Purification and Properties of Halogenated Tyrosine and Thyroid Hormone Transaminase from Rat Kidney Mitochondria*

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SUMMARY

Halogenated tyrosine transaminase has been purified 30-fold from a sonic extract of rat kidney mitochondria. Purification involves ammonium sulfate fractionation and chromatography on diethylaminoethyl cellulose and hydroxylapatite. The purified enzyme is highly specific for tyrosine analogues with halogen substituents in positions 3 or 5, for thyroid hormones, and, to a lesser extent, for naturally occurring aromatic amino acids such as tyrosine, tryptophan, and phenylalanine. The identity of the enzyme and thyroid hormone transaminase has been confirmed.

Halogenated tyrosine transaminase requires pyridoxal 5'-phosphate as a coenzyme and α-ketoglutarate as amino acceptor.

Inhibition studies indicate that a sulfhydryl group is essential for enzyme activity. 3,5-Diiodo-4-hydroxyphenylacetate has been found to be a powerful inhibitor of the enzyme, functioning as a mixed type of inhibitor.

The reaction catalyzed by this enzyme is: halogenated tyrosine + α-ketoglutarate → halogenated β-hydroxyphenylketoc acid + glutamate.

In earlier reports from this laboratory it was concluded that rat kidney mitochondria contain at least two enzyme systems concerned with the deamination of thyroid hormones, an oxidase (1) and a transaminase (2, 3). The L-amino acid oxidase has been crystallized from rat kidney mitochondria (4) and shown to deaminate thyroid hormones. During purification, the transaminase was found to resemble the enzyme which catalyzes transamination between diiodotyrosine and α-ketoglutarate (2, 3).

In the studies of the transaminase, the activities of the enzyme were estimated from the rates of degradation of 131I-labeled thyroid hormones and diiodotyrosine with the use of paper chromatography and radioautography. However, these methods are too complicated and inconvenient for the assay of enzyme activity at the many stages of purification. The present report describes the assay of transaminase activity during purification, and provides evidence which establishes the identity of the thyroid hormone transaminase with halogenated tyrosine transaminase.

EXPERIMENTAL PROCEDURE

Reagents—3,5-Diiodo-L-tyrosine, 3,5-dibromo-L-tyrosine, 3-iodo-L-tyrosine, 3,4-dihydroxy-L-phenylalanine, 3-amino-L-tyrosine, tyrosine (L and D configurations), L-phenylalanine, L-tryptophan, p-fluoro-DL-phenylalanine, 3-nitro-L-tyrosine, 3,5-dinitro-L-tyrosine, and 3,5-diido-L-4-hydroxyphenylpyruvic acid were obtained from Nutritional Biochemicals. Phenylpyruvic acid and p-hydroxyphenylpyruvic acid were purchased from Mann and from Tokyo Dainichi Seiyaku, respectively. Pyridoxal 5'-phosphate, α-ketoglutarate, oxalacetate, pyruvate, aromatic lactic acid analogues (Dl configuration), sulfhydryl reagents (o-iodosobenzoate and p-chloromercuribenzoate), crystalline protein samples (bovine blood hemoglobin, human γ-globulin, and bovine serum albumin), and glutamic dehydrogenase were obtained from Sigma. 131I-Labeled triiodothyronine and thyroxine were purchased from Abbott. 3,5-Diido-D-tyrosine was prepared from D-tyrosine by the same procedure as that employed in the iodination of L-tyrosine (5). The synthesis of 3,5-dibromo-4-hydroxyphenylpyruvic acid and 3,5-dichloro-4-hydroxyphenylpyruvic acid, based on the method of Bergmann, Zervas, and Lebrecht (6), is presented later in this report. 3-Iodo-4-hydroxyphenylpyruvic acid (7), 3,5-diido-4-hydroxyphenylpyruvic acid (8), and 3,5-diido-4-hydroxyphenylacetic acid (9) were prepared by established methods.

Assay—Transaminase activity was determined by a modific-
Keto acid (25 pmoles) was dissolved quickly in 0.25 ml of 0.1 M sodium phosphate buffer (pH 6.5). All components except 1-ketoglutarate were preincubated for 5 min at 37°C. The reaction was initiated by addition of ketoglutarate. After incubation for 10 to 210 min at 37°C, the reaction was stopped by the addition of 0.1 ml of 6 M H2SO4. A 0.7-ml aliquot was added to 2 ml of 2 M arsenate at pH 0.5 (control), and another 0.7 ml was added to 2 ml of 2 M arsenate with 1 M borate at pH 6.5 (enol-borate sample).

