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PHENYLALANINE HYDROXYLASE OF *MACACA IRUS*

PURIFICATION OF TWO COMPONENTS OF THE ENZYME

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SUMMARY

1. Phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) has been purified up to 424-fold from the liver of the Cynomolgus monkey (*Macaca irus*). This was achieved after stabilization of the enzyme during extraction and chromatography.

2. In the final stages of purification two fractions have been separated, both of which are essential for full activity.

3. Conditions for maximal activity have been established and preliminary kinetic studies have been performed. The optimum pH was found to be 7.6. The K_m for L-phenylalanine was found to be $5.7 \cdot 10^{-4}$ M and $8.5 \cdot 10^{-5}$ M for 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride.

INTRODUCTION

The conversion of phenylalanine to tyrosine has been shown to be defective in phenylketonuria[1]. The structure and characteristics of phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) need to be ascertained if this disorder and its variants are to be fully understood. This knowledge may lead to a therapeutic method more satisfactory than the presently debated[2] dietary therapy.

The mammalian enzyme has previously been purified 70–100-fold from rat liver[3,4]. The present work describes the purification of the enzyme from the liver of the Cynomolgus monkey (*Macaca irus*). This animal was chosen because it is more closely related to man than the rat. Thus, the phenylalanine hydroxylase would be expected to be more similar to that of man.

Abbreviation: ADHT, 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydrogleridine hydrochloride.

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MATERIALS AND METHODS

Chemicals

Reagent grade "Selectacel" DEAE-cellulose (Batch 2180, 0.89 mequiv/g) obtained from Brown Co., Berlin, N.H. (U.S.A.) was prepared and regenerated according to the procedure outlined by PETERSON AND SOBER[5]. The choice of cellulose is important (see RESULTS).

Brushite was prepared using a method based on that of TISELIUS *et al.*[6] and has been described[7]. Sephadex G-200 was purchased from Pharmacia, Uppsala (Sweden).

L-Phenylalanine of high purity was obtained from Halcyon Products, Melbourne (Australia). 2-Amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride (ADHT) was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wisc. (U.S.A.). ADHT was made up in distilled water and stored at −20° and was thawed not more than 4 times for use; after this the solution became cloudy and was discarded. Dithiothreitol was obtained from Calbiochem, Los Angeles, Calif. (U.S.A.). Protamine sulphate and ammonium sulphate (ultra pure) were obtained from Mann Research Laboratories, Inc., New York, N.Y. (U.S.A.). Other amino acids and fine chemicals were obtained commercially and not further purified. Inorganic chemicals were Analytical Reagent Grade and not further purified.

Monkey liver

The monkey livers were kindly provided by the Commonwealth Serum Laboratories, Melbourne. The monkeys (*Macaca irus*) were maintained on pellets and lucerne and were killed at 3–7 months after anaesthetising with ether by puncturing the diaphragm. The livers were removed and chilled until the gall bladders were removed. The livers were then stored at −20° until used.

Assay of enzyme

The reaction mixture contained 2 μmoles L-phenylalanine, 0.25 μmole ADHT, 1 μmole dithiothreitol, 10 μmoles Tris–HCl buffer and enzyme in a final volume of 0.5 ml. The final pH was 7.50.

The standard assay involved preincubation of the reaction mixture without enzyme for 1 min at 37° for temperature equilibration. The reaction was started by addition of enzyme and the mixture incubated for a further 5 min at 37° unless otherwise stated. The reaction was stopped by addition of 0.1 ml of 30% trichloroacetic acid. An unincubated control was prepared by the addition of the enzyme after the trichloroacetic acid. Protein was removed by centrifugation after standing for 10 min. This assay was used unless otherwise indicated.

Active fractions from chromatography columns were detected using the reaction mixture above, but the reaction mixture was not preincubated. Instead the enzyme was added to the reaction mixture at 4° and the reaction was started by immersion of the test tube containing the reaction mixture in a 37° waterbath. Incubation was carried out for 6 or 30 min as indicated. The reaction was stopped and the protein was removed as above. Active fractions were then pooled and the enzyme quantitated using the standard assay.

