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Phenethylamines in Brain and Liver of Rats with Experimentally Induced Phenylketonuria-Like Characteristics

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1. Phenethylamines were extracted from brain and liver of rats with phenylketonuria-like characteristics produced *in vivo* by inhibition of phenylalanine hydroxylase (EC 1.14.3.1) with *p*-chlorophenylalanine, with or without phenylalanine administration. To protect amines against oxidation by monoamine oxidase, pargyline was also administered. 2. β-Phenethylamine was the major compound found in brain and liver. β-Phenethanolamine and octopamine were also present, in lesser amounts, and the concentrations of these three amines paralleled blood phenylalanine concentrations. By comparison, tissues from control animals had only very low concentrations of these amines. 3. Small amounts of normetadrenaline, *m*-tyramine and 3-methoxytyramine were also found. 4. The inhibitors used, *p*-chlorophenylalanine and pargyline, gave rise to *p*-chlorophenethylamine and benzylamine respectively, the first via decarboxylation, the second probably by breakdown during extraction. 5. Distribution of phenethylamines in different brain regions and in subcellular fractions of rat brain cells was also investigated. The content of phenethylamine was highest in the striatum. 6. These findings are discussed in the light of changes occurring in human patients with uncontrolled phenylketonuria.

Interference with the normal metabolic functions of brain amines has been proposed as a possible biochemical mechanism that could contribute to the mental retardation of patients with uncontrolled phenylketonuria. This disorder is often accompanied by lower than normal concentrations in the blood of adrenaline and noradrenaline (Porter *et al.*, 1961) and of 5-hydroxytryptamine (Pare *et al.*, 1957), and by decreased urinary excretion of the catecholamines (Nadler & Hsia, 1961), of 5-hydroxyindolylacetic acid (Pare *et al.*, 1957), and of normetadrenaline and *p*-tyramine (Perry, 1962). Jepson *et al.* (1960) demonstrated that during treatment with a monoamine oxidase inhibitor, phenylketonuric individuals excrete up to 50 times the normal amount of β-phenethylamine per day. This implies a large increase in turnover, directly related to accumulation of phenylalanine, and is quantitatively the most striking disturbance of amine metabolism in phenylketonuria found so far.

Phenethylamine may be implicated in the mental impairment associated with phenylketonuria in three ways: (1) by formation of neurotoxic compounds, (2) by depletion of vitamin B_6, and (3) by interference with the action of the structurally related phenethylamines normally involved in neurotransmission. Phenethylamine appears to be the major precursor of phenylacetate (Edwards & Blau, 1972*c*), which Silberberg (1967) found to be toxic to rat cerebellar cells in culture. Loo & Ritman (1964) found a metabolite in the urine of phenylketonuric but not of normal individuals, later identified as a Schiff base formed from phenethylamine and pyridoxal, which produced neurotoxic symptoms in mice (Loo, 1967). The obligative synthesis of this metabolite, as a result of increased availability of phenethylamine, might lead to depletion of vitamin B_6. Deficiency of this vitamin has been shown to depress cerebral sphingolipid biosynthesis and to produce distortions of cerebral amino acid concentrations (Kurtz *et al.*, 1972). Phenethylamine itself has amphetamine-like pharmacological activity (Holtz *et al.*, 1947), depletes noradrenaline (Jonsson *et al.*, 1966) and dopamine (Fuxe *et al.*, 1967) in rat brain, and has been detected in normal rat and mouse brain (Boulton *et al.*, 1970; Mosnaim & Sabelli, 1971). It may play a normal role in neurotransmission, and abnormal amounts might disturb normal brain function. Urinary excretion of phenethylamine has been found to be diminished in depressive patients (Fischer *et al.*, 1968; Boulton & Milward, 1971).

