Tyrosine Aminotransferase

PURIFICATION AND CHARACTERIZATION

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SUMMARY

Tyrosine aminotransferase was highly purified from rat livers in which the enzyme had been induced by a glucocorticoid hormone. The preparation eluted from a DEAE-cellulose column as a single peak gave a single precipitin line by the Ouchterlony double diffusion precipitin reaction and sedimented as a single boundary in the ultracentrifuge with an s20,w of 5.9. A molecular weight of 91,000 was determined by equilibrium sedimentation. Despite these indications of homogeneity, three enzymatically active bands were observed in disk-gel electrophoresis.

The purified enzyme is yellow in solution and exhibits absorption maxima at 425 nm and 327 nm (at slightly acidic pH), which indicate an interaction of the enzyme with its cofactor, pyridoxal phosphate. Addition of tyrosine shifted the 425 nm peak to 327 nm, whereas α-ketoglutarate, the keto-acid substrate, reversed this shift. Separation of enzyme from coenzyme could be achieved by dialyzing holoenzyme with tyrosine in 0.1 M phosphate buffer. Addition of pyridoxal phosphate to apoenzyme caused return of the 425 nm peak and full reconstitution of activity. One mole of pyridoxal phosphate is bound per 94,000 g of enzyme.

Tyrosine aminotransferase has a bell-shaped pH-activity curve with a maximum near pH 7.5. The following "apparent" Michaelis constants were determined: tyrosine, 1.7 × 10⁻⁸ M, α-ketoglutarate, 7 × 10⁻⁴ M, pyridoxal phosphate, 1.7 × 10⁻⁸ M, and pyridoxamine phosphate, 1.5 × 10⁻⁷ M. Phosphate was shown to competitively inhibit the binding of the two cofactors to enzyme with Ki values of 1.1 × 10⁻³ M and 3.4 × 10⁻³ M for pyridoxal phosphate and pyridoxamine phosphate, respectively.

We have reported that the activity of the enzyme L-tyrosine-2-oxoglutarate aminotransferase (EC 2.6.1.5) can be increased by glucocorticoid hormones in a newly established line of hepatoma cells grown in tissue culture (1, 2). Some of the physiological aspects of this increase have been studied (1, 2), but little is known of the precise biochemical events responsible for it. To answer these fundamental questions, detailed knowledge of the structure of the induced enzyme will be very important. Ideally, such studies should be done with enzyme isolated from the tissue culture cells; however, it has not been technically possible up to the present time to grow sufficient quantities of cells for this purpose. Alternatively, enzyme isolated from rat liver could be studied and then compared with cell enzyme when such becomes available. Several groups have reported procedures used to prepare partially purified tyrosine aminotransferase from rat liver (3–6). In this paper we present a modification of these procedures which was used to obtain highly purified enzyme. Some of the chemical, physical, and kinetic properties of the enzyme are presented.

METHODS

Chemicals and Reagents—Triamcinolone (9-α-fluoro-16α-hydroxy-11β-prednisolone diacetate) was generously supplied by Lederle Laboratories, Pearl River, New York. Porcine kidney keto-enol tautomerase and calcium phosphate gel were obtained from Sigma. Pyridoxal-HCl, pyridoxal 5'-phosphate, α-ketoglutaric acid, dithiothreitol (Cleland's Reagent), pyridoxamine 5'-phosphate, and L-tyrosine were obtained from Calbiochem. DEAE-cellulose (Cellex-D, Lot 3204) was purchased from Bio-Rad and Sephadex G-25 from Pharmacia. Mann extra fine, enzyme grade ammonium sulfate was used. Bovine serum albumin was purchased from Armour, and 2,2',2''-nitrotriltriethanol (triethanolamine) from Eastman Kodak.

