Enzymes involved in phenylalanine metabolism in the human foetus and child

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SYNOPSIS The activities of phenylalanine p-hydroxylase, tyrosine : α -ketoglutarate and phenylalanine : pyruvate aminotransferases, and aromatic α -ketoacid reductase have been measured in liver biopsies obtained from children with a series of disorders other than phenylketonuria and from human foetuses aged 8.5 to 24 weeks. The importance of such a study in relation to the management of infants with hyperphenylalaninaemia and pregnant phenylketonuric women is discussed

In classical phenylketonuria, the absence of liver phenylalanine (Phe) p-hydroxylase results in a high level of Phe in the blood and excretion of abnormal aromatic acid metabolites in the urine (see fig). Unless treated from early infancy by a low Phe diet, progressive intellectual deterioration usually occurs. With the introduction of more sensitive screening procedures (Medical Research Council working party on phenylketonuria, 1968), not only are infants with classical phenylketonuria detected, but also a whole spectrum of children with hyperphenylalaninaemia, the significance of which is largely unknown (Bickel, 1970). This raises many problems, including whether or not dietary treatment should be used in these atypical patients.

Children born to women with untreated phenylketonuria frequently have multiple abnormalities including severe mental retardation (Yu and O'Halloran, 1970), even though they do not have phenylketonuria. With successful treatment, affected girls of normal intelligence are just reaching the age of marriage, and they will require dietary treatment during pregnancy in an attempt to protect the foetus from high levels of Phe and its metabolites in the maternal circulation. Since Phe deficiency of itself is harmful and can cause brain damage, dietary treatment presents problems (Pitt, 1967).

With these considerations in mind, a more detailed knowledge of the metabolism of Phe both in children and in the human foetus would appear essential to a fuller understanding of the problems involved. Only a few assays on Phe p-hydroxylase and the pteridine cofactor in the human subject

Fig Enzymes involved in the metabolism of phenylalanine.

(Kaufman, 1958; Justice, O'Flynn, and Hsia, 1967; Jakubovič, 1971) are available. The work presented here has established a range of values in children without phenylketonuria and in the human foetus for Phe p-hydroxylase, tyrosine : α -ketoglutarate and Phe : pyruvate aminotransferases and aromatic α -ketoacid reductase (see fig).

Materials

Liver was obtained from children when a biopsy was being taken for diagnostic purposes. None of them

had phenylketonuria but they had a variety of disorders as shown in table II.

Foetal livers from therapeutic abortions were provided by the Foetal Tissue Bank, Royal Marsden Hospital, London.

3', 5'-Diiodo-p-hydroxyphenylpyruvic acid was obtained from Calbiochem Ltd, Los Angeles, California 90054, USA, all other substrates and coenzymes from British Drug Houses Ltd, Poole, Dorset.

Methods and Results

PROTEIN ESTIMATIONS

These were performed by the biuret method on a Technicon AutoAnalyzer.

TREATMENT OF LIVER BIOPSIES

After excision all liver specimens from children were frozen immediately in liquid nitrogen, liquid n-heptane, or a solid carbon dioxide-acetone mixture and stored at -70°C . Foetal livers were usually excised within one hour of death, during which time they were maintained at $+4^{\circ}\text{C}$.

Since Phe p-hydroxylase activity appeared to deteriorate after two or three weeks' storage, this was always measured within one week of collection. All other enzymes investigated were stable for six months to one year (table I).

Enzyme	Patient	Activity	Length of Storage
Phenylalanine p-hydroxylase ($\mu\text{mole tyrosine/hr/g protein}$)	T.H.	37	1 day
		36.5	2 wk
	A.J.	31	4 wk
		23.5	1 day
		21	1 wk
	S.J.	None detected	6 mth
		15.6	1 day
		13.1	1 wk
Tyrosine : alpha-ketoglutarate amino-transferase (μmole $\text{pHPPA/min/g protein}$)	S.J.	8.8	3 wk
		85	2 days
		80.8	6 mth
	D.C.	85.5	1 day
		81	3 mth
		80.5	7 mth
Aromatic alpha-ketoacid reductase (units/g protein)	A. J.	1250	3 wk
		1220	3 mth
		1205	10 mth
	S.J.	805	1 wk
		825	2 mth
		790	7 mth
Phenylalanine : pyruvate amino- transferase ($\mu\text{mole PPA/10 min/}$ g protein)	D.C.	110	3 wk
		122	1 mth
		105	10 mth
	C.O.	286	1 mth
		275	4 mth
		268	1 yr
	A.F.	55.2	1 wk
		58	3 wk
		51.5	7 mth

