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Kidney Phenylalanine Hydroxylase from Man and Rat. Comparison with the Liver Enzyme†

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ABSTRACT: The recent observation that phenylalanine hydroxylase is present in kidney, as well as in liver, poses the question of whether the enzymes from the two organs are the same, or whether they are tissue-specific isozymes. A comparison of liver and kidney enzymes from man and rat was made on the basis of kinetic parameters and immunological reactivity. The apparent Michaelis constant for tetrahydrobiopterin and the relative rates with tetrahydrobiopterin and tetrahydro-6,7-dimethylpterin were different for the enzymes from different species, but were identical for kidney and liver enzymes of the same species, as also were the apparent Michaelis constants for phenylalanine and tetrahydro-6,7-dimethylpterin. Antibodies to rat liver phenylalanine hydroxylase reacted identically with rat liver and rat kidney enzymes. Both human kidney and human liver phenylalanine hydroxylase showed only 4% of the reactivity of the rat enzymes indicating that 4% of the antibody binding sites of the rat enzymes are present on the human enzymes. Rat liver and kidney enzymes were also compared by their elution patterns and equilibrium distribution on Bio-Gel A-5, and by the effect of phenylalanine on their sedimentation characteristics in sucrose density gradient centrifugation. Neither of these criteria, which are reflections of the molecular size, density, and shape, could differentiate between the kidney and liver enzymes. From these results a tentative conclusion can be made that phenylalanine hydroxylase in kidney and liver is the same enzyme. If tissue-specific isozymes do exist they could not be detected by any of the above criteria.

Until recently phenylalanine hydroxylase (EC 1.14.3.1) had been detected in mammals only in the liver. It was first shown to be present in kidney in the mouse in 1969 (Tourian *et al.*, 1969), and since then has also been reported in the kidney of rat and guinea pig (McGee *et al.*, 1972; Berry *et al.*, 1972).

We have found that phenylalanine hydroxylase is also present in human kidney cortex, at a level of about 20% of that found in human liver (Ayling *et al.*, 1973c, 1974). We wished to establish whether or not the liver and kidney enzymes are identical, or whether they are tissue-specific isozymes. This is of particular interest since it has been shown that in phenylketonuria, the part of the phenylalanine hydroxylating system which is defective or missing (Jervis, 1953) is the hydroxylase itself, and not the cofactor, or cofactor regenerating system (Justice *et al.*, 1967).

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In this report, kinetic and immunological properties of human liver and kidney phenylalanine hydroxylase have been determined. Since the amount of human material available was limited, the studies made with the human enzyme were confirmed and extended using phenylalanine hydroxylase from rat kidney and liver. In addition to kinetic and immunological properties, the rat kidney and liver enzymes were also compared on the basis of sedimentation characteristics in sucrose gradients and equilibrium distribution on Bio-Gel. The comparison beween species, *i.e.*, rat and man, demonstrated that the criteria by which kidney and liver enzymes were compared are sufficiently sensitive to allow discrimination between phenylalanine hydroxylases of different species.

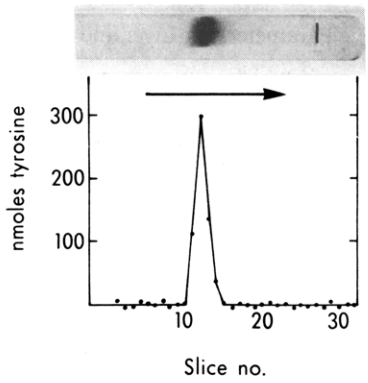

FIGURE 1: Analytical acrylamide gel electrophoresis of purified rat liver phenylalanine hydroxylase. The gel system of Ornstein and Davis was used (Ornstein, 1964; Davis, 1964) with a running gel size of 0.6 × 6.4 cm. Purified enzyme (55 μg) was applied to each gel. The arrow indicates the direction of migration. The marker, 5.4 cm from the top, in the upper gel, is the position of the indicator dye, Bromophenol Blue. This gel was stained in Coomassie Blue, while a parallel gel was cut into 1-mm slices which were assayed for tyrosine formed after 1-hr incubation (assay II).

Materials and Methods

2-Amino-4-keto-6,7-dimethyl-5,6,7,8-tetrahydropteridine (Me_2H_4Pt)[1] was purchased from Calbiochem. Tetrahydrobiopterin was generously donated by Dr. K. J. M. Andrews of Roche Products, Ltd., Hertfordshire, England. Protamine sulfate was from the Elanco division of Eli Lilly; catalase, phenylalanine, and dithiothreitol were from Sigma. Bio-Gel A-5, hydroxylapatite, acrylamide, and *N,N'*-methylenebisacrylamide were from Bio-Rad. Riboflavine and *N,N,N',N'*-tetramethylenediamine, which was redistilled, were from Eastman. Freund's adjuvant was from Difco. Human kidney and liver were supplied through the cooperation of the Departments of Surgery and Surgical Pathology at the University of California, Los Angeles, Medical School.

Assay of Enzyme Activity. Two different assay procedures were used. In each case assays were performed at 27° and pH 7.4. Unless otherwise noted assay mixtures contained 100 μmol of Tris-Cl (pH 7.4), 1 μmol of phenylalanine, 0.2 μmol of Me_2H_4Pt, 0.25 mg of catalase, and phenylalanine hydroxylase in a total volume of 1.0 ml.

