Leucine Aminotransferase

II. PURIFICATION AND CHARACTERIZATION*

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SUMMARY

Leucine transaminase was purified extensively from pig heart muscle. It appeared to be nearly homogeneous by the criteria of both starch gel and free boundary electrophoresis and ultracentrifugation. The molecular weight was found to be 75,000.

Purified leucine transaminase is yellow, and our best preparations contained 1 mole of bound pyridoxal phosphate per 75,000 g of protein. The spectrum of the enzyme has absorption maxima at 414 mμ and 320 mμ in addition to the characteristic 280-mμ peak. Addition of an amino acid substrate shifted the 414-mμ peak to 326 mμ, whereas addition of the corresponding α-keto acid partially reversed this shift. Incubation of the enzyme with an amino acid substrate plus a high concentration of phosphate yielded an inactive apoenzyme which could be reconstituted with pyridoxal phosphate. Loss of activity was correlated with a loss of absorption in the visible region. The enzyme activity was inhibited by carbonyl reagents. Reduction with sodium borohydride completely shifted the 414-mμ peak to 320 mμ and destroyed the transaminase activity. Acid hydrolysis of the reduced enzyme yielded a fluorescent compound which was tentatively identified as e-pyridoxyl lysine.

Leucine transaminase displayed a bell-shaped pH-activity curve with an apparent optimum at pH 8.3 to 8.5; however, it was most stable at pH 6 to 7. At pH 8.5 and in the presence of 0.1 M β-mercaptoethanol, this single enzyme catalyzed transamination between l-glutamate, l-leucine, l-valine, or l-isoleucine and their α-keto forms. Under arbitrary conditions, the relative rates of transamination were of the same order of magnitude with a maximum difference of 5-fold.

Direct transamination between the natural branched chain amino acids, leucine, isoleucine, and valine, occurs in a variety of biological materials (1), and evidence has been obtained for separate glutamate-aliphatic amino acid transaminases which are distinct from the glutamic-aspartic and glutamic-alanine enzymes (2-5). However, despite the abundance of evidence for branched chain amino acid transamination, none of the specific enzymes involved has been extensively purified and studied in detail. In the preceding paper, we described two colorimetric procedures to assay branched chain α-ketoglutarate transaminases (6). This paper described the isolation and some of the properties of a single, branched chain amino acid transaminase (EC 2.6.1.6) from pig heart muscle. We call it by the trivial name leucine aminotransferase.

MATERIALS AND METHODS

All chemicals were obtained from commercial sources except for α-keto-β-methylvalerate, which was synthesized from L-isoleucine (7), and pyridoxyllysine.† DEAE(A-50)-, CM(C-50)-, and G-200 forms of Sephadex were purchased from Pharmacia. Hydroxylapatite was prepared from brushite (8). Fresh pig hearts were obtained from a local slaughterhouse.

Leucine transaminase was measured routinely by its ability to catalyze transamination between L-leucine and α-ketoglutarate according to the assay method given in the preceding paper (6). However, except where noted otherwise, routine assay mixtures also contained 300 μmoles of β-mercaptoethanol per 3 ml, and units of enzyme activity were calculated from the amount of α-ketoisocaproate formed in the presence of this thiol compound. Throughout this paper, 1 unit of activity corresponds to the formation of 1 μ mole of α-ketoisocaproate per min at 37° in the presence of 0.1 M β-mercaptoethanol. Protein was determined by a modified biuret method (9); with most column fractions, protein elution patterns were estimated by the 280-mμ absorption. Absorption spectra were taken with a Cary model 14 recording spectrophotometer with a 1-cm light path.

EXPERIMENTAL PROCEDURES AND RESULTS

Purification of Enzyme

Step I: Extraction and Heating—Pig hearts, 45 pounds, were trimmed of fat, minced, and then homogenized for 40 sec (1-gal Waring Blender) with an equal volume of cold 0.05 M sodium

We wish to thank Dr. A. W. Forrey, University of Washington, Seattle, for gifts of authentic samples of both α- and e-pyridoxyl-lysine.

The abbreviation used is: CM-, carboxymethyl.

More recently, homogenization has been omitted. This leads to a more rapid filtration after heating without appreciable reduction in yield.
caproate-5.0 mM EDTA buffer, pH 6.1, to solubilize the enzyme. The homogenate (25 to 30 liters) was then poured into a 35-liter steel beaker and quickly heated to 62° with a Groen model D20 steam kettle as a 75° water bath. When the temperature reached 40°, 100 ml of neutralized 1.0 M sodium α-ketoglutarate were added. After 20 min of heating at 62° with constant stirring (water bath at 63°), the homogenate was chilled to 5° and filtered through four thicknesses of cheesecloth in a sock (100 X 20 cm); denatured material serves as a filter aid. All subsequent steps were carried out at 0–5°.

