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Note

Analysis of the isomeric tyrosines in mammalian and avian systems using high-performance liquid chromatography with fluorescence detection

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Ortho-, *meta-* and *para*-substituted phenols, many apparently endogenous, may be detected in mammalian systems. The *para* compounds will mostly have derived from *p*-tyrosine (*p*-Tyr), but the origins of the *ortho* and *meta* compounds are problematical although they could arise from the corresponding, isomeric tyrosines. Knowledge of their origins is important to the understanding of this area of mammalian biochemistry. *o*-Tyrosine and *m*-tyrosine (*o*-Tyr and *m*-Tyr, respectively) are practically unknown in the diet: small amounts are reported to occur in cocoa powder [1] for example, but no important food source is known. The endogenous *o*- and *m*-phenols therefore must arise from either hydroxylation of an aromatic substrate or dehydroxylation of a dihydroxy compound: their production and metabolism have occupied scientists for most of this century [2,3] but their origins are still obscure. A search for *o*-Tyr in man [4] was made when patients suffering from phenylketonuria were found to excrete excessive amounts of *o*-hydroxyphenylacetic acid (*o*-HPAA), but it was shown that this arose from a hydroxylation and then rearrangement (NIH-shift) of phenylpyruvate. There has been a report [5] of the occurrence of *o*-Tyr in rat liver microsomes and this has been re-examined in this paper. Among the neurotransmitter amines in the nervous system [6] it has been postulated that catecholamines, in part, and the *m*-phenolic compounds could derive from *m*-Tyr produced in vivo [7,8] but a search by gas chromatography–mass spectrometry (GC–MS) for *m*-Tyr in human plasma failed to find it [9]. Recently, several reports have appeared in the literature claiming to have found both *o*- and *m*-Tyr in human plasma [10] and serum [11] and in various rat tissues [12–14]. There is some conflict in the reports both in the compounds found and their levels [10,11]. However, the find-

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ings, if substantiated, are important and we felt that the work should be independently examined. Also we hoped that by using enzyme inhibitors we could extend the published work and discover more about the origins of the tyrosine isomers in mammalian and avian systems.

EXPERIMENTAL

Materials

Reagents were of analytical grade and mobile phase solvents of HPLC grade. The *o*-, *m*- and *p*-Tyr and DL-α-methyl-*m*-Tyr were from Sigma (Poole, U.K.). Monofluoromethyldopa (MFMD) was a gift from Merrell Dow Research Institute (Strasbourg, France). *p*-Chlorophenylalanine methyl ester (PCPA) was from Aldrich (Gillingham, U.K.).

Apparatus and conditions

The high-performance liquid chromatographic (HPLC) system consisted of a 6000A solvent delivery pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne 7125 valve injector with a 100-μl loop (Rheodyne, Berkeley, CA, U.S.A.) and a μBondapak C_{18} column (250 mm$\times$4.6 mm I.D., particle size 10 μm) protected by a guard column (12.5 mm$\times$4.6 mm) packed with the same phase (both from HPLC Technology, Macclesfield, U.K.). The fluorescence detector (Model 3000 with high-pressure cell accessory, Perkin-Elmer, Beaconsfield, U.K.) was operated at normal sensitivity and slit widths of 15 nm (excitation) and 20 nm (emission). Tyrosines were measured at wavelengths of 275 nm (excitation), 305 nm (emission), and phenylalanine (Phe) at 258 nm (excitation) and 288 nm (emission). Output was to a 10-mV pen recorder (Chessell, Worthing, U.K.). The mobile phase was 0.1% sodium chloride in 1% (v/v) acetonitrile in water adjusted to pH 3.0 with acetic acid. The system was operated isocratically at a flow-rate of 0.8 ml/min.

