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ASSAY, PROPERTIES AND TISSUE DISTRIBUTION OF *p*-HYDROXYPHENYLPYRUVATE HYDROXYLASE

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SUMMARY

Two methods for the assay of *p*-hydroxyphenylpyruvate hydroxylase (*p*-hydroxyphenylpyruvate, ascorbate: O_2 oxidoreductase (hydroxylating), EC 1.14.2.2) activity are described. (1) Chemical method: The homogentisate formed was condensed with cysteine to yield a 1,4-thiazine with an absorption maximum at 390 nm. (2) Radiochemical assay: The release of $^{14}CO_2$ from *p*-hydroxyphenyl[*carboxy*-^{14}C]-pyruvate was used as a measure of enzyme activity. Enzyme activation by certain reducing agents, pH optimum, tissue distribution, and fetal and adult enzyme activities are reported. Phenylpyruvate and 3,4-dihydroxyphenylpyruvate were substrates of the enzyme and were mutually competitive to *p*-hydroxyphenylpyruvate oxidation. The substrates, *p*-hydroxyphenylpyruvate and oxygen, are inhibitory at high concentration. Hydrogen peroxide, generated by the substrate and oxygen, may account for the observed substrate inhibition.

The enzyme is found only in liver and kidney. The significance of these findings to phenylketonuria and tyrosinemia is discussed.

INTRODUCTION

This work developed from a study of a patient with an inborn error of tyrosine metabolism[1,2]. The patient's plasma contained elevated levels of tyrosine while his urine revealed a strikingly large amount of *p*-hydroxyphenylpyruvate and its metabolic relatives[2]. A liver biopsy sample had normal amounts of *p*-hydroxyphenylpyruvate hydroxylase (*p*-hydroxyphenylpyruvate, ascorbate: O_2 oxidoreductase (hydroxylating), EC 1.14.2.2) activity but no cytosol tyrosine aminotransferase (EC 2.6.1.5) activity.

The results could be accounted for by the elevation in plasma tyrosine concentration in the absence of liver cytosol tyrosine aminotransferase leading to a sharp increase in the amount of tyrosine transaminated by other tissues. Brain, heart, and

Abbreviation: DOPA, 3,4-dihydroxyphenylalanine.

skeletal muscle are endowed with mitochondrial tyrosine aminotransferase[1,3] but conceivably lack *p*-hydroxyphenylpyruvate hydroxylase activity. The distribution of the enzymes in this manner would lead to increased amounts of the keto acid in the plasma. Unless subject to efficient renal tubular reabsorption, the *p*-hydroxyphenylpyruvate would appear in the urine.

Most earlier methods for assay of *p*-hydroxyphenylpyruvate hydroxylase determined the loss of the substrates rather than the appearance of the products[4,5]. In order to obviate this major limitation we developed two methods which measure the products of the hydroxylase and thus allow us to study the question which launched these investigations. The nature of the inhibition of the enzyme by its substrate was explored as well as substrate specificity and tissue distribution of the enzyme.

MATERIALS AND METHODS

p-Hydroxyphenylpyruvate, phenylpyruvate, snake venom *l*-amino acid oxidase *(Crotalus adamanteus)*, homogentisic acid, horseradish peroxidase (EC 1.11.1.7), and beef liver catalase (EC 1.11.1.6) were obtained from Sigma Chemical Co.; L-[*carboxy*-^{14}C]tyrosine 33.6 mCi/mM, [*carboxy*-^{14}C]phenylalanine, and DL-3,4-dihydroxy[*carboxy*-^{14}C]phenylalanine (DL-[*carboxy*-^{14}C]DOPA) from New England Nuclear; glutathione from Aldrich Chemical Co.; ascorbic acid, and 2,6-dichloroindophenol sodium salt from Eastman Organic Chemicals; and KBH_4 from Metal Hydrides.

o-Hydroxyphenylpyruvic acid lactone (m.p. 145–150 °C) was prepared in the manner described by Billek[6]. The lactone was converted to the acid by treating with 1 mM potassium phosphate (pH 7.3). *p*-Hydroxyphenylacetaldehyde was prepared by the method of Fellman[7] as modified by Robbins[8]. [*Carboxy*-^{14}C]phenylpyruvic acid derivatives were prepared by the oxidative deamination of ^{14}C-labeled amino acids with snake venom and catalase[9].