Step II: First Ammonium Sulfate Fractionation—To the filtrate from Step I, solid ammonium sulfate was added to a final concentration of 312 g per liter (53% saturation), and the precipitate was collected by centrifugation. The precipitate was then suspended in 1.5 liters of 5.0 mM sodium pyrophosphate, pH 8.5, and some of the inactive material which failed to go back into solution was removed by centrifugation.

Step III: Second Ammonium Sulfate Fractionation—The supernatant fluid from Step II was made 5.0 mM in α-ketoglutarate, and solid ammonium sulfate was added to a final concentration of 278 g per liter (47% saturation). The precipitate was again collected by centrifugation and suspended in 5.0 mM sodium pyrophosphate, pH 8.5. Any insoluble material was removed this time by centrifugation in a Beckman model L ultracentrifuge at 59,000 X g and −5°. Extensive dialysis with 5.0 mM sodium pyrophosphate, pH 8.5, was carried out preparatory to chromatography.

Step IV: DEAE-Sephadex Chromatography—Dialyzed enzyme from Step III was adjusted to pH 8.5, if necessary, and placed on a DEAE-Sephadex (A-50) column (4.0 X 30 cm) equilibrated with the dialysis buffer. As soon as the enzyme solution had passed into the column, linear gradient elution was initiated. The mixing chamber contained 1.5 liters of 5.0 mM sodium pyrophosphate, pH 8.5, and the reservoir contained an equal volume of 0.05 M pyrophosphate-0.2 M NaCl, pH 8.5. Fig. 1 shows a typical elution profile. Purification for this step was consistently 4- to 5-fold.

Step V: Hydroxylapatite Chromatography—Activity from Step IV was pooled, made 5.0 mM in neutralized α-ketoglutarate, and concentrated by the addition of 250 g of solid ammonium sulfate per liter. After centrifugation, the precipitate was dissolved in 75 ml of 0.05 M potassium phosphate, pH 7.0, and dialyzed against the same buffer. Dialyzed enzyme was then absorbed onto a hydroxylapatite column (2.5 X 15 cm) equilibrated with 0.05 M phosphate, pH 7.0. Fractionation was achieved by linear gradient elution with potassium phosphate buffer, pH 7.0. The mixing chamber contained 450 ml of 0.05 M phosphate, and the reservoir contained 450 ml of 0.28 M phosphate. Enrichment was usually 3- to 4-fold.

Step VI: CM-Sephadex Chromatography—The pooled activity from Step V was concentrated as above with solid ammonium sulfate in the presence of α-ketoglutarate, dissolved in about 30 ml of 0.05 M potassium phosphate, pH 5.8, and dialyzed against this buffer. Chromatography on a column of CM-Sephadex (C-50) (2.2 X 15 cm) equilibrated with the dialysis buffer provided further purification (2- to 3-fold). Conditions of the linear gradient elution were as follows: mixing chamber, 300 ml of 0.05 M potassium phosphate, pH 5.8; reservoir, 300 ml of 0.1 M phosphate, pH 6.8. The enzyme emerged as a sharp yellow band.

Step VII: Sephadex G-200 Chromatography—The pooled eluate from Step VI (about 30 ml) was concentrated to 3 ml by ultrafiltration (10), applied to a Sephadex G-200 column, and eluted with 0.05 M potassium phosphate, pH 7.0 (Fig. 2A). The elution pattern revealed a partial separation of at least two proteins. By careful pooling of the units in the trailing peak, ultrafiltration, and rechromatography on G-200, the elution pattern in Fig. 2B was obtained. Activity in Fig. 2B is associated with a single protein band. When the active fractions were pooled, the final specific activity was found to be 50 to 53. A specific activity of 53.0 was the highest obtained.

Table I summarizes the results of a typical purification. A key step in the purification, the heat treatment, was not included in
**TABLE I**

Purification of pig heart leucine transaminase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Units per ml</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated filtrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Heated filtrate</td>
<td>14,500</td>
<td>1.14</td>
<td>9.7</td>
<td>0.117</td>
<td>0</td>
<td>100</td>
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<tr>
<td>(NH₄)₂SO₄, 0–50% saturated</td>
<td>1,630</td>
<td>9.72</td>
<td>18.1</td>
<td>0.548</td>
<td>4.7</td>
<td>97</td>
</tr>
<tr>
<td>Dialyzed 0–47% (NH₄)₂SO₄</td>
<td>565</td>
<td>22.7</td>
<td>34.6</td>
<td>0.666</td>
<td>5.7</td>
<td>78</td>
</tr>
<tr>
<td>DEAE-Sephadex column</td>
<td>680</td>
<td>11.6</td>
<td>3.85</td>
<td>3.02</td>
<td>26.0</td>
<td>48</td>
</tr>
<tr>
<td>Hydroxylapatite column</td>
<td>310</td>
<td>16.9</td>
<td>1.42</td>
<td>11.9</td>
<td>100</td>
<td>32</td>
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<tr>
<td>CM-Sephadex column</td>
<td>30</td>
<td>104</td>
<td>3.57</td>
<td>29.2</td>
<td>252</td>
<td>19</td>
</tr>
<tr>
<td>Sephadex G-200 column</td>
<td>15</td>
<td>104</td>
<td>2.03</td>
<td>50.53</td>
<td>440</td>
<td>9.5</td>
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**TABLE II**

