THE FORMATION OF meta-HYDROXYPHENYLACETIC ACID THROUGH A DIRECT RING HYDROXYLATION REACTION IN HUMANS

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Summary

Human volunteers who ingested semi-tracer amounts (25 mg/kg) of L-[$^2H_5$]-phenylalanine excreted [$^2H_4$]-m-hydroxyphenylacetic acid in their urine. This demonstrates that direct meta hydroxylation of the phenylalanine ring can occur at some stage during its catabolism. The time course for the production of the acid was similar to that of the ortho isomer and is consistent with formation in the liver. Since the phenomenon was not observed in a child with classical phenylketonuria it is possible that the enzyme phenylalanine hydroxylase (EC 1.14.3.1), which is defective in phenylketonuria, is involved in the meta hydroxylation.

m-Hydroxyphenylacetic acid (m-HPAA) is a normal constituent of human urine. It can originate directly from the diet or be produced by gut bacteria; endogenously it may arise either through the dehydroxylation of catechol derivatives or the direct meta hydroxylation of phenylalanine or one of its metabolites (Fig. 1). m-Tyrosine has not been detected in mammals (1) although a possible metabolite, m-tyramine, is found in human urine (2) and this could be a precursor of m-HPAA. Curtius et al. (3) have demonstrated the conversion to m-HPAA of deuterium labelled phenylalanine given as an oral load to a normal subject. This did not occur in a phenylketonuric subject. These workers used racemic non-specifically 27% deuterated phenylalanine at the high dose of 200 mg/kg and this severely limited interpretation of their results. In the present study the use of semi-tracer doses of specifically labelled L-phenylalanine enables more definite conclusions to be drawn from the labelling pattern of the urinary m-HPAA. The deuterium labelled m-HPAA arises by direct meta hydroxylation of the aromatic ring, and the time course is consistent with production in

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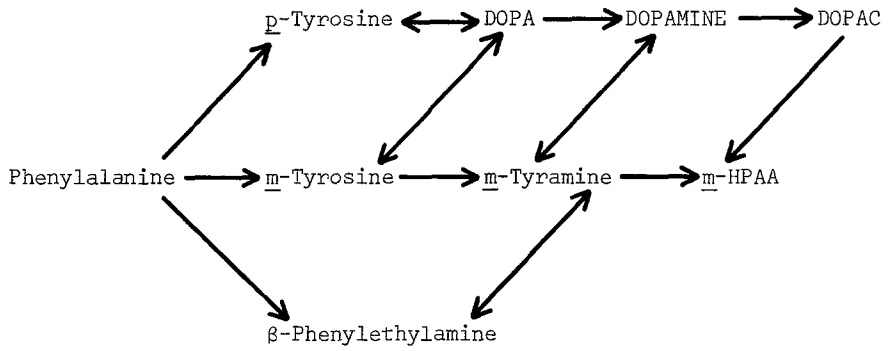

Fig. 1. Possible metabolic pathways through which m-HPAA can be derived from phenylalanine.

the liver. The failure of a phenylketonuric child with nearly normal plasma phenylalanine levels to produce a detectable amount of deuterium labelled m-HPAA following an oral load of L-[$^2H_5$]-phenylalanine supports the hypothesis that the hydroxylation step is mediated by phenylalanine hydroxylase (EC 1.14.3.1) rather than the previous suggestion (3) that tyrosine hydroxylase is the enzyme involved.

Materials and Methods

L-Phenylalanine was deuterated in the ring by exchange in 85% $^2H_2SO_4$ in ^2H_2O at 50° for 7 d after the method of Bu'Lock and Ryles (4); two cycles of exchange were used to give L-[$^2H_5$]-phenylalanine (90%) with some L-[$^2H_4$]-phenylalanine. The product purified by ion-exchange chromatography and crystallisation from aqueous alcohol contained less than 0.1% of the D-isomer. [$^2H_4$]-p-Hydroxyphenylacetic acid was prepared by Miss Rowena A. D. Jones.

Normal volunteers, maintained on a milk and water diet ad libitum from 20.00 h on the day preceding the experiment to the end of the experimental period, were given the labelled phenylalanine (25 mg/kg) in water by mouth and subsequently venous blood and urine samples were collected at regular intervals.

