Biochem. J. (1974) **139**, 741–749
Printed in Great Britain

The Isolation and Properties of Phenylalanine Hydroxylase from Human Liver

By SAVIO L. C. WOO,* SHIRLEY SU GILLAM† and LOUIS I. WOOLF
Kinsmen Laboratory, Department of Psychiatry, University of British Columbia, Vancouver 8, B.C., Canada

(*Received* 30 *November* 1973)

Phenylalanine hydroxylase was prepared from human foetal liver and purified 800-fold; it appeared to be essentially pure. The phenylalanine hydroxylase activity of the liver was confined to a single protein of mol.wt. approx. 108000, but omission of a preliminary filtration step resulted in partial conversion into a second enzymically active protein of mol.wt. approx. 250000. Human adult and full-term infant liver also contained a single phenylalanine hydroxylase with molecular weights and kinetic parameters the same as those of the foetal enzyme; foetal, newborn and adult phenylalanine hydroxylase are probably identical. The K_m values for phenylalanine and cofactor were respectively one-quarter and twice those found for rat liver phenylalanine hydroxylase. As with the rat enzyme, human phenylalanine hydroxylase acted also on *p*-fluorophenylalanine, which was inhibitory at high concentrations, and *p*-chlorophenylalanine acted as an inhibitor competing with phenylalanine. Iron-chelating and copper-chelating agents inhibited human phenylalanine hydroxylase. Thiol-binding reagents inhibited the enzyme but, as with the rat enzyme, phenylalanine both stabilized the human enzyme and offered some protection against these inhibitors. It is hoped that isolation of the normal enzyme will further the study of phenylketonuria.

Phenylketonuria is a genetically determined disease in which the enzyme phenylalanine 4-hydroxylase (EC 1.14.16.1) is absent or has very low activity. This enzyme has a wide distribution in Nature, ranging from micro-organisms (*Pseudomonas* sp., Guroff & Rhoads, 1967) to mammalian liver (for review see Kaufman, 1971) and, in some mammalian species, kidney and pancreas (Tourian *et al.*, 1969). Phenylalanine hydroxylase activity has also been found in human foetal liver (Jakubovic, 1971; Barranger *et al.*, 1972). This enzyme has been isolated in a highly purified state only from rat liver and its properties studied (Kaufman & Fisher, 1970; Gillam *et al.*, 1974).

Phenylalanine hydroxylase is unstable and its activity in human liver is rapidly lost after death (Mitoma, 1956). Thus, Friedman & Kaufman (1973) found that such a liver exhibited only 35% of the specific activity of biopsied liver even though the liver was removed and quickly frozen within 30min of death.

In the present work we have isolated this enzyme in high purity from normal human foetal liver and studied its properties. We show that the human foetal liver phenylalanine hydroxylase has very similar physical properties to those of the rat liver enzyme, and yet exhibits some characteristic kinetic differences. Livers from a full-term anencephalic infant and from an adult, obtained 1.5h after death, were also examined; the phenylalanine hydroxylase did not show any qualitative differences from the foetal enzyme.

* Present address: Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77025, U.S.A.

† Present address: Department of Biochemistry, University of British Columbia, Vancouver 8, B.C., Canada.

Experimental

Materials

Liver from human foetuses, aged 8 to 20 weeks and obtained by therapeutic abortion, was kindly provided by Dr. M. S. McBean. The liver was removed immediately after hysterotomy and kept frozen at −70°C until use. L-β-Phenylalanine was purchased from Cambrian Chemicals Ltd., Croydon, Surrey, U.K., and recrystallized twice from ammonia–ethanol. 1-Nitroso-2-naphthol was purchased from Fisher Scientific Co., Fair Lawn, N.J., U.S.A., and recrystallized from light petroleum and then from aqueous ethanol. 'Ultra pure' grade Tris $(NH_4)_2SO_4$ and sucrose were purchased from Schwartz/Mann, Orangeburg, N.Y., U.S.A. Dithiothreitol, NAD^+, sodium *p*-chloromercuribenzoate, yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase and 6,7-dimethyl-5,6,7,8-tetrahydropterin were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Ox liver catalase, twice recrystallized, was from Worthington Biochemical Corp., Freehold 2,

N.J., U.S.A. Lysophosphatidylcholine was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-α-Phosphatidylcholine was purchased from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. The tocopherol derivatives were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Sodium dodecyl sulphate was purchased from Brinkman and Co., Montreal, Canada, and recrystallized once from ethanol. Whatman DEAE-cellulose (DE22) was purchased from W. and R. Balston Ltd., Maidstone, Kent, U.K. Hydroxyapatite Bio-Gel HTP was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Sephadex G-25 and G-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide, bisacrylamide, tetramethylethylenediamine and riboflavin were purchased from Serva Feinbiochemica, Heidelberg, West Germany. Buffalo Black NBR was purchased from Allied Chemicals, Morriston, N.J., U.S.A. *p*-Chlorophenylalanine, *p*-fluorophenylalanine and 2,2′-bipyridyl were obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