Preparation of p-hydroxy[carboxy-^{14}C]phenylpyruvic acid

25 μCi L-[*carboxy*-^{14}C]tyrosine was incubated for 20 min at 37 °C with 1 mg snake venom *(Crotalus adamanteus)* and 1000 units of beef liver catalase in 3 ml 0.1 M potassium phosphate (pH 6.5). The reaction was stopped with 1 ml 0.6 M HCl and the mixture was put on a 1 cm × 3 cm Bio-Rad AG-50-X8 column (acid form, 200–400 mesh) and the product was eluted with 100 ml 2 mM *p*-hydroxyphenylpyruvate carrier. After lyophilization of the eluate the *p*-hydroxy[*carboxy*-^{14}C]phenylpyruvate residue was diluted with carrier to a specific activity of 9 μCi/mM.

Preparation of sodium [carboxy-^{14}C]phenylpyruvate

Sodium[*carboxy*-^{14}C]phenylpyruvate was prepared from 25 μCi L-[*carboxy*-^{14}C]-phenylalanine by the method described above for the synthesis of *p*-hydroxy[*carboxy*-^{14}C]phenylpyruvate. Carrier sodium phenylpyruvate was used to dilute the product to a specific activity of 18 μCi/mM.

Preparation of 3,4-dihydroxy[carboxy-^{14}C]phenylpyruvic acid

3,4-Dihydroxy[*carboxy*-^{14}C]phenylpyruvic acid was prepared from 10 μCi DL-[*carboxy*-^{14}C]DOPA by the method described above for the synthesis of *p*-hydroxy-[*carboxy*-^{14}C]phenylpyruvate. Carrier 3,4-dihydroxyphenylpyruvic acid (m.p. 185–

189 °C) was prepared by the method described by Harley–Mason and Waterfield[10] and used to dilute the product to a specific activity of 7 μCi/mM.

Preparation of tissue homogenates

Tissues were homogenized (glass tube with Teflon pestles) in 0.1 M phosphate (pH 7.3). The homogenate was centrifuged for 30 min at 15 000 × *g* and supernatants were dialyzed against 0.1 M phosphate (pH 7.3) and stored frozen. Rat liver was used throughout except where noted.

Radiochemical assay method

The enzymatic oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid and CO_2 was studied using *p*-hydroxy[*carboxy*-^{14}C]phenylpyruvic acid. The $^{14}CO_2$ evolved was trapped in hyamine and subsequently counted to determine the rate of oxidation. The oxidation of phenylpyruvic acid and 3,4-dihydroxyphenylpyruvic acid was also studied using ^{14}C-labeled substrates.

Radiochemical assay of p-hydroxyphenylpyruvate hydroxylase activity

The enzyme was assayed in a single side arm Warburg vessel closed with a number 2 rubber stopper and a number 12 serum bottle cap. A 22 gauge nichrome wire was embedded on the bottom of the stopper to form a 1.5 cm pin. An 11-mm diameter disc of Whatman number 4 filter paper was stitched on the pin and moistened with 0.025 ml 1 M hyamine hydroxide in methanol to adsorb $^{14}CO_2$.

The assay mixture (2 ml) consisted of activated enzyme* in 100 mM potassium phosphate (pH 7.3), volume 1.5 ml, in the reaction chamber, and 0.5 ml 2 mM *p*-hydroxy[*carboxy*-^{14}C]phenylpyruvate tipped in from the side arm. The assay mixture was incubated for 10 min at 37 °C in a Dubnoff metabolic shaking incubator. 1 M H_2SO_4 (0.3 ml) was injected into the side arm and was used to stop the reaction. After 10 min equilibration, the discs were placed in 19 ml Buhler's[12] solution and the amount of $^{14}CO_2$ was determined by counting in a liquid scintillation spectrometer.

Colorimetric assay method

This assay is based upon the reaction of *p*-quinones with cysteine to form yellow chromophoric 1,4-thiazine[13]. The products of the enzyme action are homogentisic acid and CO_2. Since the colorimetric method depends upon the appearance of homogentisic acid, it defines unambiguously the *p*-hydroxyphenylpyruvate hydroxylase activity of tissue extracts. Care must be taken to destroy the homogentisate oxidase (EC 1.13.1.5) activity before assay of a tissue extract. The simplest procedure for destroying homogentisate oxidase activity was to acid denature the extract as described earlier[14,15]. Adjustment of the pH of the tissue homogenate to pH 5 with 1 M acetic acid and then readjustment to pH 7.3 with 1 M NaOH eliminates all of the homogentisate oxidase activity, as shown by the total recovery of added homogentisic acid to such acid treated homogenates.

