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The Isolation and Properties of Phenylalanine Hydroxylase from Rat Liver

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Phenylalanine hydroxylase was prepared from rat liver and purified 200-fold to about 90% purity. All the enzymic activity of the liver appeared in a single protein of mol.wt. approx. 110000, but omission of dithiothreitol and of a preliminary filtration step to remove lipids resulted in partial conversion into a second enzymically active protein of mol.wt. approx. 250000. The K_m and $V_{max.}$ values of the enzyme for phenylalanine, *p*-fluorophenylalanine and dimethyltetrahydropterin were measured; *p*-chlorophenylalanine inhibited the enzyme by competing with phenylalanine. Disc gel electrophoresis at pH7.2 showed a single protein band containing all the enzymic activity, but at pH8.7 the enzyme dissociated into two inactive fragments of similar but not identical molecular weight. The molecule of phenylalanine hydroxylase contained two atoms of iron, one atom of copper and one molecule of FAD; molybdenum was absent. Treatment with chelating agents showed that both non-haem iron and copper were necessary for enzymic activity. The molecule contained five thiol groups, and thiol-binding reagents inhibited the enzyme. Catalase or peroxidase enhanced enzymic activity fivefold; it is postulated that catalase (or other peroxidase) plays a part in the hydroxylation reaction independent of the protection by catalase of enzyme and cofactor from inactivation by a hydroperoxide.

Phenylalanine is converted into tyrosine in mammalian liver by phenylalanine 4-hydroxylase acting with O_2 and a cofactor. The natural cofactor is tetrahydrobiopterin, but other tetrahydropterins are active, 6,7-dimethyltetrahydropterin being mostly used since it is commercially available (Kaufman, 1964).

In all cases of phenylketonuria and phenylalaninaemia as yet investigated enzymically, a block in the conversion of phenylalanine into tyrosine is caused by a marked decrease in phenylalanine hydroxylase activity resulting from a single mutant gene in double dose and not from the absence of cofactor (Friedman *et al.*, 1973).

The isolation of phenylalanine hydroxylase from rat liver has been described by Kaufman & Fisher (1970). These workers obtained two active fractions of mol.wts. approx. 110000 and 220000 respectively. Barranger *et al.* (1972), with a different technique, obtained three forms of phenylalanine hydroxylase from rat liver.

We report here finding two phenylalanine hydroxylases in rat liver; the molecular weights were about 110000 and 250000 respectively. However, a modified extraction procedure resulted in all of the phenylalanine hydroxylase activity of the rat liver appearing as a single, highly purified protein fraction of mol. wt. approx. 110000. The properties of the enzyme were studied.

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Experimental and Results

Materials

Crystalline catalase (1830 units/mg) and horseradish peroxidase (3075 units/mg) were purchased from Worthington Biochemical Corp., Freehold 2, N.J., U.S.A. Bio-Gel P-300 was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The other materials were as described in the following paper (Woo *et al.*, 1974).

Potassium phosphate buffers, 0.2M, were prepared by mixing 0.2M-KH_2PO_4 and 0.2M-K_2HPO_4 solutions (both prepared from the 'Certified grade' anhydrous salts from Fisher Scientific Co., Fair Lawn, N.J., U.S.A.) in relative volumes appropriate to the required pH (Gomori, 1955). The pH was checked with an Instrumentation Laboratories model 245 pH meter and, when necessary, adjusted by adding more of the KH_2PO_4 or K_2HPO_4 solution. Potassium phosphate buffers of other molarities were prepared similarly.

Adult Wistar rats were killed by decapitation; the livers were immediately removed and homogenized.

Assay for phenylalanine hydroxylase activity

The standard reaction mixture contained 0.2M-potassium phosphate (pH6.8), 5mM-phenylalanine, 0.5mM-6,7-dimethyltetrahydropterin, catalase (730 units/ml), 3mM-dithiothreitol and the phenylalanine hydroxylase preparation in a total volume of 0.25ml. The reaction was started by the addition of the tetrahydropterin and the tube was incubated for 20min at 25°C with shaking in air. The reaction was stopped by the addition of 50% (w/v) trichloroacetic acid to a final concentration of 5% (w/v.) The precipitated proteins were removed by centrifugation and the supernatant was assayed for tyrosine fluorimetrically (Waalkes & Udenfriend, 1957) by using an Aminco Bowman spectrophotofluorimeter as described in the following paper (Woo *et al.*, 1974). Protein concentrations were determined by the biuret method (Gornall *et al.*, 1949).

Purification and properties of phenylalanine hydroxylase, original method

Except where otherwise stated, all centrifugations were performed at 27000*g* for 15min in a Sorvall RC2B centrifuge with a S34 head. In fractionation steps resulting in precipitation, solutions were stirred for 15min after the last addition of precipitant before being centrifuged. All operations were carried out at 4°C unless stated otherwise.

