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BRANCHED-CHAIN AMINO-ACID AMINOTRANSFERASE OF *SALMONELLA TYPHIMURIUM*

I. CRYSTALLIZATION AND PRELIMINARY CHARACTERIZATION*

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SUMMARY

1. A protocol for the purification of transaminase B (branched-chain amino-acid:2-oxoglutarate aminotransferase, EC 2.6.1.6) from *Salmonella typhimurium* that includes the following steps: (a) heat treatment; (b) ammonium sulfate fractionation; and (c) DEAE-cellulose column chromatography is presented.

2. Crystallization of the enzyme is accomplished by an ammonium sulfate treatment of the purified preparation.

3. Results of disc gel electrophoresis provide evidence that the crystalline enzyme is highly purified and free of any significant contamination.

4. Basic enzymatic and spectral characteristics of the crystalline enzyme are presented.

INTRODUCTION

Transaminase B (L-leucine:2-oxoglutarate aminotransferase, EC 2.6.1.6) is the trivial name for the aminotransferase that catalyzes the terminal reaction in the biosynthesis of the branched-chain amino acids (isoleucine, valine and leucine) in several bacteria[1–4]. In studies on the biosynthesis of isoleucine and valine in *Salmonella typhimurium*, a procedure for the purification and crystallization of transaminase B from this organism was developed; and the details of this protocol, as well as a partial characterization of the enzyme, are presented.

EXPERIMENTAL METHODS

Materials

Commercial products utilized in the enzymatic assays include α-ketoglutarate, L-isoleucine, L-valine, L-leucine (General Biochemicals); pyridoxal, pyridoxal phos-

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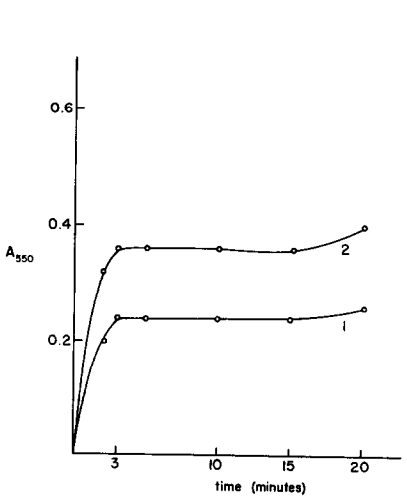
phate (Nutritional Biochemicals Corporation); pyridoxamine, pyridoxamine phosphate, pyridoxine (Sigma Chemical Co.); and 2,4-dinitrophenylhydrazine (Matheson, Coleman, and Bell). Ammonium sulfate, special enzyme grade, was purchased from Mann Research Laboratories, and diethylaminoethyl cellulose (DEAE-cellulose), medium mesh with a capacity of 1 mequiv/g, from Sigma Chemical Co. Collodion bags were obtained from Carl Schleicher and Schuell Co.

Assay

The enzymatic assay is similar to that of ICHIHARA AND KOYAMA[5]. The 3-ml incubation mixture is composed of amino donor (isoleucine, valine, or leucine) and acceptor (α-ketoglutarate) present in 13 mM concentrations, pyridoxal phosphate (0.27 mM), enzyme and 0.2 M potassium phosphate buffer (pH 7.8). The assay mixture is preincubated at 37° for 10 min before addition of enzyme. The reaction is then allowed to proceed for 10 min at 37° before it is terminated by the addition of 0.2 ml of the assay mixture to 3 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl. On addition of enzyme to the reaction mixtures that serve as blanks, 0.2 ml of the mixture is immediately added to dinitrophenylhydrazine solution. After a period of 10 min, the hydrazones of the branched-chain keto acids are separated from the hydrazone of α-ketoglutarate by extraction with 6 ml of toluene. 4 ml of the toluene layer are removed to another test tube and extracted with 4 ml of 10% Na_2CO_3 solution. 3 ml of the Na_2CO_3 layer are then removed and mixed with 0.5 ml of 20% KOH. The absorbance of this solution is measured at 550 nm (Bausch and Lomb Spectronic 20). Standard curves were prepared with known amounts of the keto acids. Specific activity is expressed as μmoles of keto acid formed per h per mg of protein.