The cuvettes were placed in a Beckman DU spectrophotometer at 23°C until maximal formation of each enol-borate complex of the keto acid had occurred. Readings were taken at the maximal absorbance of the enol-borate complex. Under the conditions employed, the following molar extinction coefficients of the main keto acid-borate complexes at optimal absorbance were obtained: 3,5-diodo-4-hydroxyphenylpyruvic acid, 8,110 at 330 nm; 3,5-dibromo-4-hydroxyphenylpyruvic acid, 9,000 at 330 nm; 3,5-dichloro-4-hydroxyphenylpyruvic acid, 9,110 at 325 nm; 3-iodo-4-hydroxyphenylpyruvic acid, 10,400 at 310 nm; 3-indolpyruvic acid, 14,000 at 332 nm; phenylpyruvic acid, 9,650 at 300 nm; and 3',3,5-triiodothyronine) were the same as the standard incubation mixture, except that 10 μmoles of thyroid hormone labeled with 131I in positions 3' and 5' were used as the substrate. At the end of 30 min of incubation the rates of degradation of these hormones were determined chromatographically as described in a previous report (2). A unit was defined as the quantity of enzyme required for the degradation of 1 μmole of thyroxine or triiodothyronine in 30 min. Specific activity was expressed in units per mg of protein.

### RESULTS

**Purification of Transaminase**

In preliminary studies it was found that the enzyme is inhibited by heavy metals and sulphydryl reagents. Therefore EDTA or 2-mercaptoethanol or both were added to all buffers used in column chromatography and dialysis. Unless otherwise noted, all procedures were performed at 0-4°C; enzyme preparations treated with ammonium sulfate were centrifuged at 20,000 × g for 20 min. The details of a typical procedure are listed in Table I.

**Disintegration of Mitochondria**—Mitochondrial sediments obtained from rat (Sprague-Dawley strain) kidneys (100 to 115 g, wet weight) were disintegrated by means of a sonic oscillator and, as described previously (4), soluble extracts were obtained by centrifugation.

**First Ammonium Sulfate Fractionation**—The soluble extract (150 to 180 ml) was fractionated by successive addition of solid ammonium sulfate and adjustment of the pH to about 7.0 with 2 N NH4OH. In each fractionation, the salt was added over a 10-min interval with continuous mechanical stirring, and stirring was continued for 30 min before centrifugation. The precipitates obtained by increasing ammonium sulfate from 0 to 0.33 and from 0.33 to 0.45 saturation manifested little transaminase activity and were discarded. The clear brownish supernatant fluid contained most of the transaminase activity present in the soluble extract. The enzyme was precipitated from the supernatant by increasing the ammonium sulfate to 0.65 saturation and adjusting the pH to about 7.0 with 2 N NH4OH. The precipitate formed was centrifuged, dissolved in 5 mM hydrochloric acid-phosphate buffer containing 1 mM EDTA and 1 mM 2-mercaptoethanol at pH 7.4, and dialyzed against 3 liters of the same buffer for 20 hours.

**DEAE-cellulose Fractionation**—The enzyme preparation was transferred to a DEAE-cellulose column (2.5 × 15 cm) pre-equilibrated with 5 mM-HCl-phosphate buffer which contained 1 mM EDTA and 1 mM 2-mercaptoethanol (pH 7.4), and then eluted with 150 ml of the same buffer. Under these conditions the enzyme passed through the column without being adsorbed. Solid ammonium sulfate was added to the eluate to 0.7 saturation of excess catalase. This was followed by spectrophotometric estimation of the keto acid formed.

A unit was defined as the quantity of enzyme required for the formation of 1 μmole of the aromatic keto acid in 30 min under the above conditions. The specific activity was expressed in units per mg of protein. Protein was usually estimated by the phenol biuret method (12), with bovine serum albumin as the standard. Unless otherwise noted, all specific activities and units of enzyme preparations were measured with 2.5 mM diiodothyronine in the standard reaction mixture.

The incubation media for thyroid hormones (thyroxine and 3',3,5-triiodothyronine) were the same as the standard incubation mixture, except that 10 μmoles of thyroid hormone labeled with 131I in positions 3' and 5' were used as the substrate. At the end of 30 min of incubation the rates of degradation of these hormones were determined chromatographically as described in a previous report (2). A unit was defined as the quantity of enzyme required for the degradation of 1 μmole of thyroxine or triiodothyronine in 30 min. Specific activity was expressed in units per mg of protein.

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total activity (μg)</th>
<th>Specific activity (μg/ml)</th>
<th>Total protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Sonic extract</td>
<td>322.0</td>
<td>1040.2</td>
<td>0.50</td>
<td>2078.0</td>
</tr>
<tr>
<td>II. First ammonium sulfate precipitate</td>
<td>43.0</td>
<td>483.0</td>
<td>0.93</td>
<td>521.2</td>
</tr>
<tr>
<td>III. DEAE-cellulose column eluate</td>
<td>35.5</td>
<td>245.0</td>
<td>2.30</td>
<td>106.5</td>
</tr>
<tr>
<td>IV. Second ammonium sulfate precipitate</td>
<td>0 to 0.45</td>
<td>6.7</td>
<td>16.0</td>
<td>12.0</td>
</tr>
<tr>
<td>0.45 to 0.58</td>
<td>8.5</td>
<td>196.0</td>
<td>2.80</td>
<td>70.0</td>
</tr>
<tr>
<td>0.58 to 0.70</td>
<td>8.5</td>
<td>7.2</td>
<td>0.36</td>
<td>19.9</td>
</tr>
<tr>
<td>V. Hydroxylapatite column eluate</td>
<td>16.0</td>
<td>62.0</td>
<td>16.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>
tion. The precipitate was collected by centrifugation, dissolved in 5 mM sodium phosphate buffer with 1 mM EDTA and 1 mM 2-mercaptoethanol at pH 6.5, and dialyzed against the same buffer (3 liters) for 20 hours.