(I) *Extraction, acid treatment and $(NH_4)_2SO_4$ precipitation.* Fresh rat liver, about 60g, was weighed, cut up into small pieces and homogenized for 1min in a Virtis homogenizer with 300ml of 0.1M-potassium phosphate, pH6.8, containing 0.1M-KCl and 5% (v/v) glycerol. Insoluble material was removed by centrifugation at 27000*g* for 30min. The pH of the supernatant fluid was adjusted to 5.0 with chilled 1M-acetic acid. After centrifugation, the supernatant was adjusted to pH7.0 with cold 1M-KOH. $(NH_4)_2SO_4$ solution, saturated at room temperature and adjusted to pH7.0, was added; the fraction that precipitated between 30 and 50% saturation was dissolved in 22ml of 0.1M-potassium phosphate buffer (pH6.8) which contained 5% (v/v) glycerol [$(NH_4)_2SO_4$ fraction].

(II) *DEAE-cellulose chromatography.* The $(NH_4)_2SO_4$ fraction was desalted by chromatography on a column (2cm×45cm) of Sephadex G-25 and the desalted solution containing 400mg of protein was applied to the top of a column (2cm×20cm) of DEAE-cellulose that had been pre-equilibrated with 0.02M-potassium phosphate (pH7.2) containing 5% (v/v) glycerol and 0.01M-phenylalanine. The

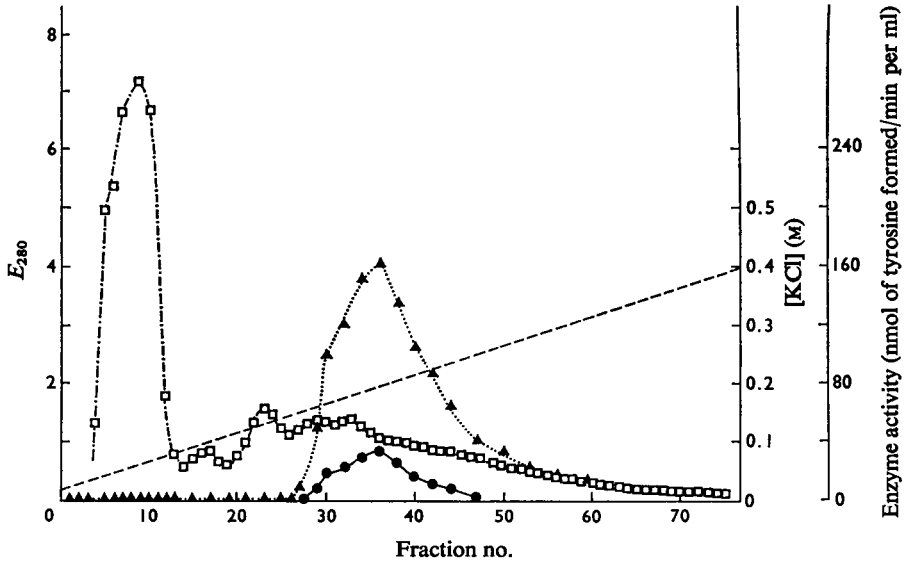

Fig. 1. *DEAE-cellulose chromatography*

The column (2cm×10cm) was pre-equilibrated with 0.02M-potassium phosphate (pH7.2) which contained 0.01M-phenylalanine and 5% (v/v) glycerol. The $(NH_4)_2SO_4$ fraction (400mg) was applied, $(NH_4)_2SO_4$ having been removed by passage through Sephadex G-25. A linear gradient of KCl, 0.01M to 0.4M (prepared from 250ml of each in the above buffer), was applied to the column; elution was at the rate of 1.2ml/min. Fractions of volume 6ml were collected. ----, Molarity of KCl applied; ▲, enzymic activity in the presence of catalase; ●, enzymic activity in the absence of catalase; □, E_{280}.

protein was eluted with a linear KCl gradient from 0.01 to 0.4M (Fig. 1). The fractions eluted between 0.17M- and 0.25M-KCl were pooled (108ml total volume) and concentrated by precipitation with an equal volume of saturated $(NH_4)_2SO_4$ solution. The recovery from DEAE-cellulose chromatography was usually greater than 80%. Substitution of 0.02M-Tris–HCl, pH7.2, for potassium phosphate, did not alter the elution profile or the salt concentrations required for eluting the enzyme activity.

As shown in Fig. 1, there was a fivefold stimulation of phenylalanine hydroxylase activity when catalase was added in the assay system. The first protein peak, eluted in fractions 3 to 12, was found to contain catalase which did not separate from phenylalanine hydroxylase in the $(NH_4)_2SO_4$ step. The addition of catalase in the assay system is therefore essential for the determination of phenylalanine hydroxylase activity after DEAE-cellulose fractionation.