Methods

Assay for phenylalanine hydroxylase. The standard assay mixture contained 0.2M-potassium phosphate, pH7.0, 5mM-phenylalanine, 750 units of catalase, 3mM-dithiothreitol, 0.5mM-6,7-dimethyl-5,6,7,8-tetrahydropterin, and various amounts of the enzyme in a total volume of 0.25ml. The cofactor was dissolved in a 30mM-dithiothreitol solution to give a concentration of 5mM, and kept frozen in 1ml portions at −70°C until use. The reaction was started by adding 0.025ml of this solution. The mixture was incubated at 25°C for 20min with mild mechanical shaking; the rate remained linear for over 30min under these conditions (see the Results section). The reaction was stopped by the addition of 0.05ml of 30% (w/v) trichloroacetic acid to the incubation mixture. Tyrosine was then determined by a modification of the nitrosonaphthol procedure of Waalkes & Udenfriend (1957). A fresh 'working' reagent was prepared by mixing equal volumes of 1.5M-HNO_3, 0.1M-$NaNO_2$ and ethanolic nitrosonaphthol (1.3mg/ml). The working reagent (0.5ml) was added to each tube and heated at 60±2°C for 20min. Distilled water (2.5ml) was then added and the tube was centrifuged for 10min at 2800rev./min in an International model HN centrifuge. The fluorescence of the clear supernatant was measured with an Aminco Bowman spectrophotofluorimeter (excitation and fluorescent wavelengths were set at 460nm and 550nm respectively). The absolute quantity of tyrosine formed was determined by comparing the fluorescence measurements with a standard tyrosine curve. One unit of activity was defined as 1μmol of tyrosine formed/min at 25°C.

Catalase was assayed at 25°C in 0.05M-potassium phosphate buffer as described by Beers & Sizer (1952). One unit of activity was defined as 1μmol of H_2O_2 decomposed/min at 25°C.

Both yeast and horse liver alcohol dehydrogenases were assayed by the method of Vallee & Hoch (1955). One unit of activity was defined as 1μmol of NAD^+ being reduced/min at 25°C.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was done as described by Ornstein (1964) and Davis (1964) in a Hoefer DE102 electrophoresis apparatus. The temperature of the chamber was regulated at 2–4°C by circulating an ice–water mixture through the chamber jacket. The gels were pre-run in the system for 30min to remove the ammonium persulphate. The samples were then applied and electrophoresis was continued at a constant current of 3mA/gel until the marker dye had reached the bottom of the gel. The gels were removed from the tubes and stained for 2h with a 0.1% (w/v) solution of Buffalo Black in 7.5% (v/v) acetic acid. They were then destained electrophoretically in a Canalco Quick Gel Destainer (no. 1801) with 7.5% (v/v) acetic acid. To identify the protein band corresponding to phenylalanine hydroxylase, a parallel gel was sliced transversely with a Canalco Lateral Gel Slicer (no. 1802) immediately after electrophoresis. Each slice, 1.5mm thick, was incubated at 4°C for 2h with 0.2ml of a complete assay mixture without the cofactor. The cofactor was then added to start the reaction as described above. By measuring the distance of migration in the gel, the protein band that showed enzymic activity was readily identified.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulphate was carried out as described by Weber & Osborn (1969). Sodium dodecyl sulphate (1%, w/v) and 2-mercaptoethanol (1%, v/v) were added to the enzyme solution and the mixture was incubated at 37°C for 2h. The mixture was then dialysed for 16h at 37°C against 500ml of 0.01M-sodium phosphate, pH7.0, containing 0.1% (w/v) sodium dodecyl sulphate and 0.1% (v/v) 2-mercaptoethanol. Samples (0.1ml) of the dialysed protein solutions were applied to the gels and electrophoresis was performed at a constant current of 8mA/gel. Staining and destaining of the gels after electrophoresis were identical with the procedure described previously.

Sucrose-density-gradient centrifugation. The 5–20% sucrose density gradients were prepared with a Hoefer SG101 gradient maker. The stock sucrose solutions and the samples also contained 0.1M-potassium phosphate, pH7.0, 10mM-phenylalanine, 0.1mM-dithiothreitol and 5% (v/v) glycerol. The gradients were centrifuged at 4°C for 18 h at 37000 rev./min in a swinging-bucket rotor, SW 39, with a

Spinco model L-2 ultracentrifuge. At the end of the run the bottoms of the tubes were carefully punctured with a needle, and fractions, each of 10 drops, were collected. A total of 34 fractions were obtained from each tube. Samples of the fractions were assayed for various enzyme activities, and the sedimentation coefficients were calculated by the method of Martin & Ames (1961).

Purification of phenylalanine hydroxylase from human foetal liver. Throughout the purification procedure, temperature was kept at 0–4°C unless otherwise specified. All buffers were freshly prepared with glass-distilled water. Protein concentration was determined by the biuret test as described by Gornall *et al.* (1949).

(1) Homogenization. About 20g of liver was thawed, cut into small pieces with a pair of scissors, and homogenized with 4 vol. of ice-cold 0.05M-potassium phosphate buffer, pH7.0, containing 0.15M-KCl, 1mM-dithiothreitol, 10mM-phenylalanine and 5% (v/v) glycerol. A Virtis model 45 homogenizer was used at one-third speed for two 15s periods, with 1min of cooling time between the periods. The homogenizing flask was chilled in an ice–water bath. This gave the 'liver homogenate'.

