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Aromatic Acids Derived from Phenylalanine in the Tissues of Rats with Experimentally Induced Phenylketonuria-Like Characteristics

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1. Aromatic acids were extracted from brain and liver of rats with phenylketonuria-like characteristics produced by administration of phenylalanine, either alone or in combination with *p*-chlorophenylalanine. The metabolism of the aromatic acids in these tissues was measured by gas chromatography. 2. At 1h after an intraperitoneal injection of L-phenylalanine (1 g/kg) in 23-day-old rats, the phenyl-lactate concentration was 2.2 μg/g in the liver and 0.43 μg/g in the brain, and the concentration of *o*-hydroxyphenylacetate was 0.26 μg/g in the liver. 3. Phenylacetate concentrations in brain and liver were 0.26 and 0.14 μg/g respectively. 4. Suckling rats produced phenyl-lactate less rapidly than weanling rats, but accumulated higher concentrations in longer-term experiments. 5. Intraperitoneal injections of phenyl-lactic acid showed that this compound could directly penetrate the blood–brain barrier, and could produce similar brain/liver ratios of phenyl-lactate to those found after phenylalanine injection. 6. Qualitative and quantitative similarities in urinary excretion of aromatic acids between the rats used in this study and human patients with uncontrolled phenylketonuria indicate that a patient with a circulating phenylalanine concentration of the order of those achieved in the experimental animal may have aromatic acid concentrations in brain and liver comparable with those found in the rats used in the present study.

The mechanism by which the deficiency of liver phenylalanine hydroxylase (EC 1.14.3.1) can produce mental impairment in patients with uncontrolled phenylketonuria is still not known. Accumulation of phenylalanine causes it to be metabolized by alternative pathways, producing abnormally large amounts of phenylpyruvate, phenyl-lactate, *o*-hydroxyphenylacetate and phenylacetate. Scheme 1 summarizes the relevant pathways of phenylalanine metabolism. These aromatic acids have been implicated in the two most frequently proposed mechanisms of pathogenesis, involving (*a*) defective myelination, as a structural irreversible defect, and (*b*) interference with the production of neurotransmitter amines as a functional defect.

Decreased cholesterol concentrations (Crome *et al.*, 1962) and impaired myelination (Alvord *et al.*, 1950) have been found in phenylketonuric brains. Phenylalanine and phenylpyruvate inhibit human pyruvate kinase (EC 2.7.1.40) *in vitro*, and phenylpyruvate inhibits hexokinase (EC 2.7.1.1); these aromatic compounds have been postulated to cause a decrease in brain glycolysis and lipid biosynthesis (Weber, 1965). Bowden & McArthur (1972) suggested that phenylpyruvate would decrease the availability of acetyl-CoA for fatty acid and cholesterol synthesis via inhibition of pyruvate decarboxylation, and thus decrease the formation of myelin. Shah (1969) showed that phenylpyruvate, phenylacetate and phenyl-lactate inhibited the formation *in vitro* of cholesterol from mevalonic acid by brain and liver extracts. Silberberg (1967) showed that phenylacetate and phenylpyruvate exerted toxic effects *in vitro* on myelinating cultures of rat cerebellum.

It has been suggested that the decrease in the concentrations of neurotransmitter amines that occurs in uncontrolled phenylketonuria may result from inhibition of decarboxylation of the aromatic amino acid precursors. Since aromatic amino acid decarboxylase activities in human brain, when measured with the substrates 5-hydroxytryptophan (Robins *et al.*, 1967) or 3,4-dihydroxyphenylalanine (Lloyd & Hornykiewicz, 1970), were found to be very low, decarboxylation might be the rate-limiting step