Extraction of enzyme and heat protection with several buffers

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Buffer, 5 mM, + 1 mM α-ketoglutarate</th>
<th>Units per ml</th>
<th>Protein</th>
<th>Specific activity</th>
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</thead>
<tbody>
<tr>
<td>Unheated</td>
<td>Sodium caproate, pH 6.0</td>
<td>0.685</td>
<td>18.9</td>
<td>0.036</td>
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<tr>
<td></td>
<td>Potassium phosphate, pH 7.0</td>
<td>0.720</td>
<td></td>
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<tr>
<td></td>
<td>Sodium pyrophosphate, pH 8.5</td>
<td>0.690</td>
<td>20.8</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Sodium maleate, pH 6.0</td>
<td>0.620</td>
<td>25.8</td>
<td>0.025</td>
</tr>
<tr>
<td>Heated</td>
<td>Sodium caproate</td>
<td>1.330</td>
<td>10.6</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Potassium phosphate</td>
<td>0.126</td>
<td>20.8</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Sodium pyrophosphate</td>
<td>0.636</td>
<td>25.8</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td>Sodium maleate</td>
<td>0.650</td>
<td>11.0</td>
<td>0.059</td>
</tr>
</tbody>
</table>

assessing the total enrichment because the yield from this step was always 2- to 3.5-fold. This increased recovery of activity upon heating the crude homogenate was not due to further enzyme solubilization from the tissue since it was also obtained with clear centrifuged supernatant fluid (Table II). A similar observation was made previously in the isolation of glutamic-aspartic transaminase from hog heart (11). Table II also shows the advantages of caproate buffers to extract the enzyme. Further study revealed that heated and unheated extracts differed not in their substrate Michaelis constants but in their apparent maximum velocities per mg of protein. The apparent heat activation could be exactly duplicated simply by the addition of 50 μg of pyridoxal phosphate to reaction mixtures containing unheated extract. Apparently 90 to 100% of the enzyme which is solubilized exists in the unheated extract as apoenzyme. In the presence of 5 mM α-ketoglutarate, added during the heat step, any pyridoxamine enzyme presumably would be converted to pyridoxal enzyme and thereby stabilized. If no α-ketoglutarate was added during heat treatment, the yield was reduced by over 90%. α-Ketoglutarate has frequently been used for heat protection in the purification of other transaminases (11-15), and it has been shown to confer heat stability to the aldehyde form per se of glutamic-aspartic transaminase (11).

**Fig. 3.** Sedimentation of leucine transaminase. The initial protein concentration was 8.7 mg per ml in 0.05 M potassium phosphate-0.1 M β-mercaptoethanol, pH 7.0. Schlieren patterns (A) and 404-nm absorbance changes across the cell (B) were taken 104 min after reaching a speed of 59,780 rpm at 6.5°C. The sharp inflection in B corresponds to the peak of the sedimenting chromoprotein. Sedimentation is proceeding from left to right across the cell.
FIG. 4. Starch gel electrophoresis. Bovine serum albumin (0.6 mg) (top) and leucine transaminase (0.4 mg; specific activity, 50.0) (bottom) were applied to Whatman paper wicks which were inserted into slots shown in the gel. A current of 8 ma was passed for 8 hours at 5°; the anode is at the left.

Protein solution. From the rate of sedimentation, an $s_{20,w}$ value of 5.1 was calculated for zero protein concentration.

Starch gel electrophoresis of purified enzyme was performed on the discontinuous buffer system of Poulik (18) except that both the gel and the electrode buffers were made 5.0 mM with respect to L-ketoglutarate. After electrophoresis, the gel block was sliced horizontally and one-half was stained with 0.05% nigrosin (19) to give the pattern in Fig. 4. Crystalline bovine serum albumin cleaved into six bands; however, the enzyme yielded a single, rather broad band which migrated toward the anode. No material was observed to migrate toward the cathode. If L-ketoglutarate was omitted from the solvent system, smearing of the transaminase occurred. From the unstained half of the gel block, it was possible to recover 8 of the 20 enzyme units applied. For this, 1-cm segments of gel were macerated and extracted with 0.05 M sodium pyrophosphate, pH 8.5. All the activity recovered was located in the same area of the gel as the stained protein band.

Purified enzyme was also examined by free boundary electrophoresis at pH 7.5 in a Tiselius apparatus (Perkin-Elmer model 3B). After 130 volts (13 mA) had been applied for 24 hours to an 11.4 mg per ml enzyme solution, the protein had migrated three-fourths of distance across the cell in the direction of the anode as a single, major band (not shown). By this criterion, purified enzyme with a specific activity of 50.0 appeared to be about 90% homogeneous.