(2) Centrifugation. The liver homogenate was centrifuged at 35000*g* in a Sorvall RC-2 centrifuge with an S34 head for 1h, and the supernatant was carefully collected by decantation through a pre-cooled glass funnel packed with glass wool to get rid of any fatty materials. The filtrate was called 'liver extract'.

(3) $(NH_4)_2SO_4$ fractionation. Concentrated NH_3 solution was added to $(NH_4)_2SO_4$ solution (saturated at room temperature), to bring the pH of a 1:20 diluted solution to 7.5 ['buffered $(NH_4)_2SO_4$']. The liver extract was gently stirred in an ice–water bath and the buffered $(NH_4)_2SO_4$ solution, at room temperature, was added at a rate of about 0.5ml/min until 26% saturation was reached. Stirring was continued for an additional 15min, and the suspension was centrifuged for 10min. To the supernatant, further buffered $(NH_4)_2SO_4$ solution was added until 44% saturation was reached. The suspension was centrifuged for 10min at 35000*g*, and the precipitate was resuspended in 8ml of cold buffer A [0.01M-Tris–HCl, pH7.2, containing 0.01M-KCl, 0.1mM-dithiothreitol, 10mM-phenylalanine and 5% (v/v) glycerol]. The suspension was called the '$(NH_4)_2SO_4$ fraction' and was kept frozen at −16°C overnight.

(4) Sephadex G-25 desalting. The $(NH_4)_2SO_4$ fraction was thawed, and applied to a Sephadex G-25 column (2.2cm×100cm), pre-equilibrated with buffer A. The column was eluted with the same buffer at a rate of 1.5ml/min. Fractions of volume 4.5ml were collected. The protein-containing fractions were pooled and called 'G-25 fraction'.

(5) DEAE-cellulose column chromatography. The G-25 fraction was applied to a DEAE-cellulose (DE22) column (1.5cm×20cm), pre-equilibrated with buffer A. The column was eluted with a linear gradient of 0.01–0.41M-KCl in buffer A at a rate of about 0.75ml/min. A total volume of 300ml of the eluting buffer was used and 4ml fractions were collected. The E_{280} of the fractions was measured with a Unicam spectrophotometer SP.500, and 0.05ml samples of the fractions were assayed for enzyme activity. The active fractions were pooled and the enzyme was precipitated by the addition of an equal volume of buffered $(NH_4)_2SO_4$. The fine suspension was centrifuged at 35000*g* for 10min and the precipitate was redissolved in 2ml of buffer B [0.1M-potassium phosphate, pH7.0, containing 0.1mM-dithiothreitol, 10mM-phenylalanine, 5% (v/v) glycerol)]; the solution was called 'DEAE fraction'. A typical elution profile is shown in Fig. 1.

(6) Sephadex G-200 gel filtration. The DEAE fraction was applied to a Sephadex G-200 column (1.5cm×90cm), pre-equilibrated with buffer B. The column was eluted with the same buffer at a rate of about 6ml/h. Fractions of volume 2ml were collected. The E_{280} of the effluent was measured, and 0.025ml samples of the fractions were used to assay for enzymic activity. Active fractions were pooled and called 'G-200 fraction'. A typical elution profile is shown in Fig. 2.

(7) Hydroxyapatite adsorption chromatography. The G-200 fraction was applied to a hydroxyapatite column (1cm diam.×1.5cm long); pre-equilibrated with buffer B. The column was eluted with the same buffer at a rate of about 0.2ml/min until the E_{280} of the effluent had decreased to zero. Fractions of volume 1ml were collected. The column was then similarly eluted successively with a 0.13M-potassium phosphate buffer, pH7.0, then with a 0.19M-potassium phosphate buffer, pH7.0, both buffers 0.1mM in dithiothreitol, 10mM in phenylalanine and 5% (v/v) in glycerol. Samples (0.025ml) of the fractions were assayed for enzymic activity. The active fractions were pooled and called 'HA fraction'. Samples (0.5ml) of this fraction were kept frozen at −70°C until used.

Results

The results of a typical preparation are shown in Table 1. The overall yield ranged from 5 to 10% in different experiments, and the enzyme was purified about 800-fold. The purified enzyme had a specific activity of 0.225 units/mg of protein under the assay conditions shown above.

Purity of enzyme

The HA fraction gave a clear single protein band on polyacrylamide-gel electrophoresis. The slices of a

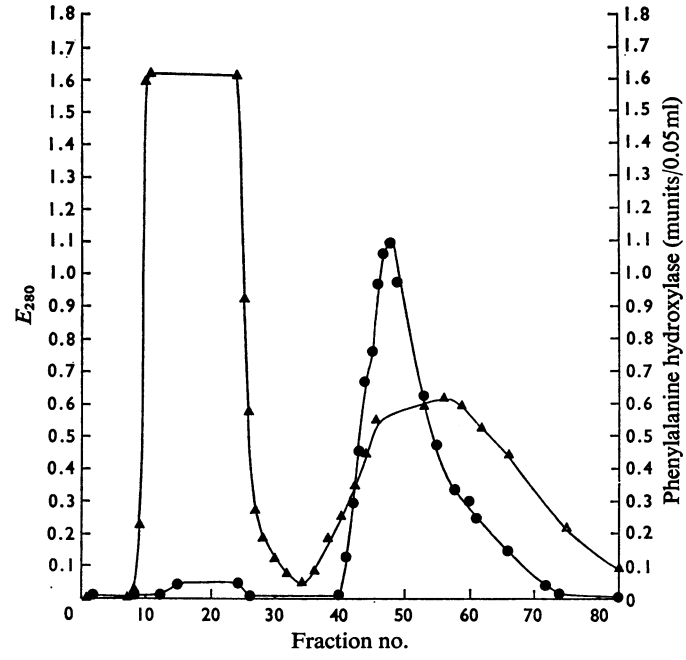

Fig. 1. *Column chromatography of phenylalanine hydroxylase from human foetal liver* [$(NH_4)_2SO_4$ *fraction*] *on DEAE-cellulose* (*DE*22)

