2,4,5-Trihydroxyphenylacetic Acid. A Metabolite of L-3,4-Dihydroxyphenylalanine†

G. H. Wada and J. H. Fellman*

ABSTRACT: Evidence is presented that 2,4,5-trihydroxyphenylacetic acid is a metabolic product of 3,4-dihydroxyphenylpyruvate, a L-3,4-dihydroxyphenylalanine metabolite. The enzyme *p*-hydroxyphenylpyruvate hydroxylase is capable of catalyzing a hydroxylation of 3,4-dihydroxyphenylpyruvate resulting in oxidative decarboxylation and alkyl migration. The chemical synthesis of 2,4,5-trihydroxyphenylacetic acid is described, and evidence for the appearance of this trihydroxy acid in the urine of three patients treated with L-3,4-dihydroxyphenylalanine is presented. A possible ambiguity of the origin of the 2,4,5-trihydroxyphenylacetic acid was noted; the trihydroxy acid can be formed nonenzymically in high pH solutions as an oxidation product of 3,4-dihydroxyphenylacetic acid.

Interest in the metabolic fate of L-DOPA[1] has expanded since the introduction of this amino acid as a therapeutic agent for the treatment of Parkinson's disease (Cotzias *et al.*, 1969; Yahr *et al.*, 1969; Mawdsley, 1970). Among the metabolic pathways investigated has been the transamination of L-DOPA to its corresponding α-keto acid. Cohen and Cammarata (1950) and Fellman and Roth (1971) observed that liver cytosol tyrosine aminotransferase (EC 2.6.1.5) can accept L-DOPA as a substrate. Furthermore, Gjessing and Borud (1965) described the detection of the transamination products of L-DOPA, 3,4-dihydroxyphenylpyruvate and 3-methoxy-4-hydroxyphenylpyruvate, in the urine of patients suffering with neuroblastoma, a tumor of the sympathetic nervous system which overproduces DOPA and its metabolites. Gjessing (1966) predicted that a trihydroxyphenylacetic acid could be formed from 3,4-dihydroxyphenylpyruvate.

The metabolism of 3,4-dihydroxyphenylpyruvate by liver *p*-hydroxyphenylpyruvate hydroxylase (EC 1.14.2.2) has been investigated by Fellman *et al.* (1971). Utilizing an enzyme assay system which follows the rate of $^{14}CO_2$ evolution from 1-^{14}C-labeled substrates (Fellman *et al.*, 1972; Lindblad, 1971; Raheja *et al.*, 1973), it was found that 3,4-dihydroxyphenylpyruvate was a substrate for avian *p*-hydroxyphenylpyruvate hydroxylase. The enzyme, purified by the method of Lindblad *et al.* (1971), retained a constant ratio of activity toward phenylpyruvate, *p*-hydroxyphenylpyruvate, and 3,4-dihydroxyphenylpyruvate. Furthermore, 3,4-dihydroxyphenylpyruvate was shown to competitively inhibit the hydroxylation of *p*-hydroxyphenylpyruvate, emphasizing the affinity of this substrate for the same enzyme. The putative product of the hydroxylation of 3,4-dihydroxyphenylpyruvate is 2,4,5-trihydroxyphenylacetic acid by analogy with the established formation of homogentisic acid from enzymatic oxidation of *p*-hydroxyphenylpyruvate. However, since the metabolism of 3,4-dihydroxyphenylpyruvate by *p*-hydroxyphenylpyruvate hydroxylase was demonstrated only by following the rate of decarboxylation of the substrate, there was no evidence for ring hydroxylation and side-chain migration. Thus, we undertook to provide evidence that the trihydroxyphenylacetic acid is, indeed, a product of this enzymatic event. In this communication, we describe: (1) the chemical synthesis and properties of 2,4,5-trihydroxyphenylacetic acid, (2) evidence for its formation from 3,4-dihydroxyphenylpyruvic acid by the enzyme *p*-hydroxyphenylpyruvate hydroxylase, and (3) the occurrence of trihydroxyphenylacetic acid in the urine of patients treated with L-DOPA.

Experimental Section

Materials. 3,4-Dihydroxyphenylpyruvic acid was prepared by the method of Harley-Mason and Waterfield (1963). 3,4-Dihydroxy[*carboxyl*-^{14}C]phenylpyruvate was prepared as previously described (Fellman *et al.*, 1971). *o*-Hydroxyphenylpyruvic acid lactone was prepared as previously described by Billek (1961). The lactone was converted to the acid by dissolving in 0.1 M KPO_4 (pH 7.3). Recrystallized catalase was purchased from Sigma Chemical Co.