We have used gas–liquid chromatography with electron-capture detection (Edwards & Blau, 1972*a*) to detect the small quantities of phenethylamines

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present in tissues, and at the same time to determine them simultaneously in brain and liver of rats with experimentally induced phenylketonuria-like characteristics (Edwards & Blau, 1972*c*). Amines were also determined in specific brain regions and in subcellular fractions of brain cells, after inhibition of monoamine oxidase, to gain further insight into the distortions of amine metabolism in uncontrolled human phenylketonuria.

Materials and Methods

General procedure

Developing male Sprague–Dawley rats (Zivic–Miller Laboratories, Allison Park, Pa., U.S.A.) were given L-phenylalanine (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) by intraperitoneal injection, alone or in combination with two prior injections of DL-*p*-chlorophenylalanine (Aldrich Chemical Co.) as previously described (Edwards & Blau, 1972*c*): *p*-chlorophenylalanine (300mg/kg) or an equivalent volume of 0.9% NaCl was given at zero time, and a second dose (150mg/kg) or an equivalent volume of 0.9% NaCl at 20h. The rats received pargyline (*N*-methyl-*N*-propynylbenzylamine; Abbott Laboratories, North Chicago, Ill., U.S.A.; 50mg/kg) at 22.5h. Phenylalanine (1g/kg), or in control groups an equivalent volume of 0.9% NaCl, was administered at 23h. At 24h animals were decapitated, the brains and livers were removed immediately, and the removed tissues were homogenized in 4vol. of ice-cold 0.01M-HCl containing 50mg of pargyline/l. Homogenates were adjusted to pH8 with 2.5M-NaOH, and the phenethylamines were extracted into butan-1-ol, and after addition of heptane, back into 0.01M-HCl. The *N*-2,4-dinitrophenyl derivatives were prepared, and all free hydroxyl groups were trimethylsilylated, and the derivatives were analysed by g.l.c. combined with electron-capture detection (Edwards & Blau, 1972*a*).

Regional distribution

Whole brains were dissected on an ice-cooled glass dish according to the procedure of Glowinski & Iversen (1966). Individual brain regions were weighed and homogenized in just enough of the HCl–pargyline solution described above to yield 1ml of supernatant solution for analysis.

Subcellular distribution

Fractions of brain tissue were obtained by a procedure modified from the method of Abdel-Latif (1966). The whole brain was homogenized with 9vol. of ice-cold 0.32M-sucrose solution (Density Gradient Grade; Schwarz/Mann, Orangeburg, N.Y., U.S.A.) by using ten vertical strokes at 900rev./min in a size A glass tissue grinder (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) fitted with a Teflon pestle machined on a lathe to give a 0.25mm radial clearance. The precipitate obtained after centrifugation at 1000*g* for 10min was washed with 5ml of the sucrose solution. The washed precipitate was suspended in 3ml of water/g of original tissue, and was designated fraction P_1. The supernatant solution was centrifuged at 14000*g* for 15min to produce the intermediate-speed supernatant fraction S_2, and the crude mitochondrial fraction. The latter was washed with 5ml of the sucrose solution and suspended in 0.32M-sucrose (3ml/g of original tissue) to give fraction P_2. A sample (2ml) of each P_2 fraction was layered on a discontinuous density gradient, prepared with 14ml each of 8 and 15% solutions of Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden; purified by the procedure of Autilio *et al.*, 1968) containing 0.32M-sucrose, in cellulose nitrate centrifuge tubes (7.6cm×2.5cm diam.; Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.). The gradients were centrifuged in a Spinco SW 25.1 rotor at 25000*g* for 45min. The myelin, synaptosomal and mitochondrial layers were removed by pipette, and 1ml samples were used for determination of phenethylamine.