Assays—Three different assays of enzyme activity were used. Assay I, a spectrophotometric assay essentially similar to that of Lin et al. (7), was used throughout the purification. The reaction mixture contained 3 μmoles of L-tyrosine, 10 μmoles of α-ketoglutarate, 0.1 μmole of pyridoxal phosphate, 70 μmoles of potassium phosphate, 0.56 mmole of sodium borate, and keto-enol tautomerase, 0.1 K unit, as defined by Knox (8). The final volume was 1.0 ml, pH 7.8. The reaction was started by adding enzyme and was followed in a Gilford recording spectrophotometer at a wave length of 310 μm at 37°C. Enzyme activity was calculated from the slope of the linear part of the curve with the
use of the molar extinction coefficient of 10,700 for the enol-borate complex of the product p-hydroxyphenylpyruvate (6). One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min under these conditions. After preliminary investigation of the kinetics with the use of purified enzyme, the reaction mixture was modified to contain 4 μmoles of tyrosine, 20 μmoles of α-ketoglutarate, 0.1 μmole of pyridoxal phosphate, 1 μmole of EDTA, 1 μmole of dithiothreitol, 0.1 K unit of tautomerase, and 0.50 μmole of sodium borate, at pH 7.6 (Assay II). In later studies, a modification of the assay described by Diamondstone was used (Assay III) (9). This is a fixed time assay which depends upon the alkali-catalyzed oxidation of p-hydroxyphenylpyruvate by molecular oxygen to p-hydroxybenzaldehyde and oxalate. Our standard reaction mixture contained 5.6 μmoles of tyrosine, 9 μmoles of α-ketoglutarate, 0.1 μmole of pyridoxal phosphate, 1 μmole of EDTA, 1 μmole of dithiothreitol, and 80 μmoles of triethanolamine, in a total volume of 0.93 ml, at pH 7.6. The reaction was started by the addition of enzyme and allowed to run for 15 min at 37°, at which time it was stopped by the addition of 0.07 ml of 10^{-3} M KOH, with rapid mixing. The reactions were read against a reagent blank (consisting of the complete reaction mixture minus enzyme) at 331 μμ in a Gilford spectrophotometer. The extinction coefficient of the product p-hydroxybenzaldehyde under these conditions is 24,900 M^{-1} cm^{-1} (in 0.1 N NaOH); however, since only 80% of the p-hydroxyphenylpyruvate is converted to p-hydroxybenzaldehyde, an "effective" extinction coefficient of 19,900 M^{-1} cm^{-1} was used (9). One unit of enzyme activity is considered to be that amount which catalyzes the formation of 1 μmole of p-hydroxyphenylpyruvate per min at 37°. Under these conditions, product increased linearly with time and the rate was linearly proportional to the enzyme concentrations up to 0.1 μg. Routinely, 0.01 to 0.05 μg of enzyme protein was used for each assay. Albumin was found to protect against the loss of enzyme activity which occurs at very low protein concentrations (see below), therefore, the purified enzyme was always diluted in a solution containing 5 mg per ml of bovine serum albumin before addition to the assay mixture.

Assay I was used throughout enzyme purification. Assay II was used for initial kinetic studies; however, during the course of these studies it was discovered, by ultraviolet absorption spectroscopy, that borate (used in high concentration in Assays I and II) formed a complex with both pyridoxal phosphate and pyridoxamine phosphate. Therefore, with the exception of the pH curve, all assays subsequent to the purification procedure were done with Assay III, which does not use borate, unless otherwise indicated.

Protein concentration was measured by a modified Biuret method in most instances (10). For very dilute solutions, ultraviolet absorption at 280 μμ or the method of Lowry et al. (11) was used. Bovine serum albumin served as standard for the colorimetric assays.

**EXPERIMENTAL PROCEDURE AND RESULTS**

**Purification Procedure**

All operations were performed at 0-5° except where noted, and the enzyme solutions were in a solution containing 2% pyridoxal phosphate, 2 × 10^{-3} M α-ketoglutarate, 10^{-3} M EDTA, and 10^{-3} M dithiothreitol. The results of a typical purification are shown in Table I.