Methods

Synthesis of 2,4,5-Trihydroxyphenylacetic Acid. A Willgerodt rearrangement of 2,4,5-tribenzyloxyacetophenone by a modified method of Wolkowitz and Dunn (1955) was used to synthesize 2,4,5-tribenzyloxyphenylacetic acid. The latter was reductively debenzylated with H_2/Pd on carbon to the 2,4,5-trihydroxyphenylacetic acid.

2,4,5-TRIBENZYLOXYPHENYLACETIC ACID. To 2 g (2.28×10^{-3} mol) of 2,4,5-tribenzyloxyacetophenone, synthesized by the method of Daly *et al.* (1965), was added 0.22 g of sulfur and 1.2 g of redistilled morpholine. The reaction mixture was refluxed gently for 7 hr and cooled to room temperature. At this point 3.8 ml of ethanol, followed by 1.6 ml of 50% NaOH, was added. The mixture was shaken vigorously and then refluxed for 42 hr. The ethanol (3.5 ml) was distilled off and 10 ml of water was added to the residue. The cooled residue was extracted with three volumes of ethyl acetate. The extract was dried over anhydrous Na_2SO_4, and the solvent was removed. The brown residue was recrystallized from ethyl acetate–hexane and yielded 1 g of product (48% yield), mp 152–154°.

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[1] The following abbreviations and trivial names are used: L-DOPA, L-3,4-dihydroxyphenylalanine; gc–ms, gas chromatographic–mass spectrometry; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide.

The product, when examined with thin-layer chromatography (tlc) on silica gel (Kodak 6060) in anhydrous ethyl ether–hexane (70:30), exhibited a major component (R_F 0.15) which gave a methyl ester with ethereal diazomethane (R_F 0.72). 2,4,5-Tribenzyloxyphenylacetophenone under the same conditions had an R_F of 0.61. The esterified product was examined with tlc on silica gel with a benzene–methanol–acetic acid (45:8:4) solvent system. Two components were resolved. The major component (R_F 0.93) was scraped from the plate and a mass spectrum was obtained on a Du Pont 21-110B high-resolution mass spectrometer. Its spectrum was consistent with the expected 2,4,5-tribenzyloxyphenylacetate methyl ester. A minor component of R_F 0.78 exhibited a mass spectrum consistent with the lactone of 2-hydroxy-4,5-dibenzyloxyphenylacetic acid.

2,4,5-TRIHYDROXYPHENYLACETIC ACID. 2,4,5-Tribenzyloxyphenylacetic acid (10 mg) was dissolved in 8 ml of anhydrous ethanol in a 10-ml serum bottle; 15 mg of 5% palladium on carbon and 0.025 ml of 2 N HCl were added and the suspension was stirred with a small magnetic stirring bar. The vial was stoppered with a rubber serum bottle cap and purified hydrogen was bubbled through the mixture. A second needle, just penetrating the rubber cap, provided a vent for the slow stream of hydrogen. The reduction was allowed to proceed for 3.5 hr. The mixture was filtered and the solvent was evaporated under a stream of nitrogen. The residue gave a single Me_3Si derivative when treated with BSTFA for 30 min at 60° and analyzed with an LKB 9000 gc–ms (see Results for details).

Avian *p*-hydroxyphenylpyruvate hydroxylase was prepared from chicken liver acetone powder by the method of Lindblad *et al.* (1971).

The enzyme activity assay was carried out using conditions employed in radiochemical procedures of Fellman *et al.* (1971).

Results

Enzymatic Formation of 2,4,5-Trihydroxyphenylacetic Acid. HYDROXYQUINONE PRODUCT. From our observations of the behavior of 2,4,5-trihydroxyphenylacetic acid in buffer solutions at pH 7.3 (the pH at which the enzymatic assays are conducted), we concluded that the trihydroxy acid is unstable and oxidizes rapidly to the hydroxyquinone. This property has been described for the trihydroxyphenyl compounds, 2,4,5-trihydroxyphenylethylamine (Senoh and Witkop, 1959; Adams *et al.*, 1972), and 2,4,5-trihydroxyphenylacetic acid in *Penicillium chrysogenum* (Isono, 1958). Figure 1 illustrates these properties. At pH 4.5 the 2,4,5-trihydroxyphenylacetic acid has an absorption maximum of 290 nm. When the pH of the solution was adjusted to 7.3, the colorless solution rapidly turned pink and the spectrum exhibited a maximum at 278 nm and the characteristic hydroxyquinone absorption peak at 480 nm. The addition of acid to this solution suppressed the dissociation of the hydroxyl group and the absorption spectrum shifted to peaks at 262 and 375 nm.