(III) *Sephadex G-200 chromatography.* Protein (81mg in 7ml) from the DEAE-cellulose step was applied to a column (1.5cm×90cm) of Sephadex G-200 which had been pre-equilibrated with the eluting buffer [0.1M-potassium phosphate, pH6.8, 0.01M-phenylalanine and 5% (v/v) glycerol]. On elution, two peaks of enzymic activity were detected (Fig. 2); fraction E_2 has a specific activity five times higher than fraction E_1. Of the total enzymic activity after fractionation on Sephadex G-200, the proportion in E_1 varied, in different preparations, between 10 and 40%.

Fraction E_1 after further chromatography on hydroxyapatite and Bio-Gel P-300 appeared still to have low specific activity as compared with fraction E_2. The possibility of the reversible dimerization of fraction E_2 to fraction E_1 was ruled out by rechromatography of the separated fractions on Sephadex G-200, each giving a single peak of unchanged retention time

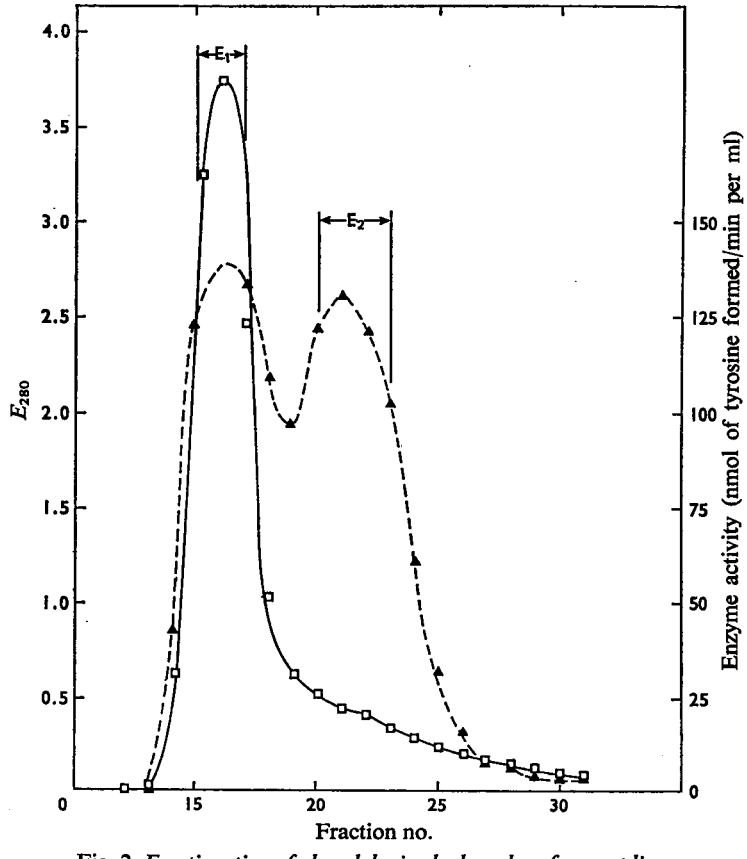

Fig. 2. *Fractionation of phenylalanine hydroxylase from rat liver*

Protein (81mg) from the DEAE-cellulose column was applied to a Sephadex G-200 column (1.5cm×90cm) which was pre-equilibrated with the eluting buffer [0.1M-potassium phosphate, pH6.8, 0.01M-phenylalanine and 5% (v/v) glycerol]. Fractions of volume 2.8ml were collected every 40min. □, E_{280}; ▲, enzyme activity.

Table 1. *Purification of phenylalanine hydroxylase from rat liver*

For details see the text.

Step	Volume (ml)	Protein (mg/ml)	Specific activity (nmol of tyrosine formed/min per mg of protein)		Recovery (%)
			Without catalase	With catalase	
Crude homogenate	220	83	1.62	1.7	100
Supernatant (27000*g*, 30min)	190	44	3.5	3.5	94
Acid precipitation	205	19	5.5	6.5	82
$(NH_4)_2SO_4$ (30–50% satn.)	22	18.5	34	36	50
DEAE-cellulose	20	6.5	25	102	40
Sephadex G-200 I E_1	2	12	14	57	15
E_2	2	6	57	280	
Sephadex G-200 II E_2	1.5	4	80	350	8

and specific activity. Sucrose-gradient centrifugation led to the same conclusion.

Fraction E_2 was further purified by rechromatography on Sephadex G-200 which removed any remaining traces of fraction E_1. Analysis of fraction E_2 by gel electrophoresis and ultracentrifugation revealed a single component.