The isotope content of the phenylalanine and of the derived urinary organic acids was determined by mass fragmentography of derivatised extracts. Samples were prepared as follows: phenylalanine was separated from the plasma by ion-exchange chromatography on a Dowex 50W X4 resin in the pyridinium form; the urinary organic acids were extracted from acidified (pH 2.5) aliquots of urine saturated with salt with successively diethyl ether and ethyl acetate. The compounds, as their trimethylsilyl derivatives, were analysed on either a modified Perkin Elmer 270 gas chromatograph-mass spectrometer using an OV-101 wall coated glass capillary column, or an AEI MS30 mass spectrometer coupled to a Pye 104 gas chromatograph containing a packed column. Column packings were 100-120 mesh Gas Chrom Q coated with 3% OV-101 in a 9' column for the urinary acids or 1.5% OV-1 in a 5' column for the phenylalanine. Single ion monitoring enabled the hydrogen form of either the molecular ion of the isomeric hydroxyphenylacetic acids or a ring containing fragment ion of the phenylalanine to be compared with the corresponding deuterated form. The smallest $^{2}H/^{1}H$ ratio which could be accurately quantitated was estimated at around 0.01 but the limit of detection of deuterium in a compound was rather less than this.

The absolute amounts of ortho- and meta-hydroxyphenylacetic acid in 24 h urine collections from normal adults or 24 h equivalent collections from phenylketonuric children were determined. Derivatised extracts of the urines containing [$^{2}H_{4}$]-p-HPAA as an internal standard were prepared and analysed as above.

Results

The presence of m-HPAA labelled with four deuterium atoms in a urine extract was shown by a complete scan taken during the elution of the appropriate gas chromatographic peak (Fig. 2). Single ion monitoring of the molecular ion peaks at m/e 300 and m/e 296 allowed measurement of the $^{2}H/^{1}H$ ratio to be made. Chromatographic conditions were chosen so that

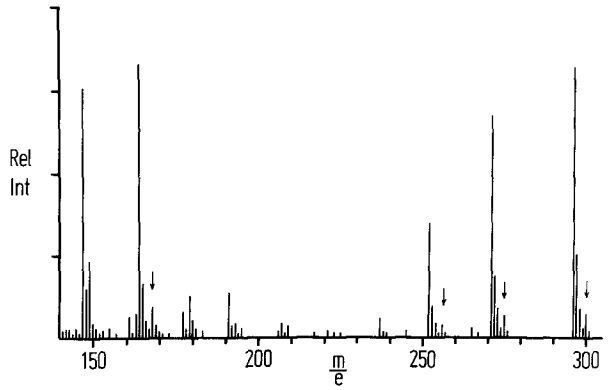

Fig. 2. Mass spectrum of m-HPAA containing [$^{2}H_{4}$]-m-HPAA taken during a gc.ms analysis of a urine extract (subject d, Fig. 3). Major deuterium labelled fragments are arrowed. The deuterated compound has a shorter retention time than the non-deuterated form and so the apparent $^{2}H/^{1}H$ ratio varies during the scan.

the isomeric acids were completely resolved: the retention times were in the order o<m<p and since the concentration in urine of the acids increases generally as o<m<p any interference due to peak tailing was minimal. Similarly the $^{2}H/^{1}H$ ratio of the plasma phenylalanine was measured by monitoring peaks at m/e 197 and m/e 192 (M-COOTMS). The results of experiments on four normal adults are shown in Fig. 3. A phenylketonuric child (F, age 6 y) on a phenylalanine controlled diet was given an oral load of L-[$^{2}H_{5}$]-phenylalanine (25 mg/kg). There was no detectable deuterium labelling in the urinary m-HPAA. o-HPAA was labelled to a maximum $^{2}H/^{1}H$ ratio of 0.95 at 1.5 h dropping to 0.33 at 7 h (cf. Fig. 3). The absolute amounts of the acids in 24 h urine collections from eight adults (4M, 4F) on a free running diet were averaged (extreme range in parenthesis): o-HPAA 0.35 (0.21-0.52) mg, m-HPAA 2.96 (0.23-9.58) mg. Five phenylketonuric children (3M, 2F, ages 3-9 y) on phenylalanine controlled diets collected urine for periods of 8 or 12 h; the 24 h equivalent excretions of the acids were o-HPAA 1.04 (0.11-2.82) mg and