These absorption spectra were used to identify the enzymatic formation of trihydroxyphenylacetic acid from 3,4-dihydroxyphenylpyruvate. The enzymatic reaction mixture contained 0.05 mg of 3,4-dihydroxyphenylpyruvate, 0.88 mg of freshly neutralized ascorbic acid, 5000 units of catalase, and 3 units[2] of purified avian *p*-hydroxyphenylpyruvate hydroxylase in a final volume of 2.0 ml (0.1 M phosphate buffer (pH 7.3) was routinely employed). A boiled enzyme control was run with all

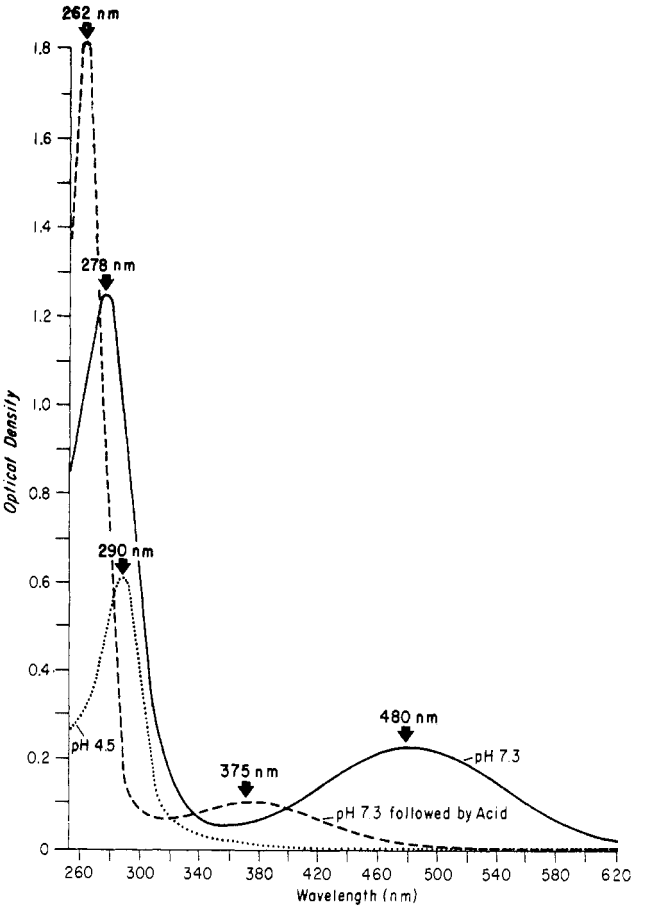

FIGURE 1: The absorbance spectra of 2,4,5-trihydroxyphenylacetic acid and the hydroxyquinone of 2,4,5-trihydroxyphenylacetic acid. 2,4,5-Trihydroxyphenylacetic acid (2.94 (10^{-6}) mol) was dissolved in 20 ml of 0.1 M KPO_4 buffer at pH 4.5 and 7.3. Then visible and uv absorbance spectra were scanned in a Cary 15 spectrophotometer. The pH 7.3 solution was acidified by 0.06 ml of 6 N HCl/ml to give a final solution of pH 2.0. Under these conditions, molar extinction coefficients found were: 2,4,5-trihydroxyphenylacetic acid (pH 4.5, ϵ_{290} = 4,014); hydroxyquinone (pH 7.3, ϵ_{278} = 9435 and ϵ_{480} = 1497; pH 2.0, ϵ_{262} = 12,245 and ϵ_{375} = 680).

experiments. The substrate was tipped in at zero time and the reaction mixture was incubated in air at 37° for 30 min. The visible adsorption spectra of these reaction mixtures were determined with a Cary 15 spectrophotometer; 0.3 ml of 2 N H_2SO_4 was added to bring the pH down to 2.0 and the precipitated protein was separated by centrifugation. The visible absorption spectra were again determined. The resulting absorption spectra were identical with those obtained with synthetic trihydroxyphenylacetic acid. The broad absorption maximum at 480 nm appeared only in the reaction vessels containing active enzyme. No change in optical density in the visible region was observed in the reaction vessels containing boiled enzyme. Again on adding acid, the 480-nm peak shifted to 375 nm, as did the synthetic hydroxyquinone of trihydroxyphenylacetic acid.

In another series of experiments, we varied the amount of enzyme which we added to the reaction mixture and demonstrated that the appearance of the peak at 480 nm was lineally related to the amount of enzyme added. The enzyme-catalyzed formation of 480-nm-absorbing product was assayed in the following system (1.0 ml): 44 μg/ml of neutralized ascorbic acid, 35 μg/ml of dihydroxyphenylpyruvate, and 10–100 μl of chicken liver supernatant (dialyzed overnight against 0.1 M

[2] Unit of enzyme activity defined as 1 μmol of *p*-hydroxyphenylpyruvate oxidized per hr.

