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DEMONSTRATION OF TWO FORMS OF PHENYLALANINE HYDROXYLASE IN HUMAN LIVER OBTAINED AT AUTOPSY

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SUMMARY

1. Phenylalanine hydroxylase (L-phenylalanine tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) has been demonstrated to be present in human liver obtained at autopsy.

2. The enzyme is shown to be present in 2 forms: (a) a 'soluble' form, which remains in the 6000 × *g* supernatant; (b) a 'particulate' form which sediments at 6000 × *g*.

3. The particulate form has been solubilised and its properties suggest that it is bound to a membrane *via* a lipid linkage.

4. Some properties of the two forms have been studied and these suggest that they are closely related.

5. It should now be possible to use human livers obtained at autopsy as a source of phenylalanine hydroxylase for purification and study.

INTRODUCTION

Phenylalanine hydroxylase (L-phenylalanine tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) has been demonstrated in biopsy samples obtained from human liver[1]. However, as it is possible to obtain only small quantities of liver in this manner, it is desirable to find an alternative source of the enzyme in sufficient amounts to study and purify. Mitoma *et al.*[2] showed that two samples of liver acquired 4 h after death were devoid of enzyme activity and attributed the lack of activity to autolysis. Liver obtained at autopsy would be such a convenient source of human phenylalanine hydroxylase that this tissue has been re-investigated. This paper reports the presence of two forms of the enzyme and describes some properties of these two forms.

Abbreviation: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine.

MATERIALS AND METHODS

2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$) was obtained from Aldrich. Dithiothreitol was supplied by Calbiochem; L-[U-^{14}C]phenylalanine was supplied by Amersham; L-phenylalanine was obtained from Merck.

The reaction mixture contained 2 μmoles phenylalanine, 0.25 μmoles $DMPH_4$, 1 μmole dithiothreitol, 50 μmoles Tris, pH 6.8, 0.1 ml of liver sample in a final volume of 0.5 ml. The assay was performed at 37 °C and stopped with 0.1 ml 30% trichloroacetic acid after 10 min then centrifuged. Unless stated otherwise, the tyrosine formed was measured fluorometrically by a variation of the method of Waalkes and Udenfriend[3]; 0.3 ml supernatant was reacted with 0.3 ml of nitrosonaphthol reagent for 30 min at 55 °C. The tubes were cooled and 1.4 ml of absolute ethanol added. The samples were measured on an Aminco-Bowman spectrophotofluorimeter. Some assays, mentioned in the text, were performed by including [^{14}C]phenylalanine in the reaction mixture and measuring radioactivity in the reaction products which were subjected to paper chromatography in two systems: (a) *n*-propanol–NH_4OH–water (8:1:1, v/v/v); (b) *n*-butanol–acetic acid–water (4:1:1, v/v/v).

Non radioactive standards of phenylalanine and *p*-tyrosine were chromatographed on the same sheet and were located by spraying with ninhydrin. The chromatograms were cut into 1-cm strips which were placed in 10 ml of Bray's solution. Radioactivity was measured in a Packard model 3003 scintillation spectrometer. Protein was measured by the procedure of Lowry *et al.*[4].

A unit of enzymic activity is defined as that amount which converts 1 μmole of phenylalanine to tyrosine per min at 37 °C.

Livers were obtained from autopsies performed up to 24 h after death upon children dying of various causes at the Royal Children's Hospital. Initially, patients who appeared to have any form of liver disease were rejected. However, after biopsy samples from babies with biliary atresia proved to show good enzyme activity, livers of patients dying of this disease were accepted. Livers were obtained 12–16 h after death from 5 adults who died suddenly in motor car accidents, through the cooperation of the pathologist to the coroner's Court. Operative liver biopsies were obtained from eight children and adults undergoing abdominal surgery for diseases which did not affect the liver.