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Enzyme Specific Activity</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>3,040</td>
<td>91,300</td>
<td>18,900</td>
<td>0.21</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>700</td>
<td>53,200</td>
<td>18,200</td>
<td>0.34</td>
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<tr>
<td>heated supernatant</td>
<td>3,500</td>
<td>9,450</td>
<td>17,500</td>
<td>1.9</td>
</tr>
<tr>
<td>Acetone fraction</td>
<td>335</td>
<td>3,630</td>
<td>11,700</td>
<td>3.0</td>
</tr>
<tr>
<td>Gel eluate</td>
<td>904</td>
<td>687</td>
<td>8,570</td>
<td>12</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>21</td>
<td>40.6</td>
<td>4,620</td>
<td>114</td>
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<tr>
<td>Crystals</td>
<td>1.3</td>
<td>27.4</td>
<td>2,860</td>
<td>104</td>
</tr>
</tbody>
</table>

**Step I: Induction and Preparation of Crude Extract**—It is known that the activity of tyrosine aminotransferase is increased in the liver of rats given glucocorticoid hormones (19). Therefore, after a 12- to 14-hour fast, 100 male Sprague-Dawley rats weighing 200 to 250 g were injected intraperitoneally with triamcinolone suspended in 0.15 M NaCl at a dose of 40 mg/100 g of body weight. This method of induction routinely gave a 20- to 22-fold increase over the basal enzyme activity. After another 9 to 10 hours of fasting, the rats were stunned by a blow to the head, decapitated, and exsanguinated. The livers were rapidly excised, minced in a meat grinder, and homogenized with 3 volumes of 0.15 M KCl which contained 10^{-3} M EDTA in an all-glass Dounce homogenizer. We found, as did Kenney (3), that homogenization with a Waring Blender yielded 60% less enzyme. The homogenate was centrifuged at 20,000 × g for 30 min in a Sorvall RC-2 ultracentrifuge. The precipitate was washed once with a volume of the homogenizing medium and centrifuged, and the wash was added to the original supernatant (crude extract).

**Step II: Ammonium Sulfate Fractionation**—Crystalline ammonium sulfate was added gradually to the crude extract to 35% saturation (199 g per liter). After stirring for 30 min, the precipitate was removed by centrifugation and discarded. Additional solid ammonium sulfate was added until 70% saturation (449 g per liter) was reached. The pH at this point was about 5.2. The mixture was stirred for 1 hour; then the precipitate was collected by centrifugation and dissolved in 0.02 M potassium phosphate, pH 7.0, containing 10^{-3} M EDTA and 10^{-3} M dithiothreitol (Buffer A). Somewhat less than a 2-fold purification with nearly complete recovery of activity was routinely achieved in this step.

**Step III: Heat Treatment**—The enzyme solution was diluted to a protein concentration of about 10 mg per ml. Pyridoxal phosphate and α-ketoglutarate were added to final concentrations of 0.2 and 2 × 10^{-3} M, respectively, and the pH was adjusted to 6.5. The solution was brought rapidly to 70° in batches of 1 liter each and maintained at this temperature, with constant stirring, for 5 min, after which it was quickly chilled in a 0.9% NaCl-ice water bath. The bulky precipitate was removed by centrifugation and the supernatant was added to the supernatant to 75% saturation (492 g per liter). The precipitate which formed was collected by centrifugation, dissolved in Buffer A, and the solution was centrifuged at 105,000 × g for 1
hour to remove insoluble material. This step usually gave a 6- to 8-fold purification with up to 90% recovery of activity.

Step IV: Acetone Fractionation—The clear solution was passed through a Sephadex G-25 column, 7 × 40 cm, to equilibrate the solution with 0.005 M potassium phosphate, pH 6.5. Acetone at −20°C was added slowly to a final concentration of 40% (by volume), and the mixture was kept at −10°C for 3 min. The precipitate was then collected by centrifugation at −10°C and dissolved in Buffer A. Insoluble material was removed by a second centrifugation. In the purification described in Table I there was 30% loss of enzyme activity with less than 2-fold purification. This step was difficult to control and gave occasional large losses of activity. Further experiments indicate that this step may be omitted.

Step V: Calcium Phosphate Gel Fractionation—The enzyme solution was again passed through a Sephadex G-25 column (7 × 40 cm) to equilibrate the solution with 0.005 M potassium phosphate, pH 7.0, free of the protecting reagents routinely used. A suspension of calcium phosphate gel was then added to give a ratio of gel to protein of 3:1, by weight. After stirring the gel eluates which contained the enzyme activity were combined and washed twice with 300 ml of 0.1 M potassium phosphate, pH 7.5. These washings contained considerable protein but little enzyme activity. The enzyme was eluted stepwise from the gel with 200 ml each of potassium phosphate buffers of 0.2 M, pH 7.5, 0.2 M, pH 8.0, and finally three times with 0.5 M buffer, pH 8.0. EDTA and dithiothreitol were added to the eluates to give final concentrations of 10−4 M. Although tedious, this step was quite reproducible and routinely gave a 4- to 6-fold purification.