A molecular weight determination was made with enzyme with a specific activity of 53.0. In Fig. 5, the log of the 404-nm absorption (log recorder deflection) is seen to be directly proportional to the square of distance from the center of rotation ($X^2$), an additional indication of chromoprotein homogeneity with respect to size. Assuming a partial specific volume of 0.74, a molecular weight of 75,000 ± 3,700 was obtained (17, 20). Accuracy by this method merely requires that the enzyme be the only chromoprotein present.

Absorption Spectra—Fig. 6 shows that the absorption spectrum of purified leucine transaminase in the visible region is characterized by maxima at 414 nm and 326 nm, respectively. With the assumption that there is 1 mole of bound pyridoxal phosphate per mole of enzyme, the molar extinction coefficient of the bound vitamin B$_6$ derivative is 7150 ± 200 at 414 nm and 5750 ± 200 at 326 nm. These are averaged values for two different enzyme preparations. Our best preparations gave an $A_{326}:A_{414}$ of 14:4.

Spectral shifts were observed in the presence of substrates. To conserve enzyme in this study, the expanded scale of the spectrophotometer was used and spectra were taken with enzyme that had not been purified beyond the CM-Sephadex column step. This material gave the same spectrum as the transaminase of Fig. 6 except that the $A_{414}:A_{326}$ ratio was slightly lower. Fig. 7 illustrates that L-leucine will decrease the absorption at 414 nm with a proportionate increase at 326 nm. L-Ketoglutarate
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1.6

Wave length, millimicrons

FIG. 6. Total absorption spectrum of leucine transaminase, 1.23 mg per ml in 0.05 M potassium phosphate, pH 7.0.

FIG. 7. Effects of substrates on the spectrum of leucine transaminase, 0.75 mg per ml in 0.067 sodium pyrophosphate, pH 8.3. Curve 1, enzyme alone; Curve 2, enzyme in 20 mM L-leucine; Curve 3, enzyme in 20 mM L-leucine plus 5 mM α-ketoisocaproate. Curve 4 was obtained also in the presence of 100 mM L-leucine-25 mM α-ketoisocaproate.

partially reversed this shift, the final relative absorbances being dependent on the ratio of keto acid to amino acid. Similar spectral changes were noted in the presence of the homologous substrate pair L-glutamate and α-ketoglutarate. Since the addition of neutralized α-ketoglutarate alone to a final concentration of 20 mM had no effect on the initial enzyme spectrum, the two absorption peaks of Fig. 6 at 326 μm and 414 μm must represent the pyridoxal form of leucine transaminase. They do not represent a mixture of phosphopyridoxal with phosphopyridoxamine enzyme.

β-Mercaptoethanol has been observed to activate the enzyme several fold (24). Enzyme, 0.08 mg per ml, was activated 3.5-fold at 37° in 0.1 M β-mercaptoethanol-0.05 M sodium pyrophosphate, pH 8.5. Spectra of activated transaminase were identical with that of the enzyme just prior to thiol treatment except for a large increase in absorption in the protein (280 μm) region and a 20% increase in the height of the 326-μm peak. L-Leucine (20 mM) at pH 8.3 imparted the same spectral shift seen in Fig. 7.

No large spectral shifts as noted with the amino acids were obtained by varying the pH. The absorption peaks at 326 μm and 414 μm did not shift their maxima positions or relative heights over the pH range 4.8 to 7.0. From pH 7.5 (about 5%) to pH 10.5 (about 20%), small decreases in the absorption at 414 μm were seen coupled with slight increases at 326 μm.

Resolution of Leucine Transaminase—Purified enzyme can be resolved by phosphate buffer in the presence of an amino acid substrate. Enzyme, 0.19 mg per ml, was incubated at 37°, pH 7.5, in phosphate buffer plus L-leucine or L-glutamate. At various times, 10-μl samples containing 0.7 unit were removed and assayed (+0.1 M β-mercaptoethanol) both with and without pyridoxal phosphate. The results are plotted in Fig. 8A and B as percentages of the activity found when the enzyme was correspondingly diluted at 0° in 0.01 M potassium phosphate and assayed immediately. Results like Curve 1 of Fig. 8A and B were obtained following incubation in 1.0 M potassium phosphate plus either 0.1 M α-ketoglutarate or 0.1 M sodium caprate, pH 7.5. Fig. 8A and B shows that (a) loss of activity occurs only in the presence of an amino donor, (b) activity is largely restored by pyridoxal phosphate, (c) high phosphate concentrations acceler-

FIG. 8. Resolution of leucine transaminase by amino acid substrates with respect to time. A, resolution by L-leucine in the presence of phosphate. Curve 1, enzyme was incubated in 1.0 M potassium phosphate, pH 7.5, then assayed + pyridoxal phosphate (50 μg per assay); Curve 2, enzyme assayed without added pyridoxal phosphate after incubation in 1.0 M phosphate-0.05 M L-leucine; Curve 3, enzyme assayed without added pyridoxal phosphate after incubation in 0.05 M phosphate-0.05 M L-leucine; Curve 4, enzyme first incubated as for Curves 2 or 3 but assayed with pyridoxal phosphate. Additional details are given in the text.
ate the loss of activity, and (d) leucine is more effective than glutamate at concentrations of about 0.05 to 0.1 M. These findings indicate that holoenzyme was resolved into apoenzyme plus pyridoxal phosphate, to the extent of 90% in Fig. 8A.