The liver samples were prepared in a manner similar to that used by previous investigators[1,5–7]. Frozen livers were partially thawed, diced, homogenized in 3 vol. of 0.15 M KCl with a glass Duall tissue grinder (Kontes), the homogenate was centrifuged at 6000 $\times$ *g* for 20 min and the supernatant used for assay.

Subcellular fractionation studies were performed by the method of Hogeboom[8] in order to separate supernatant, microsomal, mitochondrial and nuclear fractions.

RESULTS

Biopsy samples

Biopsy specimens prepared by the method described above gave the results shown in Table I in which the activity is expressed both as units per mg of protein and as units per ml of supernatant. The results are comparable to those obtained by Kaufman[1] and Justice *et al.*[6] which when converted are 0.0012 and 0.00097 units/mg

TABLE I

PHENYLALANINE HYDROXYLASE ACTIVITY IN HUMAN LIVER OBTAINED AT BIOPSY

Supernatant fraction obtained after centrifugation at 6000 × *g*. Assay performed by measuring tyrosine formed with nitrosonaphthol (see Materials and Methods).

Sample	*Activity in supernatant*	
	units/mg protein	*units/ml*
a	0.0018	0.034
b	0.0019	0.038
c	0.0012	0.024
d	0.00065	0.013
e	0.0014	0.030

of protein, respectively. The elevated values obtained in some instances may be a reflection of the differences in incubation temperatures (37°, *cf* 25 °C).

Autopsy samples

Supernatant fractions of 50 livers obtained at autopsy were prepared in a similar manner. The level of activity in these livers varied, but the majority of livers tested displayed little or no activity (Table II). Some samples did contain activity comparable to that found in biopsy samples (Samples 1, 3, 4, 12, 16, 32) and others (Samples 2, 5, 10, 11, 13, 14, 15, 17, 18, 19, 20, 24, 27, 28, 31, 33) displayed a lesser degree of activity.

TABLE II

PHENYLALANINE HYDROXYLASE ACTIVITY IN HUMAN LIVER OBTAINED AT AUTOPSY

Table showing activities in supernatant and in whole homogenate of liver obtained at autopsy. Age, underlying basic disease, and interval between death and autopsy are listed. Some patients suffered grossly inadequate respiration and/or circulation, continuously or intermittently, for many hours (or even several days) before death; this occurrence is recorded as "long period of inadequate circulation before death". Assay performed by measuring tyrosine formed with nitrosonaphthol (see Materials and Methods). N.M. = not measured.

Sample	*Age*	*Hours from death to autopsy*	*Basic disease*	*Microscopic liver abnormality*	*Long period of inadequate circulation before death*	*Enzyme activity (units/ml)*	
						Supernatant (6000 × g)	*Whole homogenate*
1	2 weeks	16	Cerebral	Fatty	No	0.022	N.M.
2	4½ weeks	15.5	Cardiac	Nil	No	0.006	0.017
3	2 months	2.5	Cerebral	Nil	No	0.020	0.025
4	14 months	11.5	Cardiac	Nil	No	0.017	N.M.
5	2 years	17	Liver (bile duct atresia)	Severe	No	0.010	0.015
6	12 days	47	Metabolic	Fatty	Yes	0	0.009
7	10 years	12	Tumour	Nil	No	0	0.010
8	22 years	12	Road accident victim	Nil	No	0	0.014
9	65 years	12	Road accident victim	Nil	No	0	0.009
10	10 days	21	Cardiac	Congested	No	0.01	N.M.
11	5 weeks	14	Cerebral	Nil	No	0.01	N.M.
12	6 months	20	Cardiac	Fatty	No	0.02	N.M.
13	4 h	4	Prematurity	Nil	Yes	0.007	N.M.