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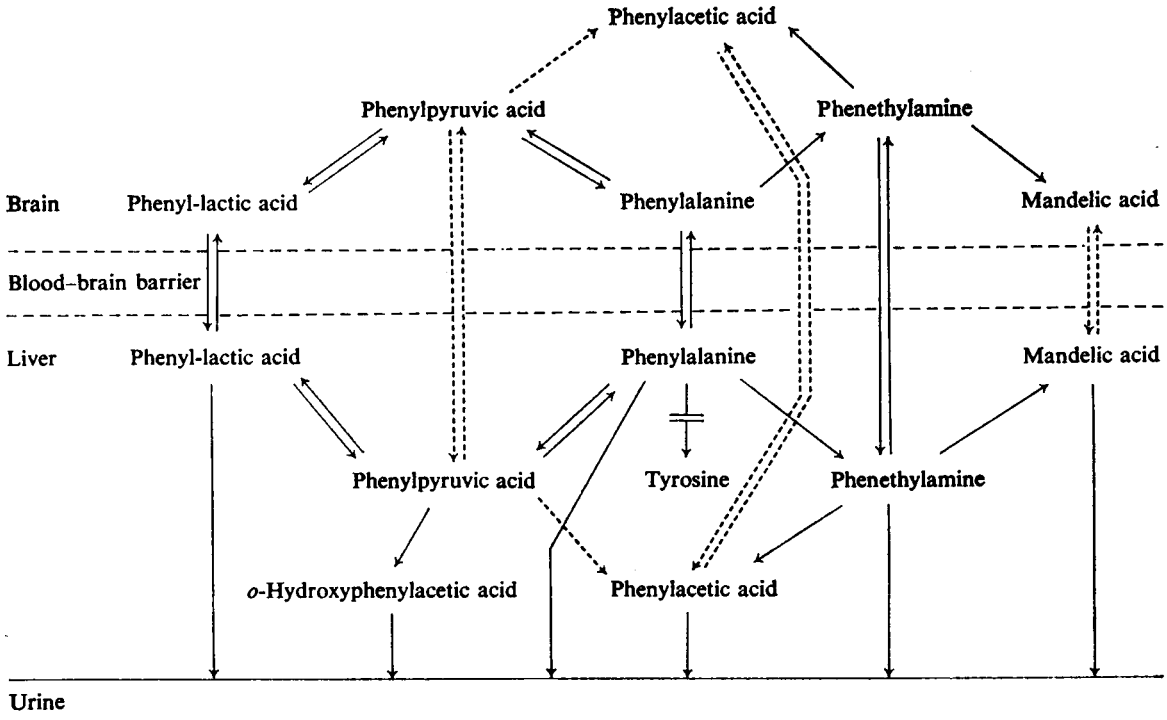

Scheme 1. *Pathways of phenylalanine metabolism in brain and liver in the phenylketonuric condition*

Solid arrows denote pathways that have been previously described or have been found in the present work; dashed arrows denote pathways that may occur, but have not been described.

in the biosynthesis of neurotransmitter amines. Fellman (1956) found that phenylpyruvate, phenyllactate and phenylacetate, in order of decreasing potency, inhibited ox adrenal dihydroxyphenylalanine decarboxylase (EC 4.1.1.26), which is the same enzyme as 5-hydroxytryptophan decarboxylase (Christenson *et al.*, 1972).

To assess the role of these aromatic acids in the production of the mental deficiency in phenylketonuria, we need to know more about the extent to which each of them may accumulate in the brain in that condition (Scheme 1). Aromatic acids are generally thought to be excluded from the brain by the blood–brain barrier, but they may cause damage during the vulnerable period when the blood–brain barrier is believed not to be fully developed. Goldstein (1961) demonstrated the appearance of phenyl-lactate in the brains of 12- and 18-day-old rats but not of older rats after the intraperitoneal injection of high doses of phenylalanine, but in these experiments phenylalanine concentrations in the blood were considerably greater than in patients with phenylketonuria.

In the present studies suckling and weanling rats were subjected to experimental procedures that produced phenylketonuria-like conditions with circulating phenylalanine concentrations of the same order as those found in untreated human patients. The concentrations of aromatic acids in brain and liver were determined, to find out to what extent these metabolites of phenylalanine were present under these conditions, and at different stages of development, and also to try to establish how they reached the brain.

Materials and Methods

Solutions

A 4% (w/v) solution of L-phenylalanine (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) was prepared by dissolving phenylalanine in 0.9% NaCl at 60°C and cooling it to 37°C just before injection. DL-*p*-Chlorophenylalanine (Aldrich Chemical Co.) was prepared as a suspension of 300mg with 3 drops of Triton X-100 (Rohm and Haas, Philadelphia, Pa., U.S.A.) in 10ml of 0.9% NaCl, by using an ultrasonic generator (Branson Sonic Power Co., Danbury, Conn., U.S.A.), just before injection. A solution

containing 3 drops of Triton X-100 in 10ml of 0.9% NaCl was used for control injections. DL-Phenyllactic acid (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was made up to 10mg/10ml in water. Pargyline (*N*-methyl-*N*-propynylbenzylamine; Abbott Laboratories, North Chicago, Ill., U.S.A.) was made up to 10mg/ml in water.