Sample preparation

Male Porton rats (nominally 150 g) were starved overnight and treated intraperitoneally (i.p.) with either isotonic saline (0.2 ml), Phe (80 mg/kg), PCPA (400 mg/kg) or Phe plus PCPA (at these doses). After a time they were killed under diethyl ether anaesthesia and blood and liver were taken for analysis. The times were: saline, Phe and Phe plus PCPA, 15 min; Phe plus PCPA, 2 h; PCPA, 2 and 4 h. A similar rat under Sagatal anaesthesia was perfused via the portal vein with isotonic saline to wash blood from the liver which was then removed for analysis.

Mice (agouti strain, male, three months, 25 g) were treated with MFMD (i.p., 100 mg/kg), killed after 15 min by cervical dislocation and tissues removed. Mice (*hph-1*/Pas, male, three months, 25 g), a strain with a defect in the pterin cofactor system involved in Phe hydroxylation, were killed and tissues removed.

An adult (at least ten months) chicken (Rhode Island x Red light Sussex strain, Gallus gallus domesticus) was killed by cervical dislocation and blood and liver were removed.

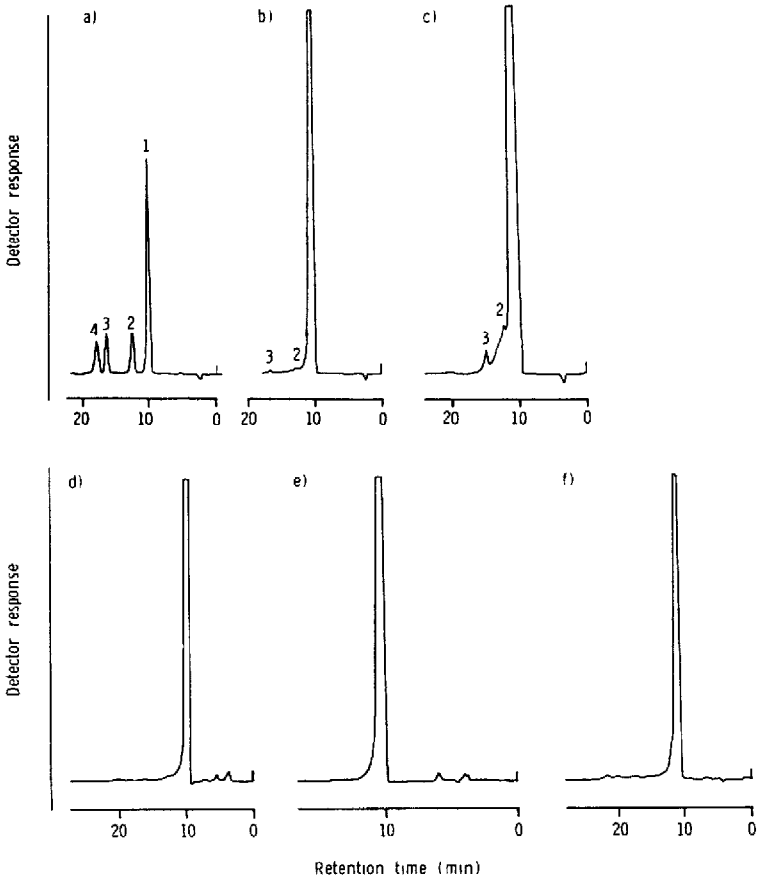

Fig. 1. (a) Standard mixture: *p*-Tyr, 2 μg/ml; *m*-Tyr, 0.4 μg/ml, *o*-Tyr, 0.4 μg/ml; Phe, 2 μg/ml. (b) Mixture of standards with *o*- and *m*-Tyr at the levels said [13] to occur in a saline-dosed starved rat: *p*-Tyr, 12.5 μg/ml; *m*-Tyr, 3 ng/ml; *o*-Tyr, 4 ng/ml. (c) Mixture of standards with *o*- and *m*-Tyr at the levels said to occur in a rat i.p. injected with Phe (80 mg/kg): *p*-Tyr, 50 μg/ml; *m*-Tyr 30 ng/ml; *o*-Tyr, 40 ng/ml (d) Plasma from a rat dosed i.p. with Phe (80 mg/kg). (e) Plasma from a saline dosed rat starved overnight. (f) Human plasma. Peaks: 1 = *p*-Tyr; 2 = *m*-Tyr; 3 = *o*-Tyr; 4 = Phe.