The column (1.5cm × 20cm) was eluted with 300ml of buffer containing a linear gradient of 0.01–0.41 M-KCl. The flow rate was 0.75ml/min and fractions of volume 4ml were collected. The E_{280} (▲) of the fractions was measured, and 0.05ml samples of the fractions were assayed for phenylalanine hydroxylase activity (●) as described under 'Methods'.

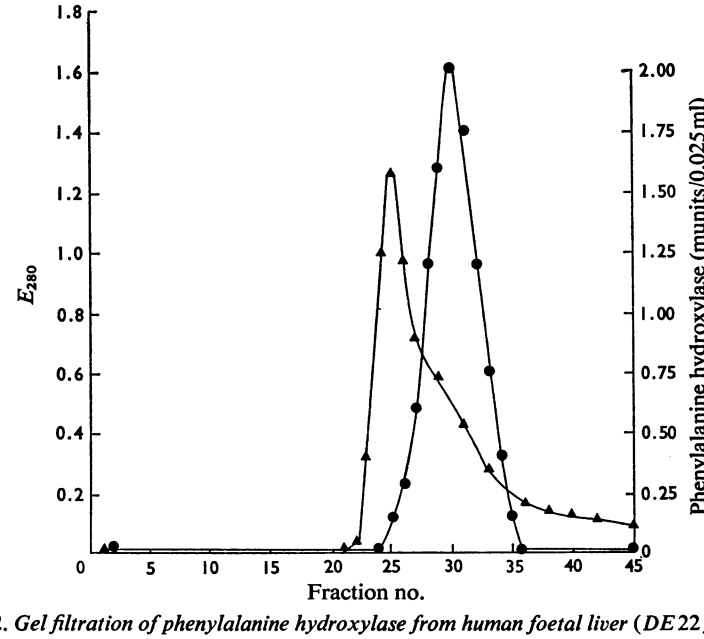

Fig. 2. *Gel filtration of phenylalanine hydroxylase from human foetal liver* (*DE*22 *fraction*)

The column (1.5cm × 90cm) of Sephadex G-200 was eluted with buffer B at a rate of 6ml/h. Fractions of volume 2ml were collected. The E_{280} (▲) of the fractions was measured and 0.025ml samples of the fractions were assayed for phenylalanine hydroxylase activity (●) as described under 'Methods'.

Table 1. *Purification of phenylalanine hydroxylase from human foetal liver*

Protein concentration was determined by the biuret method (Gornall *et al.*, 1949), and phenylalanine hydroxylase was assayed as described under 'Methods'. One unit of activity is equivalent to 1 μmol of tyrosine formed/min of incubation at 25°C under the specified conditions.

Fraction	$10^3 \times$ Specific activity (units/mg of protein)	Recovery (%)
Liver homogenate	0.28	100.0
Liver extract	0.77	98.5
$(NH_4)_2SO_4$ fraction (26–44%)	5.84	94.6
G-25 fraction	5.86	86.8
DEAE fraction	22.80	52.0
G-200 fraction	84.30	19.1
HA fraction	225.10	5.2

parallel unstained gel that exhibited enzymic activity corresponded well with the protein band in the destained gel, indicating that the only protein component of the purified enzyme preparation is the phenylalanine hydroxylase itself.

Physical properties of the enzyme

The molecular weight of phenylalanine hydroxylase was determined by sucrose-density-gradient centrifugation and by Sephadex gel filtration. A mixture of 0.15ml of phenylalanine hydroxylase, purified through the G-200 column, step 6, and 0.05ml of horse liver alcohol dehydrogenase, 10mg/ml, was carefully layered over 4.8ml of a linear sucrose density gradient, 5–20% (w/v), centrifuged, and fractions collected as described under 'Methods'. Samples of volume 0.1 and 0.03ml from each fraction of 10 drops were assayed for phenylalanine hydroxylase and alcohol dehydrogenase activities, respectively. Each enzyme gave a single, sharp, symmetrical peak of activity with no tailing off. The sedimentation coefficient of human foetal liver phenylalanine hydroxylase appears to be 5.45S, calculated as described by Martin & Ames (1961), and it corresponds to a mol.wt. of approx. 107000, calculated as described by Schachman (1959). The rat liver enzyme, by the same technique, appears to have the same sedimentation constant as the human enzyme. Molecular weight was also determined by Sephadex-gel filtration as described by Andrews (1964). Phenylalanine hydroxylase (0.5mg), purified through the G-200 step, was mixed with 0.7mg of catalase, 2.5mg of yeast alcohol dehydrogenase, 5mg of bovine serum albumin and 5mg of α-chymotrypsinogen in a final colume of 2ml. The mixture was passed through a Sephadex G-200 column (1.5cm×90cm) and eluted with buffer B as described above. The E_{280} of the eluate was measured, 0.03ml samples of the fractions were assayed for catalase and alcohol dehydrogenase activities, and 0.1ml samples were assayed for phenylalanine hydroxylase activity. The elution volumes of individual proteins were measured and plotted against their accepted molecular weight values on a logarithmic scale. All the points were in a straight line with no detectable deviation from linearity; phenylalanine hydroxylase was eluted at the volume equivalent to a protein that has a mol.wt. of 110000.

