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PURIFICATION OF BIOSYNTHETIC THREONINE DEAMINASE FROM *ESCHERICHIA COLI**

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Summary

Biosynthetic threonine deaminase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) was purified to apparent homogeneity from cell extracts of *Escherichia coli* by chromatographic procedures using valine-Sepharose, isoleucine-*N*-hexamethyleneamine-Sepharose, and hydroxyapatite with an overall yield of 40%. Analytical ultracentrifugation shows a molecular weight of 214 000. In sodium dodecyl sulfate gel electrophoresis, the enzyme migrates as a single band corresponding to a molecular weight of about 50 000. These data confirm that the enzyme is a tetramer. The sedimentation coefficient, $s^0_{20,w}$, determined by differential sedimentation experiments is 9.2 S. The enzyme shows absorption maxima at 415 and 280 nm. Determination of pyridoxal phosphate by three independent methods shows the presence of two molecules of pyridoxal phosphate per enzyme molecule, the different methods being in excellent agreement. Equilibrium dialysis experiments establish the presence of two isoleucine binding sites. The Scatchard plot suggests non-cooperativity of these sites. The association constant for isoleucine is $1.2 \cdot 10^5$ M^{-1}.

Introduction

Biosynthetic threonine deaminase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) is the first enzyme in the biosynthesis of isoleucine. The enzyme catalyzes the formation of 2-ketobutyrate from L-threonine. Studies of the allosteric properties [1] and of the role of the enzyme in the regulation of the

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Abbreviations: Ile-C_2-Sepharose, isoleucine-*N*-ethyleneamine-Sepharose; Ile-C_4-Sepharose, isoleucine-*N*-tetramethyleneamine-Sepharose; Ile-C_6-Sepharose, isoleucine-*N*-hexamethyleneamine-Sepharose.

biosynthesis of branched-chain amino acids [2,3] were hampered by difficulties in the preparation of sufficient quantities of highly purified protein. Purification of the enzyme from *Escherichia coli* by conventional methods involves laborious procedures with low yields [4].

This paper describes the purification of threonine deaminase by amino acid-Sepharose columns. This technique allows the purification of the enzyme in high yield by a simple three-step procedure. Preliminary reports of parts of this work have been published [5,6].

Experimental procedure

Materials

Amino acids (Merck), pyridoxal phosphate (Serva), alkyl diamines (Merck), Sepharose 4B (Pharmacia), L-[4,5(n)-^{3}H]isoleucine (Amersham-Buchler), hydroxyapatite (Biorad), and Dowex 1 (Carl Roth) were commercial products.

Preparation of Sepharose derivatives

α-Chloro-β-methylvaleric acid was prepared by treatment of DL-isoleucine with nitrous acid in a large excess of NaCl [7]. 10 g of α-chloro-β-methylvaleric acid were added to 40 g of ethylenediamine, tetramethylenediamine, or hexamethylenediamine at 40°C and the mixture was left for several days with stirring [8] under a seal of solid NaOH to protect it from Atmospheric CO_2. Subsequently 100 ml of water were added to the reaction mixture. The solution was applied to a column of Dowex 1-formate (5 × 10 cm). Unreacted alkylamine was eluted with water. Subsequently isoleucine-*N*-alkylamine was eluted using 1 M HCOOH. The solution was evaporated to dryness and the residual product was used without further purification.

Sepharose 4B was activated at pH 11 and 20°C by addition of 10 g of CNBr to 100 g (wet weight) of Sepharose [9]. The reaction was allowed to proceed for 10 min and the pH was maintained at 11 by addition of 6 M NaOH. The gel was washed with ice-cold 0.1 M $NaHCO_3$ (pH 9.7) and then mixed with an equal volume of 0.1 M $NaHCO_3$ containing 0.3 M valine or isoleucine or 0.1 M of the isoleucine-*N*-alkylamines, respectively. Coupling was allowed to proceed at pH 9.7 and 20°C for 24 h. Subsequently the Sepharose was washed with water and then stirred with an equal volume of a solution of 1 M ethanolamine pH 8 for 2 h.

Culture medium

The culture medium contained per liter: 0.2 g $MgSO_4$, 2 g citric acid, 2 g $Na_2HPO_4 \cdot 12H_2O$, 5 g KH_2PO_4, 2 g $NH_4H_2PO_4$, 3.4 g KOH and 2 g glucose.