It was found (see below) that two fractions (E and Y) were required for full

enzyme activity and were obtained after brushite chromatography. In this case when the enzyme was to be assayed with the standard assay the reaction was started with E (Y was in the reaction mixture during the preincubation). In the case of column fractions both components were added to the reaction mixture at 4° and incubation carried out as above.

Tyrosine was assayed as its fluorescent derivative by a modification of the methods of WAALKES AND UDENFRIEND[8] and of GUZMAN BARRON *et al.*[9] unless otherwise stated. To 0.3 ml of the solution to be assayed was added 0.3 ml of the nitroso-naphthol–nitric acid reagent[8], the mixture incubated at 37° for 45 min and then 1.4 ml of ethanol was added[9]. The relative fluorescence of the tyrosine conjugate was measured (activation 470 nm; fluorescence 555 nm; uncorrected). A standard curve was prepared with each assay. Tyrosine was added to the reaction mixture in the place of enzyme at concentrations of 0, $2 \cdot 10^{-5}$, $4 \cdot 10^{-5}$, and $8 \cdot 10^{-5}$ M and 0.1 ml trichloroacetic acid added. If the samples were read as soon as possible after the addition of ethanol the line was curved. However, if the samples were kept overnight at room temperature after the addition of ethanol the standard curve was linear.

Unit of enzyme activity

A unit of enzyme activity is that amount which converts 1 μmole of phenylalanine to tyrosine in 1 min in the standard reaction mixture at 37°. Specific activity is expressed as units/mg of protein.

Assay of protein

Protein was estimated by Folin's phenol reagent[10] using bovine serum albumin (Fraction V) as standard. Absorbance was read at 750 nm.

Preparation of purified enzyme

The purification procedure is summarized in Table I. All procedures were carried out at 4° unless otherwise stated. Component Y was not quantitated. The buffers used are outlined in Table II. Further details are as follows.

Step 1. Preparation of crude liver extract. The frozen livers were partially thawed, weighed, diced and then homogenized in a blender (half speed for 30 sec and full speed

TABLE I

PURIFICATION OF PHENYLALANINE HYDROXYLASE

The figures in parentheses represent percentage recovery of enzyme activity.

Step	*Vol. (ml)*	*Enzyme (units)*	*Protein (mg)*	*Specific activity (units/mg)*	*Purification (-fold)*
1. Crude extract	415	25 (100)	11 200	0.0022	1
2. Protamine sulphate	448	23 (92)	7 350	0.0032	1.5
3. Ethanol	218	17 (68)	3 350	0.005	2.3
4. Ammonium sulphate	48	7.7 (31)	816	0.01	4.3
5. Sephadex G-200	245	10 (40)	344	0.03	14
6. DEAE-cellulose	11.5	3.2 (13)	50	0.058	26
7. Brushite (Batch)	3.4	4.1 (16)	9.7	0.12	55
8. Brushite (Gradient)	3.4	1.5 (6)	2.5	0.6	272

TABLE II

BUFFERS USED IN THE PURIFICATION PROCEDURE

The pH values were measured at 25° after the buffer was made up.

A.	0.1 M Tris–HCl, pH 8.7, containing 10^{-2} M L-phenylalanine, 10^{-4} M EDTA
B.	Buffer A made 0.4 M with NaCl (pH 9.0)
C.	0.01 M Tris–HCl, pH 8.7, containing 10^{-3} M L-phenylalanine, 10^{-4} M EDTA, 0.025 M NaCl
D.	Buffer C made 10^{-2} M with L-phenylalanine ,pH 8.4
E.	0.01 M Tris–HCl, pH 6.8, in stabilizing solution*
F.	0.01 M potassium phosphate, pH 6.0, in stabilizing solution*
G.	0.09 M potassium phosphate, pH 7.9, in stabilizing solution*
H.	0.4 M potassium phosphate, pH 8.2, in stabilizing solution*
I.	0.3 M potassium phosphate, pH 8.2, in stabilizing solution*
J.	0.15 M potassium phosphate, pH 8.1, in stabilizing solution*
K.	0.01 M potassium phosphate, pH 7.7, in stabilizing solution*