A typical purification of phenylalanine hydroxylase is summarized in Table 1. The enzyme was purified about 200-fold by the procedures with a yield of about 10%. The extent of purification shown in the table is less than the actual purification, because there was a loss of 20–30% of the activity during each step of purification, even though the enzyme was stabilized to a marked degree by the presence of 0.01M-phenylalanine and 5% (v/v) glycerol. As shown in Table 1 and Fig. 1, catalase had a great stimulatory effect on the hydroxylase activity; catalase prevents inactivation of the hydroxylase by a peroxide formed during the reactions of 6,7-dimethyltetrahydropterin with oxygen (Kaufman, 1962, 1970; Jakubovic *et al.*, 1971), and probably plays a part in the hydroxylation reaction itself.

Modified procedure for isolating phenylalanine hydroxylase

This was similar to the procedure used for human liver (Woo *et al.*, 1974). It differed from the original method in that dithiothreitol was present during homogenization and subsequently that the supernatant from the initial centrifugation was filtered through glass wool and that the acetic acid precipitation step was omitted. The rest of the procedure was as described above, but only a single peak corresponding to the position of fraction E_2 emerged from the Sephadex G-200 column.

To check this, rat liver (40g) was homogenized as described above and divided into two equal portions: one half, (*a*), was centrifuged at 35000*g* for 1h and the supernatant decanted. To the other half (*b*) was added dithiothreitol to 1mM before centrifuging at 35000*g* for 1h, the supernatant being filtered through glass wool. The phenylalanine hydroxylase activities in the two supernatants were equal, within experimental error, each being more than 99% of that in the original homogenate. Both supernatants were taken through the original purification procedure, but in case (*b*) all precipitating solutions, eluting buffers etc. contained 0.1mM-dithiothreitol. In the final step portion (*a*) gave as usual two peaks in the positions of fraction E_1 and fraction E_2 with, respectively 6.4 and 7.2% of the activity in the original supernatant, but portion (*b*) gave a single peak in the position of fraction E_2 with 14.1% of the original activity.

Purity of the enzyme

Fractions E_1 and E_2 were analysed for purity by polyacrylamide-disc-gel electrophoresis and ultracentrifugation. The procedures and buffer solutions of Ornstein (1964) and Davis (1964) were used for disc gel electrophoresis. Electrophoresis was carried out at 4°C with current 3mA/tube. Ammonium persulphate was used as catalyst for the polymerization of the separating gels and riboflavin for the stacking gels. Ammonium persulphate was removed by pre-electrophoresis before the application of samples. The electrophoresis was carried out at pH8.7 in the glycine buffer and at pH7.2 in the potassium phosphate buffer. When the electrophoresis was run at pH7.2 with the phosphate buffer, fraction E_2 moved as a single band on the gel and all of the enzyme activity was associated with this band. However, when gel electrophoresis was carried out at pH8.7 in the glycine buffer, two major bands were observed and no enzymic activity could be detected

in either band. When fraction E_2 was run in 8M-urea gel at pH8.7 (Jovin *et al.*, 1964) four bands were detected.

In fraction E_1, most of the protein stayed on top of the gel both at pH7.2 and pH8.7. The only condition where the protein moved into the gel was after it had been treated with sodium dodecyl sulphate. Since the enzyme lost its enzymic activity under these conditions, it proved impossible to estimate the purity of fraction E_1 electrophoretically.

Sucrose-gradient-centrifugation patterns of both fractions are given in Figs. 3 and 4. The activity peak of fraction E_2 comprises about 90% of the protein (Fig. 3) and about 60% for fraction E_1 (Fig. 4). For fraction E_1 the centrifugation time was reduced to 6h at 80800*g* as it pelleted at the bottom of the centrifuge tube when the centrifugation was run for 16h as was done for fraction E_2.

Molecular weight and the prosthetic groups of the enzyme

Ultracentrifugation. Sedimentation coefficients were measured in 0.1M-potassium phosphate buffer (pH6.8) at 20°C with a Beckman ultracentrifuge model E and a photoelectric scanning absorption system (Schumaker & Schachman, 1957). Sedimentation coefficients for fractions E_1 and E_2 are 9.45S and 5.47S respectively (Table 2). The molecular weights were determined from sucrose-gradient centrifugation and from chromatography on Sephadex G-200. A mol.wt. of 110000 was obtained for fraction E_2 and 250000 for the slow-moving component of fraction E_1.