Animals

Male Sprague–Dawley rats (Zivic–Miller Laboratories, Allison Park, Pa., U.S.A.) were used. All injections were given intraperitoneally. Table 1 describes the two experimental and two control groups in terms of the schedule of administration of the various substances. In those experiments indicated, pargyline was also administered to inhibit monoamine oxidase (EC 1.4.3.4), to permit analyses of amines from the same tissue samples.

Analytical procedures

Blood from the severed neck vessels was collected on S & S no. 903 filter paper (Schleicher and Schuell, Keene, N.H., U.S.A.) by the technique of Hill & Palmer (1969). The brains and livers were quickly removed, blotted, weighed, homogenized in 4vol. of ice-cold 0.01M-HCl and centrifuged at 10000*g* for 15min. A 1.0ml sample of the decanted supernatant solution was added to 0.6g of solid NaCl and adjusted to pH1 with a drop of conc. HCl. The aromatic acids were extracted into three successive 1.0ml portions of ethyl acetate, with agitation in a vortex-type mixer and centrifugation at 1000*g* for 5min. Ethyl acetate layers were drawn off with a Pasteur pipette, combined and evaporated to dryness in a ½-dram (2.2ml) screw-top vial (Scientific Specialties Service, Randallstown, Md., U.S.A.) with a stream of dry N_2. To the residue in each vial 25μl of *NO*-bis-trimethylsilylacetamide (Pierce Chemical Co., Rockford, Ill., U.S.A.) was added, the vial was closed with its Teflon-lined cap and derivatives were allowed to form for 20min at room temperature. A sample (1.0μl) was analysed by g.l.c. on an I.D. glass U-column (200cm×0.25cm) packed with 5% OV-1 on Gas Chrom Q (80–100 mesh) (Applied Science Laboratories, State College, Pa., U.S.A.) isothermally

Table 1. *Design of the animal experiments: doses, times and sequence of injections for the four experimental groups of rats*

Animals that were also used for amine studies received in addition pargyline (50mg/kg) at $t = 22\frac{1}{2}$h. All animals were decapitated at $t = 24$h.

Group	t ... 0	20h	23h
Saline/saline	0.9% NaCl (10ml/kg)	0.9% NaCl (5ml/kg)	0.9% NaCl (25ml/kg)
p-Chlorophenylalanine/saline	*p*-Chlorophenylalanine (300mg/kg)	*p*-Chlorophenylalanine (150mg/kg)	0.9% NaCl (25ml/kg)
Saline/phenylalanine	0.9% NaCl (10ml/kg)	0.9% NaCl (5ml/kg)	Phenylalanine (1g/kg)
p-Chlorophenylalanine/phenylalanine	*p*-Chlorophenylalanine (300mg/kg)	*p*-Chlorophenylalanine (150mg/kg)	Phenylalanine (1g/kg)

Table 2. *Yield of the aromatic acids on extraction from brain homogenate or saline*

Aromatic acids were added in 0.1ml of solution, to 0.9ml either of saline or a 50% (w/v) homogenate of brain, adjusted to pH1, and extracted with three 1ml portions of ethyl acetate.

Aromatic acid	Sample	Amount added (μg)	Amount recovered (%)
Mandelic acid	Saline	1.61	96.0
	Brain	1.61	99.7
o-Hydroxyphenylacetic acid	Saline	2.49	92.0
	Brain	2.49	90.4
DL-Phenyl-lactic acid	Saline	2.89	94.1
	Brain	2.89	94.3
p-Hydroxybenzoic acid	Saline	3.36	92.4
	Brain	3.36	90.0

at 133°C (Blau, 1970). Peaks were identified by their retention characteristics, and measured quantitatively by comparing the peak heights with freshly run standards after correction for losses during extraction (Table 2), size of injected sample, dilution by homogenizing medium and instrumental attenuation.

Blood spots (0.95cm diam.) were punched out and the concentrations of phenylalanine and tyrosine were determined by the automated fluorimetric procedures of Hill *et al.* (1965) and of Hochella (1967) respectively. For the separate determination of phenylalanine and *p*-chlorophenylalanine, the amino acids were eluted from the punched-out discs with aq. ethanol (70%, v/v), and the eluates were evaporated to dryness *in vacuo*. Pivaldehyde derivatives of amino acid methyl esters were prepared and determined by g.l.c. according to the procedures of Jellum *et al.* (1969).