The assays were carried out as follows: A standard 2.0 ml reaction mixture contained 0.5 mM *p*-hydroxyphenylpyruvate, 100 mM potassium phosphate (pH 7.3), and enzyme extract which had been activated with GSH–2,6-dichloroindophenol or

* Enzyme extracts were activated before assay by the addition of an equal volume of 2.5 mM 2,6-dichloroindophenol and 52 mM GSH or 3.2 mM ascorbic acid[11].

ascorbic acid as described in the radiochemical assay procedure. Suitable controls consisting of boiled enzyme and all other reagents were run with each assay. Tubes were incubated, with shaking, in a 37 °C water bath. At the end of incubation, 0.5 ml of 800 mM sulfosalicylic acid was added and the tubes were centrifuged at 1000 × *g* for 5 min. 1 ml of the supernatant was transferred to 15 ml extraction tubes and extracted twice with 2 ml ethyl acetate. The combined ethyl acetate extracts were shaken with 1 ml of 200 mM potassium phosphate (pH 8.0). The ethyl acetate was discarded and 0.2 ml of 1 M KBH_4 added. After 10 min, 0.1 ml of 5 M acetic acid was added and the tubes shaken to destroy excess KBH_4. 0.2 ml of 200 mM cysteine and 3.0 ml of 500 mM potassium phosphate (pH 12.0) were then added and the color allowed to develop for 15 min. Tubes were well aerated with swirling and the absorbance determined at 390 nm. The amount of homogentisic acid was determined by reference to a standard curve of homogentisic acid.

RESULTS

The rate of release of $^{14}CO_2$ from the substrate *p*-hydroxyphenylpyruvate was directly related to the amount of tissue extract assayed (Fig. 1). This was supported by the results obtained employing the colorimetric method. As seen in Fig. 1, the number of μmoles of $^{14}CO_2$ released was equal to the number of μmoles of homogentisic acid formed over the range of the amount of enzyme extract studied. The variation in precision of the radiochemical assay was observed to be 1% by making 12 multiple assays of a single enzyme extract.

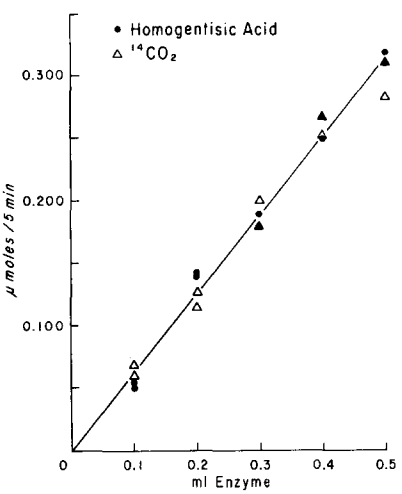

Fig. 1. Formation of CO_2 and homogentisic acid from *p*-hydroxyphenylpyruvate. A crude rat liver supernatant containing 24 mg protein/ml was used. ●—●, μmoles homogentisic acid formed/5 min; △—△, μmoles $^{14}CO_2$ formed/5 min.

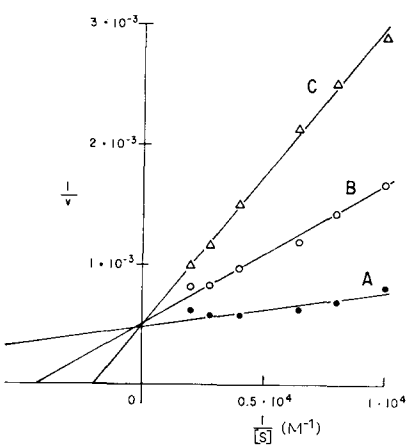

Fig. 2. Competitive inhibition of *p*-hydroxyphenylpyruvate hydroxylase by phenylpyruvate and 3,4-dihydroxyphenylpyruvate. (A) ●—●, *p*-hydroxyphenylpyruvate, no inhibitor added; (B) ○—○, *plus* phenylpyruvate, $2.5 \cdot 10^{-4}$ M; (C) △—△, *plus* 3,4-dihydroxyphenylpyruvate, $2.5 \cdot 10^{-4}$ M.

The effect of substrate concentration on the enzyme activity was studied. Inhibition of activity by high amounts of substrates has been commented upon before[11,16] and was observed in our studies (*cf.* the Lineweaver–Burk plot in Fig. 2). The mechanism of this inhibition is discussed below. From these studies a K_m for this enzyme was observed to be $5 \cdot 10^{-5}$ M. This compares with a reported value of $2 \cdot 10^{-5}$ M for rat liver extracts[5] and $5 \cdot 10^{-4}$ M for frog liver enzyme[17].

The effect of the oxygen substrate on the velocity of the reaction was also studied. K_m for oxygen was calculated from Fig. 3: K_m = 10% or $1 \cdot 10^{-4}$ M O_2 in solution. These data also show an inhibitory effect of high oxygen partial pressures.

The effect of pH on the velocity of the enzyme was studied and it was found that the pH optimum was $\simeq$ 7.5.