Iron, copper and molybdenum were determined by using a Perkin–Elmer model 303 atomic-absorption spectrophotometer with an air–acetylene flame and, respectively, iron, copper and molybdenum hollow-cathode lamps. The procedures used were those described in the instrument manual (Perkin–Elmer, 1971). The wavelengths and slit widths used were respectively, 248.3nm and 0.2mm for iron, 324.7nm and 0.7mm for copper, 313.3nm and 0.7mm for molybdenum. No molybdenum was found (sensitivity 0.1–0.2p.p.m.). Fraction E_2 contains 1 atom of copper and 2 atoms of iron per molecule of enzyme (Table 2). These metals are necessary for phenylalanine hydroxylase activity as shown by the effect of

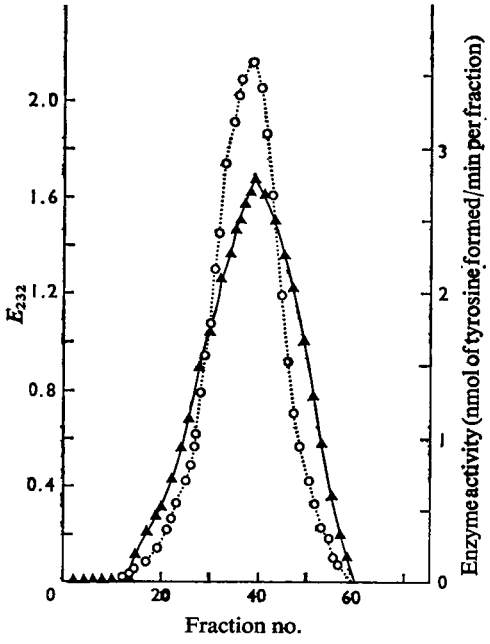

Fig. 3. *Sucrose-gradient centrifugation of fraction E_2*

Fraction E_2 (1.5mg in 0.2ml) was layered on 4.8ml of a 5–20% sucrose gradient and centrifuged in a Beckman model L preparative ultracentrifuge, with a SW39L head, at 4°C at 80800*g* for 16h. Fractions of volume 0.18ml were collected. ▲, E_{232}; ○, enzymic activity.

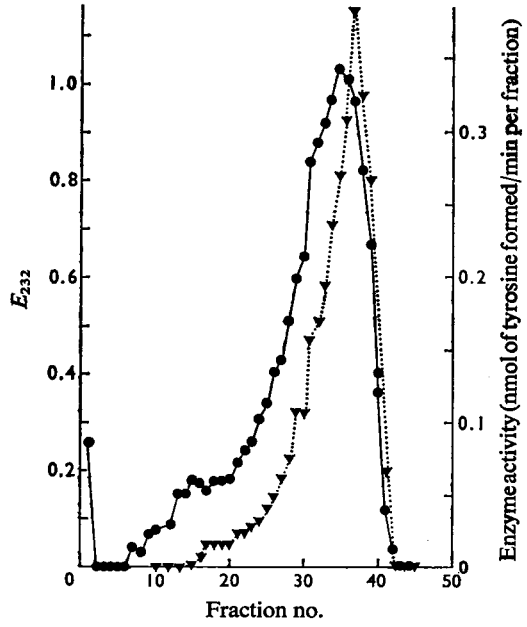

Fig. 4. *Sucrose-gradient centrifugation of fraction E_1*

Fraction E_1 (1.0mg in 0.2ml) was layered on 4.8ml of a 5–20% sucrose gradient and centrifuged in a Beckman model L preparative ultracentrifuge, with a SW39L head, at 4°C at 80800*g* for 6h. Fractions of volume 0.14ml were collected. ●, E_{232}; ▼, enzymic activity.

various metal-chelating agents on enzymic activity in the presence and absence of phenylalanine (Table 4). The participation of iron in the phenylalanine hydroxylase reaction has been described by Fisher *et al.* (1972); they found 1–2g-atoms of Fe/100000*g* of protein, measured colorimetrically.

There was a complete lack of xanthine oxidase activity at high enzyme concentration; this and the absence of molybdenum made it unlikely that the iron and copper were derived from liver xanthine oxidase present as a contaminant.

Incubation of very high concentrations of both fractions of the enzyme with L-tryptophan, 6,7-dimethyltetrahydropterin, catalase and dithiothreitol did not yield any detectable 5-hydroxytryptophan, indicating the absence of both tryptophan hydroxylase and non-specific hydroxylases.

Table 2. *Some components and properties of rat liver phenylalanine hydroxylase*

	Fraction E_1	Fraction E_2
Sedimentation coefficient	9.45S	5.47S
Molecular weight	Approx. 250000	Approx. 110000
Fe (mg/g of protein)	3.0	1.20
(atoms/molecule)	12	2
Cu (mg/g of protein)	0.6	0.5
(atoms/molecule)	2	1
FAD (mg/g of protein)	2.66	4.8
(mol/mol)	0.77	0.61
Thiol groups/molecule in	5.4	4.4
8M-urea	5.06	4.7

The purified phenylalanine hydroxylase fraction E_2 is yellowish with a small peak in its absorption spectrum at 425nm. A peak of fluorescence emission in the range 510–550nm was observed at acidic pH. The flavin content in both fractions was determined fluorimetrically (Burch *et al.*, 1948) with FMN and FAD as standards (Table 2). Since fluorescence was observed after the enzyme was precipitated with 5% (w/v) trichloroacetic acid and subsequent incubation of the supernatant solution at 37°C for 20h, it was concluded that FAD, not FMN, is the flavin nucleotide associated with phenylalanine hydroxylase (Burch *et al.*, 1948).