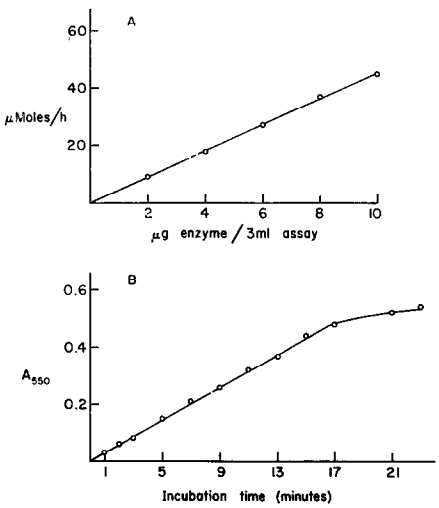


Fig. 1. Results of a time study on the formation of the hydrazone derivative of the α-keto acid analogue of isoleucine. 1, 3 μM α-keto-isoleucine in the presence of 20 mM α-ketoglutarate; 2, 5 μM α-keto-isoleucine and 20 mM α-ketoglutarate.

Fig. 2. (A) Activity with increasing amounts of enzyme in the assay. (B) Product formed in assays incubated for different time intervals.

The assay is based on the selective and time dependent extraction into toluene of monocarboxylic keto acid hydrazones from those of dicarboxylic keto acids, as described by FRIEDEMANN AND HAUGEN[6]. Studies on the assay have shown that at the concentration of α-ketoglutarate (13 mM) routinely used, a slight deviation in concentration has no effect on the colorimetric response. Neither the extraction of μmolar amounts of the hydrazone derivatives of the α-keto acid analogues of isoleucine, valine, or leucine nor the sensitivity of the assay is affected by the amount of α-ketoglutarate used in the assay. With regard to the length of time required for the formation of the monocarboxylic keto acid hydrazone, 3 min are sufficient (Fig. 1); no increase in colorimetric response is noted if hydrazone formation is allowed to continue for an additional 12 min. After 15 min an increase in absorbance is noted; therefore, a maximum of 10 min is allowed for hydrazone formation in the assay procedure. The assay produces linear results with respect to enzyme concentration (Fig. 2A) and time (Fig. 2B). At $A_{550\,nm}$ values of 0.5 or greater, however, a linear response is no longer obtained (Fig. 2B). Protein was determined by the method of LOWRY *et al.*[7]. Crystalline bovine serum albumin served as the standard.

Disc gel electrophoresis

Acrylamide gel electrophoresis is performed by the method of Ornstein and Davis, as described by DAVIS[8]. Protein samples are layered over the stacking gel in a 10% sucrose solution, and staining is carried out as described by CHRAMBACH *et al.*[9]. The apparatus used was constructed in this laboratory. The pH systems described by DAVIS[8], WILLIAMS AND REISFELD[10], and TABER AND SHERMAN[11] are used.

Growth of cells

An isoleucine–valine requiring strain *(ilvD6)* of *S. typhimurium*, deficient in dihydroxyacid dehydratase activity, is grown under conditions that favor derepression of the enzymes required for the synthesis of isoleucine and valine[12]. Cell-free extracts prepared from these cells contain 4–7-fold the transaminase B activity observed in extracts of wild-type *S. typhimurium* (Table I). None of the available evidence indicates a difference between the wild-type and derepressed enzyme. Such evidence includes: (1) relative activities for amino donors; (2) electrophoretic migration patterns at various pH values; (3) purification characteristics; and (4) elution profiles obtained from Sephadex G-200 columns. Also included in Table I are the results that show no activity in cell-free extracts prepared from two transaminase B

TABLE I

TRANSAMINATION BETWEEN α-KETOGLUTARATE AND INDIVIDUAL BRANCHED-CHAIN AMINO ACIDS BY CELL-FREE EXTRACTS OF *S. typhimurium* WILD-TYPE AND *ilv* STRAINS