Human liver (adult, female) was from a transplant donor. Human plasma was from blood taken by venepuncture and centrifuged (10 000 *g*, 30 min). Livers were homogenised in 0.25 *M* sucrose at 50% (w/v) and cell debris removed by centrifugation (10 000 *g*, 30 min). Cytosol for analysis was obtained by centrifugation of the supernatant (100 000 *g*, 1 h). Samples were deproteinised with an equal volume of 10% perchloric acid and, where necessary, de-fatted by extraction with diethyl ether.

RESULTS

Standards

Amino acid standards dissolved in 0.1 *M* hydrochloric acid (1 mg/ml) were diluted with HPLC-grade water. A plot of the fluorescence maxima ($\log_{10} I_0/I$)

TABLE I

LEVELS OF *o*-TYR AND *m*-TYR ACCORDING TO ISHIMITSU et al.

Sample	*o*-Tyr (ng/ml)	*m*-Tyr (ng/ml)	Ref.
Human plasma	0	280 ±50	10
Human serum*	3.1±0.9	2.6± 1.2	11
Rat serum	3.6±1.8	3.3± 1.9	12

*Error in paper — plasma is described.

against concentration was linear below a concentration of 50 ng/ml showing that the Beer–Lambert law is obeyed and enabling the absorptivity of the compounds to be calculated. From this and the integrated area under a chromatographic peak the absolute amount of compound responsible for the peak can be calculated and hence the recovery from the column. For amounts less than 10 ng injected the amounts seen at the detector were consistently about 70% of the quantity injected, over the period of the experiments. A proportional loss must occur since standard curves from samples run through the chromatographic system are linear over the concentration range 10–1000 ng/ml and all passed through the origin.

Quantification was from calibration curves of the tyrosines run with an inter-

TABLE II

p-TYR IN SAMPLES STUDIED FOR THIS PAPER

Species	Treatment*	Time** (h)	Concentration (mean ± S.D.) (μg/ml)	
			Plasma	Liver cytosol
Rat***	(Food ad libitum)	–	11.3±1.2	11.2±0.2
	–	–		13.6±0.17§
	–	–		7.1±0.8§§
	N-Saline	0.25	7.2±0.5	13.9±0.3
	Phe	0.25	40 ±1.1	12.1±0 8
	PCPA	2.0	6.2±0.2	9.1±0.2
	PCPA	4.0	5.9±0.2	17.4±0.5
	Phe+PCPA	0.25	62.7±0.3	20.7±0.5
	Phe+PCPA	2.0	38.0±0.8	11.8±0.1
Mouse: *hph-1*/Pas	–	–	3.0±0.2	23.0±0.1
Agouti	MFMD	0.25	5.6±0.2	7.4±0.1
Chicken	–	–	7.3±0 4	22.0±0.4
Human	–	–	13.2±0.1	20.0±0.1

*Injections i.p.: Phe, 80 mg/ml; PCPA, 400 mg/kg; MFMD, 100 mg/kg. (PCPA inhibits the enzyme which converts Phe to Tyr, MFMD inhibits the enzyme which decarboxylates dopamine.)
**After treatment.
***Starved overnight.
§Saline perfused liver.
§§Liver microsomes.

nal standard, DL-α-methyl-*m*-Tyr, at constant concentration, and plotting peak-height ratio against concentration. Because the chromatography of *o*- and *m*-Tyr in the presence of a large (1000-fold) excess of *p*-Tyr could have been altered from that when analysing the compounds alone these standards were calibrated in samples containing a large excess of *p*-Tyr. The lower limit of detection for the isomeric tyrosines was less than 1 ng/ml. To improve the sensitivity by increasing the path-length in the detector cell would increase its volume and degrade the chromatography. Stopped-flow measurements of standard solutions passed directly through the detector cell show that there is only a five-fold dilution of the compounds (measured at the peak maximum) consequent upon chromatography.