ASSAY I. Activity was measured by a spectrophotometric procedure in which the phenylalanine-dependent rate of oxidation of tetrahydropteridine to dihydropteridine was monitored at 330 nm (Ayling *et al.*, 1973b). The net molar extinction coefficient at this wavelength was 4350 M^{-1} cm^{-1}. Enzyme was added to buffer, phenylalanine, and catalase in a total volume of 0.98 ml and the reaction mixture was equilibrated for 5 min at 27°. The reaction was started by the addition of Me_2H_4Pt, 0.02 ml of a 10 mM solution, and the rate was measured in a double beam spectrophotometer against a reference cell which contained all of the components of the reaction cell, except phenylalanine.

ASSAY II. The nitrosonaphthol derivative of the product, tyrosine, was formed and measured either spectrophotometrically (Udenfriend and Cooper, 1952) or fluorometrically (Waalkes and Udenfriend, 1957). In this assay 5 μmol of dithiothreitol was included in the reaction mixture to regenerate the cofactor (Bublitz, 1969); the reaction was run with shaking for a given period of time and then stopped by the addition of 0.25 ml of 30% trichloroacetic acid. Tyrosine was determined in aliquots of the deproteinized solution. Tyrosine standards containing all of the components of the reaction mixture were run with each experiment. This procedure was used when experimental conditions were not suitable for using assay I, for example, (i) when phenylalanine was already present with the enzyme, as after sucrose gradient centrifugation in the presence of phenylalanine, (ii) when the assay solution was not clear, as in the presence of acrylamide gel, or (iii) where it was necessary to assay aliquots of enzyme containing less than 3–4 milliunits of activity. Below this level of enzyme activity the background rate due to the autoxidation of cofactor becomes higher than the enzymatic rate, and thus in assay I, which has no cofactor regenerating system, leads to large experimental errors.

One unit of enzyme activity is defined as 1 μmol of product formed/min, under standard assay conditions.

Purification of Phenylalanine Hydroxylase from Rat Liver and Rat Kidney. Sprague-Dawley rats were decapitated and the livers immediately removed and homogenized in 300 ml of 0.2 M Tris-HCl (pH 7.5)–10 mM mercaptoethanol, per 100 g of tissue, in a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 20,000*g* for 15 min, and the supernatant was treated with 100 ml of 2% protamine sulfate in 0.1 M potassium phosphate (pH 7.5), per 500 ml of supernatant. The protamine sulfate supernatant was fractionated with ammonium sulfate. The fraction which precipitates between 35 and 45% saturation with ammonium sulfate contains the phenylalanine hydroxylase activity. The active ammonium sulfate fraction was applied to a Bio-Gel A-5 column and eluted with 0.01 M Tris-Cl–10 mM mercaptoethanol (pH 7.5). The active fractions were combined and applied to a hydroxylapatite column which was eluted with a pH 7.5 potassium phosphate gradient, 0.02–0.2 M, containing 10 mM mercaptoethanol. The phenylalanine hydroxylase activity is eluted at about 0.08 M potassium phosphate. The hydroxylapatite eluate was concentrated in an Amicon ultrafiltration cell, and purified in 60-mg aliquots on a Shandon preparative acrylamide gel electrophoresis apparatus, using the gel system of Ornstein and Davis (Ornstein, 1964; Davis, 1964). The entire purification procedure was carried out at 4°.

The enzyme purified by this procedure was run on analytical acrylamide gel electrophoresis. Even when the gel was heavily loaded with protein (55 μg), staining with Coomassie Blue showed only one protein band. Another gel, run simultaneously, was cut into 1-mm slices, which were assayed for phenylalanine hydroxylase activity. A symmetrical peak of activity corresponded to the position of the protein band (Figure 1).

[1] Abbreviation used is: Me_2H_4Pt, 2-amino-4-keto-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

TABLE I: Kinetic Parameters for Liver and Kidney Phenylalanine Hydroxylase from Rat and Man.[a]

Enzyme Source	Apparent Michaelis Constants			$V_m'(H_4Bp)$ / $V_m'(Me_2H_4Pt)$
	Phenylalanine (mM)	Me_2H_4Pt (mM)	Tetrahydrobiopterin (mM)	
Rat				
Liver	0.83 ± 0.05	0.101 ± 0.014	0.023 ± 0.002	4.5
Kidney	0.95 ± 0.16	0.107 ± 0.005	0.021 ± 0.003	4.5
Human				
Liver	0.62 ± 0.06	0.154 ± 0.018	0.057 ± 0.014	2.0
Kidney	0.84 ± 0.16	0.144 ± 0.043	0.057 ± 0.014	2.0

[a] Michaelis constants were measured under standard assay conditions (assay I), except for the concentration of the variable substrate. The concentration ranges used were 0.2–4.0 mM for phenylalanine (Me_2H_4Pt, 0.2 mM), 0.02–0.5 mM for Me_2H_4Pt (phenylalanine, 1 mM), and 0.01–0.2 mM for tetrahydrobiopterin (phenylalanine, 1 mM). The last column is the ratio: (apparent maximum velocity with respect to tetrahydrobiopterin)/(apparent maximum velocity with respect to Me_2H_4Pt).