Strain	*Specific activity (μmoles/h per mg) Amino donors*			*Activity relative to isoleucine*	
	Valine	*Isoleucine*	*Leucine*	*Valine*	*Leucine*
Wild type (LT2)	5	9	10	0.6	1.1
ilvD6 (derepressed)	30	49	54	0.6	1.1
ilvE13 (derepressed)	0	0	0		
ilvE16 (derepressed)	0	0	0		

deficient strains *(ilvE13* and *16)*. Transamination between α-ketoglutarate and leucine was reported by RUDMAN AND MEISTER[1] in transaminase B deficient cells; however, under the conditions of our assay, this particular activity is not observed.

Cells are grown at 37° on a rotary shaker in 2-l erlenmeyer flasks that contain 1.3 l minimal medium[13] supplemented with $0.7 \cdot 10^{-4}$ M L-isoleucine, $0.42 \cdot 10^{-3}$ M L-valine and $0.38 \cdot 10^{-3}$ M L-leucine. After 25 h of growth the cells are harvested by centrifugation at 6000 × *g* for 20 min. The pellet is resuspended in 0.1 M potassium phosphate buffer (pH 7.4) and the centrifugation process is repeated. The packed cells are weighed, then stored in a freezer. Cells can be stored for periods up to 3 months without detectable loss of transaminase B activity.

Preparation of cell-free extract

Sonic disruption (Branson cell disruptor) is used to prepare cell-free extracts. Frozen cells are suspended in 0.1 M potassium phosphate buffer (pH 6.5; 3 ml/g cells); and the slurry, in 35-ml portions, is subjected to 3.5 min of sonic oscillation. The resultant suspension is centrifuged for 10 min at 20 000 × *g*, and the supernatant fraction obtained serves as the cell-free extract.

All fractionation procedures, unless otherwise specified, are carried out in the cold (1–5°), and the enzyme preparations are kept at similar temperatures. Potassium phosphate is the buffer referred to in the purification procedures. A Sorvall RC-2 refrigerated centrifuge is used for all centrifugations.

RESULTS AND DISCUSSION

Table II records the degree of purification achieved by the fractionation procedures described below for a representative cell-free extract prepared from 40 g of cells.

Heat treatment

15 min prior to treatment, the cell-free extract is made 2 mM with respect to α-ketoglutarate. It is critical for the following heat treatments that glass tubes are used. The extract is divided into 20-ml portions which are placed in a 60° water bath for 5 min, then chilled in ice for 15 min before centrifugation for 15 min at

TABLE II

PURIFICATION AND CRYSTALLIZATION OF TRANSAMINASE B FROM SALMONELLA

For the enzymatic data presented, α-ketoglutarate served as the amino acceptor and L-isoleucine as the amino donor. Specific activity expressed as μmoles/mg per h.

Fraction	*Protein*		*Activity*		*Purification*		*% Recovery*	
	mg/ml	*Total mg*	*Specific activity*	*Total activity*	*Per step*	*Overall*	*Per step*	*Overall*
Cell-free extract	32.3	4 716	31	146 196				100.00
Heat treatment	6.9	697	173	120 581	5.6	5.6	82.4	82.4
$(NH_4)_2SO_4$	4.0	160	730	116 800	4.2	23.5	96.8	79.8
DEAE-cellulose	0.31	36.6	2 228	81 538	3.1	71.9	69.8	55.8
Crystals	1.44	7.2	4 533	32 638	2.0	146.2	40.0	22.3
Recrystallized	1.83	3.8	4 633	17 605			53.9	

35 000 × *g*. The supernatant fraction is subjected to a second heat treatment (71° for 5 min) and the subsequent centrifugation procedure. The resultant supernatant fraction, labeled "Heat treatment" in Table II, routinely contains 80–85% of the original activity and represents a 5–6-fold purification.

Ammonium sulfate fractionations

The precipitations are carried out with a saturated ammonium sulfate solution prepared at 23° and then adjusted to pH 7.2 with NaOH. The amounts of ammonium sulfate added are specified for 23° and are not corrected for temperature.