Second Ammonium Sulfate Fractionation—The dialyzed preparation obtained from approximately 200 g of rat kidney was diluted to obtain a protein concentration of approximately 3.5 mg per ml with 5 mM sodium phosphate buffer, containing 1 mM EDTA and 1 mM 2-mercaptoethanol, and subjected to successive ammonium sulfate treatments. Finely powdered ammonium sulfate was added slowly to the above solution to 0.45 saturation. After addition of the ammonium sulfate the solution was stirred for 15 min and centrifuged. To precipitate most of the enzyme, the ammonium sulfate was increased from 0.45 to 0.58 saturation. The precipitate was collected by centrifugation. Additional ammonium sulfate was then added to the supernatant to obtain 0.65 saturation, and the precipitate was collected by the same procedure. The precipitate from each ammonium sulfate treatment was dissolved in a small amount of 0.1 M potassium phosphate buffer containing 1 mM 2-mercaptoethanol and dialyzed against 3 liters of the same buffer for 20 hours. The fraction having the highest specific activity (0.45 to 0.58) was then used for further purification.

Hydroxylapatite Column Chromatography—The enzyme preparation was transferred to a hydroxylapatite column (2.5 × 16 cm) which had been pre-equilibrated with 0.1 M potassium phosphate buffer containing 1 mM 2-mercaptoethanol, and was purified by linear gradient elution. The mixing chamber contained 250 ml of 0.1 M potassium phosphate buffer containing 1 mM 2-mercaptoethanol, pH 6.5. The reservoir held 250 ml of 0.5 M potassium phosphate buffer with 1 mM 2-mercaptoethanol at pH 6.5. The flow rate was approximately 0.5 ml per min. Typical results are shown in Fig. 1. The most active fractions, i.e., those above 15.6 in terms of units of absorbance at 280 nm, were combined. The enzyme was then precipitated by the addition of solid ammonium sulfate to 0.7 saturation. The precipitate was dissolved in 50 mM sodium phosphate buffer (containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.15 M NaCl) to obtain approximately 4 mg of protein per ml, and was dialyzed against 3 liters of the same buffer for 20 hours. The enzyme at this stage, stored at 0°C, lost 40% of its activity by the end of 1 week.

Attempts to purify this preparation further by gel filtration on a Sephadex G-100 column were unsuccessful.

Criteria of Enzyme Purity

Preliminary attempts to purify the preparation further at Stage 3 (DEAE-cellulose) by chromatography on carboxymethyl cellulose or on a hydroxyapatite column (both with stepwise elution) were uniformly unsuccessful. However, purification of the enzyme could be achieved by the method described above.

Study of the purified enzyme revealed only one peak with $s_{20,w}$ of 5.9 with spreading during sedimentation (Fig. 2), suggesting contamination by an impurity. In concentrated solution the purified enzyme was slightly yellow without observable light absorption in the visible spectrum, thereby indicating an absence of heme compounds and conjugated pyridoxal phosphate. The ultraviolet absorption of the purified enzyme (Fig. 3) shows a maximum at 281 nm and a ratio of absorbances at 280 and 260 nm of 1.75, indicating the absence of nucleotides and nucleic acids (13).
Enzyme Reaction

Reaction Product—3,5-Diiodo-4-hydroxyphenylpyruvic acid was identified as a product of the diiodotyrosine-α-ketoglutarate transamination by its absorption spectrum and chromatographic properties. To obtain enough of the product, 6 units of the enzyme (specific activity, 13 units per mg of protein) were incubated for 50 min at 37° with 6 mM L-diiodotyrosine, 10.7 mM α-ketoglutarate, 51 μM pyridoxal phosphate, 70 mM sodium phosphate buffer (pH 6.5), 0.7 mM EDTA, and 0.7 mM 2-mercaptoethanol, in a final volume of 3.0 ml. At the end of incubation the reaction mixture was acidified to pH 1.0 with H₂SO₄ and extracted three times with 4 volumes of peroxide-free ether. The pooled ether extract was washed with water and dried over anhydrous sodium sulfate. The ether was allowed to evaporate and the residue was dissolved in 3 ml of methyl alcohol. An aliquot of this solution was applied to Whatman No. 1 paper, chromatographed in pyridine-acetic acid-water (10:1:89) at 23° for 4 hours and sprayed with 2,4-dinitrophenylhydrazine and Na₂CO₃ according to the method of Shiba and Cahnmann (14). Chromatography revealed two color zones which had the same Rf values as authentic 3,5-diiodo-4-hydroxyphenylpyruvic acid (0.8) and 3,5-diiodo-4-hydroxybenzaldehyde (0.63), respectively. The latter is considered to be an artifact formed during chromatography. This interpretation is supported by the facts that (a) the absorption spectrum of the metabolite in methyl alcohol is almost identical with that of an authentic sample of 3,5-diiodo-4-hydroxyphenylpyruvic acid in the region between 250 and 305 μm and does not show the two peaks (at 260 and 345 μm) specific for an authentic sample of 3,5-diiodo-4-hydroxybenzaldehyde, and (b) an authentic sample of this keto acid is partially decomposed to the aldehyde analogue during the chromatography herein described. Other aromatic keto acids, formed enzymatically from their corresponding amino acids, were identified by the specific absorption of their enol-borate complexes.