Phenylalanine hydroxylase from rat kidney was purified in an identical manner, except for the ammonium sulfate fractionation. The fraction precipitating between 30 and 50% saturation with ammonium sulfate was used.

Phenylalanine Hydroxylase from Human Liver and Kidney. Freshly removed tissues were homogenized in a Duall homogenizer in 3 vol of 0.2 M Tris-HCl (pH 7.5). Homogenates were centrifuged at 100,000*g* for 1 hr. All of the activity was precipitated from the supernatant by addition of an equal volume of saturated ammonium sulfate solution at pH 7.5. Ammonium sulfate precipitates were dissolved in 0.1 M potassium phosphate (pH 7.4). Some tissues were frozen before use. In this case freshly removed tissue was cut into small pieces, frozen in liquid nitrogen, and stored at −15°. Frozen tissue was minced before homogenization in the Duall homogenizer. Up to 40% of the activity was lost by freezing the tissue. Due to the instability of the enzyme and the limited amount of material available, the purification procedure was not carried further than the ammonium sulfate precipitation.

Production of Rat Liver Phenylalanine Hydroxylase Antiserum. Antibodies to rat liver phenylalanine hydroxylase, which had been purified through the preparative acrylamide gel electrophoresis step, were produced in New Zealand White rabbits. Enzyme, 2.7 mg/ml of 0.05 M Tris-Cl (pH 7.5), was emulsified with an equal volume of complete Freund's adjuvant. One milliliter of this mixture was injected into each rabbit, subcutaneously at five different sites on the back. A second injection was made in the same manner after 3 weeks. Four weeks after the second treatment the same amount of enzyme, in 1.5 ml of 0.15 M NaCl (pH 7.4), was injected, without Freund's adjuvant, intravenously into the ear of each rabbit. Antibody titer was monitored by bleeding the rabbits at intervals from the ear vein. The antibody content was determined from the ability of the serum (3000*g* supernatant of blood left at room temperature for 1 hr and overnight at 4°) to precipitate rat liver phenylalanine hydroxylase activity out of solution. The maximum titer of phenylalanine hydroxylase antibodies was 6–13 days after the third injection of phenylalanine hydroxylase. Serum obtained at this time has been used for all of the immunological assays. The serum was used directly as the source of antibody, without further purification.

Results

Kinetic Comparison of Kidney and Liver Enzymes. Apparent Michaelis constants were determined for phenylalanine hydroxylase from liver and kidney of man and rat. The constants for rat liver enzyme were compared for preparations purified either through the ammonium sulfate fractionation or through the hydroxylapatite step. Since these values were identical the kinetic parameters for the enzymes from rat kidney and both human tissues were determined with ammonium sulfate fractions. In each case it was first ascertained that the activity was a linear function of the enzyme concentration over the range being used. Kinetic values were measured by assay I, varying substrate or cofactor concentration over at least a 20-fold range. The data were analyzed by a nonlinear regression graphics computer program (Dixon, 1969; Ayling and Zarky, 1974). The standard deviations for the kinetic constants given in Table I represent the closeness of fit of the data points to a curve which was calculated by computer from measurements which were made at least in duplicate. The values reported in the table are those of specific experiments in which kidney and liver constants were measured simultaneously. However, the values are in close agreement with many other determinations made at different times.

The apparent K_m for phenylalanine was determined at 0.2 mM Me_2H_4Pt and an oxygen concentration in equilibrium with the atmosphere. The values for rat liver and rat kidney, 0.83 and 0.95 mM, respectively, are not significantly different. Likewise, the K_m for human liver, 0.62 mM, is very similar to that for human kidney, 0.84 mM (Table I).

The apparent Michaelis constants for the pseudo cofactor, Me_2H_4Pt, and the natural cofactor, tetrahydrobiopterin, were measured at 1 mM phenylalanine and atmospheric oxygen, for rat and human enzymes. A comparison of the values in Table I shows that enzyme derived from rat liver could not be distinguished from rat kidney using either cofactor. With either enzyme, tetrahydrobiopterin binds 4–5 times tighter than Me_2H_4Pt, and in 1 mM phenylalanine produces 4.5 times faster maximum velocity.

Human enzymes behaved analogously to those from rat. The kidney and liver Michaelis constants are again similar, although in this instance the affinity of tetrahydrobiopterin is 2.5 times that of Me_2H_4Pt. The ratio of the apparent maximum velocities (2.0) is also lower.

Gel Filtration of Rat Liver and Kidney Enzymes. Chromatography of the 35–45% ammonium sulfate fraction of rat liver phenylalanine hydroxylase on Bio-Gel A-5 yields two peaks of enzyme activity (Figure 2a). The first is eluted after about 1.3 void volumes, and the second, which contains up to 25% of the total activity, is eluted after approximately 2 void volumes.