Thiol groups of the enzyme were determined spectrophotometrically with Ellman's reagent (Ellman, 1959) in the presence and absence of 8M-urea (Table 2).

Kinetic parameters

Kinetic properties of fractions E_1 and E_2 are shown in Table 3. With dimethyltetrahydropterin as cofactor and phenylalanine as substrate, we found no inhibition by phenylalanine. Fraction E_1 and E_2 gave identical apparent K_m values, within experimental error, but the $V_{max.}$ for fraction E_2 was about four times as high as that for fraction E_1.

p-Fluorophenylalanine served as a substrate for both fractions with a relatively slower reaction rate than for phenylalanine. This rate was so low with fraction E_1 that it was not possible to determine accurate K_m or $V_{max.}$ values.

p-Chlorophenylalanine acted as a competitive inhibitor of phenylalanine hydroxylase. The values

Table 3. *Enzyme kinetics of rat liver phenylalanine hydroxylase*

With phenylalanine as substrate, each experiment for the determination of K_m of fraction E_2 had seven different phenylalanine concentrations from 0.125–5mM. With dimethyltetrahydropterin as substrate, each experiment for the determination of K_m of fraction E_2 had seven different cofactor concentrations from 50–500μM. For further details see the text.

	Fraction E_1		Fraction E_2	
Substrate	$K_m \pm$ S.E. (mM)	$V_{max.}$ (nmol of tyrosine formed/min per mg)	$K_m \pm$ S.E. (mM)	$V_{max.}$ (nmol of tyrosine formed/min per mg)
Phenylalanine	1.42 ± 0.02	160	1.22 ± 0.14‡ 1.04 ± 0.28§	696
Dimethyltetrahydropterin	$2.84 \times 10^{-2} \pm 0.46 \times 10^{-2}$	120	$5.5 \times 10^{-2} \pm 0.4 \times 10^{-2}$|| $4.9 \times 10^{-2} \pm 0.3 \times 10^{-2}$¶	540
p-Fluorophenylalanine	1.33†	29†	2.5	150
Dimethyltetrahydropterin*	2.5×10^{-2}†	9†	2.5×10^{-2}	120

* When *p*-fluorophenylalanine was the other substrate.
† Values are approximate, see the text.
‡ With 500μM cofactor.
§ With 100μM cofactor.
|| With 5mM-phenylalanine.
¶ With 1mM-phenylalanine.

Table 4. *Effect of metal-chelating agents on activity of phenylalanine hydroxylase (fraction E_2)*

The enzyme concentrations used in the presence and absence of catalase were 60μg/ml and 200μg/ml respectively. Assays were done with and without 5mM-phenylalanine present during the 15min preincubation with the chelating agent. For further details see the text.

Metal-chelating agent	Concn. (mM)	Enzymic activity remaining (%)	
		Without 5mM-phenylalanine	With 5mM-phenylalanine
None	—	100	100
EDTA	1	95	95
CN^-*	1	94	93
2,2′-Bipyridyl*	0.1	30	50
	1	12	12
8-Hydroxyquinoline*	0.1	50	60
	1	35	26
Sodium diethyldithiocarbamate	0.1	70	80
	1	45	54
Ascorbic acid*	0.2	15	20
Dithiothreitol	5	53	78

* Assayed in the absence of catalase.

Table 5. *Effect of thiol-binding reagents on activity of phenylalanine hydroxylase fraction E_2*

The concentration of fraction E_2 was 60μg/ml. Assays were done with and without 5mM-phenylalanine present during the 15min preincubation with the thiol-binding reagent. For further details see the text.

Thiol-binding reagent	Concn. (mM)	Enzyme activity (nmol of tyrosine formed/min per mg of enzyme)		Activity remaining (%)	
		With phenylalanine	Without phenylalanine	With phenylalanine	Without phenylalanine
None	—	148	133	100	100
p-Hydroxymercuribenzoate	0.1	53	35	36	25
	1	13	0.27	9	2
N-Ethylmaleimide	1	142	133	100	97
Iodoacetic acid	1	146	140	100	100
$CuSO_4$	0.2	25	20	17	15

of K_i calculated from Lineweaver–Burk plots of $1/v$ against 1/[phenylalanine] were 2.9×10^{-3}M for fraction E_1 and 1.29×10^{-3}M for fraction E_2.