TABLE I: Inhibition of 3,4-Dihydroxyphenylpyruvate Hydroxylase Activity by *o*-Hydroxyphenylpyruvate.[a]

	o-Hydroxyphenylpyruvate		
Assay system (M)	0	10^{-6}	10^{-5}
$^{14}CO_2$/5-min evolution (cpm)	413	276	92
Per cent inhibition	0	33	78
ΔOD at 480/5 min (ΔA)	0.055	0.034	0.013
Per cent inhibition	0	38	76

[a] Enzyme activity assayed by $^{14}CO_2$ evolution and ΔOD at 480 nm. The hydroxylation of 3,4-dihydroxyphenylpyruvic acid was followed by two assay systems: (a) $^{14}CO_2$ evolution concentration of assay components and assay conditions were the same as in Figure 1 with substitution of 1-^{14}C-labeled substrate and twice the total volume. $^{14}CO_2$ was captured on a Hyamine wick as previously described (Fellman *et al.*, 1971). [b] $OD_{480\ nm}$, same conditions as in Figure 1. Additions of inhibitor were made simultaneously with substrate in both systems.

KPO_4, pH 7.3). The reaction was started by addition of substrate, using a Gilford recording spectrophotometer at 37° to follow the change in OD_{480}. A supernatant protein control in addition to a control containing all components of the assay system except the supernatant protein was run to compensate for increased turbidity experienced during incubation: $OD_{480} = \Delta A/5$ min (reaction) − {$\Delta A/5$ min (blank) + $\Delta A/5$ min (protein)}.

Finally, we studied the effect of a known inhibitor of *p*-hydroxyphenylpyruvate hydroxylase, *o*-hydroxyphenylpyruvate (Fellman *et al.*, 1971), on the rate of formation of the product which exhibited the 480-nm absorption peak and $^{14}CO_2$ evolution. The results of these experiments shown in Table I clearly demonstrate inhibition of product formation by this inhibitor.

ISOLATION OF THE PRODUCT AND IDENTIFICATION BY GC–MS. An attempt was made to unequivocally identify the formation of trihydroxyphenylacetic acid by gc–ms. The reaction mixture contained 8 units of purified *p*-hydroxyphenylpyruvate hydroxylase, 0.15 mg of 3,4-dihydroxyphenylpyruvate, 5000 units of catalase, and 1.8 mg of neutralized ascorbic acid in a final volume of 4.0 ml containing 0.1 M phosphate buffer (pH 7.3). A boiled enzyme control was run identically as above, save the addition of a heat-inactivated enzyme. The incubation at 37° in air was allowed to proceed for 30 min; 0.6 of 2 N H_2SO_4 was added, followed by a small amount of zinc dust. The pink solution turned yellow with the addition of acid and

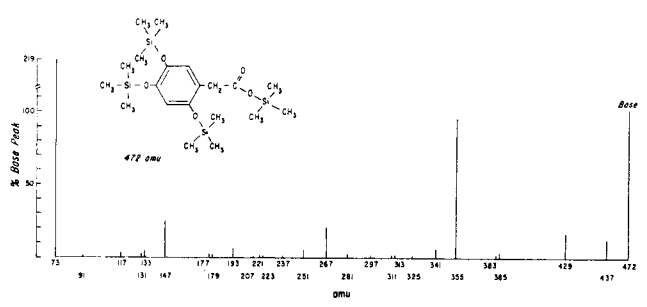

FIGURE 2: Mass spectrum of silylated 2,4,5-trihydroxyphenylacetic acid.

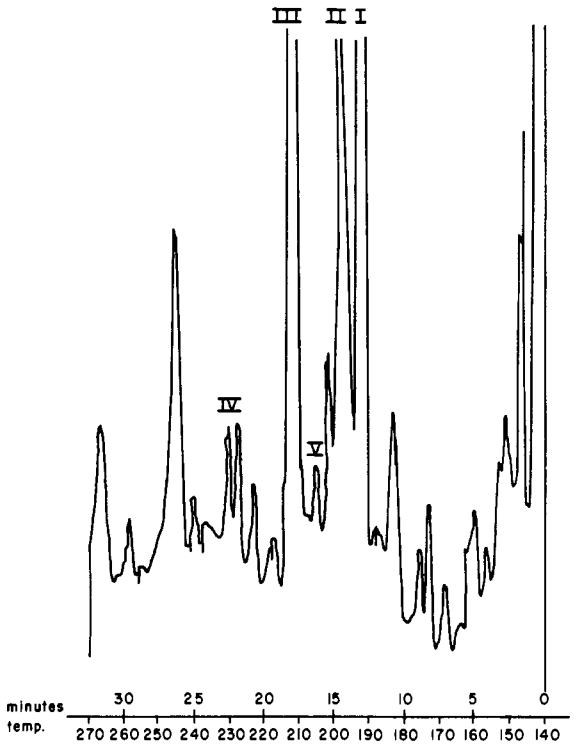

FIGURE 3: Gas chromatographic elution profile of urine from a patient undergoing L-DOPA therapy. Me_3Si derivatives of: (I) 3-methoxy-4-hydroxyphenylacetic acid, P = 326; (II) 3,4-dihydroxyphenylacetic acid, P = 384; (III) 3-methoxy-4-hydroxyphenyllactic acid, P = 428; (IV) 3-methoxy-4-hydroxyphenylpyruvic acid, P = 426; (V) 2,4,5-trihydroxyphenylacetic acid, P = 472 (P = parent mass ion).