TABLE II *(continued)*

Sample	*Age*	*Hours from death to autopsy*	*Basic disease*	*Microscopic liver abnormality*	*Long period of inadequate circulation before death*	*Enzyme activity (units/ml)*	
						Supernatant (6000 × g)	*Whole homogenate*
14	2½ years	18	Cardiac	Nil	Yes	0.005	N.M.
15	1 day	21	Cardiac	Nil	Yes	0.013	N.M.
16	2 months	8	Cerebral		No	0.03	N.M.
17	19 years	9	Muscle	Nil	Yes	0.004	N.M.
18	17 months	20	Cardiac	Nil	No	0.009	N.M.
19	3 days	7	Cardiac	Fatty	Yes	0.01	N.M.
20	6 weeks	5	Cerebral	Nil	No	0.01	0.01
21	2 years	17	Cardiac	Nil	No	0	0.006
22	9 h	9	Cardiac	Nil	Yes	0	0.007
23	6 years	8	Cerebral	Fatty	No	0	0.007
24	2 days	3.5	Cerebral	Fatty	No	0.005	0.010
25	1 day	36	Prematurity	Fatty	No	0	0.007
26	14 weeks	1.5	Metabolic	Minor	No	0	0
27	3½ years	13	Kidney	Minor	No	0.007	0.016
28	1 day	13	Cardiac	Nil	No	0.012	0.012
29	22 years	12	Road accident victim	Nil	No	0	0.005
30	55 years	12	Road accident victim	Nil	No	0	0.007
31	1 h	1	Prematurity	Not known	No	0.007	0.017
32	Stillborn	3.5	Prematurity	Not known	Yes	0.023	0.030
33	4 years	12	Metabolic	Yes	Yes	0.005	0.011
34	6 weeks	20	Cerebral	Minor	Yes	0	0
35	4 days	7	Cerebral	Nil	No	0	0
36	4 years	16	Kidney	Nil	No	0	0
37	11 months	2.5	Reye's syndrome	Fatty	Yes	0	0
38	Stillborn	18	Not known	Nil	Yes	0	0
39	7 years	5.5	Leukemia	Infiltration	No	0	0
40	10 years	12	Tumour	Nil	No	0	0
41	20 months	5	Cerebral	Nil	No	0	0
42	9 months	9.5	Cardiac	Congested	Yes	0	0
43	1.5 years	17	Cerebral	Nil	Yes	0	0
44	3 years	18	Brain	Nil	Yes	0	0
45	9 days	10	Cerebro-hepatorenal	Severe	No	0	0
46	2 years	6	Blood	Nil	No	0	0
47	10 months	13.5	Cerebral	Nil	No	0	0
48	10 years	13.5	Leukemia	Nil	No	0	0
49	5 years	2.75	Cerebral	Nil	No	0	N.M.
50	15 years	4	Muscle	Nil	Yes	0	N.M.

There appeared to be no correlation between the length of time that had elapsed after death and before the samples were obtained and the degree of activity. Those livers which showed good activity all originated from young patients, the oldest being 24 months; however not all livers from young patients displayed activity.

Though previous investigators[9] had shown that an assay of whole homogenates was inhibitory to phenylalanine hydroxylase, the complete homogenates of these samples were tested for activity (Table II). Some livers which were devoid of activity in the supernatants did possess activity when the whole homogenates were assayed (Samples 6, 7, 8, 9, 21, 22, 23, 25, 29, 30). The activity of Samples 2, 3, 5, 20, 24, 27, 28, 31, 32, 33 were not inhibited when whole homogenates were substituted for supernatants in the assay, in fact in some cases the activity was increased. The supernatant

fraction of Samples 6, 7, 8 and 9 was removed after centrifugation at 6000 × *g*. The precipitate fraction was resuspended in an equal volume of 0.01 M Tris buffer, pH 7.4. This resuspended particulate fraction contained all the enzymic activity.