Liver phenylalanine hydroxylase activity was measured by the semi-automated kinetic assay of Edwards & Blau (1971).

Results

The phenylalanine hydroxylase activity in the liver of 23-day-old rats treated with *p*-chlorophenylalanine was 0.032–0.092 μmol/min per g, i.e. 4–11% of that of the control animals (0.83 μmol/min per g). However, since the liver phenylalanine hydroxylase activity of rats is high, this amount of residual activity is probably sufficient for normal metabolism of dietary phenylalanine. This amount of inhibition does not of itself lead to increased blood phenylalanine concentration or an increased phenylalanine/tyrosine ratio in the blood, and it is therefore necessary to administer additional phenylalanine to achieve elevated blood phenylalanine concentrations and phenylalanine/tyrosine ratios (Anderson & Guroff, 1972).

The phenylalanine and tyrosine concentrations obtained for 23- and 30-day-old rats in experimental and control groups are given in Table 3. Since *p*-chlorophenylalanine gives 1.23 times the fluorescence yield of phenylalanine (Copenhaver, 1971), the amounts of these two amino acids were individually determined by g.l.c. (Jellum *et al.*, 1969). The concentration of *p*-chlorophenylalanine in the blood of 23-day-old rats treated with *p*-chlorophenylalanine was 11 mg/100ml. The corrected phenylalanine values obtained by the fluorimetric technique for the rats treated with *p*-chlorophenylalanine agreed well with those obtained by the determination of phenylalanine by g.l.c., but the fluorimetric technique was more convenient for the analysis of large numbers of samples. The corrected mean phenylalanine concentration in the blood of the 23-day-old rats that were treated with phenylalanine as well as with *p*-chlorophenylalanine was 64mg/100ml, which was not significantly higher than the mean value of phenylalanine in the blood of 23-day-old rats that were treated with phenylalanine and saline (60mg/100ml). These concentrations are within the range found in uncontrolled human phenylketonuria, in which the serum phenylalanine concentration may be as high as 79mg/100ml (Hackney *et al.*, 1968). However, the phenylalanine/tyrosine ratio for the group receiving

Table 3. *Phenylalanine and tyrosine concentrations in the blood of rats treated with various compounds as described in Table* 1

Values are given in mg/100ml, means ± S.E.M. with the numbers of rats in parentheses. Phenylalanine was determined by the method of Hill *et al.* (1965), which also measures *p*-chlorophenylalanine: *includes *p*-chlorophenylalanine, which was found, by gas chromatography, to contribute 11–13mg/100ml (see the text). Tyrosine was determined by the method of Hochella (1967).

	23-day-old rats				30-day-old rats	
	Not pargyline-treated		Pargyline-treated		Pargyline-treated	
Group	Phenylalanine	Tyrosine	Phenylalanine	Tyrosine	Phenylalanine	Tyrosine
Saline/saline	2.55 ± 0.25 ($n = 2$)	1.00 ± 0.30 ($n = 2$)	2.28 ± 0.31 ($n = 4$)	0.88 ± 0.12 ($n = 4$)	3.20 ± 0.20 ($n = 2$)	1.60 ± 0.20 ($n = 2$)
p-Chlorophenylalanine/saline	17.66 ± 4.46* ($n = 4$)	1.80 ± 0.40 ($n = 5$)	22.30 ± 4.91* ($n = 4$)	1.38 ± 0.30 ($n = 4$)	18.45 ± 3.85* ($n = 2$)	2.05 ± 0.35 ($n = 2$)
Saline/phenylalanine	58.81 ± 3.38 ($n = 4$)	6.98 ± 0.38 ($n = 5$)	59.26 ± 8.85 ($n = 5$)	7.87 ± 1.26 ($n = 7$)	53.03 ± 10.31 ($n = 3$)	9.90 ± 1.63 ($n = 3$)
p-Chlorophenylalanine/phenylalanine	77.52 ± 5.46* ($n = 6$)	2.58 ± 0.22 ($n = 5$)	76.88 ± 7.62* ($n = 8$)	3.23 ± 0.52 ($n = 8$)	123.8 ± 5.30* ($n = 4$)	2.93 ± 0.19 ($n = 4$)

Table 4. *Aromatic acids in urine in a single 9-day-old rat treated with p-chlorophenylalanine and phenylalanine*

At time $t = 0$ the animal received *p*-chlorophenylalanine (300mg/kg). At times $t = 28$, 29, 30 and 31h injections of phenylalanine (500mg/kg) were given intraperitoneally. Urine was collected at $t = 32$h, and the aromatic acids in urine were determined by the method of Blau (1970).