colorless after shaking with the zinc dust, indicating reduction of the quinone. The solution was extracted three times with an equal volume of ethyl acetate, after saturation with NaCl. The combined ethyl acetate extracts were dried briefly over anhydrous Na_2SO_4, and the solvent was evaporated under a stream of nitrogen. The dried residue was treated with 200 μl of BSTFA silylating agent at 60° for 30 min. The material was injected into the inlet port of a LKB 9000 gc–ms instrument. A 1% OV-1, 12-ft column with helium flow 38 cm³/min programmed at a 3°/min temperature rise was employed. A single product was observed to emerge at 195° whose column retention and mass spectrum were identical with that exhibited by the synthetic 2,4,5-trihydroxyphenylacetic acid silylated under identical conditions. These results are diagrammed in Figure 2. The boiled enzyme did not contain any product. Similar results were obtained with a 1% OV-25, 12-ft column. The product from the enzyme reaction emerged from the column at approximately 200° and precisely 20.04 methylene units, as did the synthetic standard. Their mass spectra were identical.

Urine Studies. We examined the urine of a Parkinson patient who was being treated with large amounts of L-DOPA. At the time of the urine collection, this patient was ingesting a total of 8 g of L-DOPA/day. The urine was collected in acid, and a sample was treated with zinc dust and extracted with ethyl acetate as above described. The silylated residue was injected into an LKB 9000 gc–ms, using an OV-25, 12-ft column with a helium flow of 18 cm³/min and a 4°/min temperature rise. A number of DOPA-derived products were observed, including prominently, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylpyruvate, 3-methoxy-4-hydroxyphenyllactic acid, and the trihydroxyphenylacetic acid (Figure 3). The latter product

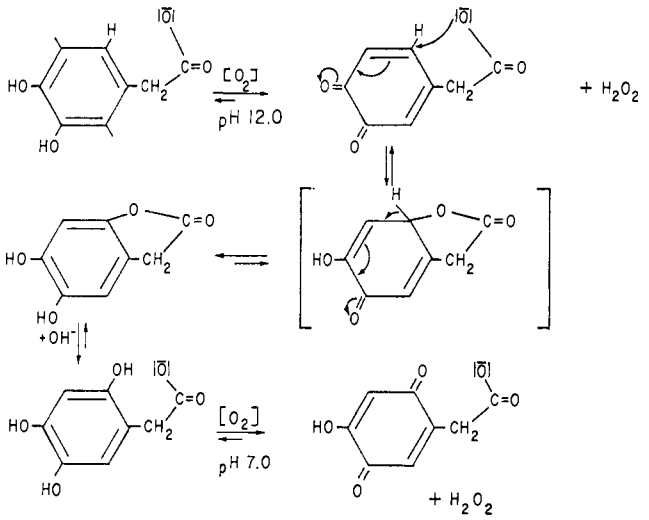

FIGURE 4: Oxidation and intramolecular addition of 3,4-dihydroxyphenylacetic acid.

emerged at approximately 200°, as did the standard synthetic 2,4,5-trihydroxyphenylacetic acid. It too was identified by its mass spectrum.

The appearance of the trihydroxyphenylacetate in two other urine samples from different patients on L-DOPA therapy was confirmed, using gc–ms analysis. These latter two patients were ingesting approximately 5 g of L-DOPA/day.

However, the matter does not rest there. After further consideration of the origin of 2,4,5-trihydroxyphenylacetic acid, we speculated that this acid could arise from oxidation and intramolecular 1,4 addition of 3,4-dihydroxyphenylacetic acid in the manner shown in Figure 4.