Growth of cells

Derepressed cells of the leucine-requiring strain *E. coli* B L 3 were obtained by growth with limiting concentrations of L-leucine [5,6]. The mutant was inoculated into 10 l of culture medium supplemented with 20 mg of L-leucine per l and incubated for 8 h at 37°C. The cell suspension was transferred to a fermenter containing 500 l of culture medium supplemented with 4.5 mg

L-leucine per l. The cells were grown for 16 h, harvested by centrifugation and stored at −20° C.

Preparation of cell extract

Cells (300 g) were thawed at room temperature and sonically treated in 450 ml of 1 M potassium phosphate, pH 8, containing 10^{-3} M each of isoleucine, EDTA and mercaptoethanol. The suspension was centrifuged for 30 min at 10 000 × *g* and 4° C. The supernatant was decanted and the sediment again sonically treated with 1 vol. of 1 M phosphate buffer. The suspension was centrifuged and the supernatants of both centrifugations were combined and dialyzed against 1 M potassium phosphate, pH 8.

Assay of threonine deaminase

The enzyme activity was measured by the procedure of Umbarger and Brown [10]. The reaction mixture contained 0.2 M potassium phosphate and 0.025 M threonine. The reaction was started by the addition of enzyme and terminated after 15 min at 37° C by the addition of 0.2 ml of 2,4-dinitrophenylhydrazine reagent [11]. 2.0 ml of 2.5 N NaOH were added after 5 min and the absorbance was measured at 440 nm. 1 unit of enzyme activity forms 1 μmol of product per min.

Protein determination

Protein concentrations were determined by the biuret method with bovine serum albumin as a standard. Protein concentrations of pure preparations were determined spectrophotometrically at 280 nm.

The extinction coefficient of threonine deaminase was determined on a refractometric basis [12,13] in a Spinco model E ultracentrifuge. Runs were performed with a filled Epon capillary-type synthetic boundary cell at a rotor speed of 8000 rev./min. A refractive increment of 4.1 fringes for 0.1% protein solutions was assumed in the calculations.

Analytical ultracentrifugation

A Spinco model E ultracentrifuge equipped with Schlieren optics and electronic temperature control was used for sedimentation velocity experiments. Runs were performed at 20° C using an An H rotor and standard single or double sector centerpieces. Rotor speed was determined for each run using a stopwatch.

Differential sedimentation experiments [14] were performed with a capillary type synthetic boundary cell. 0.24 ml of a 0.7% protein solution was added to the sectoral cavity and the cup was filled with 0.24 ml of a 0.35% solution.

Molecular weights were determined by the meniscus depletion sedimentation equilibrium method [15] using interference optics and a six-channel centerpiece.

Pyridoxal phosphate content of the enzyme

The pyridoxal phosphate content of threonine deaminase was determined by three separate methods.

Microbiological determination. Enzyme samples and solutions of pyridoxal phosphate (as standards) were hydrolyzed in 0.5 M H_2SO_4 for 2 h at 121°C. Then the solutions were adjusted to pH 7 with 2.5 M NaOH. Appropriate samples were assayed for vitamin B-6 with *Saccharomyces carlsbergensis* ATCC 9080 [16].

Phenylhydrazone of pyridoxal phosphate [17]. 0.03 ml of concentrated H_2SO_4 was added to 0.2 ml of enzyme solution containing 0.3—0.6 mg of protein. The samples were centrifuged and 0.02 ml of phenylhydrazine reagent [17] was added to 0.18 ml of the supernatant. After 10 min at room temperature, the absorbance was determined at 410 nm. Pyridoxal phosphate was used as standard.

Absorbance in 0.3 M $HClO_4$ [18]. To a sample of threonine deaminase (0.5—1.2 mg) in 0.15 ml of 0.01 M potassium phosphate (pH 7.4), 0.07 ml of 1 M $HClO_4$ was added. The mixture was allowed to stand at room temperature for 15 min. Precipitated protein was removed by centrifugation. The supernatant (0.2 ml) was analyzed for pyridoxal phosphate by measuring the absorbance at 295 nm. Pyridoxal phosphate was computed from a standard curve.