Stoichiometry of Reaction—The stoichiometry of the diiodotyrosine transaminase reaction was then determined. A reaction mixture containing 0.42 unit of enzyme (specific activity, 14 units per mg of protein), 0.31 mM EDTA, 31.3 mM sodium phosphate buffer (pH 6.0), 0.5 mM L-diiodotyrosine, 2.0 mM α-ketoglutarate, and 25.5 μM pyridoxal phosphate, in a final volume of 1.6 ml, was incubated for varying periods of time at 37° in air. The reaction mixture was tested in several replicates. The reaction was stopped by adding 0.1 ml of 6 N H₂SO₄. The aromatic keto acid formed was identified spectrophotometrically as the enol-borate complex. The acidified replicate reaction mixtures used for glutamic acid and α-ketoglutaric acid measurements were brought to the appropriate pH with 5.0 N NaOH. The formation of glutamic acid and the disappearance of α-ketoglutaric acid were determined with glutamic dehydrogenase in the presence of NAD⁺ and NADH, respectively (15). As shown in Fig. 4, the amount of 3,5-diiodo-4-hydroxyphenylpyruvic acid formed almost coincided with the glutamic acid formed (the difference was less than 10%) during 1 hour of incubation. Breakdown of 3,5-diiodo-4-hydroxyphenylpyruvate probably occurred after the compound had reached maximum levels, whereas conversion of α-ketoglutaric acid to glutamic acid continued up to 3½ hours. At the end of 3½ hours of incubation, 0.75 μmole of glutamic acid and 0.4 μmole of the corresponding keto acid were formed. Attempts to demonstrate the formation of diiodotyrosine and α-ketoglutaric acid from 3,5-diiodo-4-hydroxyphenylpyruvic acid and glutamic acid (reverse reaction) were not successful, because of spontaneous decomposition of synthetic 3,5-diiodo-4-hydroxyphenylpyruvic acid in the reaction mixture. Under similar conditions, a relationship between the formation of keto acid from dibromotyrosine (or dichlorotyrosine) and from glutamic acid was observed (Table II). The amount of the keto acid formed from dibromo- or dichlorotyrosine always exceeded by a slight amount (less than 10%) that of the glutamic acid formed until keto acid formation reached its maximum. The conversion of dibromotyrosine (or dichlorotyrosine) to the corresponding keto acid was completed during 2 hours of incubation, judging from the amount of glutamic acid formed and the quantity of halogenated tyrosine initially added. The results herein described suggest that, under the conditions employed, no significant decomposition of the halogenated keto acids occurred during 1 hour of incubation, and that the bromo- and chloroketo acids were more stable than the iodoketo acids.

Properties of Enzyme

Substrate Specificity—A number of aromatic amino acids were examined as possible substrates of the enzyme. As shown in Table III, tyrosine analogues with halogen (iodine, bromine, and chlorine) substituents in positions 3 and 5 were the most potent substrates. Under the assay conditions employed, the formation of keto acid analogues from their corresponding halogenated

![Fig. 4. Stoichiometry of the diiodotyrosine transaminase reaction.](image-url)
tyrosines was found to be proportional to the enzyme concentration in the range from 30 to 80 μg, and proved to be a linear function of time during 30-min periods. As substrate, 3-iodotyrosine was 70% as active as diiodotyrosine. The enzyme preparation exhibited very low but measurable activity toward phenylalanine, p-fluorophenylalanine, and tryptophan. Such low level, nonspecific transamination might be characteristic of this enzyme, in keeping with many other pyridoxal-dependent enzymes (16). Deamination of thyroid hormones was also catalyzed by the highly purified enzyme in the presence of added pyridoxal phosphate and α-ketoglutarate (specific activities of 164 for thyroxine transaminase and 108 for triiodothyronine transaminase), but not when these cofactors were omitted or replaced by NAD⁺. These findings suggest that there is no contamination of L-amino acid oxidase (2) or NAD⁺-dependent thyroid hormone oxidase (17) in this preparation. Transamination did not occur with the following amino acids: D-diiodotyrosine and tyrosine analogues with nitro, amino, or hydroxy substituents in positions 3 or 3 and 5. Oxalacetate, but not pyruvate, substituted to the extent of 30% of α-ketoglutarate as amino acceptor.