To examine this possibility, we dissolved 10 mg of 3,4-dihydroxyphenylacetic acid in a tube containing 2 ml of 0.1 M phosphate buffer (pH 12.0). The solution rapidly turned from yellow to red to dark red. The reaction mixture was allowed to stand in air for 2 hr. Three drops of concentrated HCl was added to acidify the solution. Zinc dust was added and the solution was extracted two times with 10 ml of ethyl acetate. The ethyl acetate extract was dried over anhydrous Na_2SO_4 and evaporated under N_2. The residue was silylated as described above and analyzed in a LKB 9000 using columns and conditions described above. A single product was observed, exhibiting column retention time and a mass spectrum identical with 2,4,5-trihydroxyphenylacetic acid. The yield of the rearrangement reaction was approximately 14% at pH 12.0, as determined by quantitation with gas chromatography using peak areas. Under otherwise identical conditions, there was no detectable product (less than nanogram amounts) when the reaction was run at pH 7.3.

Further Metabolism of 2,4,5-Trihydroxyphenylacetic Acid. If 2,4,5-trihydroxyphenylacetic acid is indeed a product of L-DOPA metabolism, an additional question remains with respect to *its* metabolic fate. Thus, we examined the following question. Would 2,4,5-trihydroxyphenylacetic acid be a substrate for homogentisate oxidase? We attempted to answer this question using manometric assay procedure (Knox, 1955). No change in oxygen uptake was observed above comparative boiled controls when 3×10^{-4} M 2,4,5-trihydroxyphenylacetic acid was added to rat liver supernatant containing 3×10^{-3} M ascorbate in 0.1 M phosphate buffer (pH 7.3) in a Warburg apparatus. Thus, rat liver appeared to be unable to oxidize the 2,4,5-trihydroxyphenylacetate as it can the analogous 2,5-dihydroxyphenylacetate.

Discussion

Our earlier studies of 3,4-dihydroxyphenylpyruvate as a substrate for liver *p*-hydroxyphenylpyruvate hydroxylase employed an assay procedure that left some doubt as to the nature of the product. This doubt has been eliminated by the results of the present studies. We have clearly established by two means the identity of 2,4,5-trihydroxyphenylacetic acid as the enzymic product of the action of *p*-hydroxyphenylpyruvate hydroxylase on the substrate 3,4-dihydroxyphenylpyruvate *in vitro*.

The smallest ambiguity remained in the possibility that the rearrangement proceeded to the alternative product 2,3,6-trihydroxyphenylacetic acid. This unlikely possibility was set aside when we compared the mass spectrum and the gas chromatographic methylene units of this isomer (prepared by alkaline oxidation of homogentisic acid in a manner similar to that described for the alkaline oxidation of 3,4-dihydroxyphenylacetic acid) to those of the enzymic product. Comparison of their respective behavior with gc–ms revealed many similarities, but some distinguishing fragment ions, diagrammed in Figure 5, and the different gas chromatographic properties (2,4,5 isomer, 20.04 methylene units; 2,3,6 isomer, 20.27 methylene units) led us to the conclusion that the enzymic product was the 2,4,5-trihydroxyphenylacetate isomer.

A number of attempts were made to demonstrate the formation of 2,4,5-trihydroxyphenylacetate from 2,4,5-trihydroxyphenylethylamine, using mitochondrial monoaminoxidase preparations. In no instance was any trihydroxyphenylacetate observed to be formed in this system (Fellman and Roth, 1973). The observation was made that 3,4-dihydroxyphenylacetic acid, a prominent DOPA metabolite, could spontaneously oxidize and rearrange to 2,4,5-trihydroxyphenylacetic acid at pH 12, but it appears that this process occurs to an insignificant extent at physiological pH or in acidified urine samples. Thus, the principal source of the 2,4,5-trihydroxyphenylacetic acid found in the urine of a patient undergoing L-DOPA therapy is most likely to be that formed by metabolism of L-DOPA through the liver tyrosine aminotransferase and the liver–kidney *p*-hydroxyphenylpyruvate hydroxylase enzyme systems.

In support of this conclusion, it is noted that Calne *et al.* (1969) found that urinary levels of *p*-hydroxyphenyllactic acid were elevated by L-DOPA therapy, suggesting *in vivo* inhibition of *p*-hydroxyphenylpyruvate hydroxylase by a DOPA metabolite. Since our past (Fellman *et al.*, 1972) and present finding show that 3,4-dihydroxyphenylpyruvate is a cosubstrate with *p*-hydroxyphenylpyruvate for the enzyme, the inhibitory metabolite could be the dihydroxyphenylpyruvate.

Relative to other L-DOPA metabolites, there were small amounts of 2,4,5-trihydroxyphenylacetic acid detected in urine samples from patients undergoing L-DOPA therapy. The quantitation of these amounts of trihydroxyphenylacetic acid and its corresponding hydroxyquinone from urine samples would be of questionable significance, because of the instability of these products. Even in frozen urine samples, it was noted that storage resulted in total loss of the trihydroxyphenylacetic acid. Using rat liver tissues in an *in vitro* system, Fellman and Roth (in preparation) have estimated the relative amount of 1-^{14}C-labeled L-DOPA metabolized through the tyrosine aminotransferase–*p*-hydroxyphenylpyruvate hydroxylase pathway *vs.* the total L-DOPA decarboxylated as 14%. This conclusion was based on the relative amounts of decarboxylation taking place in the presence and absence of α-ketoglutarate, a cosubstrate for transamination of L-DOPA, in liver homogenates.