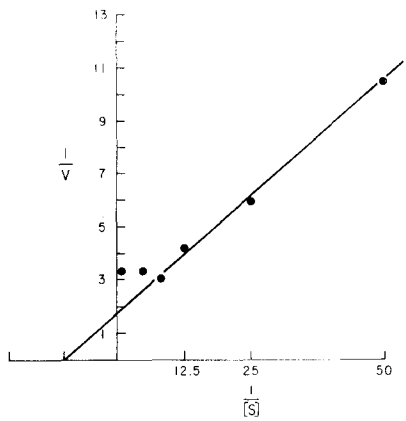

Fig. 3. *p*-Hydroxyphenylpyruvate hydroxylase. O_2 as substrate. Enzyme used was a partially purified* preparation. In addition to 2,6-dichloroindophenol–GSH activator, 1000 units of catalase was added per flask. *p*-Hydroxyphenylpyruvate concentration was 0.5 mM. $1/v$ is the reciprocal of the μmoles of homogentisic acid formed in 10 min. $1/[S]$ is the reciprocal of the percentage of O_2 present in the flask as a gas. K_m = 10% O_2 = $1 \cdot 10^{-4}$ M.

* By the method of Taniguchi and Armstrong[29]. Additional purification was attained by treatment with Sephadex G-150 and carboxy methyl Sephadex. A 15-fold increase in specific activity was achieved. This enzyme was devoid of detectable catalase activity.

Dialyzed liver homogenates are activated to maximal enzyme activity by the addition of certain reducing agents[11,18]. This characteristic requirement of *p*-hydroxyphenylpyruvate hydroxylase was demonstrated using the radiochemical assay system. Activity of the enzyme is progressively lost over a period of time when stored at −20 °C. Much of this lost activity may be recovered by treatment with the activator, reduced 2,6-dichloroindophenol.

Inhibition of the enzyme by substrate

A striking and puzzling property of *p*-hydroxyphenylpyruvate hydroxylase is its vulnerability to high concentrations of its substrate (see above). A detailed account of this phenomenon has been described earlier[4,11]. The inhibition is unusual in that it occurs after a lag period. At low substrate concentration reduced 2,6-dichloroindophenol, ascorbate, or other reducing agents will mitigate if not entirely prevent this inhibition.

From our studies it appears that the substrate, *p*-hydroxyphenylpyruvate, can generate peroxide and this substance inhibits the enzyme. Evidence that hydrogen peroxide was formed from the keto acid is the following:

(a) 10^{-3} M solutions of *p*-hydroxyphenylpyruvate at pH 7.3 generated a substance when shaken in air but not when shaken in nitrogen, which behaved like hydrogen peroxide when tested using the methods of Schales[19].

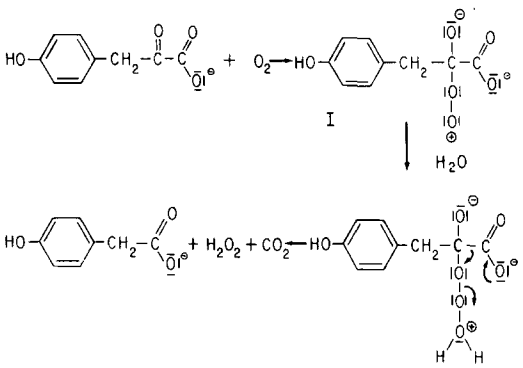

Fig. 4. Hypothetical reaction for formation of *p*-hydroxyphenylacetic acid and H_2O_2.

The nature of the reaction of oxygen with *p*-hydroxyphenylpyruvate was investigated. The proposed intermediate structure I (Fig. 4) leads to H_2O_2 *plus* CO_2 *plus* *p*-hydroxyphenylacetic acid. Oxygen is reduced to hydrogen peroxide with *p*-hydroxyphenylpyruvate oxidized to *p*-hydroxyphenylacetic acid. An alternative mechanism involves the hydration of the *p*-hydroxyphenylpyruvate and the subsequent reduction of oxygen with the decarboxylation to *p*-hydroxyphenylacetic acid. Whichever mechanism holds, the product *p*-hydroxyphenylacetic acid should be observed. Incubation of an assay mixture containing 0.5 mM *p*-hydroxyphenylpyruvate, 2.5 mM ascorbic acid, 100 mM potassium phosphate (pH 7.3) and 0.2 ml (24 mg protein/ml) crude rat liver homogenate in a final volume of 2.0 ml was carried out for 5 min and the reaction stopped by the addition of 0.5 ml of 800 mM sulfosalicylic acid. An ethyl acetate extract of the reaction mixture was made. By thin layer chromatography using silica gel plates with a benzene–methanol–acetic acid (45:8:1, by vol.) solvent system a spot was observed indistinguishable from *p*-hydroxyphenylacetic acid, R_F 0.5. Using a Beckman GC-5 gas–liquid chromatography system, the trimethylsilyl methyl ester derivative of the product was applied to an SE 30-8′ column. A material appeared having an identical methylene unit to *p*-hydroxyphenylacetic acid. Boiled enzyme preparation also contained detectable *p*-hydroxyphenylacetic acid. Indeed solutions of *p*-hydroxyphenylpyruvate in oxygen (pH 7.0) slowly generated hydrogen peroxide and *p*-hydroxyphenylacetic acid detectable by the methods described above. Furthermore in the absence of oxygen, *i.e.*, in nitrogen, no *p*-hydroxyphenylacetic acid could be observed. When the pH of the system was raised much beyond 8.0 then most of the *p*-hydroxyphenylpyruvate was oxidized by an alternate route leading to *p*-hydroxybenzaldehyde and oxalic acid[20].