Identity of Halogenated Tyrosine Transaminase and Thyroid Hormone Transaminase—In testing the possible identity of both enzymes, diiodotyrosine and thyroxine were chosen as substrates for halogenated tyrosine and thyroid hormone transaminases, respectively; transamination activities were determined at each stage in the purification. Findings are presented in Table IV. Keeping in mind the limits of accuracy of the assay, the ratio of the two activities appeared to be constant at each step of purification after fractionation with ammonium sulfate. Storage of the 30-fold purified enzyme for a full week resulted in a proportional reduction in both activities.

Effect of pH—The curves of activity with respect to pH obtained with halogenated tyrosine are shown in Fig. 5. The optimal pH was near 6.0 when diiodotyrosine was used as the substrate. This was also true in the degradation of diiodotyro-

**Table III**

*Substrate specificity of halogenated tyrosine transaminase*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>λ_max (μm)</th>
<th>Activity relative to diiodotyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-Diiodo-L-tyrosine</td>
<td>330</td>
<td>100.0</td>
</tr>
<tr>
<td>3,5-Diiodo-d-tyrosine</td>
<td>330</td>
<td>0.0</td>
</tr>
<tr>
<td>3,5-Dibromo-L-tyrosine</td>
<td>330</td>
<td>127.0</td>
</tr>
<tr>
<td>3,5-Dichloro-L-tyrosine</td>
<td>325</td>
<td>110.0</td>
</tr>
<tr>
<td>3-Iodo-L-tyrosine</td>
<td>310</td>
<td>76.5</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>310</td>
<td>3.9</td>
</tr>
<tr>
<td>3,5-Dinitro-L-tyrosine</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>3-Nitro-L-tyrosine</td>
<td>315</td>
<td>0</td>
</tr>
<tr>
<td>3-Amino-L-tyrosine</td>
<td>308</td>
<td>0</td>
</tr>
<tr>
<td>3,4-Dihydroxy-L-phenylalanine</td>
<td>335</td>
<td>0.0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>300</td>
<td>3.9</td>
</tr>
<tr>
<td>p-Fluorophenylalanine</td>
<td>298</td>
<td>3.1</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>332</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**Table IV**

*Identity of halogenated tyrosine transaminase and thyroid hormone transaminase*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity, thyroxine transaminasea</th>
<th>Specific activity, diiodotyrosine transaminaseb</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Sonic extract</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>II. First ammonium sulfate precipitate, 0.45 to 0.65</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>III. DEAE-cellulose column eluate, 0.45 to 0.58</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>IV. Second ammonium sulfate precipitate, 0.45 to 0.68</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>V. Hydroxylapatite column eluate, Enzyme at Stage V, aged 1 week</td>
<td>10.4</td>
<td>10.2</td>
</tr>
</tbody>
</table>

* Specific activities of thyroxine transaminase and of diiodotyrosine transaminase were expressed as millimicromoles of thyroxine degraded per 30 min per mg of protein and micromoles of 3,5-diodo-4-hydroxyphenylpyruvic acid formed per 30 min per mg of protein, respectively.

Since this fraction contains a large quantity of the L-amino acid oxidase that catalyzes deamination of thyroxine, the specific activity of thyroxine transaminase measured under these conditions is not a precise value (2).

Fig. 5. Effect of pH on the rate of deamination of halogenated tyrosine. The conditions of incubation and assay were the same as those described in the text, save that the pH of the reaction mixture was varied as indicated. Sodium phosphate buffer was used, except for the experiments at pH 5.8 and below and at pH 8.3, in which acetate buffer and Tris-NaOH buffer were used, respectively. The enzyme used (32 μg of protein) had a specific activity of 15.8. The mixture was incubated for 30 min at 37° with halogenated tyrosine (●—●, diiodotyrosine; ○—○, dibromotyrosine; △—△, dichlorotyrosine; ■—■, moniodotyrosine).

By partially purified transaminase (2). Also, the pH activity curves obtained with dibromotyrosine and dichlorotyrosine were similar to those obtained with diiodotyrosine, with an optimum at approximately 6.5. On the other hand, the curve for moniodotyrosine was rather broad, with a pH optimum between 7.0
and 7.5. Such differences in the optimal pH for dihalogenated tyrosines compared to monoiodotyrosine may reflect the influence of the ionization of phenolic hydroxyl groups in these compounds. The pK values for the ionization of the phenolic hydroxyl groups of diiodotyrosine and of dibromotyrosine have been reported to be approximately 6.4 (18), and the pK for dichlorotyrosine is identical with that for dibromotyrosine (19). On the other hand, the pK for the hydroxyl group in monoiodotyrosine (about 8.0) lies between that for tyrosine (10.0) and diiodotyrosine (6.4), judging from the ionization of phenolic hydroxyl groups in several iodine-substituted thyronines (18).