Step VI: DEAE-cellulose Column Chromatography—The clear gel eluates which contained the enzyme activity were combined and passed through the Sephadex G-25 column to equilibrate the solvent with 5 × 10−4 M potassium phosphate, pH 7.5, which contained EDTA and dithiothreitol, both at 10−3 M (starting buffer). The Sephadex effluent was applied directly to a DEAE-cellulose column (2 × 40 cm) which had been equilibrated with the starting buffer. After washing the column with a bed volume of starting buffer, the enzyme could be seen as a thin, bright yellow band at the top of the column.

Elution was carried out by increasing the concentration of KCl and decreasing the pH. A linear gradient apparatus was used: the mixing chamber contained 500 ml of the starting buffer and the reservoir chamber the same volume of 0.5 M KCl in 0.005 M potassium phosphate, pH 6.0, containing the same reagents as the starting buffer. As shown in Fig. 1A, the enzyme activity was eluted from the column as a single component, which corresponded well with a protein peak. Later, this step was modified with improved results. Instead of the Sephadex filtration, the calcium phosphate gel eluate was simply diluted 4-fold with triply distilled water and directly applied onto the DEAE-cellulose column. The concentration of potassium phosphate in the starting buffer was raised from 0.005 M to 0.2 M, at pH 7.6. The eluting buffer contained 0.3 M KCl and 0.2 M potassium phosphate, at pH 6.0. Under these conditions virtually all of the remaining nonaminotransferase protein was in the column wash, and the activity and the remaining protein chromatographed as a single peak (Fig. 1B).

At this stage the preparation is quite dilute, and very unstable, so the combined active fractions were immediately diluted 3-fold with starting buffer and passed through a DEAE-cellulose column (2.5 × 3 cm) equilibrated with the same buffer. The enzyme was then eluted in a concentrated solution with a small volume of the standard eluting buffer.

Depending on the degree of purity prior to this step, a 10- to 20-fold purification was obtained. Generally, recovery was 50 to 70%.

Step VII: Crystallization—The enzyme was precipitated by the addition of solid ammonium sulfate to 75% saturation and redissolved in a small volume of 0.2 M potassium phosphate, pH 7.5, containing the four protectors. A saturated solution of ammonium sulfate was added very slowly to the concentrated enzyme until the solution became slightly turbid. The solution was kept in a refrigerator for 2 days, during which time a few more drops of saturated ammonium sulfate solution were added. Most of the enzyme precipitated as fine, short needles. Although we failed to obtain better crystals in a test tube, dendritic, needle-like crystals were obtained when a drop of enzyme solution having a specific activity higher than 100 and containing 40 to 50% ammonium sulfate was kept under a cover glass on a microscope slide for several hours at room temperature (Fig. 2). Examination of these crystals by electron microscopy confirmed the crystalline structure but failed to reveal any periodicity or subunit structure. The crystals were collected by centrifugation, redissolved in a small volume of Buffer A, and kept frozen at −20°C. Since this procedure did not enhance purification, it was omitted in later preparations and the concentrated enzyme was stored at −20°C.

We routinely obtained 600- to 700-fold purification of the induced crude extract (about 12,000-fold relative to the basal, uninduced level). The highest specific activity observed was over 140. In early attempts, in which crystallization was included, only 10 to 15% of the activity was recovered, but later recovery approached 30%.
Physical Characteristics

Homogeneity of Final Preparation—A single precipitin line formed on an Ouchterlony double diffusion agar plate between the well of purified enzyme and that of the rabbit antiserum prepared against it. (The preparation of the antibody and characterization of the antibody-antigen reaction will be described in a subsequent communication.)