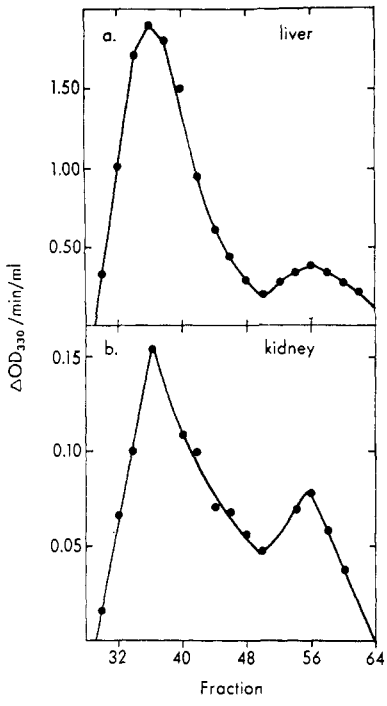

FIGURE 2: Chromatography of phenylalanine hydroxylase from rat liver and rat kidney on Bio-Gel A-5. Enzyme from both tissues was purified through the ammonium sulfate step before appliation to the column: (a) 1.2 g of rat liver enzyme (sp act. 30 mU/mg) and (b) 0.64 g of rat kidney enzyme (sp act. 7 mU/mg) were applied to a 60 × 2.5 cm column equilibrated with 0.01 M Tris-Cl–2 mM mercaptoethanol (pH 7.5). The column was eluted with the same buffer at a flow rate of 50 ml/hr. Fractions of 4.5-ml volume were collected and their activity was determined by assay I. The lower specific activity of the kidney enzyme is due to the intrinsic differences in levels of the enzyme in the two tissues (Ayling *et al.*, 1974).

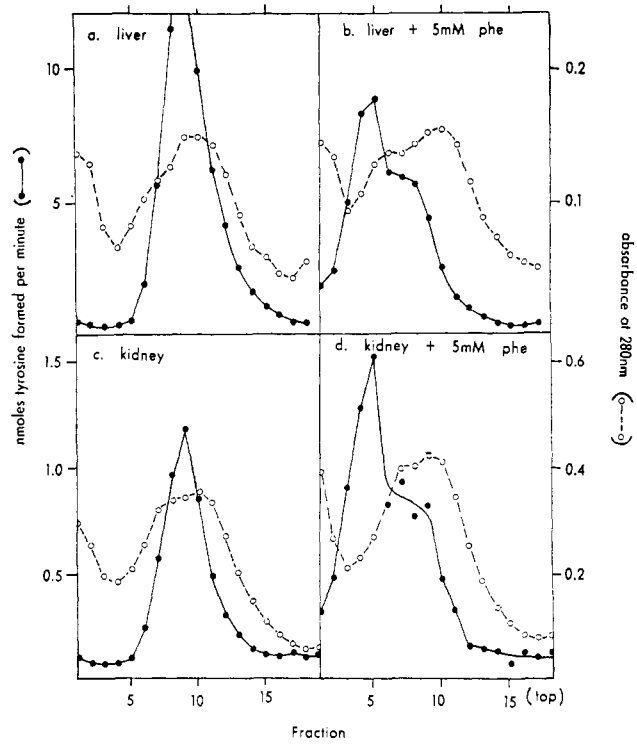

FIGURE 3: Sucrose density gradient centrifugation of rat liver and rat kidney phenylalanine hydroxylase in the presence and absence of phenylalanine. Rat liver enzyme (2 mg, sp act. 80 mU/mg) and rat kidney enzyme (5 mg, sp act. 6 mU/mg) were centrifuged on 5–20% sucrose gradients, containing 0.05 M potassium phosphate, in a Spinco SW 56 rotor at 49,000 rpm and 4°. In this experiment the sedimentation time was 10 hr. In (b) and (d) enzyme was preincubated with 5 mM phenylalanine before application to the gradient, which also contained 5 mM phenylalanine. After centrifugation, the distribution of activity was determined by assay II, with an incubation time of 10 min for the liver enzyme and 20 min for the kidney enzyme.

Enzyme from the second peak re-run on the same column under identical conditions results in an elution profile of activity similar to the initial run. Thus it appears that there is interconvertibility between two forms of the enzyme, which differ in molecular weight.

Rat kidney phenylalanine hydroxylase, purified through the ammonium sulfate step, was passed over the same Bio-Gel A-5 column as the rat liver enzyme (Figure 2b). The kidney enzyme also distributed between two forms of different molecular weight, with the majority of the activity in the higher molecular weight fractions. These two species also appear to be interconvertible since sucrose density gradient centrifugation of either gave the same sedimentation profile. Thus, both kidney and liver enzymes exhibit the same characteristics, under similar conditions of gel filtration.

Sucrose Density Gradient Centrifugation. Partially purified phenylalanine hydroxylases from rat liver and rat kidney were centrifuged in 5–20% sucrose gradients in the presence and absence of 5 mM phenylalanine. Enzyme solution was incubated for 15 min at 25°, either alone or with 5 mM phenylalanine, before application to the corresponding gradient. It has been shown that phenylalanine affects the sedimentation properties of phenylalanine hydroxylase from rat liver (Tourian, 1971).

Sedimentation profiles for the enzyme from liver and from kidney in the absence of phenylalanine are shown in Figures 3a and 3c. The addition of phenylalanine increases the sedimentation velocity of the activity peak, leaving a shoulder corresponding to the position of the peak in the absence of phenylalanine (Figures 3b and 3d). From a comparison of Figures 3a and 3b with 3c and 3d it is apparent that phenylalanine affects the sedimentation properties of the liver and kidney enzymes in an identical manner.