Localisation of enzymic activity by subcellular fractionation

The liver sample used was autopsy Sample 8 which was devoid of activity in the supernatant; the sample was homogenized in 0.25 M sucrose, layered over an equal volume of 0.34 M sucrose and centrifuged at varying speeds to separate cell components. The enzymic activity was found to be associated with the 700 × *g* pellet which is a mixture of nuclei, cell debris, and unruptured cells. Further studies were carried out in buffers of different pH and tonicity and more vigorous homogenisation; in all cases the activity was associated with the 700 × *g* pellet. Examination of this pellet by phase microscopy showed no unruptured cells or intact nuclei; the enzyme was presumably associated with the cell debris.

Demonstration that enzymic phenylalanine hydroxylase occurs in the particulate fraction

Product identification. (a) Treatment of the reaction products with nitrosonaphthol yields a fluorescent derivative, only *p*-tyrosine and tyramine fluoresce with the spectrum observed (Waalkes and Udenfriend[3]). The other possible metabolic products of phenylalanine such as *o*-tyrosine, *m*-tyrosine, phenylpyruvate or phenyllactate do not react to yield fluorescent products (Woolf *et al.*[11], Ambrose *et al.*[10]). (b) When [^{14}C]phenylalanine was used as substrate the products were subjected to chromatography in (i) *n*-propanol–NH_4OH–water (8:1:1, v/v/v); (ii) *n*-butanol–acetic acid–water (4:1:1, v/v/v).

The single radioactive product moved with an R_F identical to that of *p*-tyrosine in both systems.

Nature of hydroxylation. The conversion of phenylalanine to tyrosine could be eliminated if the cofactor ($DMPH_4$) was omitted or if the particulate fraction was replaced with a similar sample which had been boiled (Table III).

TABLE III

CHARACTERISTICS OF THE ENZYME FROM LIVER OBTAINED AT AUTOPSY

Assay mixture consisted of [^{14}C]phenylalanine (1 μmole, 0.23 Ci/mole), dithiothreitol (0.5 μmole), Tris, pH 6.8 (25 μmoles), $DMPH_4$ (0.125 μmole), enzyme sample (0.05 ml) in a total volume of 0.25 ml. The samples were incubated for 20 min at 37 °C and the reaction was stopped by adding 0.03 ml of 3 M trichloroacetic acid; after centrifugation 0.02 ml of supernatant was spotted onto the chromatogram which was developed in *n*-butanol–acetic acid–H_2O (4:1:1, v/v/v). Counts were determined as described in Materials and Methods, the figures reported have been corrected for background. The sample used was No. 3 (Table II).

Sample	*Counts in phenylalanine area*	*Counts in tyrosine area*
Supernatant (6000 × *g*)	13 080	3210
Omit $DMPH_4$	12 930	—
Boiled supernatant (6000 × *g*)	11 900	330
Particulate (6000 × *g*)	13 060	980
Omit $DMPH_4$	12 250	30
Boiled particulate (6000 × *g*)	13 690	210

Solubilisation of the particulate enzyme. Enzyme activity could not be eluted from the particulate fraction by repeated washing with buffers, however extraction has been achieved by: (a) Sodium deoxycholate; The particulate fraction was resuspended in 0.01 M Tris buffer, pH 7.4, an equal volume of 1% sodium deoxycholate was added and the mixture homogenized gently at 4 °C. The mixture was centrifuged at 20 000 × *g* and all the activity was associated with the supernatant fraction. (b) Sonic oscillation; The particulate fraction was resuspended in 0.01 M Tris buffer, pH 7.4, and treated briefly in a Mullard sonicator with 4 × 15-s bursts. The enzymic activity was associated with the 20 000 × *g* supernatant fraction.

Comparison of soluble and particulate forms

pH optimum. Both the soluble and the particulate forms showed broad pH optima near pH 7.0 which is similar to that observed in enzymes from other sources (Guroff and Ito[12], Bublitz[13]).

