The Assay of Aromatic Amino Acid Transaminations and Keto Acid Oxidation by the Enol Borate-Tautomerase Method*

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The enol tautomers of the aromatic α-keto acids combine instantaneously and reversibly with borate to form complexes of a mixed ester-anhydride structure, thereby displacing the apparent keto-enol equilibrium in favor of the enol tautomer. The enol tautomer and its borate complex have strong absorption in the 300 mλ region (1). This strong absorption is the basis of the spectrophotometric methods described here for the assay of five rat liver enzymes which catalyze either the formation or utilization of aromatic pyruvates. The principle is a general one by which reactions forming or removing aromatic α-keto acids can be followed by changes in the optical density of the enol borate.

Brief accounts have been published of the use of this method with p-hydroxyphenylpyruvate oxidase (2) and tyrosine-α-ketoglutarate transaminase (3). The present paper describes in greater detail the assays of these two enzymes and of three other enzymes: phenylalanine-pyruvate, histidine-pyruvate, and tryptophan-α-ketoglutarate transaminases. Emphasis has been placed on the identification and control of the factors affecting these enzyme activities in crude preparations of rat liver. The separation of the reactions, one from the other and from other transaminations occurring in the same preparations, will be the subject of a separate communication. With the present assays the levels of the enzymes have been measured in crude liver extracts of rats after various experimental treatments (2, 3).

EXPERIMENTAL

Materials—Keto-enol tautomerase was purified from hog kidneys as described by Knox and Pitt (1). It catalyzed the tautomerization of both phenylpyruvate and pHPP, and was free of demonstrable p-hydroxyphenylpyruvate oxidase and of tyrosine and phenylalanine transaminase activities. Partially purified preparations of p-hydroxyphenylpyruvate oxidase were kindly supplied by Dr. S. E. Hager and Dr. T. Tanaka. L-Amino acid oxidase was the crude dried venom of Agkistrodon piscivorus piscivorus obtained from Ross Allen's Reptile Institute, Silver Springs, Florida.

Phenylpyruvate (4) and indolylpyruvate (5) were synthesized by reported methods. Imidazolylpyruvate was prepared immediately before use by the complete oxidation of known amounts of histidine with the L-amino acid oxidase in the presence of catalase. All other compounds used were obtained from commercial sources. All of the amino acids used were of the L-configuration.

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Preparation of Enzymes—For the assay of all activities except triptophan transaminase, fresh rat livers were homogenized for 2 minutes in a Waring Blender with 9 ml. of 0.005 N NaOH in 0.14 M KCl per gram of tissue. The homogenate was centrifuged for 40 minutes at 13,000 to 14,000 x g. For the less active triptophan transaminase the tissue was homogenized with 4 ml. of the solvent per gram of liver, and the homogenate was centrifuged in a Spinco centrifuge at 100,000 x g for 30 minutes. The clear portion of the supernatant fraction was decanted and used within a few hours for each of the enzyme assays. All operations were carried out at 5° and the enzymes were kept in ice baths. All enzymes showed full activity during several hours under these conditions.

Identification of Reaction Products—Under the conditions of the p-hydroxyphenylpyruvate oxidase assay the disappearance of the 310 mλ absorbing enol and enol borate of pHPP did not result in the accumulation of any products absorbing at this wavelength (Fig. 1). This showed that no significant quantities of homogentisate or maleylacetacetate accumulated. This enzymic reaction leads to the formation of fumarate (malate) and acetacetate (6).

All four transamination reactions here studied required (a) the aromatic amino acid, (b) either pyruvate or α-ketoglutarate, and (c) pyridoxal phosphate. The requirement for the coenzyme was shown by a doubling in activity when pyridoxal phosphate was added to the assay systems for the transamination of tyrosine and triptophan in extracts of normal liver, and to those of phenylalanine and histidine in extracts of livers from pyridoxine deficient rats.

The aromatic α-keto acids formed from the aromatic amino acids by the enzyme transaminations were identified by the keto-enol difference spectra (Fig. 2) and by paper chromatography. The pHPP formed from tyrosine was also identified by its oxidation with p-hydroxyphenylpyruvate oxidase (Fig. 6). Alanine and glutamate in the reaction mixtures were identified by chromatography (Table 1).