Reaction of Rat Liver Phenylalanine Hydroxylase with Rat Liver Phenylalanine Hydroxylase Antibodies. In order to compare the immunological cross-reactivity of phenylalanine hydroxylase from the various sources, antiserum was obtained from rabbits which had been injected with rat liver phenylalanine hydroxylase purified through the preparative acrylamide gel electrophoresis step. Conditions were then sought which maximized the reaction of the antiserum with the rat liver enzyme. Rat liver phenylalanine hydroxylase, purified through the hydroxylapatite step, was incubated with antiserum for 30 min at 25° to allow antibody to combine with enzyme and then at 0° for increasing times so that precipitation could proceed. After 30 min at 25° the antiserum did not substantially affect the activity of the enzyme or cause precipitation. Incubation at 0°, however, did produce a precipitate. Removal of the precipitate by centrifugation resulted in a decrease in the enzymatic activity of the supernatant compared to controls in which enzyme had been incubated with serum from untreated rabbits. Precipitation was sufficiently slow, compared to the natural decay of the activity demonstrated in the control serum, that it was necessary to find conditions which stabilized the enzyme. It was discovered that the addition of pseudo cofactor (Me_2H_4Pt) and catalase, under a pure nitrogen atmosphere, allowed complete retention of enzymatic

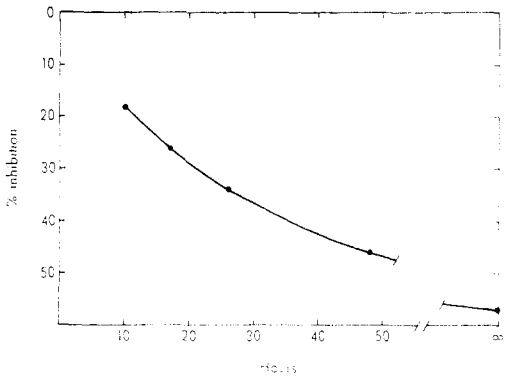

FIGURE 4: Precipitation of rat liver phenylalanine hydroxylase by antibody, as a function of time of incubation at 0°. Mixtures containing 0.01 ml of antiserum/mU of enzyme, 0.08 μmol of Me_2H_4Pt, and 0.1 mg of catalase, in a total volume of 0.4 ml, were incubated under N_2, for 30 min at 25° and then for varying times at 0°. At the end of the incubation period, antibody–antigen complex was removed by centrifugation and the supernatant assayed for activity. Controls were treated identically except that control serum was added in place of the antiserum. The end point of the precipitation, shown as ∞, was obtained by precipitating the antibody–antigen complex with ammonium sulfate, after incubation for 30 min at 25° and 3 hr at 0°.

activity in incubations of 30 min at 25° and up to 26 hr at 0°.

Despite efforts to stabilize enzyme for a time sufficient to allow precipitation, this was still not complete after 48 hr (Figure 4). Incubation times of 48 hr, or longer, proved to be impractical, as by this time up to 50% of the activity had been lost. In addition, the less pure rat kidney and human kidney and liver enzymes are more unstable than the purified rat liver enzyme. The rate of precipitation was even slower if the volume of the incubation mixture was increased, but the degree of inhibition and rate of precipitation were the same whether the incubation was in 0.1 M Tris-HCl (pH 7.4), 0.1 M potassium phosphate (pH 7.4), or 0.1 M sodium borate (pH 8.5). Since complete precipitation of the antibody–antigen complex could not be attained during the time in which there was complete retention of activity in the control, use was made of the differential precipitation of γ-globulin and phenylalanine hydroxylase by ammonium sulfate. γ-Globulins are precipitated by 33% saturation with ammonium sulfate, whereas phenylalanine hydroxylase requires higher concentrations of ammonium sulfate for its precipitation. Accordingly, incubations of antibody with antigen were made for 30 min at 25°, in the presence of Me_2H_4Pt and catalase, under N_2. Saturated ammonium sulfate, pH 7.4, was then added to a final concentration of 33%. The mixture was stirred for 15 min and centrifuged, and the precipitate discarded. The supernatants were brought to 50% ammonium sulfate, stirred, and centrifuged, and the precipitates after being dissolved in 0.1 M potassium phosphate (pH 7.4) were assayed for enzyme activity. Parallel incubations containing control serum were treated identically. In the presence of antiserum, enzyme activity was precipitated by 33% ammonium sulfate. The per cent of activity precipitated was independent of the time of incubation at 0°, and was not increased by extending the time of incubation at 25°. From this it was concluded that the combination of antibody with antigen was complete after 30 min at 25°, although the combination itself does not inhibit enzyme activity. In standardizing the procedure for handling large numbers of assays, the incubation at 25° for 30 min was routinely followed by 3 hr at 0°, before precipitating the complex with ammonium sulfate.