The subunit structure of purified phenylalanine hydroxylase (HA fraction) was investigated by polyacrylamide-gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulphate (see under 'Methods'). A single protein band was seen. Bovine serum albumin, catalase, glutamic dehydrogenase, horse liver alcohol dehydrogenase, yeast alcohol dehydrogenase and α-chymotrypsinogen were also used in parallel gels as standards. The distance of migration of each polypeptide divided by that of the marker dye gave the mobility of that polypeptide, which was linearly related to molecular weight. The migration of the single protein band detected with dissociated phenylalanine hydroxylase corresponded to a mol.wt. of approx. 54000. Therefore human foetal liver phenylalanine hydroxylase appears to contain two polypeptide chains of identical or nearly identical molecular weights, the overall molecular weight being approx. 108000.

Kinetic properties of phenylalanine hydroxylase

The purified enzyme solution was passed through a 10ml Sephadex G-25 column and eluted with 0.1M-potassium phosphate buffer, pH7.0, to remove small molecules that were added to stabilize the enzyme during purification. Enzymic activity was assayed (see under 'Methods') by using a sample of volume 2.5ml. Samples of 0.25ml were withdrawn and mixed with 0.05ml of 30% (w/v) trichloroacetic acid at various times and tyrosine was determined. Linearity of enzymic activity with respect to time was observed for the initial 30min, indicating the stability of human foetal liver phenylalanine hydroxylase under the specified conditions for at least that length of time, but there was a minor departure from linearity between 30 and 60min. The enzyme had a broad pH optimum around pH7.0.

When identical quantities of phenylalanine hydroxylase were assayed in the presence of various concentrations of phenylalanine, a typical saturation curve was obtained. With the synthetic cofactor, virtually no substrate inhibition was observed with the purified enzyme, even at a phenylalanine concentration of 20mM; with some less highly purified enzyme preparations there was slight substrate

Table 2. *Kinetics of phenylalanine hydroxylase from human foetal, neonatal and adult liver: Lineweaver–Burk plots*

Assay of enzymic activity was as described under 'Methods'; v is expressed as μmol of tyrosine formed/min per mg of protein. The foetal liver enzyme was purified through hydroxyapatite (step 7, fraction HA), that from neonatal liver was the $(NH_4)_2SO_4$ fraction (step 3) and the adult liver enzyme was the G-200 fraction (step 6); all enzyme preparations had low-molecular-weight substances removed by passage through a Sephadex G-25 column. All substrates, except *p*-fluorophenylalanine, gave straight-line double-reciprocal plots, as judged by eye.

Source of enzyme	Fixed substrate and concentration (mM)	Variable substrate, S, and concentration range (mM)	No. of points (n)	$10^4 \times$ apparent $K_m \pm$ S.E. (M)
Foetal	Cofactor, 0.5	Phenylalanine, 0.2–20	9	3.41 ± 0.11
Neonatal	Cofactor, 0.5	Phenylalanine, 0.125–20	11	3.52 ± 0.024
Adult	Cofactor, 0.5	Phenylalanine, 0.125–20	11	3.85 ± 0.011
Foetal	Phenylalanine, 5.0	Cofactor, 0.02–1.0	6	0.715 ± 0.0043
Neonatal	Phenylalanine, 5.0	Cofactor, 0.02–1.0	9	0.684 ± 0.0056
Adult	Phenylalanine, 5.0	Cofactor, 0.02–1.0	9	0.661 ± 0.00047
Foetal	Cofactor, 0.5	*p*-Fluorophenylalanine, 0.2–0.8*	4	11.3 ± 1.4

* At concentrations above 0.8 mM, the points diverged from linearity, indicating substrate inhibition.

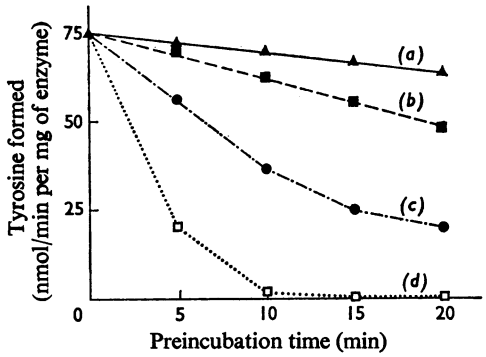

Fig. 3. *Effect of p-chloromercuribenzoate on purified foetal liver phenylalanine hydroxylase*