Determination of Aromatic Pyruvates by Keto-Enol Difference Spectrum—The enhancement of absorption of the aromatic α-keto acids by borate was used both for the identification (see previous section) and for the measurement of the α-keto acids. For these determinations the transamination reactions were carried out in phosphate or in tris(hydroxymethyl)aminomethane buffers instead of borate and the other components were added as shown in Table II.

The reaction was stopped by addition of 1.0 ml. of 20 per cent metaphosphoric acid to 3 ml. of reaction mixture. The aromatic pyruvates were measured by the following procedure. A
FIG. 1. Absorption curves of the p-hydroxyphenylpyruvate oxidase assay system before (A) and after (C) the reaction of 0.5 ml. of enzyme with 0.4 pmoles of pHPP for 20 minutes. No significant accumulation of absorbing substances occurred. Had 0.4 pmoles of homogentisate accumulated (shown in B in same medium) it would not have affected the assay, since the reaction was followed at 310 nm.

0.5 ml aliquot of the filtrate was added to 3.0 ml of 2.0 M arsenate, pH 6.5 (keto sample) and another 0.5 ml aliquot was added to 3.0 ml of 1 M borate in 2 M arsenate, pH 6.5 (enol-borate sample). The arsenate greatly accelerated the equilibration of the keto and enol tautomers with the enol borate (1). After 15 minutes the spectrum of the enol-borate solution was determined with the arsenate keto solution as its blank. The difference spectra of the four compounds, which are not identical with the simple spectra of the enol borates of these compounds, are illustrated in Fig. 2. Under the conditions used, the molar extinction coefficients (ε) of the α-keto acids in the difference spectra were: indolylpyruvate, 12,700 (332 nm); imidazolylpyruvate, 12,000 (293 nm); pHPP, 12,400 (310 nm); and phenylpyruvate, 9,150 (300 nm). By this method the products of the transamination reactions could be recognized as aromatic α-keto acids and quantitatively measured.

Continuous Spectrophotometric Enzyme Assays—The transamination of aromatic amino acids and the oxidation of pHPP were measured by continuous spectrophotometric assays in borate buffers. In the assay of p-hydroxyphenylpyruvate oxidase and of tyrosine and phenylalanine transaminases, keto-enol tautomerization of the aromatic pyruvates was made nonlimiting by the addition of purified hog kidney keto-enol tautomerase. This tautomerase did not catalyze the tautomerization of indolylypyruvate or imidazolylpyruvate (1) and was therefore omitted in the tryptophan and histidine transaminase reactions. Fortunately, the spontaneous rate of tautomerization of imidazolylpyruvate was sufficiently rapid not to be rate limiting after the initial minute or two, even with histidine transamination rates several times those observed in the present experiments. A new keto-enol tautomerase acting on indolylypyruvate, which will be described separately, was found in excess in the crude rat liver extract used for tryptophan transamination.

FIG. 2. Identification of the products of the transamination by the keto-enol difference spectra with and without borate. The curves represent the enhancement of the absorption of the compounds by the presence of borate. • — •, obtained with the deproteinized filtrate of the transamination reaction (carried out in phosphate buffer) with histidine (A), tyrosine (B), phenylalanine (O), and tryptophan (D). ○ — ○, obtained with the corresponding aromatic α-keto acid: imidazolylpyruvate, pHPP, phenylpyruvate, and indolylpyruvate respectively. The maximal difference absorption of each pair of curves were converted to 1.0 for the purpose of comparison.
TABLE I
Chromatographic identification of transaminase reaction products

<table>
<thead>
<tr>
<th>Transamination</th>
<th>Aromatic α-keto acid found</th>
<th>Aliphatic α-amino acid found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Keto acid</td>
<td>Rp</td>
</tr>
<tr>
<td>Phenylalanine-pyruvate</td>
<td>Phenylpyruvate</td>
<td>0.86</td>
</tr>
<tr>
<td>Tyrosine-α-ketoglutarate</td>
<td>pHPP</td>
<td>0.72</td>
</tr>
<tr>
<td>Tryptophan-α-ketoglutarate</td>
<td>Indolylpyruvate</td>
<td>0.37</td>
</tr>
<tr>
<td>Histidine-pyruvate</td>
<td>Imidazolylpyruvate</td>
<td>--</td>
</tr>
</tbody>
</table>

* An aliquot of the reaction mixture (no borate) was deproteinized with metaphosphoric acid, extracted exhaustively with ether, and the concentrated ether extract used for ascending chromatography. Samples from the reactions, the reaction blanks (see text) and standards were run in propionic acid-water-butanol (5:7:10) (7) for phenylpyruvate and pHPP, and in acetic acid-water (1:3) for indolylpyruvate (5). These 3 α-keto acids were detected respectively by FeCl₃, Folin-Ciocalteu, and Ehrlich’s reagents.