The ammonium sulfate solution is added slowly to the supernatant fraction obtained from the previous step until 38% saturation is reached. After 30 min of stirring, the mixture is centrifuged for 10 min at 20 000 × *g*. The supernatant fraction is then brought to 50% saturation, and the stirring and centrifugation procedures are repeated. The pellet is redissolved in 40 ml 0.05 M buffer (pH 7.8). The fraction labeled "$(NH_4)_2SO_4$" in Table II, routinely contains 75–85% of the original activity and represents an overall purification of 23–27-fold.

DEAE-cellulose column

In a 100-ml buret, a 2-g DEAE-cellulose column is prepared with 0.05 M buffer (pH 7.8). The ammonium sulfate fraction is added to the column which is then washed with 20 ml of the above mentioned buffer. The column is eluted in a stepwise fashion with increasing concentrations of NaCl. The following eluents, prepared in 0.01 M buffer (pH 7.8) are used: 0.1 M NaCl (20 ml); 0.2 M NaCl (20 ml); 0.23 M NaCl (40 ml); 0.26 M NaCl (40 ml) and 0.3 M NaCl (20 ml). 20 ml fractions are collected. A major part of the transaminase B activity is eluted in the second 20-ml portion of the 0.26 M eluent (listed as "DEAE-cellulose" in Table II). This fraction routinely contains 50–60% of the original activity and represents an overall purification of 70–80-fold.

Crystallization of the enzyme

The DEAE-cellulose fraction is concentrated in collodion bags under reduced pressure to a volume that contains a protein concentration of approx. 10 mg/ml. The concentration procedure is repeated twice, each time with a 10-ml addition of 0.01 M buffer (pH 7.8) (to reduce the concentration of NaCl in the sample). The concentrate is next adjusted with the same buffer to a protein concentration of 7.5 mg per ml, transferred to a small centrifuge tube to which solid ammonium sulfate is then slowly added until 35% saturation is achieved. The tube containing the clear solution is set in a beaker of ice which is then placed in a refrigerator. After two days in the cold, crystallization commences, as judged by the silky and opalescent appearance of the solution. By the third day, the tube contains a heavy, yellow precipitate containing transaminase B crystals. The crystals are harvested by centrifugation (10 min at 12 000 × *g*), then resuspended in an equal volume of 0.01 M buffer (pH 7.8), containing ammonium sulfate at 35% saturation. These crystals ("Crystals" in Table II) represent 22–26% of original activity and an overall purification of 140–150-fold. If compared to the activity regularly observed in wild-type extracts, it would represent a 550–600-fold purification.

The yield of crystals can be increased if the crystallization process is allowed

to continue for longer lengths of time, *e.g.* three weeks in the refrigerator results in a crop of crystals that contains 60–70% of the DEAE-cellulose activity. However, if the crystals are harvested after three days, the supernatant fraction can be returned to the refrigerator, and after one and one-half weeks a second crop of crystals can be harvested that contains 40–50% of the DEAE-cellulose activity. This second yield possesses the same specific activity as the first. Thus, by harvesting twice, the time required to obtain a majority of the enzyme as crystals is significantly shortened.