It was possible to correlate the preceding requirement for pyridoxal phosphate with a partial loss of absorption at 414 μ and 320 μm. Enzyme, 1.13 mg per ml, was first dialyzed against two 500-ml volumes of 1.0 M potassium phosphate-0.1 M glutamate, pH 7.5, for 12 hours at 25° followed by dialysis for 8 hours with 4 liters of 0.02 M phosphate, pH 7.5. This enzyme was then incubated for 5 min at 37° in 5.0 mM α-ketoglutarate-0.7 mM pyridoxal phosphate and dialyzed for 48 hours against three 1.3-liter volumes of 0.02 M potassium phosphate, pH 7.5. Enzymatic and spectral data at each step are given in Table III and Fig. 9, respectively. Because of the lability of leucine transaminase and the small initial volume (2 ml) of enzyme solution used, both the total activity and the specific activity of the reconstituted enzyme were only about one-half that of the original preparation. Incomplete loss of absorption in the visible region (Fig. 9, Curve 2) was paralleled by incomplete enzymatic resolution (Table III). The remaining bound pyridoxal phosphate may be accessible to an amino donor only after activation with β-mercaptoethanol (24).

Carbonyl Reagents—Leucine transaminase displays a sensitivity to carbonyl-binding reagents which is typical for pyridoxal phosphate enzymes (25). Inhibitory effects of these compounds are reported in Table IV as percentage of inhibition of control rates. The concentrations shown refer to those during a prior incubation of enzyme with the carbonyl reagent alone. All five compounds are seen to inhibit transamination; however, those which are known to react with carbonyl groups to form stable azomethine bonds are the most potent inhibitors.

Reduction of Enzyme-bound Pyridoxal Phosphate with Sodium Borohydride—Reduction with sodium borohydride affects both the absorption spectrum and the activity of leucine transaminase. Enzyme with a specific activity of 50.0 was treated with 5 mM borohydride-Reduction with sodium borohydride affects both absorption spectrum and the activity of leucine transaminase. Enzyme with a specific activity of 50.0 was treated with 5 mM borohydride for 40 min by the dialysis method of Matsuo and Greenberg (26) and then dialyzed for 16 hours against two 500-ml volumes of 0.02 M potassium phosphate, pH 7.5. This enzyme was then incubated for 5 min at 37° in 5.0 mM α-ketoglutarate-0.7 mM pyridoxal phosphate and dialyzed for 48 hours against three 1.3-liter volumes of 0.02 M potassium phosphate, pH 7.5. Enzymatic and spectral data at each step are given in Table III and Fig. 9, respectively. Because of the lability of leucine transaminase and the small initial volume (2 ml) of enzyme solution used, both the total activity and the specific activity of the reconstituted enzyme were only about one-half that of the original preparation. Incomplete loss of absorption in the visible region (Fig. 9, Curve 2) was paralleled by incomplete enzymatic resolution (Table III). The remaining bound pyridoxal phosphate may be accessible to an amino donor only after activation with β-mercaptoethanol (24).

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Reduction of Enzyme-bound Pyridoxal Phosphate with Sodium Borohydride—Reduction with sodium borohydride affects both the absorption spectrum and the activity of leucine transaminase. Enzyme with a specific activity of 50.0 was treated with 5 mM sodium borohydride for 40 min by the dialysis method of Matsuo and Greenberg (26) and then dialyzed for 16 hours against two 500-ml volumes of 0.01 M potassium phosphate, pH 7.2. Borohydride treatment resulted in a loss of the 414-μ absorption peak coupled with an increase in the 325-μ region (Fig. 10). The new peak at 320 μm did not change on further dialysis against 0.01 M phosphate, pH 7.2, for 48 hours. Reduced enzyme was inactive and the addition of pyridoxal phosphate restored only to 2 to 3% of the original activity. These results indicate that borohydride reduced the azomethine linkage of pyridoxal phosphate to yield a secondary amine and that essentially all of the active sites were occupied with the prosthetic group at the time of reduction. The site of pyridoxal phosphate attachment to leucine transaminase was tentatively identified as the ε-amino group of lysine.