Table I Stability of enzymes on storage at -70°C

PHENYLALANINE P-HYDROXYLASE ACTIVITY

Livers were hand-homogenized in small glass tissue grinders in 4 volumes cold 0.15 M-potassium chloride and centrifuged at 16 000 g for 15 min at 0°C . In preliminary studies assays were performed on both untreated and treated homogenate. In the latter instance 250 μl of homogenate was passed through a Sephadex G-100 column (3 cm long, 0.5 cm diameter) equilibrated with 0.15 M-KCl and eluted with the same solution to remove cofactors for other possible interfering reactions. Since assays employing the resulting homogenate gave activities varying less than 10% from those employing the untreated homogenate, the latter was used as a standard homogenate throughout the study. The incubation mixture was adapted from Justice *et al* (1967) and contained L-phenylalanine (1.0 μmole), NADH (1.25 μmole), nicotinamide (2.5 μmole), dimethyltetrahydro-pteridine (1.15 μmole), and liver homogenate (50 μl). The final volume was made up to 0.8 ml with 0.2 M-potassium phosphate buffer (pH 7.0). Incubation times used were 30 min and 60 min at 37°C . Liver homogenate was omitted from the control tube, and an additional tube containing no dimethyltetrahydro-pteridine (DMTHP) cofactor was included. The reaction was terminated by the addition of 0.8 ml 30% (wt/v) trichloroacetic acid. Standards containing 0.025 to 0.2 μmole tyrosine in 0.8 ml water were treated in the same way. The mixtures were diluted to 4 ml with water and centrifuged. Tyrosine was determined in the supernatants (0.5 ml aliquots) by the method of Udenfriend and Cooper (1952) using 0.25 ml each of 0.1% (wt/v) 1-nitroso-2-naphthol in 95% ethanol and 0.05% (wt/v) sodium nitrite in 2.66 N nitric acid. After incubating at 55°C for 30 min and cooling 1 ml water and 2.5 ml 1,2-dichloromethane were added. After mixing the tubes on a Vortex for 15 sec the upper aqueous layers were taken off and read in an Aminco SPF 125 fluorimeter at an excitation wavelength of 445 $\text{m}\mu$ and an emission wavelength of 555 $\text{m}\mu$.

TYROSINE : ALPHA-KETOGLUTARATE AMINO-TRANSFERASE ACTIVITY

The method of preparing the homogenate was the same as that used for the Phe p-hydroxylase assay; it was convenient to prepare sufficient homogenate for use in both assays and a portion was stored for 24 hours exactly at 4°C before being used in this assay. This treatment resulted in the loss of most of the p-hydroxyphenylpyruvate oxidase. Inclusion of the diethylthiocarbamate as an inhibitor of p-hydroxyphenylpyruvate oxidase in fresh preparations, or treatment with Sephadex G-100 as described above, gave activities varying less than 14% from those using aged preparations. The latter method was

therefore employed as the standard one, since the use of diethyldithiocarbamate frequently caused excessively high optical densities (as previously reported by Diamondstone, 1966). The incubation mixtures (three tubes) contained liver homogenate (10 μ l), L-tyrosine (2 μ mole), and pyridoxal phosphate (0.012 μ mole) in a total volume of 330 μ l 0.2 M-potassium phosphate buffer (pH 7.3). These were preincubated for one hour at 37°C. 10 N-sodium hydroxide (20 μ l) was then added to the control tube, followed by alpha-ketoglutarate (3 μ mole in 10 μ l 0.2 M-potassium phosphate buffer, pH 7.3). Reactions in the remaining two tubes, after the addition of 10 μ l alpha-ketoglutarate solution, were terminated after 10 and 20 min by the addition of 20 μ l 10 N-sodium hydroxide. It was very important to mix the tubes thoroughly immediately after the addition of sodium hydroxide. The optical densities of the mixtures were read at 331 m μ 25 min after the addi-

tion of sodium hydroxide. Standard mixtures of p-hydroxyphenylpyruvic acid (pHPPA, 0.05-0.25 μ mole in 340 μ l 0.2 M-potassium phosphate buffer, pH 7.3) were treated with 20 μ l 10 N-sodium hydroxide and read in the same way.