Equilibrium dialysis

Experiments were carried out in 0.25 ml cells (Dianorm, Innovativ-Medizin, Zürich) at 4°C, using Visking 20/32 tubing. 0.2 ml of solution was poured into each compartment. Equilibrium was attained in 2 h. When equilibrium was reached, the ligand concentration was determined by liquid scintillation counting in aliquots from each cell compartment. Samples of 0.01 ml were dissolved in 10 ml of scintillation cocktail (667 ml toluene, 333 ml Triton X-100, 5.5 g 2,5-diphenyloxazole, 0.1 g 1,4-bis-2-(5-phenyloxazolyl)-benzene [19]. Radioactivity was determined in a liquid scintillation counter ABAC SL 40 (Intertechnique, Paris).

Results

Elution patterns of E. coli cell extracts on different Sepharose derivatives

All buffers contained 10^{-3} M each of isoleucine, EDTA and mercaptoethanol.

Valine-Sepharose. 540 ml of crude extract were passed through a column of valine-Sepharose (diameter, 5 cm; height, 3 cm) (Fig. 1). The volume of crude extract applied was selected to saturate completely the binding capacity

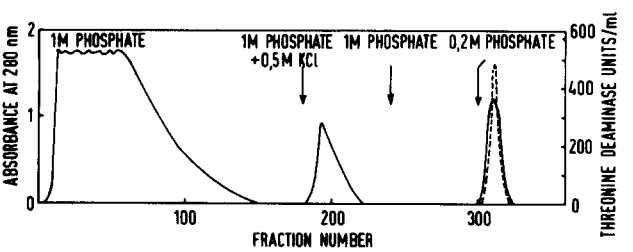


Fig. 1. Chromatography of cell extract of *E. coli* B L 3 on valine-Sepharose. Fractions of 12 ml were collected. For details see Results. ———, protein; - - - - -, threonine deaminase activity.

(10 500 units) of the column for threonine deaminase (1 ml of valine-Sepharose binds about 200 units of the enzyme). The column was washed with 1000 ml of 1 M potassium phosphate pH 8, 1000 ml of 1 M potassium phosphate pH 8 + 0.5 M KCl, and finally 500 ml of 1 M potassium phosphate pH 8. Subsequently threonine deaminase was eluted with 0.2 M potassium phosphate pH 8. Purification was 10-fold (Table I).

Isoleucine-N-alkyl-Sepharoses. Several Sepharose derivatives were prepared which contained isoleucine bound directly to the Sepharose matrix or with an interposed alkyl arm with 2, 4 or 6 C-atoms.

5 ml samples of enzyme solution (150 units) prepared by valine-Sepharose chromatography (see above) were dialyzed against 0.2 M potassium phosphate and applied to columns (1.1 × 10 cm) of each of the isoleucine-Sepharoses (Fig. 2). Threonine deaminase is retarded with respect to the bulk of protein by

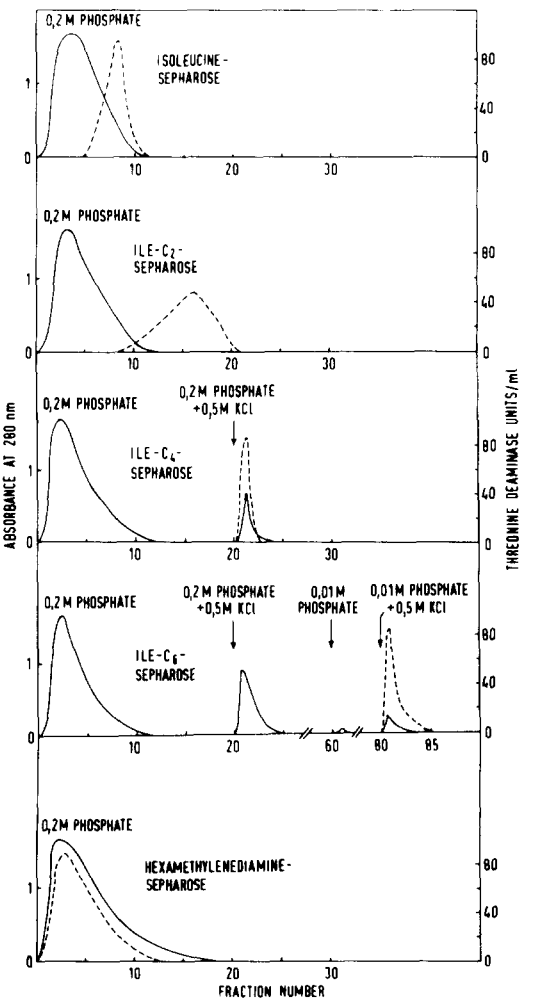


Fig. 2. Chromatography of cell extract of *E. coli* B L 3 on different isoleucine-Sepharose derivatives. Fractions of 4 ml were collected. For details see Results. ———, protein; - - - - -, threonine deaminase activity.

isoleucine-Sepharose in 0.2 M potassium phosphate (Fig. 2). Retardation is more pronounced with Ile-C_2-Sepharose, wherease Ile-C_4- and Ile-C_6-Sepharose retain the enzyme completely under the experimental conditions (Fig. 2).