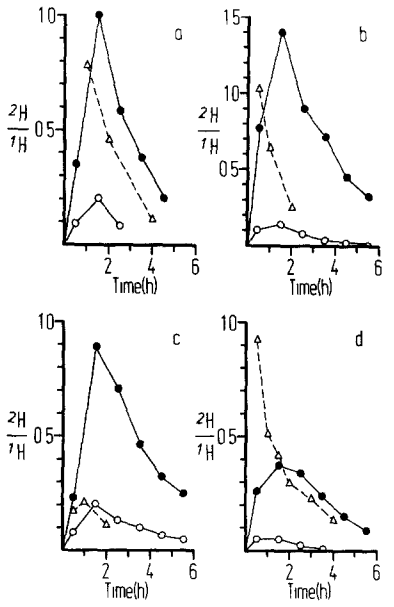

Fig. 3. Molar ratios of deuterium labelled to unlabelled compound after ingestion of L-[$^2H_5$]-phenylalanine (25 mg/kg) by four normal adults. Plasma phenylalanine Δ; urinary o-HPAA ●; m-HPAA O.

m-HPAA 2.36 (0·17-9.75) mg; for one phenylketonuric child (F, age 10 y) now off a restricted diet the amounts were o-HPAA 80.12 mg and m-HPAA 3.54 mg.

Discussion

Possible metabolic pathways leading from phenylalanine to meta hydroxylated derivatives are shown in Fig. 1. The conversion of phenylalanine to m-tyrosine by a tyrosine hydroxylase preparation from beef adrenals (1) and the production of m-tyramine from phenylalanine by a crude rat-liver extract (5) and in rats in vivo from injected β-phenylethylamine (6) all probably involve direct meta hydroxylation. Normal adults have been shown to excrete 83 ± 7 μg of m-tyramine and 4.9 ± 1.0 μg β-phenylethylamine daily (2). In the present experiments the production of [$^2H_4$]-m-HPAA from the oral L-[$^2H_5$]-phenylalanine indicates that direct meta hydroxylation is involved since the alternative routes

via catechol derivatives would give [$^2H_3$]-m-HPAA. Although some fraction of the labelled phenylalanine would have been metabolised by the catechol route, either the amount was very small (because of compartmentation effects) or its turnover time was long compared with the experimental period so that instantaneous labelling was below the limit of detection of the method.

Enzyme studies suggest that in man the liver is a major source of o-HPAA (7). The labelling patterns obtained in the present experiments support this idea as the high degree of labelling of o-HPAA compared with plasma phenylalanine in three of the four subjects indicates a strong first pass effect (Fig. 3). The time-course of excretion of the [$^2H_4$]-m-HPAA is very similar to that of the [$^2H_4$]-o-HPAA and different to that expected for peripherally derived metabolites (8). Thus it seems probable that the liver is responsible for the [$^2H_4$]-m-HPAA production and, by analogy with the rat-liver results (5) it is likely that phenylalanine hydroxylase is the enzyme involved. However, the rather low level of m-HPAA labelling found indicates that this action of phenylalanine hydroxylase is normally responsible for only a small proportion of urinary m-HPAA production. In the present study, of the approximately 1.5 g of labelled phenylalanine ingested, only about 100 µg appeared in the urine as m-HPAA. Consistent with this the overall excretion of m-HPAA by phenylketonuric children is similar to that of normal adults (cf. ref. 9). Under load conditions, however, normal subjects excrete increased amounts of m-HPAA while phenylketonuric patients do not (10). This result together with the failure of a phenylketonuric child to produce a detectable amount of labelled m-HPAA following oral deuterated phenylalanine are further evidence for the involvement of phenylalanine hydroxylase in this pathway. Curtius' suggestion (3, 10) that tyrosine hydroxylase is responsible for the *meta* hydroxylation and that this pathway is blocked by the high levels of phenylalanine or its metabolites in phenylketonuria is not tenable since I have confirmed his original observation

in a phenylketonuric child but on a controlled diet and using semi-tracer doses of phenylalanine. The peak plasma phenylalanine level following this load was only 200 μmol/l (3.3 mg/100 ml). Further proof of the pathway leading to [$^2H_4$]-m-HPAA should come from a study of the postulated intermediate amines and this work is in progress.

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