PHENYLALANINE : PYRUVATE AMINOTRANSFERASE ACTIVITY

The liver homogenates were prepared as before in 4 volumes cold 0.15 M-potassium chloride and centrifuged at 30 000 *g* for 30 minutes. Use of homogenates treated with Sephadex G-100 as described under phenylalanine hydroxylase gave results varying less than 15% from those using untreated homogenates. The latter was therefore used in the standard procedure. The incubation mixtures (three tubes) consisted of liver homogenate (20 μ l), L-phenylalanine (2.14 μ mole), and pyridoxal phosphate (0.012 μ mole) in a total volume of 320 μ l

Patient	Age	Diagnosis	Phe p-Hydroxylase (μ mole tyrosine/hr/g protein)	Tyrosine Amino- transferase (μ mole pHPPA/min/g protein)	Aromatic Alpha- ketoacid Reductase (units/g protein)	Phe Aminotransferase (μ mole PPA/10 min/g protein)
Males						
G.S.	4 wk	Septicaemia and liver abscess	15.4	47.6	1310	24
A.F.	6 wk	Obstructive jaundice	18	45.3	910	55.2
J.L.	7 wk	Biliary atresia	21.5	81	955	73
I.G.	8 wk	Giant cell hepatitis	28.4	56	970	68
R.L.	8 wk	Biliary atresia	13.1	79	2150	262
S.T.	8 wk	Biliary atresia	15.7	57.5	780	53.5
S.J.	3 mth	Biliary atresia	15.6	85	805	230
D.B.	3 mth	Hepatitis; lactose intolerance	24.5	118	1218	54.5
E.M.	3 mth	Biliary atresia	18.5	40.7	1310	67.5
J.L.	7 mth	Histiocytotic infiltration of liver and lymph nodes	39.3	65.5	1440	300
M.S.	11 mth	Abdominal cyst	37.5	200	1295	86.5
A.J.	14 mth	Glycogen storage disease	23.5	44.3	1250	104
D.L.	22 mth	Hepatosplenomegaly	53	103	1240	106
M.G.	2 yr	Appendectomy	30	27.5	—	—
D.B.	6.5 yr	Lymphocytosis and splenomegaly	6.7	189	560	63
D.C.	11 yr	Colon resection	70	85.5	2270	110
C.O.	12 yr	Portal hypertension; splenorenal shunt	48.5	222	1010	286
Mean activity $\pm$ standard deviation			28.2 $\pm$ 16.6	91 $\pm$ 58.9	1220 $\pm$ 456	121.5 $\pm$ 92
Females						
T.H.	8 wk	Biliary atresia	37	87	1780	74.5
A.T.	2.5 mth	Biliary atresia	60	153	1450	50
K.F.	3 mth	Giant cell hepatitis	17.4	22.3	1930	51
T.W.	3 mth	Biliary atresia	39.5	183	1890	109
R.S.	5 mth	Adrenal neuroblastoma with secondary liver deposits	21.5	78	1300	50
C.S.	10 mth	Neonatal hepatitis	34	138	988	152
M.K.	2.5 yr	Biliary atresia and cirrhosis of liver	21.3	47	—	—
D.F.	2.5 yr	Recurrent hepatitis; bile duct sphincteroplasty	19.3	82	620	78.5
E.M.	7 yr	Thalassaemia major and splenomegaly	22.8	41.5	1140	53
Mean activity $\pm$ standard deviation			30.3 $\pm$ 13.8	92.4 $\pm$ 54.6	1387 $\pm$ 466	77 $\pm$ 36.6

Table II Enzyme activities in children without phenylketonuria

— indicates no estimation performed.