Effect of metal-chelating agents

Because of the presence of iron and copper in the purified fraction E_2, the effect of various chelating agents was studied (Table 4). Each reagent was incubated with the purified E_2 fraction with and without phenylalanine for 15min at 25°C and the enzyme activity was assayed by adding cofactor, dithiothreitol, catalase and phenylalanine where this was not already present. Where cyanide, 2,2′-bipyridyl, 8-hydroxyquinoline and ascorbic acid were investigated, catalase was omitted because it is inhibited by these agents. EDTA and cyanide were found not to be inhibitory to phenylalanine hydroxylase. The inhibition by 2,2′-bipyridyl strongly suggests that Fe^{2+} is necessary for enzymic activity; the effect of sodium diethyldithiocarbamate suggests that Cu^{2+} may also be necessary. The inhibition by dithiothreitol in the absence of phenylalanine could be explained by the heavy-metal-complexing property of dithiothreitol. The slight protection afforded by phenylalanine raises the question whether the metal ions are involved in the binding of phenylalanine.

Effect of thiol-binding reagents

The inhibition by various thiol-binding reagents is shown in Table 5. This, together with the presence of five SH groups/molecule of enzyme, suggests that SH groups are essential for catalytic activity.

Table 6. *Effect of iron salts, catalase and peroxidase on activity of phenylalanine hydroxylase (fraction E_2)*

Each reaction mixture (0.25ml) containing 0.2M-potassium phosphate (pH6.8) and phenylalanine hydroxylase (66μg) was preincubated with ferrous ammonium sulphate or ferric ammonium sulphate and peroxidase or catalase as indicated at 25°C for 10min before the addition of phenylalanine, dimethyltetrahydropterin and dithiothreitol. The incubation and assay were as described in the Experimental and Results section.

Oxidase	Enzyme activity (nmol of tyrosine formed/min per mg of phenylalanine hydroxylase)		
	Control	Fe^{3+} (0.2mM)	Fe^{2+} (0.2mM)
None	10.24	11.34	11.02
Horse-radish peroxidase (50μg)	55	55	52
Catalase (25μg)	55	55	52.8

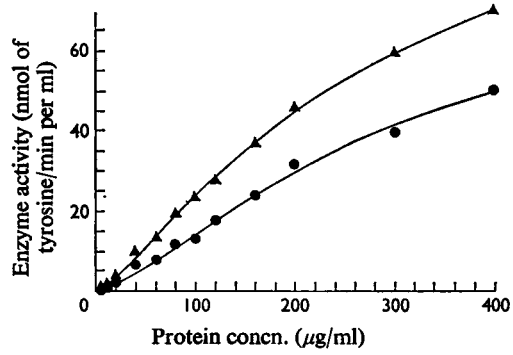

Fig. 5. *Effect of enzyme concentration and of phosphate on the enzymic activity*

The assay conditions were as described in the Experimental and Results section. The incubation was at 25°C for 10min. ▲, Enzymic activity assayed in 0.5M-potassium phosphate, pH6.8; ●, activity assayed in 0.2M-Tris–HCl, pH6.8.

Role of catalase and other peroxidase

Under our assay conditions catalase stimulates phenylalanine hydroxylation and protects the tetrahydropterin and enzyme from a peroxide formed by auto-oxidation of the tetraphydropterin (Kaufman, 1962; Jakubovic *et al.*, 1971). As shown in Table 6, horse-radish peroxidase in the presence of dithiothreitol can substitute for catalase, but horse-radish peroxidase cannot protect the enzyme from inactivation by the peroxide formed from the tetrahydropterin (Jakubovic *et al.*, 1971). Hence peroxidase and catalase have a stimulating effect on phenylalanine hydroxylation quite apart from the protective effect of catalase. Fe^{2+} and Fe^{3+} cannot substitute for peroxidase or catalase (Table 6).

Effect of ionic strength and enzyme concentrations

Fisher & Kaufman (1970) reported that when 7-methyltetrahydropterin was substituted for the natural cofactor, tetrahydrobiopterin, in the phenylalanine hydroxylase system, more than 1mol of tetrahydropterin was oxidized for each mol of tyrosine formed. They found that the ratio of tetrahydropterin oxidation to tyrosine formed depended on the enzyme concentrations and ionic strength of the reaction mixture.

The effects of ionic strength and enzyme concentrations were studied with fraction E_2. With 0.5M-potassium phosphate, there was a 30% increase in tyrosine formation as compared with the reactions carried out in 0.2M-Tris. However, this increment was found to be independent of the enzyme concentrations (Fig. 5). Since there was no great change in the specific activity of phenylalanine hydroxylase at high protein concentrations, the possibility that the enzyme may undergo reversible association at high enzyme concentrations seems unlikely. The effect of the phosphate seems to be on the cofactor (cf. Fisher & Kaufman, 1970; Woolf *et al.*, 1971).