The purity of the preparation and the sedimentation coefficient were determined with a Spinco model E ultracentrifuge equipped with schlieren optics. Enzyme solution (0.6 ml) was centrifuged in a double sector cell at 59,780 rpm at 18° and pictures were taken at 8-min intervals. The solvent contained 0.15 M KCl, 0.02 M potassium phosphate, at pH 7.0, and the four protectors. Runs were carried out at protein concentrations of 1, 2, and 4 mg per ml. The purified enzyme appeared to migrate as a single, symmetrical peak (Fig. 3) with an \( s_{20,w} \) of 5.9. However, there was a suggestion of a very small amount of a slower moving component visible in certain photographs of this experiment (e.g., Fig. 3B).

Electrophoresis on cellulose acetate strips in Tris-glycine buffer, pH 8.3, containing \( 10^{-3} \) M EDTA, \( 2 \times 10^{-3} \) M \( \alpha \)-keto glutarate, and \( 10^{-5} \) M pyridoxal phosphate, revealed a single, sharp major protein band and a faint component near the origin.

Disc-gel electrophoresis in 7.5\% polyacrylamide gel was performed by a modification of the method of Ornstein and Davis (13). The enzyme (40 \( \mu \)g) was applied on top of the spacer gel in 50\% sucrose. After the run, protein was stained with 0.25\% Amido-Schwarz in 7\% acetic acid. To locate enzyme activity, duplicate gels were immersed at room temperature in 1 ml of Assay Mixture I, also containing 1 \( \mu \)mole of DPN, 0.3 mg of crystalline glutamate dehydrogenase, 20 \( \mu \)g of phenazine methosulfate, and 0.8 mg of p-iodonitrotetrazolium violet. After an adequate color development, usually 5 to 10 min, the gel was washed and stored in 7\% acetic acid. This procedure revealed three major protein bands, all of which had aminotransferase activity, and a faint band without activity near the cathode which appeared to represent less than 5\% of the stained material.

Stability—At any stage of the purification, aminotransferase activity is stable to freezing and thawing if the total protein concentration is kept above 5 mg per ml. Purified enzyme loses 40 to 50\% of its activity over a period of months when stored frozen at concentrations of 1 to 2 mg per ml. In very dilute solutions (1 to 5 \( \mu \)g per ml) the activity is very unstable even in the cold. This inactivation can be prevented by the addition of albumin at a concentration of 5 mg per ml. Albumin also protects the dilute enzyme against inactivation by freezing and thawing. Preliminary experiments suggest that both pyridoxal phosphate and \( \alpha \)-keto glutarate enhance the inactivation of dilute enzyme at 0° or -15°, but that this effect may be prevented by the addition of albumin to the solution.

Several authors have reported that \( \alpha \)-keto glutarate protects tyrosine aminotransferase against heat inactivation (4, 6, 14, 15), and Ogasawara, studying kynurenine aminotransferase, has
shown that the combination of pyridoxal phosphate and α-ketoglutarate gives slightly better protection than the former alone (15). Holten and Kenney have noted that pyridoxal phosphate stabilizes tyrosine aminotransferase to heat (17). Fig. 4 shows that α-ketoglutarate, when added to crude liver extracts, affords good protection of aminotransferase activity against heat inactivation and that pyridoxal phosphate gives essentially complete recovery of activity when the extract is heated at 60° for 5 min in 0.1 M potassium phosphate, pH 7.0. The combination of the two reagents gives no better protection than pyridoxal phosphate or pyridoxamine phosphate alone. On the other hand, pyridoxal phosphate, but not α-ketoglutarate, protects purified apoenzyme against heat inactivation. These results show that in crude extracts α-ketoglutarate functions as a “protector” by converting the amino form (see below) of the cofactor to the aldehyde form.

Molecular Weight—The molecular weight of the enzyme was determined by short column sedimentation equilibrium (18) with the use of a Spinco model E ultracentrifuge equipped with Rayleigh interference optics. Three different enzyme concentrations, 0.5, 1.0, and 2.0 mg per ml, were centrifuged simultaneously in a 6-hole cell (18) at 17,250 rpm at 18°. The solvent was the same as that used for the sedimentation velocity analysis. Interference patterns were photographed with Kodak spectroscopic plates type II-G at 40 hours to insure that equilibrium was established. Measurements of the fringe patterns were made with a Nikon two-dimensional microcomparator. The plot of log protein concentration against the square of the distance from the center of rotation was virtually linear (Fig. 5). From these data, assuming a partial specific volume of 0.74, a molecular weight for this preparation of 91,000 was calculated.