* Stabilizing solution: 10^{-2} M phenylalanine, 10^{-4} M EDTA and 20% (v/v) glycerol.

for 30 sec) in Buffer A (3 ml/g liver). The material was then centrifuged at 10 000 × *g* for 30 min. The supernatant was poured through gauze to remove lipid. The pellet was resuspended in Buffer A (1 ml/g of initial liver weight). The mixture was re-homogenized at full speed for 30 sec and centrifuged at 10 000 × *g* for 30 min. The supernatant was pooled with the first supernatant and the extract stored at −20° till purification proceeded.

Step 2. Treatment with protamine sulphate. The crude liver extract was stirred while a 2.25% protamine sulphate solution (adjusted to pH 7 with 1 M NaOH after dissolving at 37° in distilled water) was added (1 ml/6 ml of extract) over 1 min. The mixture was stirred for a further 20 min before centrifugation at 10 000 × *g* for 45 min to remove the precipitate.

Step 3. Ethanol precipitation. The supernatant from Step 2 was stirred in an ice–salt bath until the temperature was about −1°. Ethanol (55 ml/100 ml enzyme solution) at −20° was added over 20 sec. Stirring was continued for a further 10 min. The mixture was centrifuged at 10 000 × *g* for 15 min. The precipitate was quickly resuspended (10 min) in Buffer B (half the volume of the original crude extract).

Step 4. Ammonium sulphate precipitation. The cloudy solution from Step 3 was made 50% saturated with ammonium sulphate over 1 min and stirred for a further 20 min. The mixture was centrifuged at 10 000 × *g* for 20 min. The sediment was taken up in a minimal volume of Buffer A. The insoluble material was removed by centrifugation (10 000 × *g* for 20 min) and washed several times in small volumes of Buffer A until the washes became lighter in colour. The solution was dialysed against two 2-l changes of Buffer C, each for 1 h. The procedure was carried out continuously till the end of this step and then frozen.

Step 5. Gel filtration on Sephadex G-200. The solution from Step 4 (not more than 52 ml) was chromatographed on a 4.5 cm × 120 cm column of Sephadex G-200 equilibrated with Buffer D. The flow rate was 20 ml/h and 20-ml fractions were collected during the 52-h chromatography and stored at −20°. The active fractions were detected using the 6-min assay for column fractions and emerged (V_e = 930 ml) from the column soon after a yellow peak. They were then pooled (about 260 ml) and diluted 1 in 2 in 40% (v/v) glycerol in distilled water.

Step 6. Gradient elution from DEAE-cellulose. The diluted solution from Step 5

(max. 600 mg of protein) was immediately applied (max. 450 ml/h) to a 3 cm × 20 cm column of DEAE-cellulose equilibrated with Buffer E. After application a linear gradient was generated with 400 ml of Buffer E made 0.025 M NaCl in the mixing flask and 400 ml of Buffer E made 0.125 M NaCl in the inlet flask. The rate of elution was 100 ml/h and 10-min fractions were collected. Active fractions were detected using the 6-min assay for column fractions and appeared soon after a yellow fraction and were successively concentrated using the Centriflo ultrafiltration apparatus (Amicon), and were then pooled. The final volume was 10–15 ml.

Step 7. Batch elution from brushite. A column (1.9 cm × 15 cm) was prepared for use by passing through one column volume of Buffer F. The sample from Step 6 was applied and then a yellow fraction (Component Y) was eluted (50 ml/h) with 50 ml Buffer G. Component E was eluted (50 ml/h) with 100 ml of Buffer H. For assay 0.02 ml of the most concentrated yellow fraction was added to the reaction mixture before the standard assay was performed. Component E which was not contaminated with the yellow fraction (Y) (by eye) was pooled. Yellow fractions were pooled. Component E and Component Y were concentrated separately to about 3 ml using a Sartorius ultrafiltration membrane. This process served to change the buffer of the samples to Buffer E.