Complete titration curves of rat liver phenylalanine hydroxylase with antiserum were made, keeping the enzyme concentration constant and increasing the concentration of antiserum. The total amount of serum in each incubation mixture was held constant by the addition of control serum. Three different conditions of incubation of enzyme with serum were used: (1) incubation for 30 min at 25° and 17 hr at 0°, (2) incubation for 30 min at 25° and 48 hr at 0°, and (3) incubaion for 30 min at 25° and 3 hr at 0° followed by precipitation of antibody–antigen complex with ammonium sulfate. In all cases precipitates were removed by centrifugation. Activities in the supernatants of (1) and (2) and the resuspended enzyme from the precipitated supernatant of (3) were measured by assay II. Activities were plotted against milliliters of serum/milliunit of enzyme.

Each of the above methods of precipitation produced typical titration curves (*e.g.*, Figure 5, lower curve). The results, summarized in Table II, demonstrate the inverse relationship which exists between time of incubation and the antiserum/enzyme ratio required to obtain a given inhibition. Since, due to the instability of the enzyme, incubation to completion of reaction is impractical, the amount of serum required to produce a given inhibition is dependent on the incubation time. Therefore, if reactions are not completed, a comparison of titration curves for enzymes of different tissues or species depends on the assumption that the precipitation rates are comparable.

Alternatively, antibody–antigen complex may be precipitated with ammonium sulfate. It appears from Figure 4 that precipitation with ammonium sulfate represents the end point

TABLE II: Precipitation of Rat Phenylalanine Hydroxylase by Antibodies to the Rat Liver Enzyme under Different Incubation Conditions.[a]

Incubation Conditions		ml of Antiserum/mU of Enzyme Required for 50% Inhibition	
hr at 25°	hr at 0°	Liver Enzyme	Kidney Enzyme
0.5	17	0.027	0.029
0.5	48	0.012	
0.5	∞ [b]	0.008	0.008

[a] The incubation mixtures contained 3 mU of enzyme and varying amounts of antiserum. Control serum was added to keep the total serum in each incubation constant. Me_2H_4Pt (0.08 μmol) and 0.1 mg of catalase were added to stabilize the enzyme and the mixtures were incubated under N_2. The total volume was 0.4 ml. After incubation and removal of the antibody–antigen precipitate, enzyme activity remaining was assayed by measuring tyrosine formation (assay II) after reaction times of 0, 30, and 60 min. The values are taken from titration curves, similar to that shown in Figure 5 (lower curve), in which the antiserum was varied from 0.0001 to 0.1 ml/mU of enzyme. [b] ∞ = 3 hr at 0° and then precipitated with ammonium sulfate.

of the reaction, since the amount of activity precipitated (marked ∞) is the same as that approached by an extended incubation time. Therefore, assuming that the effectiveness of precipitation with ammonium sulfate is the same in all cases, a valid comparison can be made between enzymes from different sources by this method.

Effect of Rat Liver Phenylalanine Hydroxylase Antibody on Phenylalanine Hydroxylases from Different Sources. Phenylalanine hydroxylases from rat kidney and human liver and kidney were titrated with the same antiserum as used with the rat liver enzyme. The enzymes in this study were purified through the ammonium sulfate step. The titration was over a range of milliliters of antiserum/milliunits of enzyme from 0.0001 to 0.1. Each enzyme was titrated under two different incubation conditions. In the first, the incubation was for 30 min at 25° and 17 hr at 0°, and in the second, after incubation for 30 min at 25° and 3 hr at 0°, the antibody–antigen complex was precipitated with ammonium sulfate.

Under both sets of conditions rat kidney enzyme behaves identically with the rat liver enzyme. (i) The shapes of the titration curves are the same. (ii) The rat kidney enzyme–antibody complex exhibited the same slow precipitation in the absence of ammonium sulfate as did the liver enzyme. In each case 3–4 times more serum was required to give 50% inhibition of activity after 17 hr of incubation at 0° compared to the precipitation with ammonium sulfate (Table II). (iii) Under both incubation conditions the amount of serum required to give 50% inhibition was the same for rat kidney and liver enzymes (Table II). (iv) At a ratio of ml of antiserum/mU of enzyme of 0.01, both rat kidney and rat liver enzyme were inhibited to the same extent (57%) after precipitation of the antibody–antigen complex with ammonium sulfate (Table III).