The enzyme was desalted with a G-25 column and preincubated at 25°C with 1 mM-*p*-chloromercuribenzoate with or without 5 mM-phenylalanine. Samples of the preincubation mixture were withdrawn at different times and assayed for phenylalanine hydroxylase activity as described under 'Methods'. Ingredients added in the preincubation mixture: (*a*) 5 mM-phenylalanine; (*b*) water; (*c*) 5 mM-phenylalanine and 1 mM-*p*-chloromercuribenzoate; (*d*) 1 mM-*p*-chloromercuribenzoate.

inhibition. The apparent K_m value of the enzyme for phenylalanine appeared to be $3.18 \times 10^{-4} \pm 0.16 \times 10^{-4}$ M (mean ± S.E. for four separately prepared batches of enzyme) from regression analysis in the double-reciprocal plot (Table 2). Compared with that of the rat liver enzyme, which is 1.25×10^{-3} M (Fisher & Kaufman, 1972) and 1.22×10^{-3} M (Gillam *et al.*, 1974), the human liver enzyme has a 3–4 times higher affinity for phenylalanine under our conditions. $V_{max.}$ was 108 ± 1 nmol of tyrosine formed/min per mg of enzyme.

To measure the apparent K_m value for the synthetic cofactor, the stock cofactor solution was diluted to various concentrations with a 30 mM-dithiothreitol solution, and 0.025 ml of these solutions were used to start the hydroxylation reaction with constant quantities of the purified enzyme and phenylalanine. A typical saturation curve was again observed and the double-reciprocal plot, by regression analysis, gave an apparent K_m value of $7.02 \times 10^{-5} \pm 0.17 \times 10^{-5}$ M (mean ± S.E. for four separately prepared batches of enzyme). No inhibition was observed, under our assay conditions, even at 1.0 mM cofactor concentration (Jakubovic *et al.*, 1971). $V_{max.}$ was 153 ± 0.6 nmol of tyrosine formed/min per mg of enzyme.

Phenylalanine hydroxylase was also prepared from the liver of a full-term anencephalic infant obtained at the moment of death through the courtesy of Dr. F. Cockburn and Dr. J. W. Farquhar. The enzyme was purified through $(NH_4)_2SO_4$ fractionation (step 3). The liver of a 30-year-old male, with no history of liver disease, who was killed in an automobile accident, was removed, immediately chilled and then frozen in an acetone–dry ice bath within 1.5 h of death. Phenylalanine hydroxylase was prepared to the G-200 stage from a portion of this liver (see under 'Methods'). Molecular weights of the enzyme from full-term and adult livers were identical with that of the human foetal liver enzyme, obtained by using the Sephadex-gel-filtration method described by Andrews (1964). Phenylalanine and 6,7-dimethyltetrahydropterin gave typical saturation curves with both enzyme preparations as obtained previously for the foetal liver enzyme. By using the double-reciprocal plots, the apparent K_m values for phenylalanine were 3.5×10^{-4} M for the full-term infant and 3.8×10^{-4} M for the adult preparations respectively (Table 2). For the synthetic cofactor apparent K_m

Table 3. *Effect of metal-chelating agents on purified human foetal liver phenylalanine hydroxylase*

The enzyme was desalted with a G-25 column, eluted with 0.1M-potassium phosphate buffer, pH7.0. It was pre-incubated with the metal-chelating agents at 25°C for 15min and 0.05ml samples of the mixtures were assayed for phenylalanine hydroxylase activity as described under 'Methods'.

Additions in preincubation	Concn. (mM)	Activity remaining (%)
Water		100
EDTA	1	96
CN^-*	1	95
2,2'-Bipyridyl*	0.1	83
	1	53
Sodium diethyldithiocarbamate*	1	42

* Catalase was omitted in the reaction mixture because it is inhibited by these compounds.

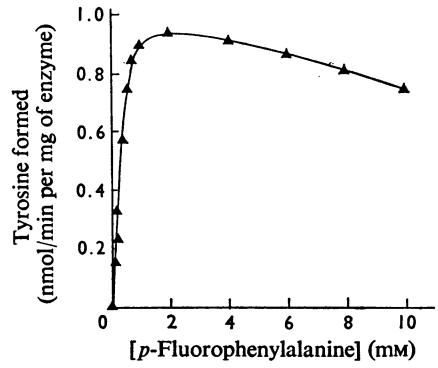

Fig. 4. *Activity of purified foetal liver phenylalanine hydroxylase plotted against increasing concentrations of p-fluorophenylalanine*

The enzyme was passed through a G-25 column before incubation. For details see the text.

values were 6.8×10^{-5}M and 6.6×10^{-5}M for the full-term infant and the adult preparations respectively (Table 2). These values are, within experimental error, identical with those found for the foetal liver enzyme.

The effect of *p*-chloromercuribenzoate (Fig. 3) indicates that the human foetal liver enzyme also requires one or more free thiol groups for enzymic activity. All activity was lost by preincubating the enzyme with the inhibitor for 10min (Fig. 3*d*). When the same experiment was performed with the exception that the preincubation mixture also contained 5mM-phenylalanine, a slower rate of inactivation by the inhibitor was observed (Fig. 3*c*). Fig. 3(*b*) shows the relative instability of the human foetal liver enzyme at 25°C in the absence of phenylalanine, and that 5mM-phenylalanine is capable of stabilizing it to a large extent (Fig. 3*a*).