Step 8. Gradient elution from brushite. A column (1.9 cm × 15 cm) was prepared as in Step 7. Both components from Step 7 were treated similarly. The sample was applied and a linear gradient was generated with 200 ml Buffer F in the mixing flask and 200 ml Buffer I (for E) or Buffer J (for Y) in the inlet flask. The rate of elution was 50 ml/h and 10-ml fractions were collected. Active fractions were detected with the 30-min assay for column fractions and concentrated by ultrafiltration (Sartorius), dialysed to Buffer K and stored at −20°.

Gradient elution of the two components from one column of brushite

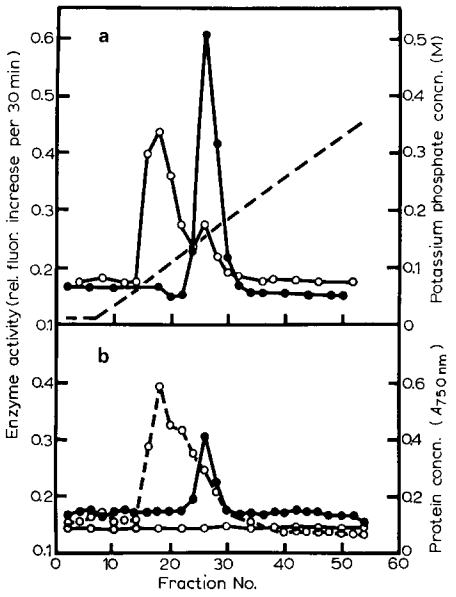
A column (1.9 cm × 13.5 cm) was prepared for use as in Step 7 of the purification procedure. The sample was purified up to the end of Step 6 and applied to the column. The sample (8 ml) contained 1.4 units of activity and 20 mg of protein. A gradient was generated with 200 ml Buffer F in the mixing flask and 200 ml Buffer H in the inlet flask. The rate of elution was 50 ml/h and 10-ml fractions were collected. Fractions were assayed using the procedure for column fractions (Fig. 1).

Methods for electrophoresis

Acrylamide gel (7.5%) electrophoresis was carried out with a continuous buffer system as used by HJERTÉN *et al.*[11]. The buffer used was 0.05 M sodium bicarbonate pH 9.4. Electrophoresis was carried out at 0.5 mA/tube for 30 min and then 6 mA/tube for 2 h. Gels were either stained with 1% Amido Schwartz in 7% acetic acid for 1 h and destained in 7% acetic acid (Fig. 2) or cut into sections for assay of activity as detailed in Table IV.

Identity of products

A reaction mixture was prepared containing twice the normal concentrations of ADHT and L-phenylalanine (see above). Components E (14 μg) and Y (75 μg) were added to two lots of the reaction mixture (each 0.25 ml). One was kept as an unincubated control and the other was incubated for 2 h. A control reaction mixture



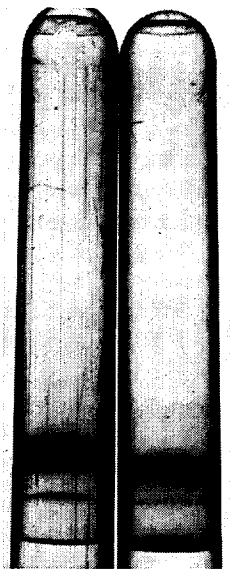


Fig. 1. Gradient elution of the two components from brushite. (a) ○—○, assay of column fractions (0.1 ml) with 0.05 ml Tube 26 added to standard reaction mixture; ●—●, assay of column fractions (0.1 ml) with 0.1 ml Tube 18 added to standard reaction mixture; — — —, approximate concentration of potassium phosphate buffer (Buffer H). (b) ○—○, assay of in-incubated column fractions (0.1 ml); ●—●, assay of column fractions (0.1 ml) without addition; ○— — —○, protein concentration assayed with 1 ml of column fraction.