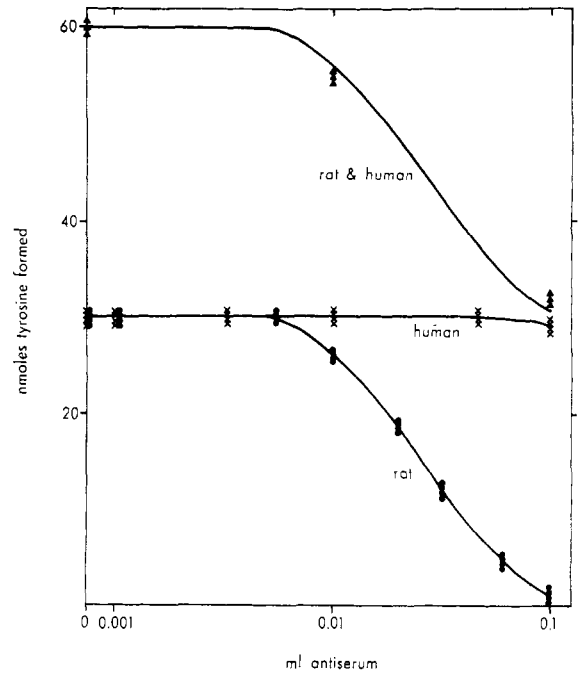

FIGURE 5: Effect of human liver enzyme on the inhibition of rat liver enzyme by rat liver enzyme antibodies. One milliunit of either rat liver enzyme, human liver enzyme, or 1 mU of each, per indicated volume of antiserum, was incubated under N_2 for 30 min at 25° and 17 hr at 0°, in the presence of 0.08 μmol of Me_2H_4Pt, and 0.1 mg of catalse in a total volume of 0.4 ml. Control serum was added to keep the total serum in each incubation mixture constant. At the end of incubation, the antibody–antigen complex was removed by centrifugation and the supernatant assayed for phenylalanine hydroxylase activity (assay II). The two lower curves, obtained from incubating antibody with either rat liver enzyme or human liver enzyme, have been used to calculate the upper curve (rat and human). Data points on this curve were obtained from incubating antibody with rat and human liver enzyme together.

TABLE III: Effect of Rat Liver Phenylalanine Hydroxylase Antibody on Phenylalanine Hydroxylase from Different Sources.[a]

Enzyme Source	% Inhibition at ml of Antiserum/mU of Enzyme = 0.01 (%)	ml of Antiserum/mU of Enzyme Required for 50% Inhibition
Rat liver	57	0.008
Rat kidney	57	0.008
Human liver	0	0.2
Human kidney	0	0.2

[a] Enzyme, 3 mU, was incubated with varying concentrations of antiserum for 30 min at 25° and 3 hr at 0°. The antibody–antigen complex was then precipitated by 33% saturation with ammonium sulfate. Enzyme activity remaining in the supernatant was assayed after precipitation with 50% ammonium sulfate. Other experimental conditions were the same as for Table II, except with the human enzymes the titration curves were extended to 0.2 ml of serum/mU of enzyme. In this case 1.5 mU of enzyme was used.

In contrast, the reaction of human kidney and liver phenylalanine hydroxylases with antibody to the rat liver enzyme was remarkably different from that of the enzymes from rat liver and kidney. After 30 min at 25° and 17 hr at 0°, precipitation of human phenylalanine hydroxylases was not detected with up to 0.1 ml of antiserum/mU of enzyme even though at this level there was almost complete precipitation of both rat phenylalanine hydroxylases. Attempts to precipitate the antibody–antigen complex with ammonium sulfate at a ratio of ml of antiserum/mU of enzyme of 0.01, which gave 57% precipitation of both rat enzymes, also failed to produce any precipitation of the human enzymes (Table III). As the milliliters of antiserum/milliunits of enzyme ratio was increased some inhibition of activity was observed, after precipitation of the antibody–antigen complex with ammonium sulfate. The inhibition reached 33% at a level of 0.1 ml of antiserum/mU of enzyme. Extending the titration curve to even higher levels of antiserum showed that 0.2 ml of antiserum/mU of enzyme was required to achieve 50% inhibition, with both the human liver and human kidney enzymes (Table III). This is 25 times more antibody than required to give 50% inhibition of the rat enzymes.

We wished to ensure that the observed differences in antigenicity between the human and rat enzymes were indeed due to differences in the properties of the enzymes and not due to some experimental artifact. For example, the lower reactivity of the human enzyme with rat liver antibodies could have been due to (1) nonspecific complexing of the antibodies, since the human enzymes used were not highly purified, (2) degradation of the antibodies by proteolytic enzymes present in the human enzyme preparations, or (3) the presence of a large amount of

enzyme which had lost its catalytic activity, but still retained its antigenic activity, and therefore the ratio of antiserum/enzyme was lower than presumed. Any of the preceding factors although possible are unlikely. (1) The rat kidney enzyme was only purified to the same crude state as the human enzymes and yet showed identical antigenicity to the more highly purified rat liver enzyme. (2) Although antibodies might be degraded during long incubation times the effect would not likely be significant for the short incubation time preceding precipitation of the antibody–antigen complex with ammonium sulfate. (3) The human kidney enzyme used for the immunological experiments was prepared from fresh surgically removed kidney which had one of the highest specific activities for phenylalanine hydroxylase that we have observed for human kidney. During the course of purification and storage more than 50% of the original total catalytic activity was retained. However, to ensure that the observed antigenic properties of the human enzyme were not due to any of the above factors, or other possible artifacts, the inhibition of rat enzyme by antibodies was determined in the presence of the human enzyme. Figure 5 illustrates the results of such an experiment. The two lower curves show the effect of antibody on rat liver enzyme and human liver enzyme when incubated separately with increasing concentrations of antiserum. The incubation time in this case was 17 hr, and no inhibition of the human enzyme was detected with up to 0.1 ml of antiserum/mU of enzyme. The upper curve is that expected from the direct summation of the two lower curves. The data points were obtained from incubations to which both the human and rat liver enzymes were added. The observed values fall onto the expected curve. Thus, it appears that the human enzyme preparation contains nothing which interferes with antibody activity.