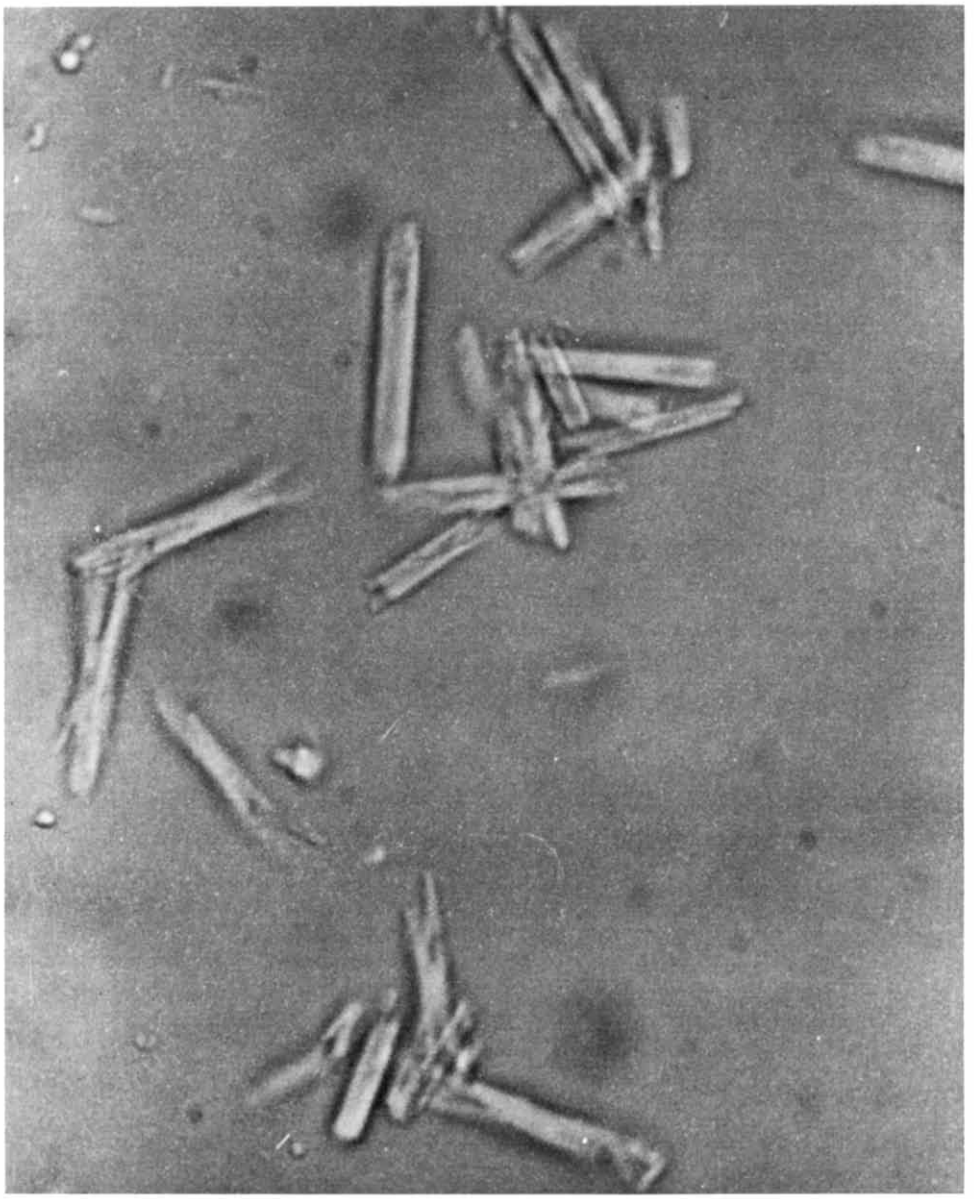


Fig. 3. Transaminase B crystals photographed at 1200 × magnification.

Second crystallization

To examine the purity of the crystals obtained, they are subjected to the following recrystallization procedure. The preparation of crystals is centrifuged for 10 min at 12 000 × *g*; the supernatant fraction is decanted, and the crystals are redissolved in 0.01 M buffer (pH 7.8) (one-half the original volume). The crystallization procedure, as described above, is repeated. As seen in Table II, no significant difference is noted between the specific activity of the original crystals and the recrystallized preparation. As for the first crystallization, increased lengths of time in the cold increase the yield. Fig. 3 is a photograph of the crystals obtained after recrystallization.