Absorption Spectra—Spectroscopic analyses were carried out in a Cary model 14 spectrophotometer in quartz cells with a 1.0-cm light path. The complete absorption spectrum of the enzyme is shown in Fig. 6. In addition to the peak at 327 μ, which has been attributed to a phosphopyridoxalaldimine group (19-22), an absorption peak at 327 μ was observed.
The latter is near the wave length for absorption of derivatives of the phosphopyridoxal form when the aldimine bond is saturated, and for the phosphopyridoxamine form of the enzyme (23, 24). The characteristic absorption maximum at 277 m\(\mu\), contributed by aromatic amino acid residues, was also seen. The absorption ratio, \(A_{277}/A_{425}\), was 8.9 at pH 7.0, which is lower than such ratios for other aminotransferases except alanine aminotransferase (22). Assuming that only 1 mole of pyridoxal phosphate is bound per mole of enzyme (see below), this ratio indicates a high degree of purity of the preparation.

Resolution and Reconstitution—Upon the addition of 0.005 M L-tyrosine, the 425 m\(\mu\) peak virtually disappears and the 327 m\(\mu\) peak was markedly increased (Fig. 7). This shift could be reversed by the addition of 0.02 M \(\alpha\)-ketoglutarate (not shown). When the tyrosine-treated enzyme was dialyzed for 18 hours at 0-5\(^\circ\) against 0.1 M potassium phosphate buffer at pH 6.0, the peak at 327 m\(\mu\) was no longer detected (Fig. 8), suggesting the resolution of the coenzyme from the enzyme. This method was used to prepare apoenzyme which had no detectable enzymatic activity in the absence of added cofactor.

![Figure 7](image.png)

**Fig. 7.** Absorption spectra of tyrosine aminotransferase with (-----) or without (-----) L-tyrosine, 0.005 M. The buffer was 0.1 M potassium phosphate, pH 6.0; the enzyme concentration was 2.3 mg per ml.

![Figure 8](image.png)

**Fig. 8.** Resolution and reconstitution of tyrosine aminotransferase. The enzyme (1.53 mg per ml) treated with tyrosine (see Fig. 7) was dialyzed for 12 hours against 0.1 M potassium phosphate, pH 6, at 0\(^\circ\) (-----). To this apoenzyme solution, \(\alpha\)-ketoglutarate, 0.003 M (-----) and pyridoxal phosphate, 10\(^{-6}\) M (-----) were added in succession (Fig. 9). Addition of the 5'-phosphates of pyridoxal or pyridoxamine gave 100% reconstitution of activity, but pyridoxal hydrochloride had no such effect, nor did the latter inhibit the reconstitution by pyridoxal phosphate (Fig. 9).

Addition of \(\alpha\)-ketoglutarate to the apoenzyme caused no change in the absorption spectrum but addition of pyridoxal phosphate resulted in the reappearance of the 425 m\(\mu\) peak (Fig. 8). This peak clearly represents bound cofactor since the free coenzyme exhibits absorption maxima at 330 and 388 m\(\mu\).

Pyridoxal Phosphate Content—Two different methods were used to estimate the amount of pyridoxal phosphate bound to the enzyme. The purified enzyme, in a solution containing 2 \(\times\) 10\(^{-4}\) M phosphate, was dialyzed extensively against 0.02 M potassium phosphate, pH 7, containing 0.08 M KCl, to remove unbound cofactor. Aliquots of the dialyzed preparation were then precipitated with an equal volume of 0.6 N perchloric acid to remove the bound cofactor from the enzyme. The precipitate was washed once with 0.3 N perchloric acid and the wash was added to the 0.6 N perchloric acid supernatant. The free pyridoxal phosphate in the perchloric acid was then reacted with KCN to form the highly fluorescent cyanohydrin (24). The average value determined in seven such experiments gave 1 mole of pyridoxal phosphate bound per 93,900 g of enzyme.