Discussion

Phenylalanine hydroxylase from rat or human kidney could not be distinguished from the corresponding liver enzyme when compared on the basis of (i) apparent Michaelis constants for phenylalanine, Me_2H_4Pt and tetrahydrobiopterin, and the relative rates with the two cofactors, or (ii) immunological cross-reactivity to rat liver phenylalanine hydroxylase antibodies. These criteria were sufficiently sensitive to demonstrate species differences. The enzymes from the two rat tissues were also indistinguishable when further compared by (iii) the elution pattern and equilibrium distribution on Bio-Gel A-5 and (iv) the effect of phenylalanine on the sedimentation velocity in sucrose density gradient.

The rat kidney enzyme is less stable than the liver enzyme in homogenate supernatants, and this has been taken as evidence for different forms of the enzyme in the two tissues (Murthy and Berry, 1971). However, the instability appears to be a property of the extract, rather than of the enzyme, since after purification through the hydroxylapatite step, enzymes from both tissues exhibit similar stability.

The significance of the kinetic comparisons between tissues can be gauged by the differences between species. The Michaelis constants for phenylalanine and the pseudo cofactor, Me_2H_4Pt, are almost identical for human and rat, but there is more than a twofold species difference in the apparent K_m for the natural cofactor, tetrahydrobiopterin, and in the ratios of the rate with tetrahydrobiopterin to the rate with Me_2H_4Pt (Table I). The apparent maximum velocity with tetrahydrobiopterin is 4.5 times higher than with Me_2H_4Pt for the rat enzymes and two times higher for the human enzymes. The faster rate with tetrahydrobiopterin compared to Me_2H_4Pt is in agreement with our earlier observations with phenylalanine hydroxylase from rat liver (Ayling *et al.*, 1973a). This is at variance with Kaufman and coworkers who report the opposite relationship in the relative rates with the two cofactors (Fisher and Kaufman, 1972; Friedman and Kaufman, 1973). Possible causes for this discrepancy have been discussed previously (Ayling *et al.*, 1973a).

Antiserum to rat liver phenylalanine hydroxylase reacted identically with rat liver and rat kidney enzyme. On the other hand 25 times more antiserum was required to precipitate either the human kidney or human liver enzyme. These results indicate that 4% of the rat enzyme antibody binding sites are common with those of the human enzyme. Since enzymes were compared on the basis of units of catalytic activity an alternative, but less likely, interpretation is that the antibody binding sites are the same for both species, but that the turnover number of the human enzyme is 25 times lower than the turnover number of the rat enzyme. In either case, it is of significance that the human liver and human kidney enzymes are indistinguishable with respect to their cross-reactivity with rat liver phenylalanine hydroxylase antibodies.

The species difference which we have observed is in contrast to the recent report of Friedman *et al.* (1972), who found that antibodies to rat liver phenylalanine hydroxylase reacted equally well with phenylalanine hydroxylases from rat kidney, guinea pig, mouse, and human liver and also with bovine adrenal and rat brain tyrosine hydroxylases. The reason for this discrepancy may be due to the method used for antibody production. Friedman *et al.* produced antibodies in sheep by intramuscular injection of an emulsion of homogenized polyacrylamide gel containing phenylalanine hydroxylase and Freund's adjuvant. The antibodies which we used were produced in the rabbit after a final intravenous boost of native phenylalanine hydroxylase (*i.e.*, not emulsified). Thus, these antibodies may recognize fewer sites than antibodies produced to enzyme which has first been emulsified in Freund's adjuvant.

Although differing in specificity the phenylalanine hydroxylase antibody content of our rabbit serum is almost identical with that of Friedman's sheep serum when compared on the basis of units of activity precipitated per milliliter of serum. It appears that phenylalanine hydroxylase has relatively low antigenicity. The specific activity of pure phenylalanine hydroxylase is of the order of 1 U/mg of enzyme. The minimal amount of serum required to give 50% precipitation was 0.008 ml of antiserum/mU of enzyme. Thus, 1 ml of antiserum can precipitate about 0.1 mg of rat liver phenylalanine hydroxylase.

The use of rat liver phenylalanine hydroxylase antibodies for the isolation of mutant human enzyme is impractical when the reactivity with normal human enzyme is 25 times less than with rat enzyme. The method of Friedman *et al.* (1972) produced antibodies to rat liver enzyme which reacted equally well with human enzyme. However, the lack of specificity of the antibodies may diminish their usefulness in the isolation of mutant phenylalanine hydroxylases. Since antibodies to rat liver phenylalanine hydroxylase cross-reacted identically with the rat kidney enzyme, it seems reasonable to expect the same cross-reactivity between the human liver and kidney enzymes. Enzyme from human kidney, which is more available than human liver (Ayling *et al.*, 1974), could thus be used for the production of antibodies, allowing the study of mutant enzymes from phenylketonuric liver. It is as yet unknown whether kidney phenylalanine hydroxylase is also affected in

any forms of phenylketonuria. If the kidney and liver enzymes are identical it would be expected that enzymes from both tissues would be similarly affected at least in the types of phenylketonuria which result from a mutation in the structural gene for phenylalanine hydroxylase.

It has previously been reported that rat liver phenylalanine hydroxylase exists as two (Kaufman and Fisher, 1970) or three (Barranger *et al.*, 1972) different isozymes. If this is the case, the results reported above indicate that the same isozymes exist in the kidney as in the liver. However, further physical and chemical studies are required before a conclusion can be made.

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