Effect of Substrate Concentration—Fig. 6 indicates that diiodotyrosine inhibited the activity of the enzyme at low concentrations, whereas the other compounds tested did not. The inhibitor constant (K') for a possible enzyme-diiodotyrosine complex and the excess of diiodotyrosine can be calculated from data obtained when I/V is expressed as a function of S (see inset in Fig. 6) in the appropriate equation (20) assuming this to be of the competitive type. The intercept on the base line gives a value for K' of 4.0 × 10^-3 M. This is about three times greater than the K', for diiodotyrosine and the enzymes. K' and V_{max} values for halogenated tyrosines, calculated from intercepts and slopes of the lines in Fig. 6, are summarized in Table V. By extrapolation of these data, the turnover number of the enzyme (aromatic α-keto acid formed, in moles per min per mole of enzyme) was 97.3 with diiodotyrosine, 202.6 with dibromotyrosine, 206.2 with dichlorotyrosine, and 141.8 with monoiodotyrosine, assuming that the enzyme used is of a high degree of purity and has a molecular weight of 80,000 (see below).

Effect of Pyridoxal Phosphate and α-Ketoglutarate—In contrast to tyrosine α-ketoglutarate transaminases from dog liver (21) and from rat brain (22), but like the purified tyrosine α-ketoglutarate transaminase from rat liver (23), the enzyme was not inhibited by high concentrations (2 × 10^-2 M) of α-ketoglutarate (Fig. 7A). The purified enzyme was pyridoxal phosphate-dependent and did not exhibit transaminase activity in the absence of this coenzyme. Lineweaver and Burk (24) plots (see insets, Fig. 7, A and B) yielded K' values of 4.1 × 10^-4 M and 3.7 × 10^-4 M for pyridoxal phosphate and α-ketoglutarate, respectively.

Inhibition—Several aromatic compounds were examined as possible inhibitors of diiodotyrosine transaminase. 3,5-Diiodo-4-hydroxyphenylpyruvic acid formed per 30 min; S, molar concentration of α-keto acid or of pyridoxal phosphate.

FIG. 6. Relation of concentration of halogenated tyrosine to the formation of the corresponding keto acid by purified enzyme during an interval of 30 min. The conditions of the incubation and assay were the same as those in the text, save that pH of sodium phosphate buffer was fixed at the pH which was optimal for the activity of each transaminase (see Table V). Enzyme with a specific activity of 16 (protein, 25 μg) was used. The initial velocity, V, is expressed as molar concentration of aromatic keto acid formed, in moles per min per mole of enzyme. For explanation of symbols see Fig. 5.

\[
\frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{S}{K_i V_{\text{max}}}
\]

FIG. 7. Effect of varying concentrations of A, α-ketoglutarate and B, pyridoxal phosphate on diiodotyrosine transaminase activity. In each experiment the standard reaction mixture was employed, save that the concentration of the component indicated was varied and the pH of the buffer was fixed at 6.2. Enzyme with a specific activity of 16 (protein, 23 μg) was used. The initial velocity, V, is expressed as molar concentration of 3,5-diido-4-hydroxyphenylpyruvic acid formed per 30 min; S, molar concentration of α-keto glutaric acid or of pyridoxal phosphate.
centrations necessary to produce 50% inhibition (Table VI). In the same table, it can be seen that the acetic acid analogue which may be one of the metabolic products of diiodotyrosine in rat kidney or liver produced 50% inhibition at a lower concentration than that of o-iodosobenzoate. Inhibition by the above sulfhydryl reagents could be almost completely prevented by cysteine at 1.0 \times 10^{-4} \text{ M}, whereas that exerted by the acetic acid analogue was not affected by this concentration (untabulated data).

The nature of the inhibition manifested by 3,5-diiodo-4-hydroxyphenylacetate was studied in detail by the methods of Lineweaver and Burk (24) and Hunter and Downs (25). The kinetics of this inhibitor appear to be of the mixed type (Fig. 8.1). The equation for mixed type inhibition proposed by Friedenwald and Maengwyn-Davies (26) was used to determine the inhibitor constants from data plotted by the method of Lineweaver and Burk.

\[ \frac{V_{\text{max}}}{V} = \left( \frac{1 + K_i}{S} \right) \left( 1 + \frac{K_i}{S + \alpha K_s} \right) \]  

where \( I \) and \( S \) are defined as molar concentrations of the inhibitor and substrate, \( V \) is velocity in the presence of the inhibitor, \( K_i \) represents inhibitor constants for the enzyme-inhibitor complex, and \( \alpha \) is an interaction factor.

\[ V_{\text{max}} \text{ and } K_i \text{ (assuming } K_s \text{ to be } K_{\text{app}}) \text{ can be easily calculated from the data of an uninhibited reaction. Insertion of the data for diiodotyrosine (Fig. 8A) into Equation 1 yielded values of } 4.1 \times 10^{-4} \text{ m for } K_i \text{ and approximately } 10 \text{ for } \alpha. \text{ When dibromotyrosine was used as substrate under the same conditions,} \]

**Table VI**

_Effect of some aromatic compounds and sulfhydryl reagents on diiodotyrosine transamination_