From Ile-C_6-Sepharose, threonine deaminase activity was recovered as follows: 0.2 M phosphate + 0.5 M KCl elutes the bulk of bound protein, but no threonine deaminase activity. Threonine deaminase is subsequently eluted by 0.01 M potassium phosphate + 0.5 M KCL (Fig. 2). This procedure results in some 20-fold purification of the enzyme.

The same type of experiment was performed with hexamethylenediamine-Sepharose without the ligand isoleucine. Threonine deaminase is not bound by this adsorbent under the experimental conditions (Fig. 2).

Purification of the enzyme

Buffers contained 10^{-3} M each of isoleucine, EDTA and mercaptoethanol, and 10^{-4} M pyridoxal phosphate unless otherwise stated. Table I shows a typical experiment.

Valine-Sepharose chromatography. Dialyzed cell extract (540 ml) was applied to a column of valine-Sepharose (diameter, 5 cm; height, 3 cm). Elution was as described above.

Ile-C_6-Sepharose. Active fractions from valine-Sepharose were pooled and applied to a column (2.5 × 6 cm) of Ile-C_6-Sepharose. The column was washed with 300 ml of 0.2 M potassium phosphate pH 8, 500 ml of 0.2 M potassium phosphate pH 8 + 0.5 M KCl, and 200 ml of 0.01 M potassium phosphate pH 7.4. Threonine deaminase was subsequently eluted by 0.01 M potassium phosphate pH 7.4 + 0.5 M KCl. Fractions were combined and concentrated by ultrafiltration using type PM 30 or PM 10 membranes (Amicon). The solution was dialyzed against 0.01 M potassium phosphate pH 7.5.

Hydroxyapatite chromatography. The enzyme solution was applied to a column of hydroxyapatite (2 × 5 cm) which was equilibrated and developed with 0.01 M potassium phosphate pH 7.4. EDTA was omitted from buffer in this step. Threonine deaminase is not retained by hydroxyapatite. Active fractions were pooled and concentrated. The enzyme was stored at −20° C. 50% loss of enzyme activity was observed after 3 weeks.

Analytical gel electrophoresis

Threonine deaminase showed one band after polyacrylamide gel electrophoresis in sodium dodecyl sulfate gel [20] corresponding to a molecular

TABLE I

PURIFICATION OF THREONINE DEAMINASE

Fraction	Volume (ml)	Enzyme (units)	Protein (mg)	Specific activity (units/mg protein)	Yield (%)
Cell extract	540	10530	9200	1.1	100
Valine-Sepharose	252	7560	705	10.7	72
Ile-C_6-Sepharose	13.1*	5150	33	155	54
Hydroxyapatite	9.1*	3650	16	230	38

* Volume after concentration.

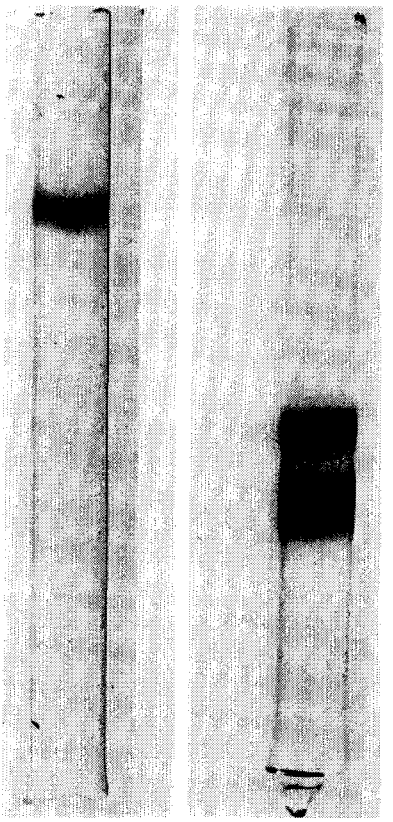


Fig. 3. Polyacrylamide gel electrophoresis of purified threonine deaminase. Left, sodium dodecyl sulfate gel; right, standard gel. Migration was downward.

weight of about 50 000 (Fig. 3). Gel electrophoresis in the standard system at pH 9.5 [21] showed two protein bands (Fig. 3) which both exhibited catalytic activity as shown by dissection of gels followed by enzyme activity determination. This may be a consequence of dissociation of the enzyme into dimers at alkaline pH [4].