Discussion

By our original method, which is similar to, though not identical with, that of Kaufman & Fisher (1970), we isolated two apparent forms of phenylalanine hydroxylase, E_1 and E_2, with properties resembling those reported by Kaufman & Fisher (1970). A modified technique gave a single fraction, E_2; this did not appear to result from destruction or loss of fraction E_1, but rather from either preventing the artifactual conversion of some of fraction E_2 into fraction E_1 or from the dissociation of fraction E_1 to fraction E_2. Against the second possibility is the relative mildness of the additional steps in the modified procedure and the difficulty in dissociating isolated fraction E_1 to yield fraction E_2. In the following paper (Woo *et al.*, 1974) we discuss the evidence that fraction E_1 is a complex of fraction E_2 with inert molecules, possibly lipid or lipoprotein in nature; the evidence points to this complex arising during the isolation procedure rather than occurring *in vivo*, both in human and rat liver. The finding of

three isoenzymes by Barranger *et al.* (1972), by using a different isolation technique, suggests that fraction E_2 may form more than one discrete complex with different inert substances.

The rat liver phenylalanine hydroxylase, E_2, isolated by either the original or the modified procedure appears to be about 90% pure and to have a mol.wt. of approx. 110000. Its properties are reported. Although, on electrophoresis at pH8.7, it gave evidence of yielding two subunits of mol.wt. approx. 54000, these had different mobilities suggesting they were non-identical. We cannot explain the appearance of four bands on electrophoresis in 8M-urea gel at pH8.7. The presence of a single copper atom/unit of 110000 daltons (Table 4) is further evidence that the molecule is not a dimer. The presence of copper and FAD (Table 4) has not previously been reported in phenylalanine hydroxylase. Both copper and non-haem iron appear to be essential to enzymic activity; if FAD is also a necessary part of the molecule, then loss of this cofactor may partly account for the instability of phenylalanine hydroxylase during isolation.

Peroxidases, including catalase, plainly play a very important part in the action of phenylalanine hydroxylase (Table 6, Fig. 1). This suggests that a protein-bound hydroperoxide of the tetrahydropterin cofactor may be an intermediate, as suggested by Mager & Berends (1965) and Woolf *et al.* (1971). Further work is necessary to investigate this.

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References

Barranger, J. A., Geiger, P. J., Huzino, A. & Bessman, S. P. (1972) *Science* **175**, 903–905

Burch, H. B., Bessey, O. A. & Lowry, O. H. (1948) *J. Biol. Chem.* **175**, 457–470

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427

Ellman, G. C. (1959) *Arch. Biochem. Biophys.* **82**, 70–77

Fisher, D. B. & Kaufman, S. (1970) *Biochem. Biophys. Res. Commun.* **38**, 663–669

Fisher, D. B., Kirkwood, R. & Kaufman, S. (1972) *J. Biol. Chem.* **247**, 5161–5167

Friedman, P. A., Fisher, D. B., Kang, E. S. & Kaufman, S. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 552–556

Gomori, G. (1955) *Methods Enzymol.* **1**, 143

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

Jakubovic, A., Woolf, L. I. & Chan-Henry, E. (1971) *Biochem. J.* **125**, 563–568

Jovin, T., Chrambach, A. & Naughton, M. A. (1964) *Anal. Biochem.* **9**, 351–369

Kaufman, S. (1962) in *Oxygenases* (Hayaishi, O., ed,.) pp. 129–180, Academic Press, London and New York

Kaufman, S. (1964) *J. Biol. Chem.* **239**, 332–338

Kaufman, S. (1970) *J. Biol. Chem.* **245**, 4751–4759

Kaufman, S. & Fisher, D. B. (1970) *J. Biol. Chem.* **245**, 4745–4750

Mager, H. I. X. & Berends, W. (1965) *Rec. Trav. Chim. Pays-Bas Belg.* **84**, 1329–1343

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349

Perkin–Elmer (1971) *Analytical Methods for Atomic Absorption Spectrophotometry*, Norwalk, Conn.

Schumaker, V. N. & Schachman, H. K. (1957) *Biochim. Biophys. Acta* **23**, 628–639

Waalkes, T. P. & Udenfriend, S. (1957) *J. Lab. Clin. Med.* **50**, 733–736

Woo, S. L. C., Gillam, S. S. & Woolf, L. I. (1974) *Biochem. J.* **139**, 741–749

Woolf, L. I., Jakubovic, A. & Chan-Henry, E. (1971) *Biochem. J.* **125**, 569–574