Results

Determination of phenethylamines in brain and liver

The g.l.c. patterns of amine derivatives obtained from brain and liver extracts of experimental and control rats were composed of up to eight different peaks, and the amines corresponding to these peaks were identified on the basis of the chromatographic properties of the derivatives on three different columns (Table 1). Other means of identification were not possible because the amounts of amines in the tissues were so small. No peaks were seen under these conditions in tissue samples from rats not treated with pargyline, and even when larger samples from such rats were analysed at lower attenuations only very small peaks were seen. This indicates that all eight peaks correspond to amines that are substrates for monoamine oxidase, and are normally maintained at very low concentrations in tissues. The accumulation of amines determined in the tissues of pargyline-treated animals is related to the relative turnover rates of these compounds, since the turnover rates of pharmacologically active amines may be a more significant index of their effectiveness than their endogenous concentration (Maas, 1970; Neff *et al.*, 1969; Persson & Waldeck, 1970). *p*-Chlorophenethylamine was found in brain and liver extracts from rats treated

Table 1. *Identification of unknown amines by comparison of g.l.c. retention times of derivatives with the same data for derivatives of known amines run under identical conditions on three different columns*

N-2,4-Dinitrophenylamine *O*-trimethylsilyl ether derivatives were used. Analyses were done on 1.8m-long U-shaped Pyrex columns (2mm internal diam.) containing three different stationary phases, silicones OV-17, OV-25 and OV-210, each at 1%, supported on 80–100 mesh Gas-Chrom Q. Helium at 125ml/min was carrier gas, and a ^{63}Ni electron-capture detector with 135ml of P-10 purge gas [Ar+CH_4 (90:10)]/min was used at 210°C (Edwards & Blau, 1972*a*).

		Retention times of known and unknown amines relative to phenethylamine							
		OV-17 (230°C)		OV-17 (250°C)		OV-25 (250°C)		OV-210 (230°C)	
Peak	Amine	Unknown	Known	Unknown	Known	Unknown	Known	Unknown	Known
A	Benzylamine	0.73	0.75	0.75	0.76	0.76	0.75	0.74	0.72
A′	β-Phenethanolamine	—	0.96	0.88	0.89	0.80	0.83	—	1.00
B	β-Phenethylamine	1.00	1.00	0.98	1.00	1.00	1.00	0.97	1.00
C	Octopamine	1.92	1.91	1.66	1.69	1.47	1.37	2.00	1.93
C′	*m*-Tyramine	—	1.78	1.77	1.66	1.54	1.54	1.54	1.55
D	*p*-Chlorophenethylamine	2.31	2.21	2.17	2.07	2.12	2.04	2.34	2.41
E	Normetadrenaline	2.58	2.64	2.24	2.28	—	2.18	2.24	2.28
F	3-Methoxytyramine	3.35	3.36	2.89	2.97	2.72	2.71	—	2.62
	β-Phenethylamine (min)	5.6		2.9		5.0		2.9	

Table 2. *Concentrations of β-phenethylamine in brain and liver of 23-day-old rats in the experimental and control groups*

At time $t = 0$ animals were injected intraperitoneally with *p*-chlorophenylalanine (300mg/kg) or an equivalent volume of 0.9% NaCl. At $t = 20$h a second injection of *p*-chlorophenylalanine (150mg/kg) or equivalent volume of 0.9% NaCl was given. At $t = 22.5$h all rats were given pargyline (50mg/kg) and at $t = 23$h phenylalanine (1g/kg) or an equivalent volume of 0.9% NaCl was injected. Animals were killed at $t = 24$h for determination of the amines. Values are given as means±S.E.M., for the numbers of animals in parentheses.

Group	Phenethylamine (μg/g)		Brain/liver ratio of β-phenethylamine
	Brain	Liver	
Saline/saline (2)	0.06±0.01	0.07±0.01	0.88±0.05
p-Chlorophenylalanine/saline (2)	0.21±0.09	0.25±0.16	1.01±0.26
Saline/phenylalanine (6)	0.95±0.32	1.31±0.34	0.87±0.08
p-Chlorophenylalanine/phenylalanine (6)	2.38±0.36	1.74±0.28	1.41±0.20

with *p*-chlorophenylalanine, and was presumably formed *in vivo* by decarboxylation of *p*-chlorophenylalanine (Edwards & Blau, 1972*b*). Benzylamine was produced from pargyline by the extraction procedure, and was therefore found in the tissues of all rats that had received pargyline, but we cannot tell whether it also occurs normally.