(b) It had been reported that *p*-hydroxyphenylpyruvate hydroxylase activity could be separated into two fractions A and B, which individually had little activity but added together had vigorous activity[16]. Later these investigators showed that one of these fractions could be replaced by catalase and suggested a specific role for the enzyme in the peroxidative oxidation of the substrate. Following this report other investigators questioned these findings[15]. They demonstrated that hydrogen peroxide was not required for the enzymatic reaction and that catalase would stimulate the activity only under conditions of high substrate and high oxygen such as used in Warburg manometry. We examined this question and indeed found that as the catalase was separated by protein fractionation from the *p*-hydroxyphenylpyruvate hydroxylase activity, the activity was lost. The effect of catalase on the rate of reaction is seen in Fig. 5. Horseradish peroxidase acted similarly to catalase in protecting the *p*-hydroxyphenylpyruvate hydroxylase activity from inhibition (Fig. 5).

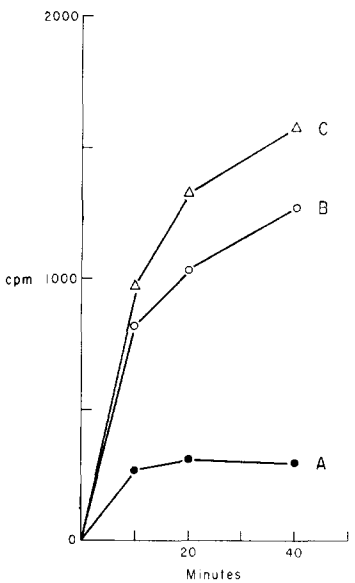

Fig. 5. Effect of catalase and horseradish peroxidase on the rate of the reaction. Enzyme was partially purified (see Fig. 3) from rat liver supernate. Ascorbic acid (3.2 mM) was the activator. *p*-Hydroxyphenylpyruvate concentration was 1.2 mM. (A) ●—●, enzyme and substrate; (B) ○—○, enzyme and substrate *plus* 0.5 mg crude horseradish peroxidase per flask; (C) △—△ enzyme and substrate *plus* 1000 units purified catalase per flask.

(c) Other observations relevant to the peroxide generating activity of the substrate were the inhibition studies using *o*-hydroxyphenylpyruvate. This material was investigated because we reasoned that the *o*-hydroxy group would interfere with oxygen addition and thus peroxide formation would not occur. Thus, this material should not exhibit non-competitive inhibition when preincubated with the enzyme. Furthermore, *o*-hydroxyphenylpyruvate should not be a substrate for the enzyme. Both properties were observed. The substance was, however, an excellent competitive inhibitor of the enzyme. Finally, the non-enzymic oxidation of *o*-hydroxyphenylpyruvate did not occur in alkaline solution as it did for the *p*-hydroxyketo acid. When

a solution of 10^{-5} M *o*-hydroxyphenylpyruvate was allowed to react at pH 9.3 in 100 mM phosphate, no alteration in the absorption spectrum was observed. The single, symmetrical peak at 328 nm remained unchanged over a period of 90 min. A comparable sample of *p*-hydroxyphenylpyruvate was rapidly oxidized to the *p*-hydroxybenzaldehyde[20].

