.5 \cdot 10^{-4}$

activity recovered in the eluate from the total activity applied. In the case of Adsorbent A, no detectible enzyme activity was bound. Adsorbents B and C bound 9.5 and 35 enzyme units per ml of gel, respectively. If the results are expressed in terms of moles of enzyme bound per mole of adsorbent cofactor, Adsorbent C was 12 times more efficient than Adsorbent B in binding tyrosine aminotransferase.

As a practical matter, tyrosine aminotransferase will not bind to any of the adsorbents in the presence of free pyridoxal phosphate, nor will the enzyme bind if it has been carried through the "heat step"[1,2], in which the enzyme solution is incubated at 65 °C in the presence of pyridoxal phosphate and α-ketoglutarate, even if the enzyme solution is subsequently dialyzed. If the enzyme is resolved to the apoenzyme form by incubation with tyrosine followed by dialysis, binding is enhanced. It should be noted that tyrosine aminotransferase prepared from hepatoma tissue culture cells grown in a medium rich in pyridoxal, does not bind as well as rat liver tyrosine aminotransferase.

Elution

The composition of the buffer used to elute the adsorbent tyrosine aminotransferase from Adsorbent B or C was empirically determined. In general, the requirements for elution are high salt, pH extreme (either acidic or basic) and the presence of excess cofactor in the buffer (Table II). It is also necessary that the buffer contain

TABLE II

EFFECT OF BUFFER COMPOSITION ON ELUTION OF TYROSINE AMINOTRANSFERASE FROM AFFINITY COLUMNS

The additions listed were added to Tris–KCl–$MgCl_2$ buffer (pH 10), containing 0.03 M β-mercaptoethanol, 0.5 M NaCl and 0.01 M pyridoxal phosphate. In every case the amount of eluting buffer used was equal to the column volume. The enzyme elutes at the front of the buffer between pH 8.8 to 9.2. Recovery (%) was calculated by determining the amount of enzyme which appeared to bind (as in Table I) and dividing that into the amount recovered. This calculation assumes no loss of enzyme activity prior to elution.

Additions	*Recovery of enzyme activity (%)*
Albumin (2.5 mg/ml)	85–90
Sucrose (0.15 M) *plus* spermine (0.002 M)	60–75

albumin, or sucrose and spermine which presumably act as protective agents for the enzyme. In the case of Adsorbent C, NaCl, albumin and cofactor in Tris–KCl–$MgCl_2$ buffer at pH 10 will elute 90% of the bound enzyme. If albumin is replaced by sucrose and spermine, the recovery ranges between 60 and 75%. The only major difference between Adsorbents B and C is that for the former 80% of the bound enzyme is eluted with a buffer having a pH of 4, while Adsorbent C requires buffers of alkaline pH for successful elution.

An example of enzyme adsorption to and elution from Adsorbent C is shown in Fig. 2. For this experiment hepatoma tissue culture cells were grown in the presence of ^3H-labeled mixed amino acids and 10^{-6} M dexamethasone phosphate for 24 h (1 generation). An extract from these cells was passed through a column containing Adsorbent C. After applying the extract, the column was washed with Tris–KCl–$MgCl_2$