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### Table III

**Activity of leucine transaminase, resolved and reconstituted**

Enzyme in this experiment was not purified beyond the CM-Sephadex step. Experimental details are given in the text.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Specific activity</th>
<th>Total units</th>
<th>Percentage of resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyridoxal-5'-P</td>
<td>Pyridoxal-5'-P</td>
<td>Pyridoxal-5'-P</td>
</tr>
<tr>
<td>Original ..........</td>
<td>28.0</td>
<td>28.5</td>
<td>61.0</td>
</tr>
<tr>
<td>After dialysis against glutamate-phosphate</td>
<td>20.5a</td>
<td>2.5</td>
<td>43.0</td>
</tr>
<tr>
<td>After incubation with pyridoxal-5'-P and dialysis</td>
<td>10.2</td>
<td>10.5</td>
<td>27.3</td>
</tr>
</tbody>
</table>

* To 3.0 ml of assay solution, 50 μg were added. Activity was measured in 0.1 M β-mercaptoethanol. Changes in the protein concentration of the original material were taken into account by measurement of the absorption at 280 and 260 μm (113).

a Preincubation of the enzyme with pyridoxal-5'-P did not increase the activity more than 5%.

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### Table IV

**Inhibition of leucine transaminase by carbonyl reagents**

Samples of enzyme, 7.3 μg, were incubated with carbonyl reagent in 0.05 M sodium pyrophosphate, pH 8.3, for 15 min at 37°. Transamination was then initiated by the addition of a 1-leucine-α-ketoglutarate solution, pH 8.3, and was determined as usual. β-Mercaptoethanol was omitted in these assays, however.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
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<tbody>
<tr>
<td>Hydroxylamine</td>
<td>1 x 10^-4</td>
<td>82</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>1 x 10^-7</td>
<td>24</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>1 x 10^-8</td>
<td>10</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>3 x 10^-2</td>
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</tr>
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<td>Phenylhydrazine</td>
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<tr>
<td>Thiosemicarbazide</td>
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<td>9</td>
</tr>
<tr>
<td>Sodium bisulfite</td>
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<td>23</td>
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</table>

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FIG. 9. Partial spectral resolution of leucine transaminase. Curve 1, enzyme, 1.13 mg per ml in 0.02 M potassium phosphate, pH 7.0; Curve 2, enzyme of Curve 1 after dialysis against 1.0 M potassium phosphate-0.1 M glutamate, then 0.02 M phosphate: Curve 3, enzyme of Curve 2 after incubation with pyridoxal phosphate and dialysis; Curve 4, enzyme of Curve 3 + L-leucine (0.02 M); Curve 5, spectrum of 2.5 X 10^-3 M pyridoxal phosphate in 0.02 M phosphate, pH 7.0. Additional details are given in the text.
Fig. 10. Absorption spectrum of leucine transaminase before (A) and after (B) reduction with sodium borohydride. Curve A, spectrum of the enzyme at 0.87 mg per ml in 0.01 M potassium phosphate, pH 7.2. Curve B, the same solution after treatment with sodium borohydride. Details of the reduction are given in the text.

Enzyme (45 mg; specific activity, 32.0) was reduced with sodium borohydride, dialyzed extensively against distilled water, and then hydrolyzed for 18 hours in 6 M HCl at 110° in an evacuated sealed tube. The entire hydrolysate was taken to dryness by evaporation with N,N gas, dissolved in a small volume of water, and then streaked on Whatman No. 3MM paper for descending chromatography in butyl alcohol-acetic acid-water (4:1:5). Descending chromatography yielded several fluorescent bands, only one of which (Rf 0.08) was quenched by ammonia fumes and which again gave a positive test for 3-hydroxypyridines. A blue color is given by 3-hydroxypyridines (27). This band was eluted with water and concentrated by evaporation to identify the material further. When subjected to two-dimensional chromatography in the system of Turano et al. (28), it chromatographed like authentic ε-pyridoxyllysine and yielded a single fluorescent spot which was quenched by ammonia fumes and which gave a blue color test. Upon paper strip electrophoresis at pH 7.5 in 0.01 M potassium phosphate (29), this material migrated toward the cathode the same distance as ε-pyridoxyllysine as judged by finding a single fluorescent spot which was quenched by ammonia fumes and which again gave a positive test for 3-hydroxypyridines.

Effects of pH

The marked variation of leucine-α-ketoglutarate transaminase activity with pH under standard assay conditions (6) is depicted in Fig. 11. In A, enzyme was first activated by a 15-min incubation at 37° in 0.1 M β-mercaptoethanol-0.1 M sodium pyrophosphate, pH 8.3, before 0.1-ml aliquots (0.8 unit) were removed and assayed (24). In B, enzyme (3.2 units) was assayed directly in the absence of β-mercaptoethanol. Both thiol-activated and untreated enzyme have apparent optima at pH 8.25. In Tris-HCl buffer, the maximum is shifted slightly to about pH 8.5. Pyrophosphate stimulates enzyme which was not treated with β-mercaptoethanol (3- to 4-fold less active) 20% at pH 8.25.