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-Diiodo-4-hydroxyphenylacetic acid</td>
<td>6.25 \times 10^{-3}</td>
<td>100</td>
</tr>
<tr>
<td>3,5-Diiodo-4-hydroxyphenylacetic acid*</td>
<td>2.50 \times 10^{-4}</td>
<td>50</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetic acid</td>
<td>6.25 \times 10^{-3}</td>
<td>64</td>
</tr>
<tr>
<td>3,5-Diiodo-4-hydroxyphenyl-L,L-lactic acid</td>
<td>6.25 \times 10^{-3}</td>
<td>100</td>
</tr>
<tr>
<td>4-Hydroxyphenyl-L,L-lactic acid</td>
<td>6.25 \times 10^{-3}</td>
<td>10</td>
</tr>
<tr>
<td>Phenyl-L,L-lactic acid</td>
<td>6.25 \times 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td>Indole-3-L,L-lactic acid</td>
<td>6.25 \times 10^{-4}</td>
<td>0</td>
</tr>
<tr>
<td>o-Iodosobenzoate</td>
<td>6.3 \times 10^{-6}</td>
<td>50</td>
</tr>
<tr>
<td>4-Chloromercuribenzoate</td>
<td>4.5 \times 10^{-6}</td>
<td>50</td>
</tr>
</tbody>
</table>

* The enzyme (0.41 unit with specific activity of 11.0), which had been dialyzed exhaustively against 0.02 M sodium phosphate buffer (pH 6.5), was incubated with the inhibitor in the standard reaction mixture (pH 6.2) containing 2.5 mM diiodotyrosine for 30 min at 37°C. The enzyme was added to the reaction mixture as the last component after the usual 5 min of preincubation. The concentration of the inhibitor to produce 50% inhibition was calculated from the data obtained when \(-\log \text{ concentration (molar)} \text{ of inhibitor is expressed as a function of percentage inhibition.}

**Fig. 8.** Inhibition of A, diiodotyrosine transamination and B, dibromotyrosine transamination by 3,5-diiodo-4-hydroxyphenylacetate. The conditions of the incubation and assay were the same as those in the legend to Fig. 6, save that the inhibitor was added to the reaction mixture at pH 6.2. Numbers over straight lines represent the concentration of the inhibitor. Velocity, \( V \), is expressed as the molar concentration of aromatic keto acid formed from the corresponding amino acid per 30 min, with or without added inhibitor, \( K_i \), molar concentration of halogenated tyrosine; \( K_s \), dissociation constant of the enzyme-diodotyrosine complex (assumed to be \( K_{\text{app}} \)) and \( \alpha \), interaction factor. \( K_i \) values plotted on the vertical axis in A and B insets are those obtained from Equation 1 in the text.

The inhibition by 3,5-diiodo-4-hydroxyphenylacetate also appeared to be of the mixed type. From the data obtained with two different concentrations of the inhibitor (Fig. 8B), \( K_i \) and \( \alpha \) were found to be 7.4 (±1.2) \times 10^{-3} \text{ m and approximately } 2, \text{ respectively. There are many examples in the literature in which the inhibitor is classified as competitive or as noncompetitive when the } \alpha \text{ value is as small as 10 or as large as 2 (26). Data plotted by the method of Hunter and Downs (25) (see inset, Fig. 8, A and B) indicate that the effect of 3,5-diiodo-4-hydroxyphenylacetate on the diiodotyrosine transaminase (Curves 1, Fig. 8A) and that on dibromotyrosine transaminase (Curves 1 and 2, Fig. 8B) are, practically, competitive and noncompetitive, respectively. From these findings, it can be tentatively concluded that the effect of the inhibitor on the transaminase consists of a reduction in the binding affinity of diiodotyrosine which is greater than that observed with dibromotyrosine and that the inhibitor is not conjugated to the binding site of the substrate in the enzyme molecule. The former statement is further supported by the large difference in the } K_m \text{ values of both substrates and the same enzyme (Table V).}

**Molecular Weight**—Gel filtration of the purified enzyme was studied with a view to estimating its molecular weight. The enzyme in 0.02 M sodium phosphate buffer (pH 7.4) with 0.15 M NaCl was added to a column (2.5 x 50 cm) of Sephadex G-100, calibrated according to the method of Andrews (27) with crystalline samples of human \( \gamma \)-globulin, bovine serum albumin contaminated with its dimer, and bovine blood hemoglobin. Elution was performed with the same buffer and fractions were collected and assayed for enzyme activity. When 10 units of enzyme
were added to the column, the elution volume of the enzyme suggested a molecular weight of about 80,000.

Synthesis of Some Halogenated Aromatic Pyruvic Acids

3,5-Dichloro-4-hydroxyphenylpyruvic Acid—3,5-Dichloro-L-tyrosine (3 g), prepared by the method of Zeynek (28), was suspended in ethyl acetate (60 ml) and refluxed with chloroacetyl chloride (1.7 ml) for 80 min. After unreacted amino acid was removed by filtration, the filtrate was concentrated by means of an air current and the resulting crystalline N-chloroacetyl-3,5-dichloro-L-tyrosine was collected by filtration. Recrystallization from a minimum amount of hot ethanol by dilution gave 2.0 g of colorless plates, m.p. 188°-190°.

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The reaction mixture was then transferred into ice water, and brownish crystalline material appeared. This was collected, washed thoroughly with water, and dried over P2O5 under reduced pressure. The yield was 1.2 g of the oxazolone of α-acetylamino-β-(4-acetoxo-3,5-dichlorophenyl)acrylic acid (m.p. 162-164°), which was not further purified.