Fig. 2. Electrophoresis of the purified components. Left hand gel: 22 μg Component E (424-fold purified); Right hand gel: 72 μg purified Component Y. Top of gel is the cathode.

(without enzyme) was prepared with L-tyrosine (1 μmole) in the place of L-phenylalanine. Controls and incubated reaction mixtures were immersed in a boiling-water bath for 5 min. Protein was removed by centrifugation. Samples (0.05 ml) of the supernatant were chromatographed on Whatman No. 3 in *n*-butanol–acetic acid–water (120:30:50, v/v/v). Chromatography (ascending) was carried out for 4 h and the chromatograph stained with ninhydrin (0.2% in 95% ethanol) and nitroso-naphthol[12].

RESULTS

Influence of pH on extraction and stability of enzyme

The pH of the buffer used to extract the liver was found to greatly influence the activity of phenylalanine hydroxylase obtained in the extract (Table III). The pH was also found to influence the stability of the enzyme during storage (Table III).

The purification procedure

The progress of one of many purifications is shown in Table I.

The low total activity of Step 4 material is not typical. The decreased total activity at Step 6 may be due to partial removal of Component Y (see below), as the result was typical.

TABLE III

THE EFFECT OF pH ON EXTRACTION AND STABILITY OF PHENYLALANINE HYDROXYLASE

A single liver was diced and divided into portions and extracted as in MATERIALS AND METHODS. Enzyme activity is given as relative fluorescence increase per 5 min for 0.1 ml of the extracts using the unmodified tyrosine assay[8]. All results except those marked* were obtained by duplicate assays.

	Buffer for extraction	
	0.1 M Tris-HCl, pH 8.7, plus 10^{-2} M L-*phenylalanine*	*0.1 M Tris-HCl, pH 6.8, plus 10^{-2} M* L-*phenylalanine*
pH of extract (approx.)	7.5	6.0
Protein concentration of extract (mg/ml)	43	38
Activity of extract	0.09	0.04
Activity of extract after 18 h at 5°	0.11	0.02
Activity of extract after 18 h at −20°	0.11*	0.05*

The behaviour of phenylalanine hydroxylase on gel filtration indicated a molecular weight of approximately 130 000. This figure was obtained by reference to the graphs obtained by ANDREWS[13]. Some eluates from Sephadex G-200 columns showed evidence of a high molecular weight enzyme activity which was close to the void volume.

When EDTA (10^{-4} M) was added to the second of two similar Sephadex G-200 steps (Step 5) the recovery was nearly doubled. This would indicate that the enzyme was susceptible to denaturation by metals.

The choice of DEAE-cellulose proved important as the product from one maker did not separate the phenylalanine hydroxylase from a large peak of yellow protein material. The material stated in the methods retarded the phenylalanine hydroxylase more than the large protein peak but results varied from batch to batch. The fact that the yellow material was held less strongly than phenylalanine hydroxylase on the adsorptive brushite may indicate that the difference between the celluloses is due to non-ionic adsorption.

Preparations purified up to the end of Step 5 needed EDTA and glycerol when chromatographed on DEAE-cellulose and brushite to prevent rapid loss of activity.

When material from the yellow peak (Y) from Step 7 was added to fractions from the gel filtration step (5) and from the DEAE-cellulose step (6) no stimulation of activity of the phenylalanine hydroxylase from Sephadex was observed, but a slight stimulation was observed on the trailing edge of the peak of phenylalanine hydroxylase from DEAE-cellulose.

Component E from the final step was purified 424-, 315-, 135- and 275-fold in the last four procedures carried out. Unless otherwise stated the 315-fold purified preparation was used in experiments with purified enzyme. All these preparations showed a slight yellow-orange colour and all showed some activity without addition of Y.