Age (weeks)	Phenylalanine <i>p</i> -Hydroxylase (μ mole tyrosine/hr/g protein)	Tyrosine : Alpha-ketoglutarate Amino-transferase (μ mole pHPPA/min/g protein)	Aromatic Alpha-ketoacid Reductase (units/g protein)	Phenylalanine : Pyruvate Aminotransferase (μ mole PPA/10 min/g protein)
Male				
12.5	25.8	7.5	780	36.3
14	11.4	2.85	895	34.6
14	22	5.4	780	10.3
15	28.8	10.3	—	—
18	20	8.4	910	—
21	8.3	11.7	1170	20.8
22	10.8	9.6	1055	15
24	1.67	6.7	843	14.2
Mean activity $\pm$ standard deviation	16 $\pm$ 9.4	7.8 $\pm$ 2.8	919 $\pm$ 145	22 $\pm$ 11
Female				
8.5	15.8	—	—	—
10	16.6	3.3	497	31.2
14.5	6.8	9.7	865	12.1
15	8.95	6.9	763	7.9
16	5.2	24.5	—	—
17	13.3	11.6	910	43.5
18	12	6	890	30
18	20.8	12.1	760	27.8
18	21.7	7.6	1150	40
19	18.7	7.5	—	—
20	10.3	7.9	405	—
20	10.3	7	401	41.5
Mean activity $\pm$ standard deviation	13.4 $\pm$ 5.4	9.5 $\pm$ 5.3	738 $\pm$ 256	29.2 $\pm$ 17.5

Table III Enzyme activities in human foetal livers

— indicates no estimation performed

0.2 M-potassium phosphate buffer (pH 7.7). These were preincubated at 37°C for one hour. Ten N-sodium hydroxide (20 μ l) was then added to the control tube, followed by pyruvate (3 μ mole in 10 μ l 0.2 M-potassium phosphate buffer, pH 7.7). The remaining two tubes, after the addition of 10 μ l pyruvate solution, were incubated at 37°C for 20 and 40 min, the reaction being terminated by the addition of 20 μ l 10 N-sodium hydroxide. It was very important to mix the tubes thoroughly immediately after the addition of sodium hydroxide. The optical densities of the mixtures were read at 315 m μ within two or three min of the addition of sodium hydroxide. Standard mixtures of phenylpyruvic acid (PPA, 0.05 to 0.5 μ mole in 3.2 ml 0.2 M-potassium phosphate buffer, pH 7.7) were treated with 200 μ l 10 N-sodium hydroxide and read in the same way.

AROMATIC ALPHA-KETOACID REDUCTASE

Livers were homogenized as before and spun at 6000 *g* for 20 minutes. The method used was adapted from Weber and Zannoni (1966). Use of homogenates treated with Sephadex G-100 as described under phenylalanine hydroxylase gave results varying less than 12% from those using untreated homogenates. The latter was therefore used in the standard procedure. The reaction mixture (contained in 1 cm microcuvettes) consisted of liver homogenate (5 μ l), 0.2 M-potassium phosphate buffer containing 1 mmol EDTA per litre (pH 6.5) (20 μ l), water (250

μ l), sodium dodecyl sulphate (SDS), (0.001 μ mole in 1 μ l) and p-chloromercuribenzoate (0.025 μ mole in 5 μ l). These reagents were mixed and left at 25°C for 30 minutes. Then NADH (0.1 μ mole in 10 μ l) and 3', 5'-diiodo pHPPA (0.2 μ mole in 5 μ l of a solution containing 150 mmol sodium carbonate and 50 mmol sodium bicarbonate per litre) were added to initiate the reaction, and the decrease in optical density followed at 25°C at 340 m μ for 5 min, readings being taken at 30-sec intervals. The NADH was omitted from the control solution, and the test solutions were assayed with and without sodium dodecyl sulphate. One unit of activity was defined as the quantity of enzyme causing an optical density decrease of 1 per min under the conditions stated for the assay. The initial rate of decrease of optical density was always used.

AGREEMENT BETWEEN DUPLICATES AND REPRODUCIBILITY OF ASSAYS

Agreement between duplicates within individual enzyme assays was usually excellent. The variation in the phenylalanine hydroxylase assay was usually nil, but occasionally variations of between 0.1 and 0.5 fluorimeter units were found. These are so small that no significant differences can be deduced from the standard graphs. Duplicates in the tyrosine transaminase assay never varied more than 0.01 optical density units. This produces almost negligible differences in the activity: for example, 11.8 instead

of 11.6 μ moles pHPPA per min per g protein in liver from the female foetus aged 17 weeks. Duplicates in the phenylalanine transaminase assay were also excellent except in subjects K.F., R.S., and D.L. where a variation of 0.01 in optical density caused activities to vary from 49 to 53, 46.5 to 53.5, and 103 to 109 μ moles PPA per 10 min per g protein respectively. Mean results for these three subjects are given in table II. Since aromatic alpha-ketoacid reductase activities are based on a rate of change of optical density, the occasional optical density value that fell outside the straight line could be ignored, and duplicates were all in good agreement.