The crude oxazolone (1 g) was refluxed in a mixture of 6 N HCl (10 ml) and glacial acetic acid (40 ml) for 5 hours to remove the acetyl group in position 4 and to open the unsaturated oxazolone ring. The hydrolysate was then allowed to cool overnight. The resulting keto acid was collected and recrystallized from hot glacial acetic acid. The yield was 0.35 g of colorless needles, m.p. 196° (decomposed).

C8H16OClN
Calculated: C 40.45, H 3.09, N 4.29
Found: C 40.23, H 3.16, N 4.22

N-Chloroacetyl-3,5-dichlorotyrosine (1 g) was dissolved in dry pyridine (8 ml) and treated with acetic anhydride (16 ml) at room temperature for 40 min. The reaction mixture was then transferred into ice water, and brownish crystalline material appeared. This was collected, washed thoroughly with water, and dried over P2O5 under reduced pressure. The yield was 1.2 g of the oxazolone of α-acetylamino-β-(4-acetoxo-3,5-dichlorophenyl)acrylic acid (m.p. 162-164°), which was not further purified.

The crude oxazolone (1 g) was refluxed in a mixture of 6 N HCl (10 ml) and glacial acetic acid (40 ml) for 5 hours to remove the acetyl group in position 4 and to open the unsaturated oxazolone ring. The hydrolysate was then allowed to cool overnight. The resulting keto acid was collected and recrystallized from hot glacial acetic acid. The yield was 0.35 g of colorless needles, m.p. 196° (decomposed).

C8H16OCl
Calculated: C 43.36, H 2.43, Cl 28.47
Found: C 43.39, H 2.57, Cl 28.56

3,5-Dibromo-4-hydroxyphenylpyruvic Acid—3,5-Dibromo-L-tyrosine (2 g) suspended in ethyl acetate (50 ml) was acetylated with chloroacetyl chloride (10 ml). Concentration of the reaction mixture yielded crystalline material. Recrystallization of the material from a mixture of ethanol and water gave 1.45 g of long colorless plates, m.p. 181-182°.

C8H16OBrzClN
Calculated: C 31.95, H 1.91, Br 47.34
Found: C 31.98, H 1.79, Br 47.29

DISCUSSION

The purity of the transaminase herein described is uncertain in view of the data presented. The enzyme has been purified approximately 30-fold, beginning with a soluble extract of rat kidney mitochondria. This is the same order of magnitude of purification as that attained with the mammalian L-amino acid oxidase from the same extract (4). At this stage of purity the enzyme shows a single peak in an ultracentrifuge, with spreading during sedimentation, and is free of nucleic acids or nucleotides.

Investigations of substrate specificity and the effects of sulfhydryl reagents indicate that the enzyme is highly specific for halogenated tyrosines and has a strict requirement for sulfhydryl groups for activity. It is interesting that inhibition by 3,5-diiodo-4-hydroxyphenylacetate is neither purely competitive nor purely noncompetitive, but is, rather, of the mixed type. This inhibitor is probably not bound to catalytic sites but is attached to an adjacent region of the enzyme.

Shiba and Cahmann (14) have reported the breakdown of 3,5 diiodo 4 hydroxyphenylpyruvic acid to 3,5-diiodo-4-hydroxybenzaldehyde and other products in alkaline media. The author has confirmed that all of the dihalogenated 4-hydroxyphenylpyruvates are unstable in aqueous solutions even in sodium phosphate buffer at pH 6.0 to 7.5. It seems, however, that such keto acids formed enzymatically under the conditions used in the present report are considerably more stable. On the basis of spectrophotometric analyses of the ether extracts of the reaction mixtures and the stoichiometry of the reaction, the conversion of the keto acids to their corresponding benzaldehyde and phenylacetic acid homologues appears to be negligible under the conditions used for the enzyme assay, excepting perhaps during prolonged incubation. Knox (29) has reported that 4-hydroxyphenylpyruvic acid formed by tyrosine α-ketoglutarate transaminase is probably in the keto form, while the crystalline keto acid may be in the enol form which would then be converted to the keto form in aqueous solution. This is likely to be the case in the keto acids derived from halogenated tyrosines. Probably, the crystalline halogenated keto acids, when dissolved in buffer at nearly physiological pH, are easily decomposed owing to the instability of their enol forms in aqueous solution.

The following is presented as the general reaction catalyzed by the transaminase.

Halogenated tyrosine + α-ketoglutarate →
halogenated p-hydroxyphenylpyruvic acid + glutamate

The reversibility of this reaction has not been demonstrated. A large number of purified enzymes are found to be inhibited by thyroxine in vitro and in vivo (30). Therefore, one of the physiological roles of this transaminase might be to convert the excess of thyroid hormones in tissue to less active components, such as their corresponding keto acids.

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Purification and Properties of Halogenated Tyrosine and Thyroid Hormone Transaminase from Rat Kidney Mitochondria

Minoru Nakano


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