Substrate affinities. The results obtained when phenylalanine was varied while $DMPH_4$ was kept constant at $5 \cdot 10^{-4}$ M are shown in Fig. 1. Both the soluble and particulate forms had similar affinities for phenylalanine, the K_m apparent being $5 \cdot 10^{-4}$ M. This value is in reasonable agreement with data obtained by Kaufman[1] for human biopsy samples. Similarly Fig. 2 shows the results when $DMPH_4$ was varied while phenylalanine was kept constant at $4 \cdot 10^{-3}$ M, the K_m apparent for both soluble and

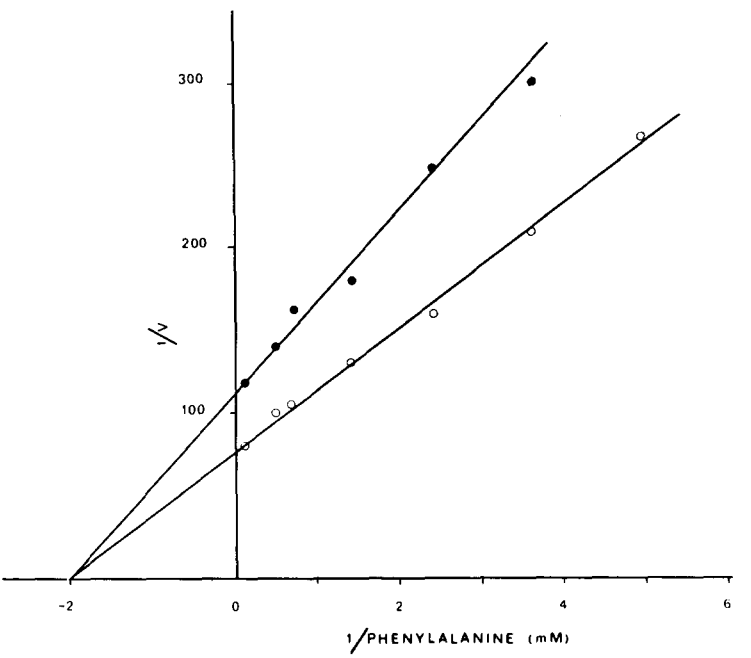

Fig. 1. The effect of L-phenylalanine concentration on the rate of tyrosine production (double-reciprocal plot). Assay conditions used were as described in Materials and Methods; $DMPH_4$ level was held constant at $5 \cdot 10^{-4}$ M. Enzyme samples used were: (1) soluble enzyme (supernatant fraction 700 × *g*) Sample 3 (Table II) ○—○; (2) particulate enzyme (resuspended precipitate fraction 700 × *g*) Sample 8 (Table II) ●—●. Velocity is expressed as μmoles of tyrosine formed per min per ml of enzyme sample at 37 °C.