Acid	Concentration (μg/ml)
Phenyl-lactic acid	287
Phenylpyruvic acid	68
o-Hydroxyphenylacetic acid	5.3
p-Hydroxyphenylacetic acid	4.9
Mandelic acid	3.0

phenylalanine and saline was only 8.6, whereas the same ratio for the group that had received phenylalanine as well as *p*-chlorophenylalanine was 24.7. The phenylalanine/tyrosine ratio of the latter group more closely approximated to the 20:1 ratio typical of the human phenylketonuric condition. Similar blood concentrations of phenylalanine and tyrosine were found in groups of 8-, 14- and 30-day-old rats, except that the blood phenylalanine concentrations of the oldest rats treated with *p*-chlorophenylalanine were significantly higher.

The aromatic acid patterns in urine of the rats with experimentally induced phenylketonuria were qualitatively very similar to those found in human phenylketonuria (Blau, 1970). Table 4 gives a typical result for such an analysis on a sample of urine from a 9-day-old rat treated with *p*-chlorophenylalanine 9h after the last of four hourly injections of phenylalanine (0.5g/kg). All of the same aromatic acids could be detected in the blood spots except phenylpyruvic acid, which Blau (1970) found to be subject to loss by oxidation when samples were collected on filter paper. However, phenylpyruvic acid could be determined in plasma, which was obtained in sufficient quantities from adult rats and from rabbits, and the concentration of phenylpyruvic acid was 23% of the value for phenyl-lactic acid 1h after an injection of phenylalanine.

Brain and liver extracts each produced characteristic aromatic acid patterns. The peaks were identified on the basis of retention values obtained from both isothermal and programmed g.l.c. Phenylalanine loading specifically produced peaks in liver samples which corresponded to *o*-hydroxyphenylacetate, and produced peaks in brain and liver samples which corresponded to phenyl-lactate.

Table 5 shows the concentrations of phenyl-lactate in the brain and liver and of *o*-hydroxyphenylacetate in the liver 1h after the administration of phenylalanine (1g/kg) to 23-day-old rats pre-treated with *p*-chlorophenylalanine. It was not possible to detect *o*-hydroxyphenylacetate in the brain; in the liver its concentration was about one-quarter of that of phenyl-lactate, but this was near the minimum detectable amount, so its presence in brain cannot be ruled out entirely.

Phenylacetate was determined by g.l.c. at a lower temperature; small amounts were found in both brain and liver extracts from phenylalanine-injected but not from control rats. The concentrations of phenylacetate in 23-day-old rats 1h after a single phenylalanine injection were 0.26μg/g in brain and 0.14μg/g in liver. In brain the phenylacetate/phenyl-lactate ratio was 0.16–0.3, but in liver it was only about 0.04–0.1. Phenylacetate was not detected, or was detected in very much smaller amounts in rats pre-treated with pargyline, so that most of it must come from the action of monoamine oxidase on phenethylamine.

Experiments were done to study the formation and distribution of phenyl-lactate over longer periods of time. Multiple injections of phenylalanine (0.5g/kg) given at 2h intervals beginning 1h after the second injection of *p*-chlorophenylalanine resulted in blood phenylalanine concentrations that were the same as those produced by a single injection of 1g/kg. Rats were killed 1h after the second, fourth and sixth injection of phenylalanine; other rats received only the first two injections of phenylalanine, but were killed at the same time as those receiving six injections. Table 6 shows that both the tissue concentrations and the brain/liver ratios of phenyl-lactate were greater in 23-day-old rats given multiple injections of phenylalanine than in those given only a single injection (Table 5), although the phenylalanine values were similar in both cases. Somewhat greater concentrations of phenyl-lactate were found in the tissues of 9-day-old rats that were similarly treated with multiple injections of phenylalanine, even though phenyl-lactate could not be detected in the tissues of 8- and 14-day-old suckling rats 1h after a single injection of phenylalanine. Further, phenyl-lactate was still detected in both brain and liver tissues of 9-day-old but not of 23-day-old rats 9h after the last phenylalanine injection.