Analytical ultracentrifugation

In the analytical ultracentrifuge, the enzyme migrated as a single, symmetrical boundary (Fig. 4). Differential sedimentation experiments [14] yielded a sedimentation coefficient, at infinite dilution ($s^0_{20,w}$), of 9.2 S and a concentration dependence of the sedimentation coefficient with k = 0.009 l/g. This k value is in the range found for other globular proteins [22].

The molecular weight of threonine deaminase was determined by meniscus depletion sedimentation equilibrium [15] at 5° C. Measurements at rotor speeds of 13 000 and 14 000 rev./min gave linear plots of log c versus r^2 corresponding to molecular weights shown in Table II. A partial specific volume of 0.74 was used in the calculation of molecular weights. From the results, we conclude that the molecular weight of threonine deaminase is close to

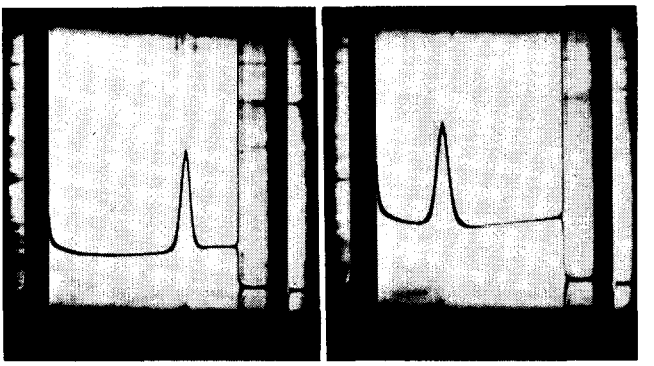


Fig. 4. Sedimentation pattern of threonine deaminase. A 0.9% solution of the enzyme (spec. act., 230 units/mg) in 0.1 M potassium phosphate pH 7.4 was centrifuged in a single sector cell at 20° C. Photographs were taken at a bar angle of 70° (left) and 60° (right) 24 min and 56 min after attaining a rotor speed of 61 500 rev./min. Sedimentation is from right to left.

214 000 in reasonable agreement with the value (204 000) reported by Calhoun and co-workers [4].

Absorption spectrum of purified threonine deaminase

The purified enzyme exhibits a marked yellow colour. The absorption spectrum in 0.1 M potassium phosphate, pH 7.4, shows a maximum at 415 nm characteristic for pyridoxal phosphate-dependent enzymes. Dialysis of threonine deaminase against 0.05 M Tris · HCl, pH 8.5, resolves the enzyme from its coenzyme [4] with concomitant disappearance of the absorption maximum at 415 nm (Fig. 5).

On the basis of refractometric protein determinations in the analytical ultracentrifuge [12,13], the absorbance of a 0.1% solution of threonine deaminase in 0.1 M potassium phosphate, pH 7.4, at 280 nm is 0.84 (light path, 1 cm).

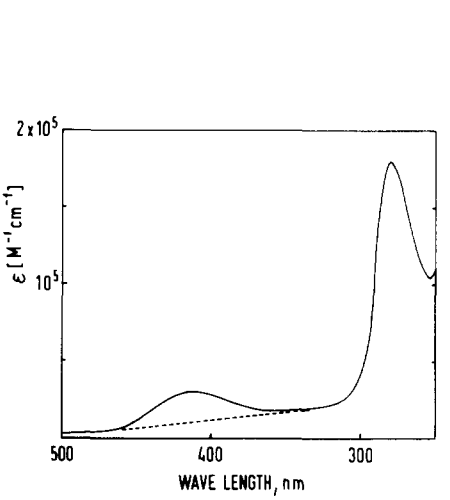
Pyridoxal phosphate content of threonine deaminase

The pyridoxal phosphate content of the enzyme was determined by three different procedures as described under Methods. In close agreement the data show the presence of two molecules of pyridoxal phosphate per molecule of threonine deaminase (Table III). The same value was reported for threonine deaminase of *Salmonella typhimurium* [23]. In contrast, degradative threonine deaminase of *E. coli* was shown to have four active sites per tetramer [24].