The gradient elution of the two components from brushite

Gradient elution of material of Step 6 from brushite resulted in the elution of

a phenylalanine hydroxylase peak (E) with a large loss of activity (Fig. 1b). This activity was greatly stimulated when material from the early yellow fractions (Y) was added to all fractions before enzyme assay (Fig. 1a). The peak of this stimulating activity was found by addition of material from the phenylalanine hydroxylase peak to all fractions before assay (Fig. 1a). The stimulating activity of Y was lost on boiling and was not replaced by an addition of the same weight of bovine serum albumin. When ADHT was made up in dithiothreitol (10^{-2} M) to ensure that no ADHT was oxidized in solution and then used as substrate for E without Y being added, there was no stimulation of activity when compared with an assay mixture containing ADHT having been made up in distilled water.

Purity of the components

Results of electrophoresis of the two components are shown in Fig. 2. Three slow moving impurities are present in E and one faint fast moving impurity moves in the region of Y. Until Component E is purified to homogeneity it will not be known for certain whether the remaining activity and the colour are due to the presence of Y. The one major impurity found in Y could be the same as one of the impurities of E.

TABLE IV

LOCATION OF ENZYME COMPONENTS AFTER ELECTROPHORESIS

Samples were components purified to the end of Step 8. The amounts added for assay and applied to the gel were 22 μg E and 72 μg Y. After electrophoresis portions of gel were cut out equal in thickness to twice that of the yellow band (by eye). The first cut was made at the anode edge of the yellow band and the second on the cathode side to include the region occupied by E (Fig. 2) (assay Tubes 2 and 4). The equivalent portion was cut out of the gel containing E (assay Tubes 1 and 3) and two blank gels (assay Tubes 5 and 6). A further blank portion was cut from the anode end of a gel containing Y (assay Tube 7). The components of the reaction mixture as given in MATERIALS AND METHODS were added in double quantity in 0.75 ml instead of 1.0 ml. This volume was just sufficient to cover the gel portions. Incubation was carried out for 60 min at 37°.

Assay tube	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Component cut from gel	E	Y	E	Y	None	None	None
Component added for assay	Y	E	—	—	Y	E	—
Rel. fluorescence after 60 min	0.35	0.93	0.20	0.17	0.16	0.49	0.17

Location of the enzyme activities after electrophoresis

Both components were found to be active after electrophoresis (Table IV) and were located at or close to the heavily stained regions of protein.

Identity of product

With phenylalanine as substrate an extra ninhydrin staining spot was present in the chromatogram of the incubated reaction mixture but not in that of the unincubated control. This extra spot corresponded in R_F to the spot in the chromatogram of the tyrosine containing control. When similar chromatograms were stained with nitrosonaphthol reagent a distinct pink spot was found in the chromatogram of the incubated reaction mixture but not in that of the unincubated control. A similar spot of similar R_F was found in the chromatogram of the tyrosine containing control. No spot corresponding to tyrosine was found in an incubation mixture from which ADHT was omitted.

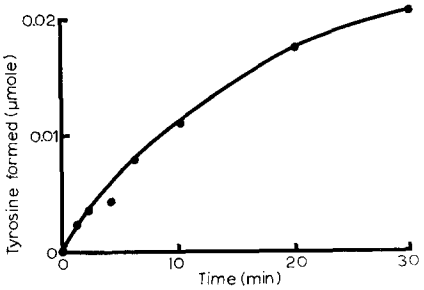
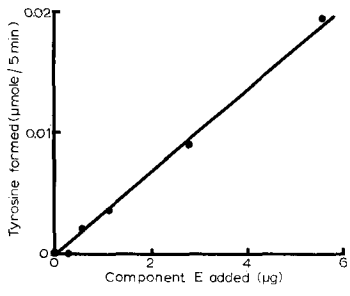


Fig. 3. Rate of formation of tyrosine.