† Extent of the reaction, as determined by the keto-enol difference spectrum.
‡ Aliquots of the reaction mixtures were applied directly to salt-treated paper for chromatography of the amino acids by the technique of McMenamy et al. (8). Alanine was separated in 20 hours and glutamic acid in 90 hours. Amounts were estimated from the density of the ninhydrin spots in comparison with standards run on the same paper.
§ Not higher than the trace amount present in the reaction blank.
¶ Determined by the difference spectra method.

The conditions and the reagents for the assay of p-hydroxyphenylpyruvate oxidase and the transaminases are summarized in Table II. For the assay of p-hydroxyphenylpyruvate oxidase, a solution of sodium pHPP (keto form), the borate buffer, and tautomerase were mixed and allowed to equilibrate for 5 minutes before the reaction was started by addition of the oxidase. In the assayed transaminases, the liver extract was preincubated for 3 to 5 minutes with 30 μg. of pyridoxal phosphate to assure maximal activity. Then 2.0 ml. of a solution of the amino acids in the appropriate borate buffer, other components of the system, water, and finally pyruvate or α-ketoglutarate to initiate the reaction were added to give a final volume of 3.5 ml. and a final borate concentration of 0.57 M. The optical blank for each reaction was a similar mixture with one of the reacting components omitted (Table II, Footnote 3).

The reactions were run at 25 ± 1°C in 1 cm. quartz cells in a Beckman model DU spectrophotometer equipped with a photomultiplier or in a Beckman model DK spectrophotometer with circulating water constant temperature jackets. The reactions were followed by determination of the optical densities at appropriate wave lengths (Table II) for 5 or 10 minutes with p-hydroxyphenylpyruvate oxidase and for 10 or 20 minutes with the transaminases. The rates were expressed as μoles of substrate reacting per milliliter of enzyme per 10 minutes, calculated from the observed optical density changes and the extinction coefficients of the enol borates in the assay system (Table II).

The major difference between this spectrophotometric assay and the previously used manometric assay is the lower concentration of substrate required by the present method. Addition of activators, like ascorbic acid and 2,6-dichlorophenolindophenol, was unnecessary (9). The more sensitive spectrophotometric assay permitted the determination of the dissociation constant (Kₐ) of the enzyme (Table III). This value was determined graphically with the assumption that the keto form of pHPP was the substrate. About 60 per cent of the total

### Table II

<table>
<thead>
<tr>
<th>Reaction</th>
<th>λ</th>
<th>ρH</th>
<th>Tautomerase</th>
<th>Substrate</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxyphenylpyruvate oxidase</td>
<td>310 μm</td>
<td>8050</td>
<td>8.2</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Transaminases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine-α-ketoglutarate</td>
<td>310</td>
<td>8050</td>
<td>8.0</td>
<td>0.5</td>
<td>80</td>
</tr>
<tr>
<td>Phenylalanine-pyruvate</td>
<td>310</td>
<td>5800</td>
<td>8.2</td>
<td>0.5</td>
<td>160</td>
</tr>
<tr>
<td>Histidine-pyruvate</td>
<td>310</td>
<td>5500</td>
<td>8.2</td>
<td>0.5</td>
<td>160</td>
</tr>
<tr>
<td>Tryptophan-α-ketoglutarate</td>
<td>328</td>
<td>14000</td>
<td>8.2</td>
<td>20</td>
<td>120</td>
</tr>
</tbody>
</table>

* Molar extinction coefficient of the equilibrium mixture of the aromatic α-keto acid and borate under the conditions of the assay at the given wave length (λ).
† Final pH of 0.57 m borate reaction mixture. The pH of the 1 m stock solution was about 0.3 units lower.
‡ Substance omitted from blank reaction mixture. The activities observed in the absence of pyruvate or α-ketoglutarate were always less than 5 or 10 per cent of the activities of the complete systems.