Other substrates and inhibitors

Several aromatic keto acids were studied as substrates and/or inhibitors of *p*-hydroxyphenylpyruvic hydroxylase. Phenyl[*carboxy*-^{14}C]puruvate behaved as a substrate in a manner similar to *p*-hydroxyphenylpyruvic acid. The applicability of the radiochemical procedure to assay the phenylpyruvate hydroxylase activity is seen in Fig. 6. The observed K_m for this substrate was $6 \cdot 10^{-5}$ M. A study of the inhibitory effect of phenylpyruvate on the activity of the *p*-hydroxyphenylpyruvate hydroxylase was undertaken. Phenylpyruvate was added to incubation mixtures containing *p*-hydroxy[*carboxy*-^{14}C]phenylpyruvate, and the evolution of labeled CO_2 was followed. This data was plotted by the method of Lineweaver–Burk and is given in Figs 2 and 7. These substrates were mutually competitive when added simultaneously but each behaved as non-competitive inhibitors when preincubated with the enzyme before substrate was tipped in.

3,4-Dihydroxyphenylpyruvate was also studied as a substrate and inhibitor of *p*-hydroxyphenylpyruvate hydroxylase activity. Again the velocity of the hydroxylase reaction was directly related to the amount of tissue extract used (Fig. 6). The observed K_m for the enzyme with this substrate was $5 \cdot 10^{-5}$ M. The inhibitory

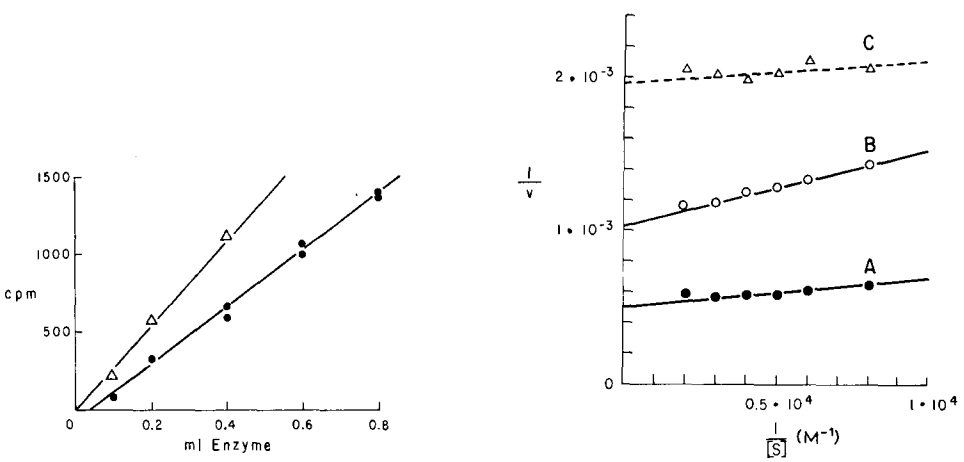

Fig. 6. Relationship of amount of enzyme to velocity of reaction. △—△, phenylpyruvic acid (0.5 mM) as substrate. Enzyme was partially purified rat liver supernate activated with 2,6-dichloroindophenol–GSH as described in the methods section. Incubation time was 10 min. ●—●, 3,4-dihydroxyphenylpyruvic acid (0.5 mM) as substrate. Enzyme was rat liver supernate activated with 2,6-dichloroindophenol–GSH. Incubation time was 10 min.

Fig. 7. Non-competitive inhibition of *p*-hydroxyphenylpyruvate hydroxylase by phenylpyruvate and 3,4-dihydroxyphenylpyruvate. Enzyme was rat liver supernatant (0.1 ml containing 32 mg protein/ml) activated with 2,6-dichloroindophenol–GSH as described in the methods section. Enzyme was incubated with inhibitor 5 min at 37 °C before addition of substrate. Incubation time after substrate addition was 5 min. (A) ●—●, no inhibitor; (B) ○—○, phenylpyruvate, $6.7 \cdot 10^{-5}$ M; (C) △---△, 3,4-dihydroxyphenylpyruvate, $1.7 \cdot 10^{-5}$ M.

activity of 3,4-dihydroxyphenylpyruvate for *p*-hydroxyphenylpyruvate hydroxylase activity was also investigated as described for the phenylpyruvate. We observed a profound non-competitive inhibition when the 3,4-dihydroxyphenylpyruvate was preincubated before labeled *p*-hydroxyphenylpyruvate was tipped in (Fig. 7). However, these substrates were mutually competitive when simultaneously tipped in (Fig. 2). The non-competitive inhibition observed when these substrate-type inhibitors are preincubated with the enzyme but competitive when added simultaneously with substrate, was also consistent with the peroxide mechanism for substrate inhibition described above.