Effect of order of addition of Components E and Y to assay mixture

The amount of activity shown by a fixed amount of E and Y depended on the order of addition to the reaction mixture. If the reaction mixture was preincubated with Y and the reaction started with E a greater enzyme activity was observed than with the reverse sequence. Activity was 2-, 3-, 3- and 10-fold greater in four experiments. It is not known if this effect is due to inhibition or instability of E in the reaction mixture or to activation of Y in the reaction mixture.



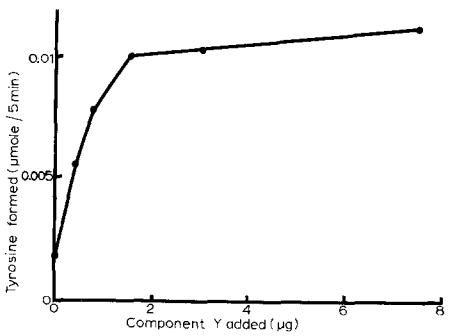


Fig. 4. Activity of Component E assayed with 32 μg Component Y added.

Fig. 5. Activity of Component Y assayed with 2.75 μg Component E added.

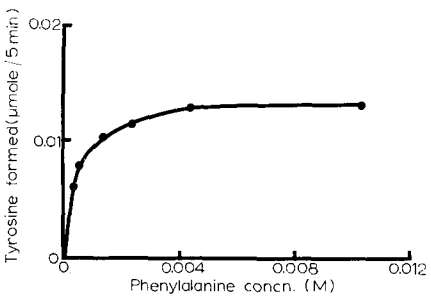
Effect of dithiothreitol on enzyme activity

The effect of dithiothreitol at $2 \cdot 10^{-3}$ M (MATERIALS AND METHODS) was to stimulate activity over the no addition level by 10, 56 and 90% in three experiments. This finding is in agreement with that of BUBLITZ[14].

Enzyme kinetics

(a) Rate of reaction. The rate of reaction can be seen to be gradually decreasing with time (Fig. 3).

(b) Effect of concentration of enzyme. A linear relationship exists between amount of Component E added and enzyme activity and a typical plot is shown (Fig. 4). Component E alone has some activity (Fig. 5) and is stimulated about 4-fold by Component Y.



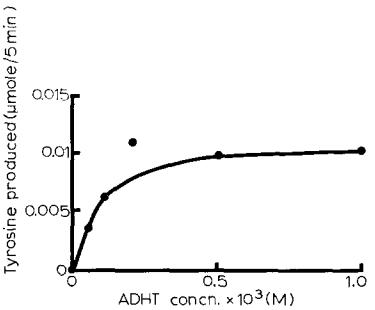


Fig. 6. Phenylalanine hydroxylase activity as a function of phenylalanine concentration. L-Phenylalanine concentrations are corrected for the phenylalanine present in the enzyme solutions for stabilization.

Fig. 7. Phenylalanine hydroxylase activity as a function of ADHT concentration.

(c) Effect of substrate. The enzyme obeys Michaelis–Menton kinetics with L-phenylalanine as substrate (Fig. 6) and with ADHT as substrate (Fig. 7). The apparent K_m values were found to be $4 \cdot 10$, $5 \cdot 10^{-4}$ and $8 \cdot 10^{-4}$ M and $8 \cdot 10^{-5}$ and $9 \cdot 10^{-5}$ M, respectively.

(d) Effect of pH. The optimum pH was found to be close to 7.6 (Fig. 8).

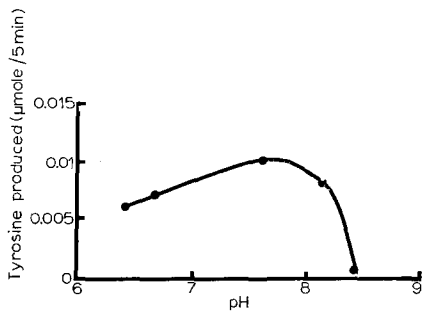


Fig. 8. Phenylalanine hydroxylase activity as a function of pH (424-fold purified Component E).