Phenyl-lactic acid was injected into rats to try to determine whether the cerebral phenyl-lactate might originate in the liver and subsequently enter the brain via the blood–brain barrier. In a preliminary experiment phenyl-lactic acid (1g/kg body wt.) was injected intraperitoneally into an 8-week-old rat. After 1h the animal was killed and the phenyl-lactate concentration in tissues and body fluids was determined (Table 7). Although most of the phenyl-lactate was found in the urine, a significant amount was detectable in the brain, where its concentration was 7.5% of that in the plasma. This is much more

Table 5. *Tissue concentrations of phenyl-lactate and o-hydroxyphenylacetate in 23-day-old rats treated with p-chlorophenylalanine and phenylalanine*

Rats received *p*-chlorophenylalanine (300mg/kg) at time $t = 0$, phenylalanine (1g/kg) at $t = 23$h and were killed at $t = 24$h. Values are corrected for recovery and are given as mean±S.E.M. determined for five rats.

Phenyl-lactate (μg/g)		Brain/liver ratio	Liver *o*-hydroxyphenylacetate (μg/g)	Liver *o*-hydroxyphenylacetate/ phenyl-lactate ratio
Brain	Liver			
0.726±0.096	2.90±0.45	0.304±0.082	0.607±0.201	0.240±0.066

Table 6. *Tissue concentrations of phenyl-lactate after multiple intraperitoneal injections of phenylalanine and pre-treatment with p-chlorophenylalanine*

All rats were injected with *p*-chlorophenylalanine (300mg/kg) at time $t = 0$, and multiple injections of phenylalanine were started at $t = 24$h. Values given are ranges with the number of rats in parentheses.

Age (days)	Phenylalanine administration and times of sampling	Phenyl-lactate concentration (μg/g)		Brain/liver ratio
		Brain	Liver	
23	Multiple injections of 0.5g/kg at 2h intervals			
	1h after last injection	1.2–4.4 ($n = 6$)	2.3–15.4 ($n = 6$)	0.29–0.64 ($n = 6$)
	9h after last injection	<0.4 ($n = 2$)	<0.4 ($n = 2$)	
9	Multiple injections of 0.5g/kg at 2h intervals			
	1h after last injection	1.0–9.2 ($n = 3$)	9.2–28.1 ($n = 3$)	0.17–0.48 ($n = 3$)
	9h after last injection	2.0 ($n = 1$)	2.7 ($n = 1$)	0.74 ($n = 1$)

Table 7. *Distribution of phenyl-lactate in the tissues and body fluids of an 8-week-old rat after a single injection of phenyl-lactic acid*

Phenyl-lactate was determined in tissues and body fluids of an 8-week-old rat 1h after an intraperitoneal injection of phenyl-lactic acid (1g/kg).

Tissue	Phenyl-lactate concentration (μg/g or μg/ml)	% of plasma concentration
Brain	12.7	7.5
Liver	166	97.6
Kidney	437	257
Spleen	146	85.9
Plasma	170	100
Urine	35700	21000

than could be accounted for by the phenyl-lactate content of blood remaining in the brain at the time it was homogenized, since the brain contains an average of only 2% blood by weight (Weil-Malherbe *et al.*, 1959). We were unable to determine whether phenyl-lactate entered brain cells or was only in the extracellular fluid, but Silberberg (1967) found certain aromatic acids (though not phenyl-lactic acid) to be toxic to rat cerebellar cells in tissue culture, indicating that such acids can enter brain cells.