SPECIAL CONDITIONS FOR INDIVIDUAL ENZYME ASSAYS

p-Hydroxyphenylpyruvate Oxidase—Under the conditions outlined in Table II, the p-hydroxyphenylpyruvate oxidase activity of the crude liver preparations was similar to that found by measurement of oxygen uptake. The reaction was further characterized by its unusually high temperature coefficient and by its complete inhibition with DDC (11). The supply of oxygen in the spectrophotometric assay was not limiting, since 0.5 ml. of enzyme completely oxidized the 0.4 μmole of pHPP present during a 20 minute assay as shown by the absorption spectra before and after the reaction.

The major difference between this spectrophotometric assay and the previously used manometric assay is the lower concentration of substrate required by the present method. Addition of activators, like ascorbic acid and 2,6-dichlorophenolindophenol, was unnecessary (9). The more sensitive spectrophotometric assay permitted the determination of the dissociation constant (Kₐ) of the enzyme (Table III). This value was determined graphically with the assumption that the keto form of pHPP was the substrate. About 60 per cent of the total

1 The abbreviations used are: pHPP for p-hydroxyphenylpyruvate; EDTA, ethylenediaminetetraacetate; and DDC, diethylidithiocarbamate.
pHPP was present under these conditions as the nonabsorbing keto tautomer and 40 per cent as the strongly absorbing enol borate complex, calculated from the extinction coefficients of reaction mixtures and of the individual tautomers (1).

Tyrosine-α-ketoglutarate Transaminase—The presence of p-hydroxyphenylpyruvate oxidase in crude liver extract prevented the stoichiometric accumulation of pHPP formed from tyrosine. If additional purified p-hydroxyphenylpyruvate oxidase was added to this reaction mixture, accumulation of pHPP was almost completely suppressed (Fig. 6, Curve B). Since the rate of transamination of tyrosine was measured by the formation of pHPP in this assay, it was necessary to inhibit the endogenous p-hydroxyphenylpyruvate oxidase. This was achieved by the use of DDC. In the presence of this chelating agent, even the addition of exogenous p-hydroxyphenylpyruvate oxidase did not affect the transaminase assay (Fig. 6, ○, Curve A). That DDC did not affect the activity of the tyrosine-α-ketoglutarate transaminase itself was demonstrated by the identity of the transamination rate in the presence or absence of the chelating agent under anaerobic conditions, where pHPP could not be further metabolized.

Phenylalanine-pyruvate Transaminase—Of the four transaminase reactions studied in the crude liver extract, the phenylalanine system was least subject to side reactions. There was no degradation of phenylpyruvate by the crude liver extract as shown by the stability of the optical density at 310 μm of added phenylpyruvate for 30 minutes.

His&-line-pyruvate Transaminase—The action of histidase on histidine with the formation of urocanic acid in crude liver ex-
Assays of Transamination and Oxidation

One component was varied, and the others held at the usual assay concentrations.

Values were determined from Lineweaver-Burke plots. Only a few activity data are presented.

**Transaminases**

- **p-Hydroxyphenylpyruvate oxidase**
  - Concentration giving half-maximal activity.

**Disassociation constants of enzymes and substrates**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amino acid</th>
<th>α-Keto acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxyphenylpyruvate oxidase</td>
<td>M</td>
<td>2 × 10⁻⁴†</td>
</tr>
</tbody>
</table>

† Concentration giving half-maximal activity. All other Kₘ values were determined from Lineweaver-Burke plots. Only one component was varied, and the others held at the usual assay concentrations.

**DISCUSSION**

The assay methods described here were developed for the determination of the enzyme activities in crude liver preparations, where control of side reactions was often necessary, but they are applicable to purified preparations without major changes.

A spectrophotometric method based on the slight absorption difference between the aromatic amino acid and its α-keto acid in the absence of borate was used by Cammarata and Cohen (11) to study the transamination of phenylalanine and phenylpyruvate, and presumably this was the basis of the determinations reported by Litwack (12). In the absence of borate was used by Cammarata and Cohen (11) to study the transamination of phenylalanine and phenylpyruvate, and presumably this was the basis of the determinations reported by Litwack (12).

In this assay the α-keto acid was omitted from the blank. Tryptophan was added to both the reaction mixture and the blank so that products formed from tryptophan via the tryptophan peroxidase pathway and absorbing at the wave length would be canceled out in the assay.