The other six peaks correspond to amines formed endogenously by the metabolism of phenylalanine and tyrosine, and were of interest in the context of our studies on phenylketonuria. Phenethylamine concentration showed the greatest increase in brain and liver after phenylalanine administration. Phenethanolamine was detected in both brain and liver in amounts up to 10% of the phenethylamine concentrations. Normetadrenaline, *m*-tyramine and 3-methoxytyramine were found in the brain extracts at concentrations of approx. 0.1μg/g each. These quantities were too small to measure accurately under the conditions used, so that we cannot determine whether the concentrations of these amines were affected by administration of *p*-chlorophenylalanine or phenylalanine. Octopamine concentrations on the other hand, although too low for quantitative determination, were generally increased after the administration of either *p*-chlorophenylalanine or phenylalanine.

Table 2 shows that 1h after administration of phenylalanine to 23-day-old rats that had been treated with *p*-chlorophenylalanine and pargyline, concentrations of phenethylamine in brain and liver were 41 and 27 times respectively those of brain and liver from control animals that had received neither inhibitor. The mean brain/liver ratio of phenethylamine in the *p*-chlorophenylalanine-treated rats was 1.4 after 1h, and this ratio could be increased to 2.1 after repeated injections of phenylalanine at 2h intervals over a period of 10h.

Regional distribution of phenethylamines

The chromatographic patterns obtained from specific brain regions of rats pretreated with *p*-chlorophenylalanine and pargyline and injected with phenylalanine, showed that phenethylamine was rather uniformly distributed among the different brain regions, but the concentrations were somewhat higher in the striatum (Table 3). *m*-Tyramine and 3-methoxytyramine (Table 1, peaks C′ and F respectively) were found to be major amine constituents in the striatum; smaller amounts of *m*-tyramine were found in the hippocampus and of 3-methoxytyramine

Table 3. *Distribution of β-phenethylamine in specific regions of rat brain*

Rats (23 days old) were pretreated with *p*-chlorophenylalanine and pargyline as described in Table 2 and in the text, and injected intraperitoneally with phenylalanine (1g/kg) 1h before being killed for determination of amines. Each value is the mean of three determinations, of which two were on brain areas pooled from two rats each.

Brain region	Concn. of phenethylamine (μg/g)
Cerebellum	3.1
Medulla	2.7
Hypothalamus	3.3
Striatum	4.5
Midbrain	2.9
Hippocampus	3.9
Cortex	3.2
(Liver)	3.5

Table 4. *Distribution of β-phenethylamine in subcellular fractions of rat brain*

Rats (23 days old) were pretreated with *p*-chlorophenylalanine and pargyline as described in Table 2 and in the text. In Expt. (1) the animals were injected intraperitoneally with phenylalanine (1 g/kg) 1 h before being killed for determination of the amines. Fraction P_1 was not washed. Each value is the mean of two determinations. In Expt. (2) phenylalanine (1 g/kg) was injected at $t = 23$, 24, 25, 26 and 27 h, and the animals were killed at $t = 28$ h for determination of the amines. Fraction P_1 was washed with 5 ml of sucrose solution. Each value is the mean ± S.E.M. of three determinations.

	Concn. of phenethylamine (ng/g of whole brain)	
Fraction	Expt. (1)	Expt. (2)
Nuclear fraction (P_1)	382 ± 25	390 ± 70
Supernatant (S_2)	1552 ± 103	2747 ± 446
Crude mitochondrial fraction (P_2)	Up to 15	Up to 80
Myelin (A)	54 ± 7	Trace
Synaptosomes (B)	Up to 61	Trace
Mitochondria (C)	Not detectable	Not detectable

in the hypothalamus, midbrain and cortex. The concentrations of these amines in specific brain regions were too low to permit quantitative measurement, and we were thus unable to determine to what extent concentrations of *m*-tyramine and 3-methoxytyramine in whole brain were changed by the administration of phenylalanine. Small amounts of tyramine were also detected in the medulla, cerebellum and hypothalamus of both control and experimental rats.