Leucine transaminase also shows a variation in stability with pH upon incubation for 1 hour at 37°. Although maximum stability (about 85%) exists in the pH range 6 to 7, the pH optimum for activity is independent of the pH of maximum stability when one uses a 10-min incubation time. Similar stability and activity curves were found with chicken liver leucine transaminase (30). Both concentrated and dilute solutions of the purified enzyme lost activity (about 20% in 1 week) at pH 7 when stored at 5°; however, concentrated solutions which had been frozen for 1 month at -10° retained 90% of their original activity.

Enzymatic Evidence for Single Transaminase and Substrate Specificity

Several lines of evidence show that a single enzyme (leucine transaminase) catalyzes transamination between α-ketoglutarate and all three of the branched chain amino acids. Purified enzyme of specific activity 32.0 was about equally active with L-leucine, L-valine, or L-isoleucine plus α-ketoglutarate as determined by the standard assay procedures described in the preceding paper (6), in the presence of 0.1 M β-mercaptoethanol. The absolute requirements for each reaction are shown in Table V. Absorbance reference blanks were prepared by treating 3.0 ml of water with 2,4-dinitrophenylhydrazine and making the appropriate extractions. Transaminase activities between α-ketoglutarate and the branched chain amino acids were determined at each step throughout two separate purifications. Table

![Fig. 11. Variation of transaminase activity with pH at substrate concentrations used in routine assays. A, enzyme was activated with β-mercaptoethanol as described in the text. B, untreated enzyme. Buffers were: △—△, potassium phosphate, ionic strength 0.2; ○—○, 0.067 M sodium pyrophosphate; and □—□, 0.067 M Tris-chloride. The pH of each buffered reaction mixture was measured at 37°.](http://www.jbc.org/)

### Table V

<table>
<thead>
<tr>
<th>Omission from the complete system</th>
<th>L-Leucine</th>
<th>L-Isoleucine</th>
<th>L-Valine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.05</td>
<td>1.13</td>
<td>0.84</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.005</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amino acid</td>
<td>0.008</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
VI shows the averaged relative activities which remained constant throughout a 450-fold enrichment as well as the apparent 3-fold heat activation step discussed earlier. Activation by β-mercaptoethanol (24) was not specific for leucine-α-ketoglutarate transamination. After CM Sephadex chromatography (Step VI), the addition of 0.1 M β-mercaptoethanol caused increases in activity for transamination with α-ketoglutarate by the following factors: 1-leucine, 3.0; L-isoleucine, 2.4; and L-valine, 3.5. The foregoing data plus the homogeneity (about 90%) of enzyme preparations with a specific activity of 50 clearly established that we were dealing with a single transaminase. It was therefore of interest to examine its substrate specificity. Various combinations of amino acids plus α-keto acids were tested under arbitrary conditions. Reaction mixtures contained 45 μmoles of amino acid, 45 μmoles of α-keto acid, 100 μmoles of β-mercaptoethanol, 50 μmoles of sodium pyrophosphate, and enzyme (2 to 8 μg) in a total volume of 1.0 ml, pH 8.5. Reactions were initiated by the addition of enzyme and were terminated after 10 min at 37° with 50 μl of 97% formic acid. The extent of the leucine-α-ketoglutarate reaction was proportional to both time and enzyme concentration under these conditions until 5 μmoles of substrate were consumed. Several samples of the reaction mixtures (10 to 50 μl) were then streaked onto Whatman No. 1 paper (31), and separation of amino acid products from reactants was achieved by: (a) ascending paper chromatography in the hyperphase of a 4:5:1 mixture of n-butyl alcohol-water-glacial acetic acid, (b) paper strip electrophoresis at pH 4.0 in a solvent consisting of 23 ml of glacial acetic acid and 6 ml of pyridine per liter of water, or (c) descending paper chromatography with a solvent consisting of 23 ml of glacial acetic acid and 6 ml of pyridine.\(\text{NH}_3\)SO₄ and dialysis. Method c was used only to separate leucine from isoleucine (32). After separation, amino acid products from reactants were determined by a modification of the quantitative ninhydrin procedure of Kray, Harris, and Entemann (31). Alkali was omitted from the ninhydrin reagent, and the papers were sprayed twice. Standard curves were prepared for each substrate combination which yielded visually detectable amounts of amino acid product. Product amino acid, 5 μmoles, was added to 1 ml of the corresponding reaction mixture, and 10- to 50-μl samples were streaked on paper for separation. In the case of L-glutamate added to the leucine-α-ketoglutarate assay mixture, absorbance at 575 μm was proportional to the amount of glutamate up to a reading of 0.9 (0.25 μmole) and triplicate determinations agreed within 6%.