The tryptophan-α-keto glutarate transaminase reaction followed zero order kinetics for the first 5 to 10 minutes of reaction, and then the reaction rate decreased. When indolylpyruvate was added to such a crude liver extract a fairly constant decrease of about 0.2 optical density units per ml. of enzyme per 10 minutes was observed at 328 mμ. The decrease in the apparent rate of tryptophan transamination with time observed with crude liver extracts was probably due to the slow action of an indolylpyruvate removing enzyme. In an ammonium sulfate fractionated tryptophan transaminase preparation, the apparent transamination rate did not decrease in the 20 minute assay period. The enzymic breakdown of indolylpyruvate in the crude system was not inhibited by EDTA or anaerobiosis. No products were detected by spectrophotometric or paper chromatographic methods. The rate of breakdown of indolylpyruvate in the crude system amounted to 15 to 30 per cent of the apparent transamination rate shown in Fig. 3.

The structural requirement for the spectrophotometric enol borate assay is a keto acid whose enol tautomer is conjugated and absorbing at the wave length would be canceled out in the assay. The wave lengths chosen also permit the use of high concentrations both of substrates and of crude enzyme preparations to obtain maximal activities.

The discovery of a new keto-enol tautomerase acting on indolylpyruvate was made by heating the crude liver extract at 60°C for 5 minutes and presumably this was the basis of the determinations reported by Litwack (12). In the absence of borate was used by Cammarata and Cohen (11) to study the transamination of phenylalanine and phenylpyruvate, and presumably this was the basis of the determinations reported by Litwack (12). In the absence of borate was used by Cammarata and Cohen (11) to study the transamination of phenylalanine and phenylpyruvate, and presumably this was the basis of the determinations reported by Litwack (12).
ruvate, in addition to the original tautomerase active with phenylpyruvate and pHPP, suggests that there may be an even wider occurrence of such enzymes. In the absence of the tautomerase, aromatic pyruvates can be measured by the difference spectrum method with arsenate as the catalyst for the tautomeration.

With the present method, transaminations with both pyruvate and α-ketoglutarate have been shown for tryptophan, tyrosine, and phenylalanine. The reaction of histidine with α-ketoglutarate was very weak or absent in rat liver extracts. A number of similar transaminations catalyzed by bacterial extracts have also been assayed by the same method.

Where comparison was possible the properties of the enzymes studied with the present method agreed in general with those reported in other assays. We have made a detailed comparison of similar transaminations catalyzed by bacterial extracts have been used to develop measurements of the activities of enzymes which either form or break down the aromatic α-keto acids. The enzymes studied were: p-hydroxyphenylpyruvate oxidase, tyrosine-α-ketoglutarate transaminase, phenylalanine-pyruvate transaminase, histidine-pyruvate transaminase, and tryptophan-α-ketoglutarate transaminase.

The existence of transaminations of phenylalanine, histidine and tryptophan with pyruvate or α-ketoglutarate have previously been reported. These usually depended on the recognition of one or both the reaction products in crude tissue extracts (11, 15-18). One of the advantages of the continuous assay employed here was the ease with which the several reactions could be defined and their characteristics determined.

The immediate purpose of this study was to measure the activities of the transaminases and p-hydroxyphenylpyruvate oxidase in unfractionated tissue extracts under controlled conditions and in such a manner that comparisons of the activities of the enzymes in animals in different physiological states could be made. The methods described appear to be adequate for this purpose, with the reservation that tryptophan and histidine transaminations are actually somewhat more rapid than observed, and that changes in the rates of disappearance of indolylpyruvate or imidazolylpyruvate can modify the apparent rate of transamination determined in this way.

**SUMMARY**

Continuous spectrophotometric assays for five enzymes based on the keto-enol tautomeration and the strong ultraviolet absorption of the enol borate complexes of aromatic α-keto acids have been described. This property of the borate complexes has been used to develop measurements of the activities of enzymes which either form or break down the aromatic α-keto acids. The enzymes studied were: p-hydroxyphenylpyruvate oxidase, tyrosine-α-ketoglutarate transaminase, phenylalanine-pyruvate transaminase, histidine-pyruvate transaminase, and tryptophan-α-ketoglutarate transaminase.

Optimal conditions for the activities of these enzymes which may be used for the assay of these enzymes either in crude liver extracts or in partially purified preparations are described.

**Acknowledgments**—The authors wish to thank Professor A. B. Hastings for helpful comments and Mr. Eli Messinger for carrying out preliminary experiments in the tryptophan-α-ketoglutarate transaminase assay.

**REFERENCES**

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