Catalytically inactive apoenzyme, prepared as described under "Enzyme Cofactor Interaction," was titrated spectrophotometrically with pyridoxal 5'-phosphate in 0.002 M potassium phosphate buffer, pH 7.0, containing 0.01 M \(\alpha\)-ketoglutarate. This procedure takes advantage of the spectral shift of pyridoxal phosphate coincident with protein binding (i.e. 388 m\(\mu\) to 425 m\(\mu\)).
Reconstitution of apoenzyme activity. A, 2 x 10^-4 M pyridoxamine 5'-phosphate. B, 2 x 10^-4 M pyridoxal 5'-phosphate. C, no addition at time zero, 2 x 10^-4 M pyridoxal 5'-phosphate at time indicated by the arrow. D, 2 x 10^-4 M pyridoxal HCI at time zero, 2 x 10^-4 M pyridoxal 5'-phosphate at time indicated by the arrow. The initial "rates" in C and D are equal to that when no apoenzyme is added to the reaction mixture. Tyrosine aminotransferase activity, in milliunits, is shown on the ordinate, time after starting the reaction on the abscissa.

Tyrosine aminotransferase reactions are complicated, multistep processes. Their complete kinetic analysis requires that velocity-substrate relationships for a given reactant be studied at several concentrations of the other substrates. In the present work this was not done and the dependence of velocity on substrate concentration was evaluated only at saturating levels of the other substrates. Therefore, the reactant concentrations at which half-maximal velocity was achieved are referred to as the "apparent" Michaelis constant, or $K_m$.

A comparison of the apparent Michaelis constants calculated by the double reciprocal plot method of Lineweaver and Burk (25) for both Assays II and III is shown in Table II. The saturation curve and inverse plot for tyrosine is shown in Fig. 11A. The apparent $K_m$ for $\alpha$-ketoglutarate is 7 x 10^-4 M in Assay III and about three times lower in Assay II. No significant substrate inhibition was noted when up to 2 x 10^-3 M $\alpha$-ketoglutarate was used (Fig. 11B). Kenney also failed to see such inhibition (3), in contrast with the situation found in dog liver tyrosine aminotransferase, as reported by Cannelakis and Cohen (26).

**TABLE II**

<table>
<thead>
<tr>
<th>Factor limited</th>
<th>Assay II</th>
<th>Assay III</th>
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</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>1.67 x 10^-3 M</td>
<td>1.43 x 10^-3 M</td>
</tr>
<tr>
<td>$\alpha$-Ketoglutarate</td>
<td>2.1 x 10^-4 M</td>
<td>6.7 x 10^-4 M</td>
</tr>
<tr>
<td>Pyridoxal phosphate</td>
<td>1.1 x 10^-4 M</td>
<td>1.67 x 10^-4 M</td>
</tr>
<tr>
<td>Pyridoxamine phosphate</td>
<td>4.5 x 10^-4 M</td>
<td>1.54 x 10^-2 M</td>
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</table>
DISCUSSION

Tyrosine aminotransferase has been purified 600- to 700-fold from crude extracts of rat livers in which the enzyme has been induced by prior administration of a glucocorticoid hormone. This preparation appears homogeneous by several different criteria, but small amounts of contaminating enzymatically inactive material were seen on paper and disc gel electrophoresis. In addition, three enzymatically active bands were seen by the latter method, and their significance is unknown at present. This could result from aggregation during the preparative or disk gel electrophoresis procedures. However, Martinez-Carrion et al. (28) have reported that a glutamate-aspartate aminotransferase preparation, which appeared homogeneous by ultracentrifugation and free boundary electrophoresis, could also be separated into three electrophoretic components on starch gel which, although identical with respect to amino acid composition, pyridoxal phosphate content, and reaction with antibody, differed in specific activity and spectral properties. A similar situation might exist with tyrosine aminotransferase.

The absorption spectrum of the purified enzyme indicates that pyridoxal phosphate is bound to the protein and two different quantitative methods show that 1 mole of pyridoxal phosphate is bound per 94,000 g of enzyme. This value is similar to those of several other highly purified mammalian pyridoxal phosphate-containing enzymes such as phosphorylase (1 mole/125,000 g) (29), alanine aminotransferase (1 mole/85,000 g) (21), serine transhydroxymethylase (1 mole/83,000 g) (30), and leucine aminotransferase (1 mole/75,000 g) (15).