Subcellular distribution of phenethylamine

Table 4 shows that phenethylamine was found predominantly in the supernatant fraction of homogenates of brains obtained from rats that had been pretreated with *p*-chlorophenylalanine and pargyline and injected with phenylalanine. However, a considerable amount of phenethylamine remained in the nuclear fraction even after washing, although we do not know how much of this phenethylamine is specifically localized in the nuclear fraction and how much attaches to occluded cytoplasm (Whittaker, 1965). Only small amounts of phenethylamine were detected in the crude mitochondrial layer, in the myelin and in the synaptosomal pellet, but none was found in the mitochondrial pellet, even when repeated injections of phenylalanine were given to rats to produce very high concentrations of phenylalanine in the blood.

Discussion

Jepson *et al.* (1960) found increased urinary excretion of phenethylamine in patients with phenylketonuria who were receiving monoamine oxidase inhibitors, and although these authors were unable to determine what proportion of the presumed increase in turnover of phenethylamine was contributed by the central nervous system, it was concluded that such an increased turnover was occurring there (Oates *et al.*, 1963). The present study provides direct evidence of increased phenethylamine turnover in the central nervous system of animals with experimentally induced hyperphenylalaninaemia comparable in extent with that seen in human patients with uncontrolled phenylketonuria (Table 2). The phenethylamine brain/liver ratio in the *p*-chlorophenylalanine-treated animals also receiving a monoamine oxidase inhibitor was greater than 1, and increased when high concentrations of phenylalanine were maintained in the blood by repeated injections. We do not know how much of the higher brain concentrations of phenethylamine was contributed by local synthesis and how much by entry into the brain of phenethylamine formed in other tissues; Nakajima *et al.* (1964) found that phenethylamine could cross the blood–brain barrier and could be concentrated in the brain. However, phenethylamine formed in phenylalanine-injected rats may also be produced in the brain itself, since aromatic L-amino acid decarboxylase activity in the brain is about 14% of that in the liver (McCaman *et al.*, 1965).

During inhibition of monoamine oxidase activity, factors tending to increase brain phenethylamine concentration are local synthesis and entry of peripherally synthesized amine; factors that tend to decrease phenethylamine concentration in the brain are exit into blood and cerebrospinal fluid (and renal clearance from the bloodstream), and flux into other metabolic pathways (such as β-hydroxylation and metabolic conjugation). The parallel increase of tissue phenethylamine and blood phenylalanine

concentrations in animals treated with pargyline, together with the very small amounts of phenethylamine found in the tissues of animals not so treated, indicate that oxidation by monoamine oxidase is the major pathway of phenethylamine metabolism. The accumulation of the amine in the tissues of animals under inhibition of monoamine oxidase activity is therefore an index, though not a precise measure, of the rate of turnover, and supports the inference that blood phenylalanine concentration and turnover of phenethylamine parallel one another. A patient with uncontrolled phenylketonuria, with an abnormally high blood phenylalanine concentration, presumably has a correspondingly increased rate of phenethylamine turnover, and we may speculate how this might affect the central nervous system and lead to mental impairment. The permanent nature of this impairment in affected patients has led to a search for damage to structural elements in the brain, and interference with myelin synthesis and maturation, and with the myelination of nerve fibres, has been implicated (see Crome, 1971). In this connexion the toxic effects of the Schiff base formed between pyridoxal and phenethylamine (Loo, 1967) may be significant, not only through obligatory depletion of vitamin B_6 via formation of this complex with increased supplies of phenethylamine, but also because the complex has been shown to have anti-vitamin B_6 activity (Loo & Ritman, 1967). Vitamin B_6 deficiency was found to result in serious interference with myelin synthesis in young rats by Kurtz *et al.* (1972), and additionally to cause severe distortions of amino acid metabolism, as might be expected from the fact that this vitamin is a cofactor to so many of the enzymes of amino acid metabolism.

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