TABLE II

MOLECULAR WEIGHT OF THREONINE DEAMINASE

Initial protein concentration (mg/ml)	Apparent molecular weight	
	13000 rev./min	14000 rev./min
0.8	215500	213700
0.6	213200	212900
0.48	212600	



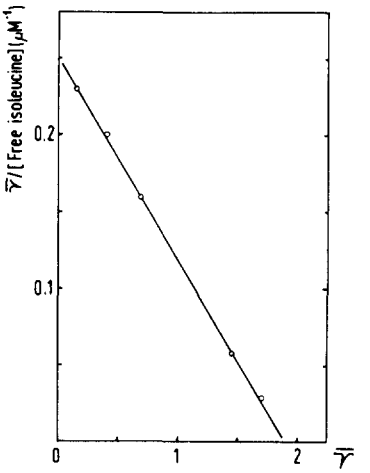


Fig. 5.. Absorption spectrum of threonine deaminase. Native enzyme in 0.1 M potassium phosphate pH 7.4 with 10^{-3} M isoleucine, EDTA and mercaptoethanol (———); apoenzyme after dialysis against 0.05 M Tris · HCl, pH 8.5 (- - - - - -).

Fig. 6. Scatchard plot of L-[^{3}H]isoleucine binding to threonine deaminase (equilibrium dialysis at 4° C; protein concentration, 0.2%). γ is the number of mol of isoleucine bound per mol of threonine deaminase.

Equilibrium dialysis

Binding of the feedback inhibitor of biosynthetic threonine deaminase, isoleucine, to the purified enzyme was studied by equilibrium dialysis with L-[^{3}H]isoleucine. The experiments were performed at 4° C in 0.1 M potassium phosphate pH 7.4. Enzyme concentrations were 0.2—0.3%. Fig. 6 shows a Scatchard plot indicating two isoleucine binding sites per threonine deaminase tetramer. The association constant is $1.2 \cdot 10^5$ M^{-1}. The linearity of the Scatchard plot indicates non-cooperative behavior of the isoleucine binding sites.

TABLE III

DETERMINATION OF PYRIDOXAL PHOSPHATE

Method	Mol of pyridoxal phosphate per mol of threonine deaminase
Microbiological (*Saccharomyces carlsbergensis*)	1.8 2.0
Absorbance of the phenylhydrazone of pyridoxal phosphate	2.0 2.1
Absorbance of pyridoxal phosphate in 0.3 M $HClO_4$ (295 nm)	2.0 2.1

Discussion

Purification of threonine deaminase by conventional methods involves laborious procedures with low yields [4]. The utilization of amino acid-Sepharose columns provides a simple three-step procedure yielding disc-electrophoretically pure enzyme with an overall yield of 40%.

We have shown previously that chromatography of threonine deaminase on valine-Sepharose using high phosphate concentrations is not due to specific interactions of the bound amino acid with threonine deaminase. Indeed we have found that separation of threonine deaminase from the bulk of protein can be also achieved by glycine-Sepharose, although glycine has presumably no specific affinity for threonine deaminase [5]. Thus it is not astonishing that chromatography on valine-Sepharose using descending gradients of phosphate buffer can be used for the purification of proteins without any specific affinity to valine as emphasized recently by Rimerman and Hatfield [25]. The observed adsorption effects with valine-Sepharose may be due to ion exchange and/or hydrophobic effects.

The affinity of threonine deaminase to Ile-C_6-Sepharose depends from the ionic environment in a rather complex manner. The enzyme binds tightly to the column in phosphate buffer from 0.01 M to 1.0 M, whereas it is rapidly eluted by 0.5 M KCl.

Thus binding is not only a function of the ionic strength but specific interactions with the buffer ions must be considered. Detailed studies of this phenomenon are presently being undertaken. From the present evidence we conclude that the binding of threonine deaminase to Ile-C_6-Sepharose is due to specific interaction with the Sepharose-bound isoleucine.

In the tetrameric state, threonine deaminase of *E. coli*, as the *Salmonella typhimurium* enzyme [23,26] binds two molecules of pyridoxal phosphate and isoleucine, respectively. Present evidence favours the concept that the four subunits of the enzyme are identical. On this basis threonine deaminase appears to be a further example of 'half-of-the-sites reactivity'; this phenomenon may be explained (1) by negative cooperativity ('induced fit' hypothesis) or (2) by a pre-existent non-equivalence among the subunits [27].

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