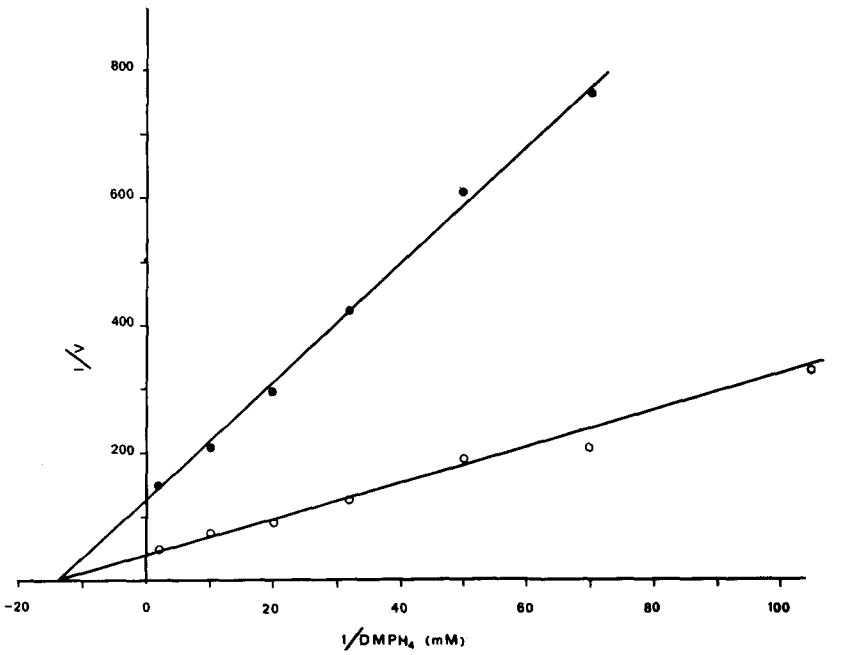

Fig. 2. The effect of $DMPH_4$ concentration on the rate of tyrosine production (double reciprocal plot). Assay conditions used were as described in Materials and Methods; L-phenylalanine level was held constant at $4 \cdot 10^{-3}$ M. Enzyme samples used were: (1) soluble enzyme (supernatant fraction 700 × *g*) Sample 3 (Table II) ○—○; (2) particulate enzyme (resuspended precipitate fraction 700 × *g*) Sample 8 (Table II) ●—●. Velocity is expressed as μmoles of tyrosine formed per min per ml of enzyme sample at 37 °C.

particulate forms being $7 \cdot 10^{-5}$ M. This value is also in good agreement with that obtained for human biopsy liver by Kaufman[1].

DISCUSSION

These results demonstrate that human livers obtained at autopsy do possess phenylalanine hydroxylase activity and that this enzyme may exist in two forms. Previously Freedland[14] had shown that phenylalanine hydroxylase from biopsy samples of human liver exists in the 100 000 × *g* supernatant. This present study gives evidence for an alternative form of the enzyme which exists in the particulate fraction of *postmortem* livers.

When the activities of all the *postmortem* livers studied were compared there is a surprising lack of relationship between the time elapsed between death and autopsy, and the distribution of the enzyme in the two forms. Over 50 *postmortem* liver homogenates were examined of which 14 contained activity only in the supernatant fraction, 10 contained activity only in the particulate fraction, another eight livers contained both soluble and particulate forms. The remaining livers lacked activity altogether; but it was noted that several of these patients had been kept alive on respirators for many hours before death was pronounced.

It is not yet clear whether these two forms of the enzyme exist *in vivo*, or whether the particulate form appears only under postmortem conditions. However, a

biopsy sample from one adult has been found to possess appreciable activity in the particulate form (J. M. Connellan, unpublished). The observation that only livers from young cadavers possess solely soluble enzyme and in quantities comparable to those observed in biopsy sample may indicate that variant forms of the enzyme exist (as for haemoglobin) the form found in younger patients being more resistant to destruction or change. The enzyme tyrosine transaminase (EC 2.6.1.5), which is closely related to phenylalanine hydroxylase on the pathway of metabolism, has been shown to exist in a soluble and particulate form (Canellakis and Cohen[15]). Fellman *et al.*[16] have recently studied this enzyme in liver biopsy samples from a patient with tyrosinaemia and found negligible activity in the soluble fraction but normal mitochondrial tyrosine transaminase activity.

The release of the particulate form of the enzyme into the supernatant by treatment with bile salts or by sonication indicates that the enzyme is bound to a membrane *via* a lipoprotein linkage. The liberated soluble enzyme may still be associated with a smaller amount of lipid *i.e.* the enzyme may function as lipoprotein. Fisher and Kaufman[17] have recently shown that rat liver phenylalanine hydroxylase is activated by the phospholipid lysolecithin and inhibited by lecithin; the authors suggest that these lipids may regulate the *in vivo* activity of the hydroxylase. The results presented in this paper which demonstrate a lipid bound form of the enzyme to be present in human liver from autopsy emphasises the importance of lipid to the character of this enzyme.

The similarity of the properties of the two forms so far examined (pH optima, K_m apparent for phenylalanine and $DMPH_4$) indicates that the enzymes are closely related.

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