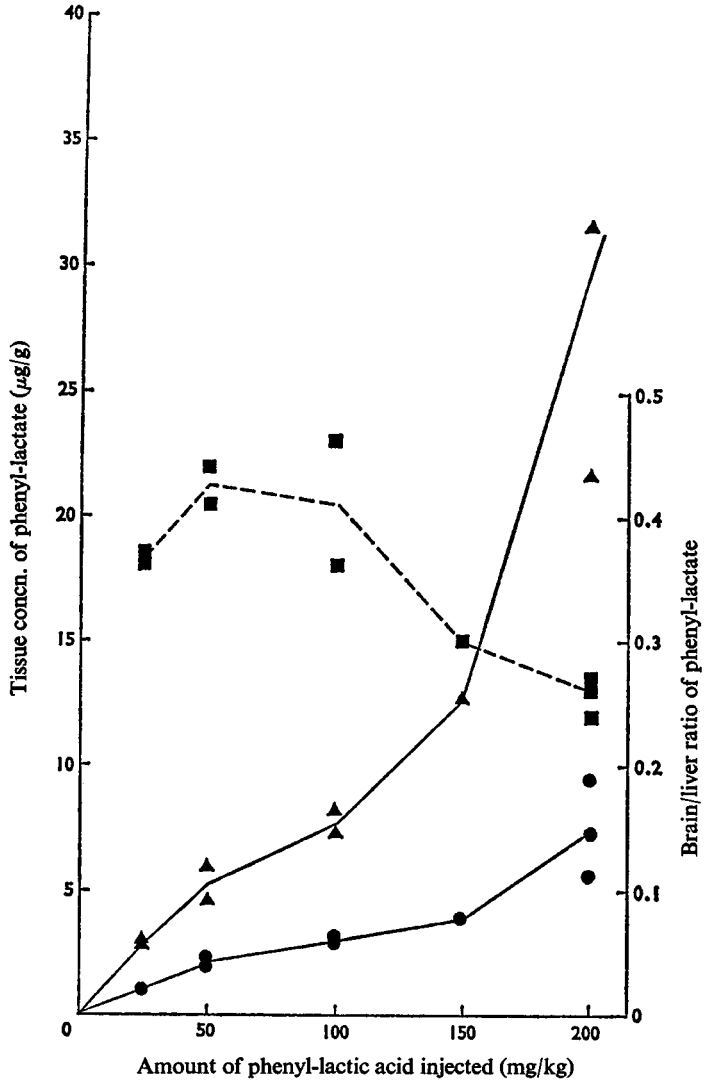

Fig. 1. *Distribution of injected phenyl-lactic acid in the brain and liver of 23-day-old rats*

Concentrations were determined 1 h after intraperitoneal injection of varying doses of phenyl-lactic acid. ●, Brain phenyl-lactate concentration; ▲, liver phenyl-lactate concentration; ■, brain/liver ratio of phenyl-lactate concentration.

The phenyl-lactic acid which was administered produced only minimal amounts of phenylpyruvate; the phenylpyruvate concentration in urine was only 0.6% of that of phenyl-lactate. No increase of phenylalanine was detectable in blood or tissues after phenyl-lactic acid injection. It therefore appeared unlikely that phenyl-lactate was metabolized in the 'reverse direction' to either phenylpyruvate or phenylalanine, which might then have entered the brain to be re-metabolized to phenyl-lactate.

Fig. 1 shows that both brain and liver phenyl-lactate concentrations in 23-day-old rats increased almost linearly over the range of doses from 25–200 mg of phenyl-lactic acid/kg. These curves pass through the origin, indicating the lack of a threshold of its entry into both tissues. The brain/liver ratios of phenyl-lactate over this range are similar to those produced by the endogenous formation of phenyl-lactate after phenylalanine injections (Table 5). The phenyl-lactate concentrations in the brain and liver

1 h after an injection of phenyl-lactic acid (25 mg/kg: see Fig. 1) were approximately the same as those produced by an injection of phenylalanine (1 g/kg) as shown in Table 5.

Discussion

Goldstein (1961) found that experimentally induced hyperphenylalaninaemia in rats leads to the appearance of phenyl-lactate in the brains of 12- and 18-day-old but not older rats, and only after they were given five injections of phenylalanine (1 g/kg) at 2 h intervals, which gives phenylalanine concentrations as high as 174 mg/100 ml 2 h after the last injection; the maximal concentrations were reached 1 h after injection, and were probably even greater. The method we used was sensitive enough to detect 0.1 μg/g of tissue, an amount one-fiftieth of the minimum quantity detectable by Goldstein (1961). We were therefore able to measure quantitatively concentrations of phenyl-lactate in the brains of rats whose circulating phenylalanine concentrations were 60–80 mg/100 ml, which much more resembled those found in untreated human phenylketonuria. We found measurable quantities of phenyl-lactate in the brain of weanling but not of suckling rats 1 h after they were given a single injection of phenylalanine. However, the brain concentrations of phenyl-lactate of 9-day-old rats became even greater than those of the weanling rats after 12 h of multiple injections of phenylalanine. These results may explain why Goldstein (1961) found phenyl-lactate in the brains of only the suckling rats, the phenyl-lactate concentrations in the weanling rats being below what he was able to detect. However, in Goldstein's (1961) experiments the blood phenylalanine concentrations continued to increase during the course of the repeated injections, so it was not clear whether the appearance of phenyl-lactate in the brain was due to a threshold of phenylalanine concentration or to a time-lag before the phenyl-lactate was produced. Our experiments with multiple injections of phenylalanine were designed to maintain phenylalanine concentrations within the same range as that produced by a single injection, and the results indicate that more phenyl-lactate may accumulate in the tissues of suckling than of weanling rats. Relatively high concentrations of phenyl-lactate were found in the tissues of suckling rats 9 h after multiple injections of phenylalanine, by which time the blood concentration of phenylalanine was almost back to normal. These results suggest that phenyl-lactate is initially formed more slowly in suckling rats than in weanling rats. The higher concentrations of phenyl-lactate in the tissues of the suckling rats during longer-term experiments may in part be caused by substrate induction of an enzyme, such as phenylalanine aminotransferase, which is involved in the synthesis of phenyl-lactate and which may initially not show much activity in the immature animal. Phenyl-lactate catabolism or excretion may be less efficient in the less-mature animal, and this also would explain the higher tissue phenyl-lactate concentrations observed even after the phenylalanine concentration in the blood was almost back to normal.