An extension of this argument was made by testing catechol as an inhibitor of the enzyme activity. When $2 \cdot 10^{-4}$ M catechol was preincubated with the rat liver enzyme extracts for 5 min there was an approximately 90% inhibition of the enzyme activity. When this amount of catechol was added to the enzyme simultaneously with the substrate (*p*-hydroxyphenylpyruvate), no inhibition was observed. Since catechol can autooxidize to form hydrogen peroxide[19] the pre-exposure of the enzyme to catechol would be expected to cause an inhibition of the type described above.

o-Hydroxyphenylpyruvate was not a substrate but behaved only as a competitive inhibitor of *p*-hydroxyphenylpyruvate. *p*-Hydroxyphenylacetaldehyde was not a substrate for the enzyme and lacked inhibitory activity when tested at a concentration of 10^{-3} M. The judgment that both *p*-hydroxyphenylacetaldehyde and *o*-hydroxyphenylpyruvate were not substrates was based upon the lack of any new product formed which could be detected by paper chromatography and the absence of a hydroquinone or catechol product detectable by condensation with cysteine to form thiazines.

Tissue distribution

The enzyme activity was determined in a number of different organs and species. These are given in Table I. No enzyme activity could be observed in any tissue other than liver and kidney. The development of the activity in human, monkey,

TABLE I

TISSUE DISTRIBUTION OF *p*-HYDROXYPHENYLPYRUVATE HYDROXYLASE ACTIVITY IN A NUMBER OF SPECIES

Supernatant fractions obtained from homogenates of tissues indicated, are reported. No activity was found in non-supernatant fractions (see text). n = number of animals or human subjects assayed. The values represent average specific activities (μmoles/mg protein per h) $\pm$ standard deviations. 0.00 = <0.001 μmole/mg protein per h.

Species	*n*	*Liver*	*Kidney*	*Heart*	*Muscle*	*Brain*
Rat	4	1.00 ± 0.03	0.17 ± 0.03	0.00	0.00	0.00
Monkey						
Maternal	7	2.25 ± 0.52	1.23 ± 0.13	0.00	0.00	0.00
Neonatal 0–2 days	6	0.98 ± 0.45	0.29 ± 0.17			
Human						
Adult	2	0.60 ± 0.11	0.12 ± 0.05	0.00	0.00	—
Fetal 13–28 weeks	7	0.00	0.00			
Salmon (Coho)	3	1.43 ± 0.12	—	—	—	—
Opossum						
Maternal	1	1.14	—	—	—	—
Neonate (8 g)	6	0.77 ± 0.07	—	—	—	—

and opossum tissue was studied. These results (Table I) confirm those of others who showed the absence of hydroxylase activity in early fetal life[21,22].

Subcellular distribution

Separation of crude rat liver homogenates in 0.25 M sucrose[23] showed clearly that all of the enzyme activity resided in the cytosol. No activity could be detected in the nuclear, mitochondrial or microsomal fractions.

DISCUSSION

Early studies of *p*-hydroxyphenylpyruvate hydroxylase determined the loss of substrates[4,5]. The methods described above measure the appearance of products and are thus intrinsically more precise and more sensitive.

Ideally, the assay of enzymes requires that saturating amounts of substrates are employed. In the instance cited here the enzyme is subject to a profound substrate inhibition from both *p*-hydroxyphenylpyruvate and oxygen. This inhibition is probably never of biological consequence since in the normal mammalian state oxygen partial pressures in excess of 100 mm Hg in liver and kidney tissues never occur. Also the activity of the tyrosine aminotransferase responsible for producing *p*-hydroxyphenylpyruvate in the liver and kidney is rate limiting[24]. In fact, the inhibition by substrates in the case examined here is a contrivance resulting from the laboratory methods conventionally used in enzyme assay, *i.e.*, 21% oxygen with saturating amounts of *p*-hydroxyphenylpyruvate.

The inhibitory effects of both substrates, oxygen and *p*-hydroxyphenylpyruvate, can be understood from our results. Catechol, a hydrogen peroxide generator, was found to be a non-competitive inhibitor when preincubated with the enzyme *plus* air. 3,4-Dihydroxyphenylpyruvate also behaved in this manner. However when 3,4-dihydroxyphenylpyruvate was simultaneously added with *p*-hydroxy[*carboxy*-^{14}C]-phenylpyruvate to the enzyme, competitive inhibition of the enzymes was observed. These findings coupled with experiments showing the detection of hydrogen peroxide and *p*-hydroxyphenylacetic acid from solutions of *p*-hydroxyphenylpyruvate support the view that "substrate inhibition" is due to peroxide formation leading to enzyme inactivation. The fact that the addition of catalase or horseradish peroxidase to purified enzyme can mitigate the inhibition further supports this proposal.