Biological samples were analysed for *o*-, *m*- and *p*-Tyr and the presence of Phe was also established (Fig. 1). In no sample tested (rat, mouse, chicken, human) was any compound found that could be unequivocally assigned as *o*- or *m*-Tyr: *p*-Tyr and Phe were always present. A rat brain homogenate was incubated at 37°C with Phe and a pteridine co-factor according to a published method [15]: no *o*- or *m*-Tyr could be detected in the reaction mixture. No peaks attributable to *o*- or *m*-Tyr could be found in a sample prepared from rat adrenals. Similarly when rat liver microsomes were analysed no *o*-Tyr was found (cf. ref. 5). An unknown compound with a similar retention time to *o*-Tyr appears on the chromatogram. This is not *o*-Tyr, but may be the compound found by other authors [5] using thin-layer chromatography (TLC) and described as *o*-Tyr by them. Several experiments with enzyme inhibitors were carried out in both rats and mice: no *o*- or *m*-Tyr could be detected in samples taken.

A sample of plasma with *o*- and *m*-Tyr added to levels stated in the literature showed new peaks at the expected retention times for the added compounds. Occasionally peaks with similar but not identical retention times to the *o*- and *m*-isomers were seen in the assays. Spiking the samples with authentic material invariably showed double peaks which confirmed that there had been no shift in the retention times resulting from some arcane property of the sample.

The reported levels of *o*- and *m*-Tyr in rat and man are given in Table I. The levels of *p*-Tyr found in our samples are shown in Table II.

DISCUSSION

Animals produce a variety of phenolic compounds among which the endogenously produced *ortho*- and *meta*-substituted phenols could arise from the corresponding isomeric tyrosines, presumably produced via an unknown hydroxylation step from Phe [4,8,16]. Phe hydroxylase (EC 1.14.3.1) converts Phe to *p*-Tyr, but it has not been shown directly to produce *m*-Tyr, at least in vivo [17]. There seems no doubt though that enzyme systems do exist which can produce *m*-Tyr in vitro [15,18,19] and possibly *o*-Tyr, as well [15,20], from Phe as a substrate. Also there may be an alternative route to *o*- and *m*-Tyr by synthesis from an otherwise produced *o*- or *m*-phenol [21]. All together it seems entirely reasonable that *o*- and *m*-Tyr [22] could be produced enzymatically in vivo.

In the absence of an enzyme system the possibility of non-enzymatic hydroxylation must be considered. Non-specific hydroxylation of aromatic systems me-

diated by an oxygen source has a considerable history [23] and the reactions and mechanisms have been studied to recent times [24]. Whether or not the conditions exist in vivo for the non-enzymatic formation of tyrosines is unknown. What is known is that when these compounds are given per se no metabolites other than those ordinarily observed in the urine are observed [8,9,21] except possibly when large doses are given [25]. Urinary metabolites which could result from these putative amino acids are produced in quantities from around 100 μg to several milligrams per day. Therefore if the metabolites derive from the amino acids several milligrams of each of these must be formed each day and a detectable amount should exist in the blood (but see ref. 8). It has been claimed that this is so [10–13] in both the rat and man. Unfortunately we are unable to confirm these reports either in the present work or in earlier work with GC–MS [8]. We can readily measure the plasma concentrations detected by Ishimitsu et al. [10–13] and detailed in the results sections. In fact these concentrations would be detectable by a conventional amino acid analyser. We do not find similar concentrations in our samples. In a number of samples we obtained peaks with similar retention times to *o*- and *m*-Tyr. Spiking the samples with authentic *o*- and *m*-Tyr invariably showed that the unknown peaks were not these compounds. However, their particular fluorescence indicates that these and other unknown compounds giving peaks on the chromatogram are aromatic and probably phenolic compounds. The majority of our samples tested had no peaks at or near the retention times of *o*- and *m*-Tyr.

We hope that our failure to detect *o*- and *m*-Tyr in a variety of samples of animal origin will stimulate others to investigate this interesting and biochemically important matter.

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