Preincubation with metal-chelating agents gave results very similar to those obtained with rat liver phenylalanine hydroxylase (Gillam *et al.*, 1974). The enzyme was inhibited to various degrees by different metallic-ion-chelating agents (Table 3).

The purified human foetal liver phenylalanine hydroxylase could also utilize *p*-fluorophenylalanine as substrate. Enzymic activity was linear with respect to the concentration of *p*-fluorophenylalanine up to approx. 1mM; higher concentrations became inhibitory (Fig. 4). With the double-reciprocal plot, the apparent K_m value for *p*-fluorophenylalanine, calculated from non-inhibitory concentrations, was approx. 10^{-3}M, as compared with 3.2×10^{-4}M with phenylalanine itself. $V_{max.}$ for *p*-fluorophenylalanine was 22±2.1nmol of tyrosine formed/min per mg of enzyme as compared with 108±1 for phenylalanine.

The activity of the enzyme was assayed, by using various concentrations of phenylalanine, in the presence and absence of 0.5mM-*p*-chlorophenylalanine. In the double-reciprocal plots, the values of the intercepts on the $1/v$ ordinate were identical whereas those on the 1/[S] abscissa differed, indicating competitive inhibition; the apparent K_i value for *p*-chlorophenylalanine was calculated to be 1.1×10^{-3}M. This value is similar to that found for the rat liver enzyme, which was 8.5×10^{-4}M (Gillam *et al.*, 1974).

Because Fisher & Kaufman (1972) reported a stimulatory effect of lysophosphatidylcholine on the activity of phenylalanine hydroxylase preparations, with tetrahydrobiopterin as cofactor, six different concentrations of lysophosphatidylcholine, from 0.1 to 2.0mg/ml, dissolved in water, were added to the assay mixture. Purified enzyme and synthetic cofactor were used. Lysophosphatidylcholine was very slightly, but probably significantly, inhibitory: the curve for reaction velocity plotted against lysophosphatidylcholine concentration was a straight line, the equation being $v = 122.95-(1.155\pm0.45)$[lysophosphatidylcholine] nmol of tyrosine formed/min per mg of enzyme, the concentration of lysophosphatidylcholine being in mg/ml; $P = 0.05$ (probability that the departure from zero slope occurred by chance). A similar experiment with phosphatidylcholine (six concentrations from 0.5–10mg/ml dispersed in water by sonication) showed more marked and highly significant inhibition: $v = 121.45-(3.2\pm0.10)$[phosphatidylcholine].

It has been reported by Brase & Westfall (1972) that crude preparations of rat liver enzyme were stimulated by derivatives of vitamin E and by sodium dodecyl sulphate. We find that when the purified

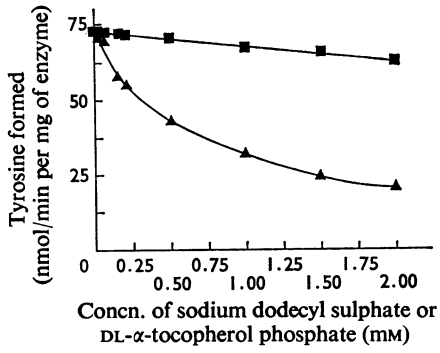

Fig. 5. *Effect of various concentrations of* DL *α-tocopherol phosphate and of sodium dodecyl sulphate on activity of purified foetal liver phenylalanine hydroxylase*

The enzyme was passed through a G-25 column. (▲) DL-α-Tocopherol phosphate; (■) sodium dodecyl sulphate.

human foetal liver enzyme was used, sodium dodecyl sulphate was slightly inhibitory, and DL-α-tocopherol phosphate was highly inhibitory over a range of concentrations (Fig. 5).

Discussion

Phenylalanine hydroxylase has been isolated from human foetal liver, purified and its properties studied. It seems to consist of two polypeptide chains, each with a mol.wt. of approx. 54000, which is very similar to the enzyme purified from rat liver by Kaufman & Fisher (1970). The human enzyme is rapidly inactivated at pH values below 6.0, differing from rat phenylalanine hydroxylase in this respect; this introduced difficulties in isolating human phenylalanine hydroxylase which were not encountered in the rat preparation. Kaufman & Fisher (1970) and Barranger *et al.* (1972) extracted multiple forms of phenylalanine hydroxylase from the livers of rats and humans respectively; some of these had molecular weights in the region of 200000–250000, leading Kaufman & Fisher (1970) to suggest a tetrameric form of the enzyme. In our earlier work (Gillam *et al.*, 1974), we also found two forms of phenylalanine hydroxylase in our preparations from rat and human foetal liver. Changes in the homogenization step and, in particular, filtration through glass wool after centrifugation (step 2), resulted in all of the phenylalanine hydroxylase activity of the liver appearing in a single molecular species with respect to both Sephadex-gel filtration and sucrose-density-gradient centrifugation. Both methods yielded only one symmetrical activity peak corresponding to a mol.wt. of approx. 108000. This finding does not appear to be the result of loss of the higher-molecular-weight material, since virtually all of the phenylalanine hydroxylase activity of the liver homogenate was recovered in the liver extract (step 2, see Table 1). This, together with the stimulatory effects of various surfactants on cruder preparations (Fisher & Kaufman, 1972; Brase & Westfall, 1972; Connellan & Danks, 1973) and their inhibitory effects on purified human preparations, suggests that the human foetal liver enzyme does not aggregate into a tetramer form, and the substances of higher molecular weight represent aggregates of phenylalanine hydroxylase with foreign material, some of it of a lipid nature.