Opportunities for studying the reproducibility of enzyme assays over several days were severely limited by the small amount of material available, but some indications that reproducibility was fairly good can be seen in table I, which sets out the activities of some specimens during long periods of storage.

Results of the enzyme assays are set out in tables II and III.

Discussion

The concentrations of enzymes were similar in male and female fetuses and apart from Phe p-hydroxylase showed no change with age. The results did suggest that Phe p-hydroxylase might decrease in activity as the male fetuses became older, but until more data are available this finding is inconclusive. Whereas the mean activity for Phe p-hydroxylase in children was 29.2 μ mole per hr per g protein, it was only 14.7 in the foetal liver. Low activity of Phe p-hydroxylase was observed by Jakubovič (1971) in the livers of seven human fetuses aged 11 to 20 weeks, but though both cofactor and reductase were demonstrated no detailed investigations were performed and no adult tissues were included for comparison. There exists, therefore, a diminished but still fairly active system for the conversion of Phe to tyrosine in the foetus. The system is a complex one involving a pteridine cofactor and dihydropteridine reductase, in addition to the Phe p-hydroxylase. The cofactor and dihydropteridine reductase were measured in foetal rat liver by Brenneman and Kaufman (1965), who found less activity than in the adult rat, whereas the hydroxylase activity was not diminished at all. Although Kenney and Kretchmer (1959) reported that the cofactor and dihydropteridine reductase were present in the foetal rat, they found that Phe p-hydroxylase activity was somewhat lower than in the adult. Recent work by McGee, Greengard, and Knox (1972) supports the latter finding. We are now studying the activities of the cofactor and dihydropteridine reductase in human

liver, and the additional data should give a clearer picture of the foetal enzyme system in man.

The activities of the two aminotransferases, particularly the tyrosine : alpha-ketoglutarate enzyme, were much lower in the foetus than in the child. It has been known for some time that amino acid concentrations in the foetal liver (Ryan and Carver, 1966) and circulation (eg, Crumpler, Dent, and Lindan, 1950) are much higher than those in the maternal liver and circulation and this could well be a reflection of the relative inability of the foetal amino-transferases to metabolize the amino acids further. Foetal rat liver is also deficient in tyrosine aminotransferase activity (Sereni, Kenney, and Kretchmer, 1959). It is possible that a number of the other aminotransferases in foetal liver are also somewhat inactive, although the tyrosine enzyme does appear to be particularly late maturing. Our results suggest that dietary control of the intake during pregnancy in the woman with phenylketonuria will have to be strict if high levels of Phe in the foetus are to be avoided.

Four of the boys (R.L., S.J., J.L., and C.O.) had very high phenylalanine aminotransferase activities and of these C.O. also had an abnormally raised tyrosine aminotransferase activity. Since R.L., S.J., and C.O. also had very high serum GOT and GPT activities, these phenylalanine aminotransferase activities should probably not be regarded as normal. If these four values are omitted from the calculation of the mean activity, it becomes 68 ± 40 μ mole per 10 min per g protein which corresponds more closely with that of 77 ± 36.6 obtained for the livers from girls.

Neither the present study nor the reported work of Weber and Zannoni (1966, 1968) have elucidated the reason for the non-linearity of the change of substrate concentration with time in the assay for aromatic alpha-ketoacid reductase. The activities in the present work are based, as are those of Weber and Zannoni (1966) on the initial rate of disappearance of NADH. The results show that the enzyme is quite active in the foetus (about 65% of the activity in the child) unlike the activities of the two transaminases.

This study has provided values for some liver enzymes involved in the metabolism of Phe by the foetus and the child. It suffers from the inevitable limitation that all the children were ill and liver biopsy was performed for this reason, but none of them had phenylketonuria and the data provide more valid reference values than would be obtained from studies in animals. Such information may eventually help to elucidate those patients at present considered to have 'hyperphenylalaninaemia' or 'atypical' phenylketonuria.

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