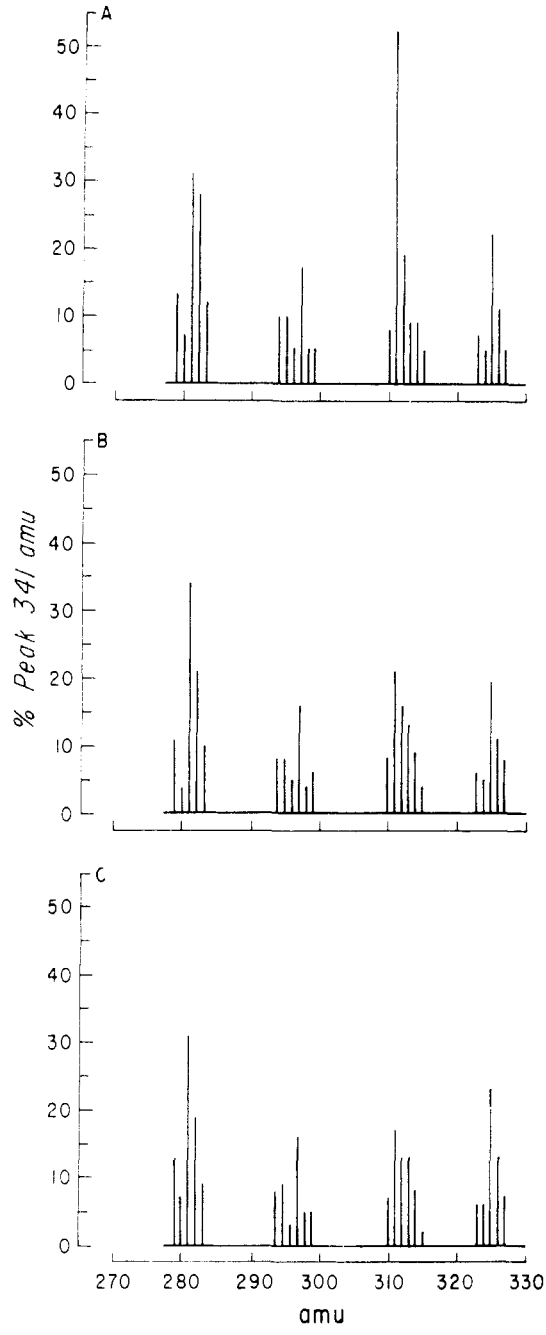

FIGURE 5: Mass spectral ion fragmentation patterns distinguishing 2,4,5- and 2,3,6-trihydroxyphenylacetic acid: (A) 2,3,6 isomer (from homogentisate oxidation and rearrangement); (B) product from hydroxylation of 3,4-dihydroxyphenylpyruvate; (C) 2,4,5 isomer (synthesized standard).

The establishment of 2,4,5-trihydroxyphenylacetate as a metabolic product of DOPA metabolism in man is a matter of some consequence, since the trihydroxyphenyl moiety is known to be quite reactive. 2,4,5-Trihydroxyphenylethylamine (6-hydroxydopamine) has been shown to be toxic either as a result of its capacity to undergo oxidation to a reactive *p*-quinone (Saner and Thoenen, 1970) or by *in situ* generation of semiquinone and superoxide radicals (Adams *et al.*, 1972; Heikkila and Cohen, 1973) and of hydrogen peroxide (Heikkila and Cohen, 1971). The identical capacity exists for the trihydroxyphenylacetic acid. Elevation of blood urea nitrogen and serum transaminases have been reported in patients treated with DOPA (McDowell *et al.*, 1970). Furthermore, there have been reports of decreases in aromatic L-amino acid decarboxylase activity of rat liver (Dairman *et al.*, 1971) and catechol *O*-methyltransferase activity of rat liver and kidney (Sharpless *et al.*, 1973) during chronic administration of L-DOPA. These effects are attributed to lower enzyme levels rather than the presence of inhibitors or lack of adequate levels of cofactors. The same enzymes in other tissues were unaffected by the same treatment. These phenomena could be explained by an increased enzyme degradation, caused by a toxic DOPA metabolite, such as 2,4,5-trihydroxyphenylacetic acid. The 2,4,5-trihydroxyphenylacetic acid, generated by liver and kidney *p*-hydroxyphenylpyruvate hydroxylase during DOPA metabolism, could explain the selective inactivation of the enzymes in these two tissues.