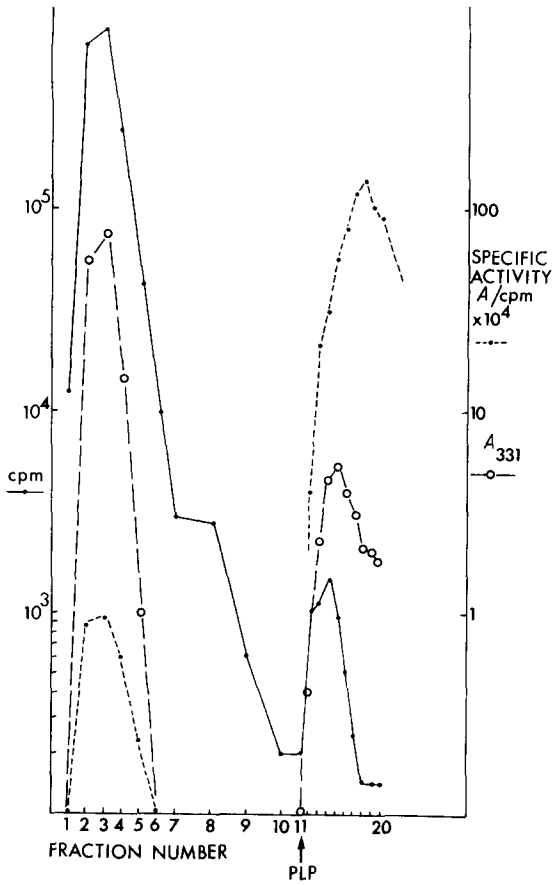

Fig. 2. Chromatographic profile of tyrosine aminotransferase. A 100 000 × *g* supernatant fraction was prepared from 1.3 l of cells at $8 \cdot 10^5$ cells/ml, grown for 20 h in the presence of 500 μl mixed ^{3}H-labeled amino acids and 10^{-6} M dexamethasone phosphate. 2 ml of this, containing 1.02 enzyme units of tyrosine aminotransferase and $1.66 \cdot 10^6$ cpm were passed trough a 2-ml column of Adsorbent C. The column was washed with 12 ml Tris–KCl–$MgCl_2$ buffer, eluted with 2 ml of the albumin containing elution mixture (Table III) and further washed with 4.5 ml Tris–KCl–$MgCl_2$ buffer. Fractions were collected (1–7, 1 ml, 8–10, 2 ml, and 11–20, 0.5 ml) and the total amount of enzyme activity (as $A_{331\ nm}$) and trichloroacetic acid-precipitable cpm were determined for each fraction. The specific activity plotted is $A_{331\ nm}$/cpm × 10^4 and the starting specific activity was 1.1. The cpm were determined by precipitating 0.05 ml of each fraction with 1 ml 10% trichloroacetic acid, dissolving the precipitate in 0.5 ml NCS and counting in a Packard Tri-Carb liquid scintillation counter with an efficiency for ^{3}H of 25%. PLP = pyridoxal 5′-phosphate.

buffer and eluted with the albumin elution mixture shown in Table III. The chromatographic profiles (Fig. 2) show total trichloroacetic acid-precipitable radioactivity, total enzyme activity, and enzyme specific activity (in arbitrary units) plotted against fraction number. In this case, it was necessary to use radioactivity as a measure of total protein since the elution was carried out in the presence of albumin. Except for a lower yield, the results are the same when albumin is replaced by sucrose and spermine and the protein is measured directly. As the enzyme in this preparation was not

resolved to the apoenzyme form some tyrosine aminotransferase does not bind. The bound tyrosine aminotransferase elutes in a relatively sharp band, the maximal specific activity of which is 125 times, and the average specific activity 100 times greater than that of the starting material. This can be improved to an average specific activity which is at least 125 times greater than the starting material by following the Tris–KCl–$MgCl_2$ buffer wash with a wash of 0.5 M KCl in Tris–KCl–$MgCl_2$ buffer (1 column volume) before eluting. This is the procedure now used routinely.

The nature of the other proteins which bind and are eluted with tyrosine aminotransferase has not been examined. It was originally supposed that other pyridoxal phosphate requiring enzymes would bind, but as discussed in another part of this paper, this may not be the case.

TABLE III

PURIFICATION OF RAT LIVER TYROSINE AMINOTRANSFERASE

Summary of enzyme purification scheme, details of which are given in the text.

Fraction	*Total tyrosine aminotransferase activity (units)*	*Specific activity (units/mg protein)*	*Purification (-fold)*
100 000 × *g* supernatant	2000	0.6	
Pyridoxamine-*P* Long arm (C)	1100	75	125
Sephadex G-200	500	400	650

Tyrosine aminotransferase purification

One of the enzyme purification schemes we have used is illustrated in Table III. Rat livers were prepared as described in Materials and Methods, and 300 ml of 100 000 × *g* supernatant fluid was passed (overnight) through a 25-ml column of Adsorbent C. The column was then washed with 100 ml Tris–KCl–$MgCl_2$ buffer, and eluted with 40 ml of the sucrose- and spermine-containing buffer (Table III) adjusted to pH 10. The eluted enzyme in a volume of 25 ml and having a pH of 9 was concentrated by pressure dialysis against Tris–KCl–$MgCl_2$ buffer to a volume of 1.5 ml. This was passed through a 1 cm × 40 cm Sephadex G-200 column equilibrated with 10^{-5} M pyridoxal 5′-phosphate and 10^{-3} M β-mercaptoethanol in Tris–KCl–$MgCl_2$ buffer. The enzyme activity emerged in a single peak. The peak fractions were concentrated by pressure dialysis against Tris–KCl–$MgCl_2$ buffer. The overall purification was 650-fold with a 25% yield and final specific activity of 400 units/mg. This compares favorably with the highest specific activity reported of 500 units/mg[2].