### Table VI

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Relative activity ratio (leucine:isoleucine:valine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated filtrate</td>
<td>0.88:1:0.93</td>
</tr>
<tr>
<td>Heated filtrate</td>
<td>0.93:1:0.93</td>
</tr>
<tr>
<td>After 0-47% (NH₃)SO₄ and dialysis</td>
<td>0.95:1:0.88</td>
</tr>
<tr>
<td>DEAE-Sephadex column eluate</td>
<td>0.98:1:0.88</td>
</tr>
<tr>
<td>Hydroxyapatite column eluate</td>
<td>0.91:1:0.87</td>
</tr>
<tr>
<td>CM-Sephadex column eluate</td>
<td>0.86:1:0.83</td>
</tr>
<tr>
<td>Sephadex G-200 column eluate</td>
<td>0.89:1:0.87</td>
</tr>
</tbody>
</table>

### Table VII

#### Transamination with α-ketoglutarate

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Isoleucine (allo-free)</td>
<td>100</td>
</tr>
<tr>
<td>L-Valine</td>
<td>90</td>
</tr>
<tr>
<td>DL-Alloiso-leucine</td>
<td>75</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>66</td>
</tr>
<tr>
<td>L-Norvaline</td>
<td>44</td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>22</td>
</tr>
<tr>
<td>L-α-Aminobutyrate</td>
<td>18</td>
</tr>
<tr>
<td>DL-α-Aminopimelate (0.045 M)</td>
<td>12</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>11</td>
</tr>
<tr>
<td>S-Methyl-L-cysteine</td>
<td>8</td>
</tr>
<tr>
<td>DL-α-Aminoadipate (0.015 M)</td>
<td>7</td>
</tr>
<tr>
<td>nL-Allylglycine</td>
<td>6</td>
</tr>
</tbody>
</table>

Glycine, L-alanine, L-lysine, L-phenylalanine, L-tryptophan, D-alloiso-leucine, D-valine, D-leucine, L-threonine, L-histidine, L-arginine, L-cysteine, DL-homocysteine, β-aminoisobutyrate, γ-aminoisovalerate, α-aminobutyrate, and glutamine were also present in hog heart. Table VII confirms the absence of activity for either pyruvate or oxalacetate and demonstrates that leucine transaminase will catalyze transamination between any of the branched chain amino acids. It is interesting that L-isoleucine is more reactive than its D allo isomer since transamination does not alter the configuration of the β-carbon atom (33). These results establish the absolute requirement for an α amino group in the l configuration and rule out significant contamination by glutamic-alanine (34) and glutamic-aspartic (11) transaminase which are also present in hog heart.

### Table VIII

#### Transamination between branched chain amino acids and glutamate

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>α-Keto-glutamate</th>
<th>Oxalacetate, pyruvate</th>
<th>α-Keto-isovalerate</th>
<th>α-Keto-caproate</th>
<th>α-Keto-β-methyl valerate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Isoleucine</td>
<td>100 &lt;3</td>
<td>66</td>
<td>108</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>L-Valine</td>
<td>90 &lt;3</td>
<td>60</td>
<td>41</td>
<td>38</td>
<td>77</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>66 &lt;3</td>
<td>66</td>
<td>41</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>66</td>
<td>60</td>
<td>41</td>
</tr>
</tbody>
</table>
the natural branched chain amino acids or glutamate and the
keto acid homologue of any other member in this group. Whether
the specificity data in Tables VII and VIII reflect
differences in Michaelis constants, maximum velocities, or both
is not known. They probably reflect maximum velocities since
0.045 m concentrations of L-leucine and α-ketoglutarate were
found to saturate the enzyme at pH 8.5.

**DISCUSSION**

Leucine transaminase is comparable to other pyridoxal phos-
phate enzymes with respect to its content of the vitamin B₆
derivative (1 mole/75,000 g) and its absorption in the 400-mu
region. All the pyridoxal phosphate enzymes thus far purified,
except L-aspartic-β-decarboxylase (35), have a yellow form with
absorption at 410 to 430 mu. Quantitatively, the molar inten-
sity of this absorption for leucine transaminase (ε₄₃₀ = 7150)
is close to that reported for other vitamin B₆ enzymes: (a) glutamic-
aspartic transaminase, ε₄₃₀ = 6700 at 430 mu (36); (b) cystathio-
ablein, ε₄₃₀ = 8900 at 427 mu (37); (c) serine transhydroxymethylase,
ε₄₃₀ = 7920 at 430 mu (29). The shape of the absorption spec-
trum of leucine transaminase closely resembles that of α-
alanine-n-glutamate transaminase from Bacillus subtilis which also has
been found to contain 1 mole of pyridoxal phosphate per mole of
enzyme (38). By reference to the valine imines, absorption at
410 to 430 mu has been attributed to a hydrogen-bonded aldimine
(39). Absorption near 330 mu has been ascribed to
the azomethine bond of pyridoxal phosphate has been
reported to be more reactive than its free carbonyl group.

In addition, the azomethine bond of pyridoxal phosphate has been
found to saturate the enzyme at pH 8.5.

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