Detoxification of the trihydroxyphenylacetic acid might occur through O-methylation by catechol *O*-methyltransferase, in the same way that 2,4,5-trihydroxyphenylethylamine is converted to 2,4-dihydroxy-5-methoxyphenylethylamine (Daly *et al.*, 1961). However, the failure of O'Gorman's effort at finding the 2,4-dihydroxy-5-methoxyphenylacetic acid in urines of patients receiving L-DOPA therapy (O'Gorman *et al.*, 1970) may indicate the inability of catechol *O*-methyltransferase to O-methylate this substrate before it undergoes rapid autooxidation to the hydroxyquinone.

Rapid autooxidation to the hydroxyquinone may also explain the *in vitro* evidence that homogentisate oxidase cannot further metabolize 2,4,5-trihydroxyphenylacetic acid.

Acknowledgments

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Computer Analysis of the Two-Substrate Reaction Catalyzed by Yeast and Bovine Transaldolase†

Ekkehard Kuhn and Karl Brand*

ABSTRACT: Kinetic studies with transaldolase (EC 2.2.1.2) from *Candida utilis* and bovine mammary gland were carried out in order to obtain further information about the different reaction mechanisms of the yeast and the animal enzyme. The data were fitted to different rate equations with a digital computer using a general parameter estimation program. By this method for each enzyme a reaction sequence could be established which is consistent with the data obtained from chemical and kinetic studies. The kinetics of yeast transaldolase indicate a Ping Pong mechanism. No satisfactory fit can be obtained if an ordered ternary complex mechanism is assumed since systematic deviations are always observed between the models and the data. Thus the catalysis of yeast transaldolase proceeds mainly with formation of a binary complex which can be attributed to the dihydroxyacetone enzyme complex formed during incubation of the enzyme with its substrate D-fructose 6-phosphate. The reaction therefore occurs in a stepwise rather than in a concerted manner. On the other hand, the kinetic data obtained with bovine transaldolase could not be explained by a linear mechanism, but the data were in best agreement with a model in which a Random Bi Bi mechanism was assumed. Thus aldol cleavage reaction seems not to involve formation of a binary complex, but to proceed *via* a ternary enzyme–substrate intermediate. No covalent label could be found to be attached to the enzyme protein by the borohydride technique in previously published experiments. This fact suggests that Schiff base formation between enzyme and substrate is not required for the catalytic mechanism in the case of the bovine enzyme. The kinetics of the reaction indicate the formation of a ternary complex by random order combination of substrates with the enzyme protein.

In a previous paper (Kuhn and Brand, 1972) a purification procedure for transaldolase (D-sedoheptulose 7-phosphate: D-glyceraldehyde dihydroxyacetone transferase, EC 2.2.1.2) from bovine mammary gland and some physical properties of this enzyme as well as its modification with sodium borohydride have been described. Using sodium borohydride reduction, Horecker *et al.* (1963a) have shown that yeast transaldolase incubated with uniformly labeled [^{14}C]fructose 6-phosphate forms a stable inactive enzyme–dihydroxyacetone intermediate. Chemical degradation of this intermediate yielded N^6-β-glyceryllysine, indicating the structure of the enzyme–substrate complex being a Schiff base containing dihydroxyacetone linked to the ϵ-amino group of a lysine residue at the active site of the enzyme. In analogous experiments carried out with bovine transaldolase no labeled amino acid derivative could be detected and no label could covalently be incorporated into the enzyme after borohydride treatment (Kuhn and Brand, 1972). From these results it has been concluded that in the case of the animal enzyme the aldol cleavage reaction proceeds *via* a mechanism different from the Schiff base mechanism of yeast transaldolase.

In the present work the kinetics of yeast as well as bovine mammary gland transaldolase are described in detail. The results are based on a computer analysis of initial velocity data. Using a general Fortran program designed to estimate the parameters of nonlinear mathematical models the coefficients of simple as well as more complex rate equations could be determined. Thus the adequacy of different branching and nonbranching two-substrate reaction mechanisms could be proved and for each enzyme a reaction sequence could be established which is consistent with the kinetic data and the data obtained from chemical experiments.

Materials and Methods

D-Fructose 6-phosphate, D-erythrose 4-phosphate, NADH, α-glycerophosphate dehydrogenase, triosephosphate isom-

† From the Max-Planck-Institut für Ernährungsphysiologie, 46 Dortmund, Rheinlanddamm 201, Germany. *Received May 1, 1973.* Dedicated to Professor Dr. Th. Wieland, Heidelberg, on the occasion of his 60th birthday.

* Present address: Physiologisch-Chem. Institut der Universität Erlangen, 8520 Erlangen, Egerlandstr. 7, West Germany.