A protein of the size estimated for tyrosine aminotransferase (90,000) is quite likely to be composed of subunits. The finding of only 1 mole of cofactor bound for 94,000 g of protein suggests that these subunits are not identical. However, it is possible that the ratio was underestimated because of incomplete resolution of the cofactor during production of the apoenzyme.

The absorption maximum seen at 425 mp is assumed, by analogy to other aminotransferases, to represent the phosphorylaldehyde form of the enzyme. Although absorption at 327 mp could be due to the phosphopyridoxic acid form, the derivative formed between enzyme, cofactor, and tyrosine will also absorb at this wave length. Under the conditions of these experiments the latter is probably the predominant form. The cofactor can easily be removed from the enzyme by dialysis against phosphate buffer after treatment with L-tyrosine. Similarly, Scardi et al. (31) have shown that glutamate-aspartate aminotransferase can be resolved by phosphate ion only when it is in the phosphopyridoxaminic form, and Wada and Snell (32) showed that this form is in general more readily displaced from that enzyme than is the phosphopyridoxyl group. The binding of the phosphopyridoxic form is presumed to be primarily ionic (93), while the binding of the aldehyde form is both ionic and covalent, and therefore much stronger.

The resolution of the cofactor from the enzyme is completely reversible, as shown by spectral and activity studies (cf. Figs. 8 and 9). The 5'-phosphates of both pyridoxal and pyridoxamine were equally effective in completely restoring activity to the apoenzyme, while pyridoxal HCl had no effect, as in the case of the glutamate-aspartate (34) and d-alanine-d-glutamate (35) aminotransferases.

The pH activity curve of tyrosine aminotransferase reported here agrees with that reported by others (3, 36), and activity was not significantly different in a variety of different buffers. No attempt to determine the true Michaelis constants for the components of the tyrosine aminotransferase reaction was made in this study. The apparent $K_m$ values presented herein are

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**Fig. 11.** Effect of substrates on tyrosine aminotransferase activity. Saturation curves and double reciprocal plots are shown. The substrate concentrations were varied as indicated; other conditions are as described for Assay III in "Methods." The concentration of the apoenzyme solution used was 1 mg per ml. Enzyme (0.02 $\mu$g) was used in each assay and the activity is expressed as units per milliliter of enzyme solution. A, effect of tyrosine concentration. B, effect of $\alpha$-ketoglutarate concentration.

**Fig. 12.** Effect of pyridoxal 5'-phosphate on tyrosine aminotransferase apoenzyme activity. Cofactor concentration varied as indicated; other conditions are as described for Assay III in "Methods." The concentration of the apoenzyme solution used was 1 mg per ml. Enzyme (0.02 $\mu$g) was used in each assay and the activity is expressed as units per milliliter of enzyme solution. A, saturation curve and double reciprocal plot. B, double reciprocal plots of various pyridoxal 5'-phosphate concentrations at: 1, no added potassium phosphate; 2, 0.1 M potassium phosphate; 3, 0.4 M potassium phosphate.

Table II shows that the apparent $K_m$ values for pyridoxal phosphate (1.67 x 10$^{-8}$ M) and for pyridoxamine phosphate (1.54 x 10$^{-7}$ M) are 100 and 30 times lower, respectively, in Assay III than in Assay II. These striking differences between the kinetic constants observed with Assays II and III are probably largely due to the interaction of borate (used only in Assay II) with the cofactor, as noted above in "Methods."
in good agreement with those found by others for tyrosine (3, 6, 36) and \( \alpha \)-ketoglutarate (3, 36). However, the apparent \( K_m \) for pyridoxal phosphate (Assay III) was distinctly lower than that calculated by Kenney (3), Litwack, Winicov, and Squires (36), and ourselves with Assay II. This is undoubtedly accounted for by the fact that all of these determinations were done in the presence of phosphate buffer in concentrations considerably greater than the \( K_i \) for the phosphate inhibition of enzyme activity. As mentioned above, the formation of the cofactor-borate complex probably also contributed to the spurious values obtained from Assay II.

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