These results indicate that in chronic hyperphenylalaninaemia, such as occurs in untreated human phenylketonuria, phenyl-lactate may accumulate in the tissues during early stages of development. Since the period of brain development from 14 to 18 days of age in the rat corresponds to the period from 6 to 12 months of age in the human during which the irreversible damage is thought to occur (Lo *et al.*, 1970), high concentrations of phenyl-lactate in the brain during this critical period might contribute to the permanent mental impairment. Phenyl-lactate can also directly enter the brain from the peripheral circulation. The brain/liver ratios of phenyl-lactate are very similar whether it is produced from elevated blood phenylalanine concentrations or whether phenyl-lactic acid is injected directly into the peritoneal cavity. Although we injected racemic phenyl-lactic acid, this would not affect the tissue distribution of phenyl-lactate if its entry into the brain was by a passive mechanism, which appears to be likely because of the failure of the brain concentrations to reach a saturable limit (Fig. 1). A small proportion of the phenyl-lactate found in the brain after intraperitoneal injections of phenylalanine might be produced within the brain from the increased concentrations of phenylalanine. Both the aminotransferase (Fuller, 1970) and the aromatic α-oxo acid reductase (Zannoni & Weber, 1966), which could catalyse the metabolic formation of phenyl-lactate from phenylalanine, are present in the brain.

Phenylacetate was present in small but detectable amounts in the brains and livers of phenylalanine-injected rats. It may be produced by the action of monoamine oxidase on phenethylamine, and could also arise from phenylpyruvate by an, as yet, unknown mechanism. The decreased phenylacetate concentrations found in the tissues after inhibition of monoamine oxidase activity with pargyline indicate that the former pathway is the major source of phenylacetate in rats with experimentally induced phenylketonuria, and therefore phenylacetate excretion may be usable as an index of phenethylamine turnover. The finding of somewhat larger quantities of phenylacetate in the brain than in the liver suggests that a significant portion of the phenylacetate may be synthesized within the brain. Phenylacetate is known to be toxic, and could produce detrimental effects on mental function in human phenylketonuria (Silberberg, 1967).

Aromatic acid metabolites of phenylalanine reach measurable and significant concentrations in the brain

of rats with experimentally induced phenylketonuria-like states, and some of these acids may be formed locally, whereas others, in particular phenyl-lactate, may be formed elsewhere, and reach the brain from the peripheral circulation. An analogous situation may be inferred for the human brain in patients with uncontrolled phenylketonuria, although its significance in causing mental deficiency is not yet clear. More-sensitive techniques, such as the use of electron-capture detection, will be needed to study smaller amounts of aromatic acids in the tissues, including the phenyl-lactate concentrations in suckling rats after a single injection of phenylalanine, concentrations of *o*-hydroxyphenylacetic acid in the brain, if present, and concentrations of other metabolites present in low concentrations in the tissues, but (like mandelic acid) known to be excreted in the urine of patients with phenylketonuria (Blau, 1970). Ultimately we want to know at what concentrations these compounds may be detrimental to mental development, whether their brain concentrations are high enough in phenylketonuria to account for some of the damage, and if so, whether such damage may be avoided if these compounds can be prevented from being formed, or from entering the brain, by direct means additional to or instead of dietary phenylalanine restriction.

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