A number of modifications have been tried to further simplify this procedure. Two of the most useful have been the concentration of the enzyme in the peak fractions from both columns by $(NH_4)_2SO_4$ precipitation and the use of DEAE-cellulose chromatography[2] instead of Sephadex G-200. Both of these result in more rapid purification, with equivalent yields of enzyme of the same specific activity described in Table III.

Polyacrylamide gel electrophoresis of the product of the purification procedure has consistently shown several bands, despite the high specific activity (Fig. 3). The

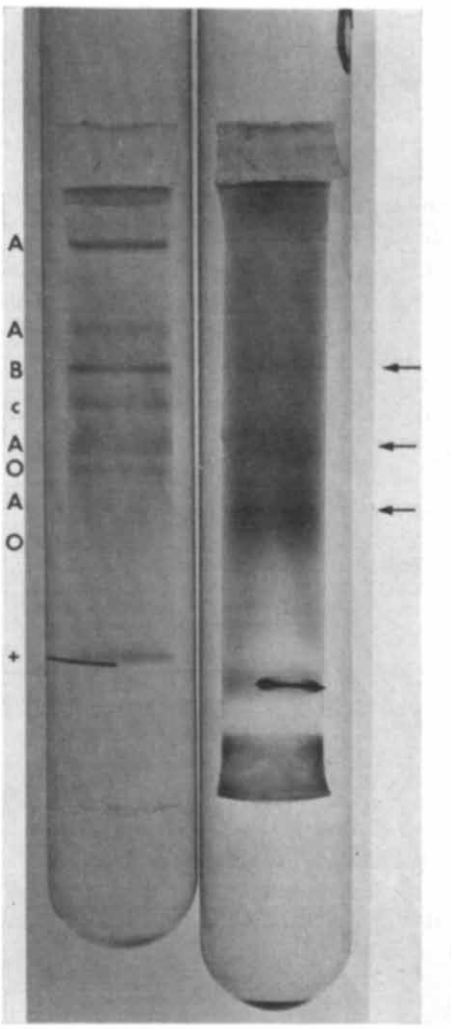

Fig. 3. Polyacrylamide gel electrophoresis of purified tyrosine aminotransferase. 6% gels which were run as described in Materials and Methods. 60 μg of protein were put in the left hand gel and it was stained for protein; 1 μg of protein was put on the right hand gel and this was stained for enzyme activity. The anode and the front are indicated by +. The three enzymatically active bands are indicated by arrows.

two faster moving A bands and the B band always show enzyme activity when analyzed by histochemical stain. The two slower moving A bands and the c band occasionally show activity. The two bands labeled o have never shown activity and are presumed to be contaminants or catalytically inactive subunits. Several different gel concentrations have been run and Ferguson plots[14] from these data have been obtained for each of the protein staining bands. On the basis of these preliminary data it is possible to speculate that the proteins represented by the A bands may be aggregates of the same enzyme (or subunits) with molecular weights of approx. 100 000, 150 000,

200 000 and 400 000. Band B is probably an isoenzyme with a molecular weight of about 100 000. Band c on the basis of occasional enzyme stain may also be an isozyme with a molecular weight of about 300 000. The two faster moving A bands and B are present in unfractionated rat liver.

DISCUSSION

Tyrosine aminotransferase can be purified at least 100-fold from a crude enzyme preparation in a single step by means of affinity chromatography. The operation is simple, provides a good yield, and can be accomplished in a short period of time. It is suitable for preparation of the enzyme on a large scale. It can also be used on very small amounts of enzyme as an adjunct to immunoprecipitation or polyacrylamide gel electrophoresis, where it is helpful to clean up the preparation prior to analysis. In addition, it is an advantage of this method of tyrosine aminotransferase purification which is apparently unique that several different forms or types of the enzyme are co-purified. Thus, it should be particularly useful in the study of hormone induction of isozymic forms of tyrosine aminotransferase.

Such affinity columns are also potentially useful in purifying other pyridoxal enzymes. Collier and Kohlhaw[15] for example, have been able to demonstrate binding of pig heart aspartate aminotransferase to pyridoxamine adsorbents. We have examined the chromatographic behavior of the bacterial apoenzymes, D-serine dehydratase, tryptophanase and arginine decarboxylase, which were gifts of Dr E. Snell. None of these enzymes bind under the same conditions used for tyrosine aminotransferase binding. We have also examined the binding of these enzymes in potassium phosphate buffer at the pH which is optimal for enzyme activity without achieving adsorption. Other variables unsuccessful in aiding adsorption were time of exposure to the gel (3 min–180 min) and temperature (5 °C, 25 °C and 37 °C). While it may be that the enzymes tested are unable to bind to these columns, it is quite possible that the conditions appropriate for their binding were not used.

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