In the mouse phenylalanine hydroxylase activity has been reported in kidney and pancreas as well as in liver (Tourian *et al.*, 1969). This has been confirmed for mouse kidney (S. L. C. Woo, unpublished work). However, we found no activity in human foetal kidney or gut.

Friedman & Kaufman (1973) have demonstrated an apparent K_m value for phenylalanine of 1.6mM, by using the synthetic cofactor and the enzyme partially purified from human autopsy liver; however, with the purified enzymes from human foetal liver as well as full-term infant and adult human liver, we found the apparent K_m value for phenylalanine to be 3.2×10^{-4}–4.0×10^{-4}M. The reason for this difference is not clear.

The inhibition of the human enzyme by thiol-blocking agents and by metal-chelating agents suggests it requires one or more free thiol groups and one or more metallic ions to remain functional, which is similar to the rat enzyme. Phenylalanine partly protects the enzyme against *p*-chlormercuribenzoate (Fig. 3); since phenylalanine has no known effect in protecting free thiol groups, this suggests that either one or more essential SH groups are located close to the binding site for phenylalanine and are occluded when phenylalanine binds to the enzyme, or the binding of phenylalanine induces an allosteric conformational change of the enzyme, such that the essential SH groups and the metal ions are no longer available for the blocking agents. It was shown by Gillam *et al.* (1974) that the rat liver enzyme contains 2 atoms of iron, 1 atom of copper, and 4–5 free SH groups per 110000 daltons. Fisher *et al.* (1972) had also shown that the rat liver enzyme contained 1–2 atoms of iron/100000 mol.wt. Whether the metals in the human enzyme are also iron and copper is not yet established, but the results in Table 3 suggest close similarity of the rat and human enzymes in this respect.

The preparations from human foetuses of 8–20 weeks gestation, a full-term anencephalic infant and an adult were similar in their apparent K_m values for phenylalanine, *p*-fluorophenylalanine and dimethyltetrahydropterin, freedom from substrate inhibition, broad pH optimum near 7.0 and competitive inhibition by *p*-chlorophenylalanine. Above all, each of

the three sources yielded, as far as our techniques could resolve the matter, a single molecular species of mol.wt. approx. 108000. This strongly suggests that a single form of phenylalanine hydroxylase is present in human liver throughout life. This is important in regard to phenylketonuria and its variant forms.

This work would not have been possible without the co-operation of Dr. M. S. McBean who arranged for the collection of foetal tissues. Foetal tissues were also supplied by the Vancouver General Hospital. Dr. J. W. Farquhar and Dr. F. Cockburn very kindly sent liver from an anencephalic full-term infant. The Department of Pathology of the Royal Columbian Hospital kindly supplied the adult liver. Mr. G. Dong gave expert technical assistance. The investigation was financially supported by grants from the Medical Research Council of Canada and the National Genetics Foundation Inc. to L. I. W.

References

Andrews, P. (1964) *Biochem. J.* **91**, 222–223
Barranger, J. A., Geiger, P. J., Huzino, A. & Bessman, S. P. (1972) *Science* **175**, 903–905
Beers, R. F. Jr. & Sizer, I. W. (1952) *J. Biol. Chem.* **195**, 133–140
Brase, D. A. & Westfall, T. C. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1185–1191
Connellan, J. M. & Danks, D. M. (1973) *Biochim. Biophys. Acta* **293**, 48–55
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Fisher, D. B. & Kaufman, S. (1972) *J. Biol. Chem.* **247**, 2250–2252
Fisher, D. B., Kirkwood, R. & Kaufman, S. (1972) *J. Biol. Chem.* **247**, 5161–5167
Friedman, P. A. & Kaufman, S. (1973) *Biochim. Biophys. Acta* **293**, 56–61
Gillam, S., Woo, S. L. C. & Woolf, L. I. (1974) *Biochem. J.* **139**, 731–739
Gornall, A. C., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–756
Guroff, G. & Rhoads, C. A. (1967) *J. Biol. Chem.* **242**, 3641–3645
Jakubovic, A. (1971) *Biochim. Biophys. Acta* **237**, 469–475
Jakubovic, A., Woolf, L. I. & Chan-Henry, E. (1971) *Biochem. J.* **125**, 563–568
Kaufman, S. (1971) *Advan. Enzymol. Relat. Areas Mol. Biol.* **35**, 245–319
Kaufman, S. & Fisher, D. B. (1970) *J. Biol. Chem.* **245**, 4745–4750
Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
Mitoma, C. (1956) *Arch. Biochem. Biophys.* **60**, 476–484
Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349
Schachman, H. K. (1959) *Ultracentrifuge in Biochemistry*, Academic Press, New York
Tourian, A., Goddard, J. & Puck, T. T. (1969) *J. Cell. Physiol.* **73**, 159–170
Vallee, B. L. & Hoch, F. L. (1955) *Proc. Nat. Acad. Sci. U.S.* **41**, 327–338
Waalkes, T. & Udenfriend, S. (1957) *J. Lab. Clin. Med.* **50**, 733–736
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412