DISCUSSION

The two components of the enzyme system described in this work appear to be different from those described by MITOMA[15] and KAUFMAN[16] as the addition of substrate amounts of the reduced pteridine (ADHT) eliminates the need for pteridine reductase[17], the second component in their work.

Recent work has shown a stimulation of activity by catalase[14,18]. In the present work both components elute from Sephadex G-200 in a form with a molecular weight close to 130 000. However, as several mammalian catalases have molecular weights of 225 000–250 000[19], it seems unlikely that Y is catalase. Besides, Y has a marked yellow colour. The yellow component (Y) may be related to one of the stimulatory factors described by BESSMAN AND HUZINO[20] obtained after chromatography of rat liver extracts on calcium phosphate gel. The present work has indicated that Component E may well be inactive when purified to homogeneity. Work is in progress

to characterize E and Y. Implications of these results for the understanding of phenylketonuria are evident.

Preliminary studies with the ultracentrifuge indicate a molecular weight much higher than 130 000 (see RESULTS) and also indicate variable molecular weights. This, together with the high molecular weight component seen during gel filtration may indicate an association–dissociation phenomenon. The work of GUROFF AND RHOADS[21] indicated a molecular weight of 300 000 for the rat liver enzyme but their diagram (Fig. 3) indicated that a lower molecular weight form may have been present. Thus, a similar association–dissociation may be present in the rat liver enzyme.

The rat liver enzyme has been reported[3,4] as being purified up to 100-fold. The best purification of E in the present work was 424-fold. As the rate of reaction is not linear up to 5 min (Fig. 3), this figure is not strictly accurate but serves as a guide for purification.

TABLE V

COMPARISON OF APPARENT K_m VALUES FOR SUBSTRATES IN THIS WORK AND WORK WITH OTHER LIVER ENZYMES

	Animal	*Substrate (M)*		*pH*	*Temp.*
		L-Phenylalanine	*ADHT*		
Present work	Monkey	$4 \cdot 10^{-4}$, $5 \cdot 10^{-4}$, $8 \cdot 10^{-4}$	$8 \cdot 10^{-5}$, $9 \cdot 10^{-5}$	7.5	37°
CHRISTENSEN[22]	Rat	$1 \cdot 10^{-3}$	—	6.8	25°
GUROFF AND RHOADS[21]	Rat	$3 \cdot 10^{-4}$–$7 \cdot 10^{-4}$	$5 \cdot 10^{-4}$	7.3	30°
MITOMA[15]	Rat	$2 \cdot 10^{-4}$	—	7.0	37°
BUBLITZ[14]	Rat	$1.12 \cdot 10^{-3}$	$6.7 \cdot 10^{-5}$	7.2	25°
TOURIAN *et al.*[26]	Mouse	$5.0 \cdot 10^{-4}$	—	7.0	25°
		$2.4 \cdot 10^{-4}$ (cell suspension)	—	7.0	37°
		$6.2 \cdot 10^{-4}$ (cell suspension)	—	7.0	25°
KAUFMAN[27]	Human	$1 \cdot 10^{-3}$	$5.7 \cdot 10^{-5}$	6.8	25°

This study reveals no time lag (Fig. 3) in the rate of reaction as reported in earlier work[22] with rat liver enzyme. Also the enzyme activity curve (Fig. 4) is linear and this differs from other work[22–24] with rat liver enzyme. No evidence was found of inhibition of enzyme activity by higher levels of phenylalanine as has been found[4,15,21,25] with rat liver preparations. These differences may well be due to the difference in the source of enzyme.

The addition of glycerol and EDTA to buffers allows chromatography and may well stabilize the unstable rat liver enzyme[4].

The apparent K_m values for L-phenylalanine and ADHT are similar to values obtained by other workers for other liver enzymes (Table V).

Work with the rat liver enzyme has in some cases[15,16] indicated that ferrous ion was stimulatory. Preliminary results with the monkey enzyme indicate that it is inhibitory in agreement with the results of GUROFF AND RHOADS[21] with the rat liver enzyme.

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