The results selected for Table II are those obtained with isoleucine as the amino donor and α-ketoglutarate as the amino acceptor. Comparable assays, using

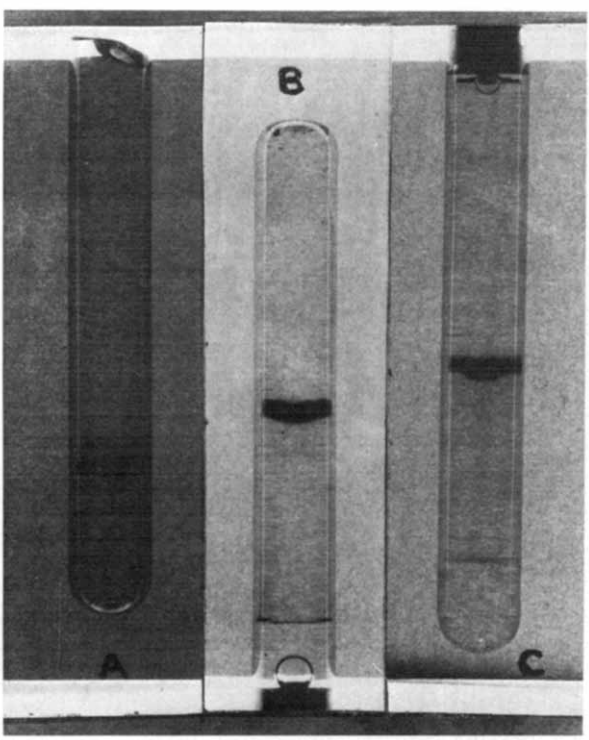


Fig. 4. Photographs of disc gels stained with Coomassie blue after electrophoresis of crystalline preparation of transaminase B at the following pH values: (A) 6.6; (B) 7.5; (C) 9.5. 30 μg of protein were applied to the gels. To present comparable photographs of the gels, it was necessary to invert Photograph B, which has the top of the gel at the bottom of the tube.

leucine and valine as the amino donors have been routinely included in the study, and the relative specific activities of 1:1.1:0.6 for isoleucine, leucine, and valine, respectively, remain constant throughout the purification and crystallization procedures.

The homogeneity of the crystalline preparation has been ascertained by the use of disc gel electrophoresis. As depicted in Fig. 4, a single band is obtained when

TABLE III

EFFECT OF DIFFERENT SUBSTRATES, OMISSIONS OR ADDITIONS ON ACTIVITY OF CRYSTALLINE ENZYME

The assays were carried out in 0.2 M Tris buffer (pH 7.8) as described in EXPERIMENTAL METHODS. EDTA (tetrasodium salt) and *o*-phenathroline were present at $1 \cdot 10^{-3}$ M concentrations; the cations at $1 \cdot 10^{-4}$ M. All additives were preincubated with enzyme for 4 h at 37° prior to assay.

NH_2 donor	*NH_2 acceptor*	*Coenzyme*	*Addition to assay*	*% Maximal activity*
I. Change in NH_2 donors:				
L-Isoleucine	α-Ketoglutarate	Pyridoxal phosphate	None	100
L-Leucine	α-Ketoglutarate	Pyridoxal phosphate	None	113
L-Valine	α-Ketoglutarate	Pyridoxal phosphate	None	64
II. Omissions from assay:				
L-Isoleucine	None	Pyridoxal phosphate	None	0
None	α-Ketoglutarate	Pyridoxal phosphate	None	0
L-Isoleucine	α-Ketoglutarate	None	None	70
III. Additions to assay:				
L-Isoleucine	α-Ketoglutarate	Pyridoxal phosphate	*o*-Phenathroline	100
L-Isoleucine	α-Ketoglutarate	Pyridoxal phosphate	EDTA	84
L-Isoleucine	α-Ketoglutarate	Pyridoxal phosphate	$CoCl_2$	90
L-Isoleucine	α-Ketoglutarate	Pyridoxal phosphate	$HgSO_4$	100

the enzyme is subjected to electrophoresis at three different pH values (6.6, 7.5, and 9.5). A range of protein concentrations (up to 400 μg) also yields a single band. Thus, as judged by this criterion, the procedures described above yield highly purified transaminase B that is free of any significant contaminants. More extensive studies on the apparent homogeneity of the crystalline preparation are currently in progress.