Many of the previously described characteristics of the rat liver *p*-hydroxyphenylpyruvate hydroxylase were confirmed using both the radiochemical and colorimetric assay procedures. The inhibition of the enzyme by high substrate concentration, the activation of the enzyme by reduced 2,6-dichloroindophenol, the pH optimum have all been described by previous methods. The apparent K_m for the substrate oxygen has not been previously reported. Perhaps the clearest display of the versatility of the radiochemical procedure was the study of the mutually inhibitory substrates. The radiochemical method allowed the study of the activity of the enzyme employing for example *p*-hydroxyphenylpyruvate as substrate and phenylpyruvate as inhibitor. The observed competitive inhibition, when both substrate and "inhibitor" are simultaneously added to the enzyme, is consistent with the view that a single enzyme is responsible for the oxidation of both substances[25]. A biological extension of this competition by substrates in tyrosinemia and phenylketon-

uria has been observed. In tyrosinemia, elevated excretion of phenylpyruvate was observed when the *p*-hydroxyphenylpyruvate increased[2,26] and in phenylketonuria, the elevated urinary excretion of *p*-hydroxyphenylpyruvate[27] has been reported. The presence of the hydroxylase enzyme in mammalian liver and kidney but not in other tissues can account for the appearance of *p*-hydroxyphenylpyruvate in the urine of patients with tyrosinemia due to transaminase deficiency[28].

Finally, we confirm the absence of prenatal *p*-hydroxyphenylpyruvic hydroxylase activity in fetal liver and kidney. All monkey neonate liver and kidney possessed active enzyme and although the number of neonates examined was small we observed a progressive postpartum increase in enzyme activity.

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REFERENCES

1 J. H. Fellman, P. J. Vanbellinghen, R. T. Jones and R. D. Koler, *Biochemistry*, 8 (1969) 615.
2 N. G. Kennaway and N. R. M. Buist, *Ped. Res.*, 5 (1971) 287.
3 J. E. Miller and G. Litwack, *J. Biol. Chem.*, 246 (1971) 3234.
4 B. N. LaDu and V. G. Zannoni, *J. Biol. Chem.*, 217 (1955) 777.
5 E. C. C. Lin, B. M. Pitt, M. Civen and W. E. Knox, *J. Biol. Chem.*, 233 (1958) 668.
6 G. Billek, *Monatsh.*, 92 (1961) 352.
7 J. H. Fellman, *Nature*, 182 (1958) 311.
8 J. H. Robbins, *Arch. Biochem. Biophys.*, 114 (1966) 576.
9 A. Meister, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 4, Academic Press, London and New York, 1957, p. 404.
10 J. Harley-Mason and W. R. Waterfield, *Tetrahedron*, 19 (1963) 65.
11 V. G. Zannoni and B. N. LaDu, *J. Biol. Chem.*, 234 (1959) 2925.
12 D. R. Buhler, *Anal. Biochem.*, 4 (1962) 413.
13 R. Kuhn and I. Hammer, *Ber. Chem. Ges.*, 84 (1951) 91.
14 W. E. Knox and M. LeMay-Knox, *Biochem. J.*, 49 (1951) 686.
15 S. E. Hager, R. I. Gregerman and W. E. Knox, *J. Biol. Chem.*, 225 (1956) 935.
16 B. N. LaDu and V. G. Zannoni, *Nature*, 177 (1956) 574.
17 T. Laskowska-Klita, *Acta Biochim. Polon.*, 16 (1969) 35.
18 M. N. D. Goswami and W. E. Knox, *J. Chron. Dis.*, 16 (1963) 363.
19 O. Schales, *Ber. Chem. Ges.*, 71 (1938) 441.
20 C. H. Doy, *Nature*, 186 (1960) 529.
21 N. Kretchmer, S. Z. Levine and H. McNamara, *Am. J. Dis. Child.*, 93 (1957) 19.
22 M. N. D. Goswami and W. E. Knox, *Biochim. Biophys. Acta*, 50 (1961) 35.
23 G. S. Hogeboom, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 1, Academic Press, London and New York, 1955, p. 16.
24 E. C. C. Lin and W. E. Knox, *J. Biol. Chem.*, 233 (1958) 1186.
25 J. H. Fellman, T. S. Fujita and E. S. Roth, *Biochim. Biophys. Acta*, 268 (1972) 601.
26 L. R. Gessing, in L. R. Gessing, *Symp. on Tyrosinosis*, Norwegian Monograph on Medical Science, Universitats-Fordaget, Oslo, 1953, p. 61.
27 R. J. Boscott and H. Bickel, Scand., *J. Clin. Lab. Invest.*, 5 (1953) 380.
28 J. H. Fellman, N. G. Kennaway, N. R. M. Buist and R. E. Swanson, *Clin. Chim. Acta*, 39 (1972) 243.
29 D. Taniguchi and M. D. Armstrong, *J. Biol. Chem.*, 238 (1963) 4091.