The results presented in Table III show that the relative activities of the three branched-chain amino acids when assayed with the crystalline enzyme are the same as those observed with cell-free extracts (Table I). If either the amino donor or acceptor is absent, no activity is observed; however, lack of the coenzyme (pyridoxal phosphate) does not result in a marked loss of activity. This latter result is interpreted to mean that the coenzyme is tightly bound to the enzyme. The presence of the chelating agent, *o*-phenathroline, has no effect on the activity; and only a slight inhibition is noted when EDTA is added to the assay. Thus, no involvement of a metal ion is indicated. Of a number of cationic species tested, only Co^{2+} produces an effect on activity, and it is doubtful if the 10% inhibition observed after a 4-h incubation period with the enzyme is significant. Cations found to exert no effect on the activity include Mg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Ni^{2+}, Be^{2+}, Cu^{2+} and Hg^{2+}. The lack of an effect by $1 \cdot 10^{-4}$ M Hg^{2+}, as well as by $1 \cdot 10^{-3}$ M *p*-hydroxymercuribenzoate (not shown), implies that no sulfhydryl group is required for activity.

The pH curves for the three branched-chain amino donors are presented in Fig. 5. Each of the amino acids produces a characteristic curve, but all show a broad pH optimum in the range of pH 7.8. Less activity (about 10%) is observed when Tris is the buffer used. With regard to the coenzyme moiety of the enzyme, the absorption spectrum of the enzyme is typical of enzymes that contain pyridoxal phosphate[14–16] with major peaks at 230 and 280 nm and smaller coenzyme-specific peaks at 320 and 425 nm (these latter peaks are shown in Fig. 6). As depicted in the

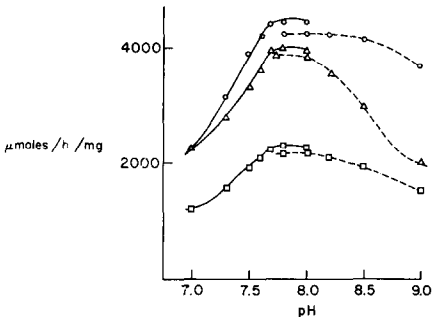


Fig. 5. pH curves for the branched-chain amino acids (amino donors) in assays with α-ketoglutarate as amino acceptor. ○, leucine; △, isoleucine; □, valine. —, potassium phosphate buffer; - - -, Tris buffer.

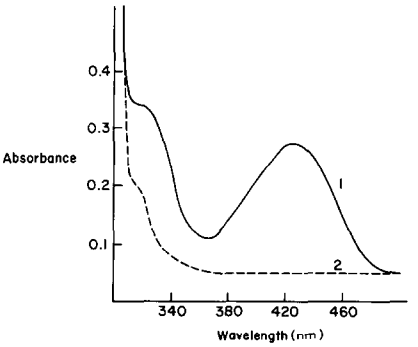


Fig. 6. Spectra of enzyme before and after dialysis. (1), native holoenzyme (2 mg protein per ml) in 0.2 M potassium phosphate buffer (pH 7.8); 2, dialyzed enzyme (apoenzyme) in same buffer. Analyses carried out with the use of a Cary Model 15 recording spectrophotometer. Procedure for dialysis is given in the text.

same figure, overnight dialysis against 0.05 M Tris buffer (pH 8.5) (containing 0.5 mM dithiothreitol, 0.5 mM EDTA and 0.1 mM isoleucine) essentially removes the coenzyme-specific peaks. The apoenzyme has no detectable activity without added pyridoxal phosphate. The results of attempts to restore the activity of the apoenzyme by preincubation with pyridoxal phosphate and related compounds are presented in Table IV. 70% of the activity is restored after a 10 min preincubation period with pyridoxal phosphate; and if a longer period of preincubation is allowed, 100% of the original activity is regained. Of the pyridoxal analogues tried, only pyridoxamine phosphate is capable of restoring activity but only after prolonged incubation with the apoenzyme. These results provide good evidence that, like other aminotransferases, the coenzyme moiety is pyridoxal phosphate.

Other results of spectral analyses are presented in Figs. 7 and 8. As seen in Fig. 7, the spectrum of the holoenzyme at pH 8.0 is affected by the presence of the amino donor, isoleucine (Curves 1 and 2). The peak at 425 nm is attributed to a quinoid structure, presumably the result of Schiff base formation between pyridoxal

TABLE IV

RESTORATION OF THE ACTIVITY OF THE APOENZYME

Apoenzyme preincubated with 100 μM quantities of coenzyme in 0.2 M Tris buffer (pH 8.0) prior to assay.

Coenzyme	*% Maximal activity*
I. 10-min preincubation	
Pyridoxal phosphate	70
II. 4-h preincubation	
Pyridoxal phosphate	100
Pyridoxamine phosphate	85
Pyridoxal	0
Pyridoxamine	0
Pyridoxine	0

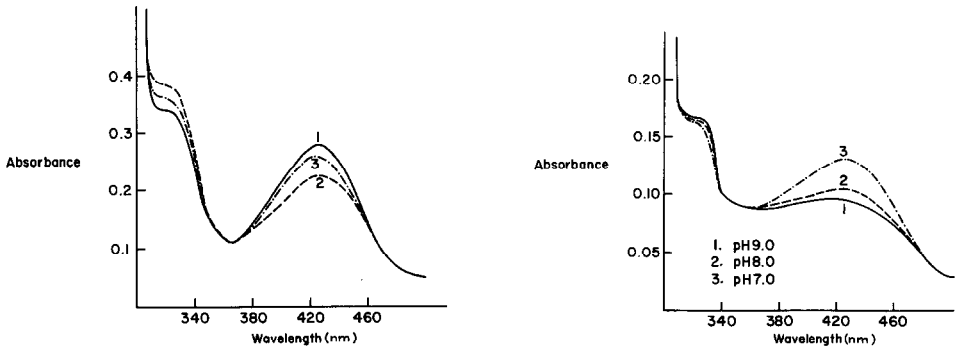


Fig. 7. Effect of substrates on spectrum of holoenzyme. 1, spectrum of native holoenzyme (2 mg protein per ml) in 0.2 M potassium phosphate buffer (pH 7.8); 2, spectrum of enzyme in presence of 100 μM L-isoleucine; 3, spectrum of enzyme in presence of 100 μM each of L-isoleucine and α-ketoglutarate.

Fig. 8. Spectra of native holoenzyme (1.5 mg protein per ml) at three pH values. 1, in 0.2 M Tris buffer (pH 9.0); 2, in 0.2 M Tris buffer (pH 8.0); 3, in 0.2 M potassium phosphate buffer (pH 7.0).

phosphate and the ε-amino group of lysine; whereas, the peak at 320 nm is attributed to a tetrahedral form of the carbon attached to the ring in position 4 (refs. 14, 17). The spectral changes caused by the addition of amino donor to the holoenzyme can be explained as a decrease in the amount of coenzyme in the Schiff base form (425 nm) with a concomitant increase of the coenzyme in the pyridoxamine form (320 nm). Addition of amino acceptor to the holoenzyme and amino donor mixture (Curve 3 of Fig. 7) results in a partial restoration of the aldehyde form of the holoenzyme. As depicted in Fig. 8, the spectrum of transaminase B is pH dependent. Although similar results are obtained with the spectra of aspartate aminotransferase[18] and alanine aminotransferase[17], the branched-chain amino-acid aminotransferase from mammalian sources does not exhibit this phenomenon[14].

This initial study on the branched-chain amino-acid aminotransferase of Salmonella has been concerned with the crystallization of the enzyme and an investigation of the more basic characteristics of the enzyme, reaction, and assay. Because the available literature about this particular enzyme is sparse, a number of detailed studies are needed to develop a more complete description of this aminotransferase and to elucidate more clearly its role in metabolism. Kinetic studies of the reaction have been carried out with the enzyme from two species of Salmonella and will appear as a separate report.

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